Bcr4 is a Chaperone for the Inner Rod Protein in the *Bordetella* Type III Secretion System

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Running Head: Bcr4 is a Chaperone for Inner Rod in *Bordetella* T3SS

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Abstract

Bordetella bronchiseptica injects virulence proteins called effectors into host cells via a type III secretion system (T3SS) conserved among many Gram-negative bacteria. Small proteins called chaperones are required for stabilizing some T3SS components or localizing them to the T3SS machinery. In a previous study, we identified a chaperone-like protein named Bcr4 that regulates T3SS activity in *B. bronchiseptica*. Bcr4 does not show strong sequence similarity to well-studied T3SS proteins of other bacteria, and its function remains to be elucidated. Here, we investigated the mechanism by which Bcr4 controls T3SS activity. A pull-down assay revealed that Bcr4 interacts with BscI, based on its homology to other bacterial proteins, to be an inner rod protein of the T3SS machinery. A pull-down assay using truncated Bcr4 derivatives and secretion profiles of *B. bronchiseptica* producing truncated Bcr4 derivatives showed that the Bcr4 C-terminal region is necessary to interact with BscI and to activate the T3SS. Moreover, the deletion of BscI abolished the secretion of type III secreted proteins from *B. bronchiseptica* and the translocation of a cytotoxic effector into cultured mammalian cells. Finally, we showed that BscI is unstable in the absence of Bcr4. These results suggest that Bcr4 supports the construction of the T3SS machinery by stabilizing BscI. This is the first demonstration of a chaperone for the T3SS inner rod protein among the virulence bacteria possessing the T3SS.

41 **Importance**

42 The type III secretion system (T3SS) is a needle-like complex that projects outward from
43 bacterial cells. *Bordetella bronchiseptica* uses the T3SS to translocate virulence proteins
44 into host cells. Our previous study reported that a protein named Bcr4 is essential for the
45 secretion of virulence proteins from *B. bronchiseptica* bacterial cells through the T3SS.
46 Because other bacteria lack a Bcr4 homologue, the function of Bcr4 has not been
47 elucidated. In this study, we discovered that Bcr4 interacts with BscI, a component of the
48 T3SS machinery. We showed that a *B. bronchiseptica* BscI-deficient strain was unable to
49 secrete type III secreted proteins. Furthermore, the deletion of Bcr4 led to BscI instability in
50 *B. bronchiseptica*. These results suggest that Bcr4 stabilizes BscI to allow construction of
51 the T3SS in *B. bronchiseptica*.

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61 **Introduction**

62 The genus *Bordetella* consists of Gram-negative bacteria that infect the respiratory tracts
63 of humans and animals. *Bordetella pertussis* causes a severe coughing attack called
64 whooping cough in humans (1, 2). *Bordetella bronchiseptica* causes atrophic rhinitis in pigs
65 and kennel cough in dogs (1, 2). These *Bordetella* spp. harbor a virulence factor secretion
66 apparatus called the type III secretion system (T3SS).

67 The T3SS consists of a basal body that penetrates the inner and outer membranes of the
68 bacteria and a needle structure that protrudes outside the bacteria, and is conserved in
69 many Gram-negative bacteria such as *Yersinia*, *Salmonella*, and *Pseudomonas*. *B.*
70 *bronchiseptica* uses the T3SS to inject virulence factors, called effectors, into host cells to
71 disrupt the physiological functions of host cells (3). Once the basal body and the export
72 apparatus of the T3SS are completed, the type III secreted proteins are secreted in a fixed
73 order. First, the components of the needle structure and the inner rod that ties the needle
74 structure to the basal body are secreted. After the needle is completed, translocators are
75 secreted, which form small pores in the host cell membrane to create a pathway for
76 effectors. Finally, the effectors translocate into the host cell via the T3SS (4, 5). BopB (6),
77 BopD (7), and Bsp22 (8) function as translocators, while BteA (also referred to as BopC) (9,
78 10), BopN (11, 12), and BspR (also referred to as BtrA) (13, 14) function as effectors in *B.*
79 *bronchiseptic*a. Bsp22 is located at the tip of the needle and bridges the needle to the
80 pore-forming factors embedded in the plasma membrane (15). BteA has been shown to

81 cause membrane-disrupting cytotoxicity in mammalian cells (10). BspR is a regulator that
82 represses transcription of the *bteA* gene and genes on the *bsc* locus, where genes
83 encoding type III secreted proteins and components of T3SS are located (14, 16). The
84 BscF and BscI proteins of *B. bronchiseptica* have 18.4% and 18.3% identity with the
85 *Yersinia* needle YscF and inner rod YscI, respectively, suggesting that they are the needle
86 and inner rod of the *Bordetella* T3SS.

87 In addition, many type III secreted proteins have unique chaperones that are involved in
88 stabilizing the substrate and preventing premature polymerization of the substrate in the
89 bacterial cytosol, and then in efficiently transporting the substrate to the T3SS machinery
90 (17-20). For example, PscE and PscG function as chaperones of the needle PscF in
91 *Pseudomonas*. These chaperones stabilize PscF in the bacterial cytosol and are involved
92 in PscF secretion through the T3SS (21). In bacteria such as *Yersinia* and *Pseudomonas*,
93 an inner rod chaperone is thought to exist, because the inner rod has the properties of
94 being secreted out of the bacterial cell and polymerizing (22, 23). However, a chaperone for
95 the inner rod has not been reported.

96 So far, a chaperone-like protein called Bcr4 has been identified in *B. bronchiseptic*a (24).
97 In this study, we attempted to identify factors that interact with Bcr4 in order to investigate
98 how Bcr4 is involved in the T3SS regulation.

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101 **Results**

102 **Bcr4 binds to BscI, an inner rod protein of the *Bordetella* type III secretion system.**

103 The results of a previous study suggested that Bcr4 is a chaperone for components of
104 the type III secretion system (T3SS) (24). T3SS chaperones are known to be involved in
105 substrate stability and efficient transport of substrates to the T3SS machinery (17, 19, 20).
106 In addition, it is generally known that the genes of these chaperones are localized adjacent
107 to genes encoding their substrates (25). On the *B. bronchiseptica* S798 chromosome, the
108 genes encoding BcrH2, BscI, BscJ, and BscK are located in the vicinity of the *bcr4* gene
109 (Fig. 1A), and these proteins are predicted to function as a translocator chaperone, inner
110 rod, inner membrane ring, and ATPase cofactor, respectively (5, 26). Based on the above,
111 we hypothesized that Bcr4 binds to either BscI, BscJ, or BscK. To test whether Bcr4 binds
112 to BscI or BscK, we added *E. coli* lysates containing the V5-tagged target proteins (BscI-V5
113 or BscK-V5) to Strep-Tactin beads with the purified Strep-tagged Bcr4 (Bcr4-Strep), and
114 then performed the pull-down assay. Supernatant fraction (Sup) and pellet fraction (Pellet)
115 samples were prepared, separated by SDS-PAGE, and subjected to Western blotting with
116 antibodies against V5 (Fig. 1B). The results showed that the V5 signal was detected in the
117 Pellet sample of the BscI-V5, but not in that of BscK-V5 (Fig. 1B). These results suggest
118 that Bcr4 binds to BscI, an inner rod protein of the *Bordetella* T3SS.

119 Next, to reconfirm that Bcr4 binds to BscI, a pull-down assay was performed by adding *E.*
120 *coli* lysate containing V5-tagged Bcr4 (Bcr4-V5) to Strep-Tactin beads with the purified

121 Strep-tagged BscI (BscI-Strep) or BteA N-terminal 1–312 amino acids region
122 (BteA-N-Strep). As a result, the V5 signal was detected in the Pellet sample of the
123 BscI-Strep, but was hardly detected in that of the BteA-N-Strep (Fig. 1C). These results
124 strongly suggested that Bcr4 binds to BscI.
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126 **The C-terminal region of Bcr4 is required for the binding of Bcr4 to BscI and T3SS**
127 **activity.**
128 Next, to investigate the Bcr4 region responsible for the binding to BscI, we produced
129 Strep-tagged full-length Bcr4 (Bcr4-FL-Strep), Bcr4 lacking amino acids region 58–109
130 (Bcr4Δ58-109-Strep), and Bcr4 lacking amino acids region 110–173 (Bcr4Δ110-173-Strep)
131 in *E. coli* (Fig. 2A). A pull-down assay was performed by adding *E. coli* lysate containing
132 V5-tagged BscI (BscI-V5) to Strep-Tactin beads with the purified Bcr4-FL-Strep,
133 Bcr4Δ58-109-Strep, or Bcr4Δ110-173-Strep. The Sup and Pellet samples were then
134 prepared, separated by SDS-PAGE, and subjected to Western blotting using antibodies
135 against V5 (Fig. 2B). As a result, the V5 signal was detected in the Pellet sample of
136 Bcr4-FL-Strep, but not in those of Bcr4Δ58-109-Strep and Bcr4Δ110-173-Strep. These
137 results suggest that the BscI-binding domain is located in the 58–173 amino acids region of
138 Bcr4.
139 To determine the region of Bcr4 required for the function of T3SS, we produced
140 full-length FLAG-tagged Bcr4 (Bcr4-FL-FLAG), Bcr4 with the 5 C-terminal amino acids

141 deleted (Bcr4Δ169-173-FLAG), Bcr4 with the 10 C-terminal amino acids deleted
142 (Bcr4Δ164-173-FLAG), and Bcr4 with the 15 C-terminal amino acids deleted
143 (Bcr4Δ159-173-FLAG) (Fig. 2A) within a Bcr4-deficient strain (ΔBcr4)
144 (ΔBcr4+Bcr4-FL-FLAG, ΔBcr4+Bcr4Δ169-173-FLAG, ΔBcr4+Bcr4Δ164-173-FLAG and
145 ΔBcr4+Bcr4Δ159-173-FLAG, respectively). Whole cell lysates (WCL) and Sup samples
146 were prepared from these strains and separated by SDS-PAGE. Western blottings were
147 then carried out using antibodies against Bcr4, FLAG, BteA (an effector and a type III
148 secreted protein) or BopD (a translocator and a type III secreted protein) (Fig. 2C). Since
149 the antibody against Bcr4 was generated using its 18 C-terminal amino acids (amino acids
150 (aa) 156–173) as the antigen peptide, it may not recognize the Bcr4 partial deletion mutant
151 proteins used in this experiment. Therefore, we also performed Western blotting using
152 antibodies against FLAG (Fig. 2C). As a result, when anti-Bcr4 antibodies were used
153 against the WCL samples, signals were detected in the wild-type, ΔBcr4+Bcr4-FL-FLAG
154 and ΔBcr4+Bcr4Δ169-173-FLAG, but almost no signals were detected in
155 ΔBcr4+Bcr4Δ164-173-FLAG and ΔBcr4+Bcr4Δ159-173-FLAG (Fig. 2C). On the other hand,
156 when anti-FLAG antibody was used, signals were detected in ΔBcr4+Bcr4Δ164-173-FLAG
157 and ΔBcr4+Bcr4Δ159-173-FLAG (Fig. 2C). These results confirmed that the Bcr4 partial
158 deletion mutant proteins were produced in the ΔBcr4 strain. When anti-BteA or anti-BopD
159 antibodies were used against the Sup samples, signals were detected in the wild-type,
160 ΔBcr4+Bcr4-FL-FLAG, ΔBcr4+Bcr4Δ169-173-FLAG and ΔBcr4+Bcr4Δ164-173-FLAG,

161 respectively, but not in ΔBcr4 or Bcr4Δ159-173-FLAG (Fig. 2C). These results suggest that
162 the region required for the T3SS function is located in the 159–163 amino acids region of
163 Bcr4.
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165 **BscI is required for the function of the T3SS in *B. bronchiseptica*.**
166 BscI is a homologue of YscI, an inner rod of the *Yersinia* T3SS, and YscI is essential for
167 the function of the T3SS (Wood, JB, 2008). To confirm that BscI is required for the function
168 of the *B. bronchiseptica* T3SS, the *B. bronchiseptica* S798 wild-type, BscI-deficient strain
169 (ΔBscI), BscI-complemented strain (ΔBscI+BscI), and BscN-deficient strain (ΔBscN) were
170 cultured in SS medium. WCL and Sup samples were prepared, and these samples were
171 separated by SDS-PAGE, followed by Western blotting using antibodies against BscI, BteA,
172 BopD or FHA (filamentous hemagglutinin, an adhesion factor) (Fig. 3). As a result, no
173 signals of BscI were detected in the WCL samples of all strains used here (Fig. 3). The BscI
174 signal was detected in the Sup of ΔBscI+BscI, and a faint BscI signal was detected in the
175 Sup of the wild-type (Fig. 3). The BteA and BopD signals were detected in the Sup of the
176 wild-type and ΔBscI+BscI, but not in those of ΔBscI and ΔBscN (Fig. 3). As expected, the
177 FHA signals were detected in the WCL and Sup samples of all strains used here (Fig. 3).
178 These results suggest that in *B. bronchiseptica*, BscI is required for the function of the
179 T3SS and is secreted out of the bacterial cell.
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181 **BscI plays an important role in the function of the *B. bronchiseptica* T3SS during**

182 **infection of cultured mammalian cells.**

183 To further investigate whether BscI is required for the construction and function of the

184 T3SS in *B. bronchiseptica*, we infected L2 cells (a rat lung epithelial cell line) with the

185 wild-type, ΔBscI, ΔBscI+BscI or ΔBscN and measured the number of Bsp22 (a translocater,

186 and a component of the filament-like structure; Fig. 6) signals detected on L2 cells by

187 immunofluorescence microscopy. For the efficient detection of Bsp22 signals, we cultured

188 the bacteria in iron-depleted SS medium, which has been shown to increase the amount of

189 type III secreted protein secreted by *B. bronchiseptica* (27). L2 cells were infected with

190 bacteria for 1 h at a multiplicity of infection (MOI) of 2000. Then, Bsp22 and F-actin were

191 stained with antibodies against Bsp22 and Rhodamine-Phalloidin, respectively (Fig. 4A). As

192 a result, the fluorescence signals of Bsp22 were detected on the L2 cells infected with the

193 wild-type or ΔBscI+BscI, but not on the L2 cells infected with ΔBscI or ΔBscN (Fig. 4A, B).

194 We also infected L2 cells with the wild-type, ΔBscI, ΔBscI+BscI or ΔBscN for 1 h at an MOI

195 of 200 and measured the amount of lactate dehydrogenase (LDH) released into the

196 extracellular medium. The results showed that LDH was detected in the medium of L2 cells

197 infected with the wild-type or ΔBscI+BscI, but not in the medium of L2 cells infected with

198 ΔBscI or ΔBscN (Fig. 4C). These results suggest that BscI is required for the construction of

199 the T3SS and induction of T3SS-dependent death of mammalian cells in *B. bronchiseptica*.

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201 **Bcr4 is required for the stability of BscI in *B. bronchiseptica*.**

202 As mentioned above, it was suggested that the interaction between Bcr4 and BscI is
203 necessary for the function of the T3SS (Fig. 2). Therefore, to investigate whether Bcr4 is
204 involved in the stability of BscI, we cultured the *B. bronchiseptica* wild-type, Bcr4-deficient
205 strain (ΔBcr4, T3SS-inactive strain) (24), BspR-deficient strain (ΔBspR,
206 T3SS-overproducing and -oversecreting strain) (14) or BspR- and Bcr4-deficient strain
207 (ΔBspRΔBcr4, a T3SS-overproducing but -inactive strain) (24) in SS medium. The prepared
208 WCL samples were separated by SDS-PAGE, and Western blotting was performed using
209 antibodies against BscI, BscJ (a protein composing the inner membrane ring of the T3SS),
210 and Bcr4. As a result, the BscJ signals were detected in the WCL samples of ΔBspR and
211 ΔBspRΔBcr4 (Fig. 5). On the other hand, the BscI signal was detected in ΔBspR but not in
212 ΔBspRΔBcr4 (Fig. 5). These results suggest that Bcr4 is necessary for the stability of BscI
213 in *B. bronchiseptica*.

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221 **Discussion**

222 In the present study, we found that deletion of BscI in *B. bronchiseptica* abolished the
223 extracellular secretion of type III secreted proteins (Fig. 3, 4). In addition, when Bcr4 was
224 deleted in the *B. bronchiseptica* BspR-deficient strain, BscI (a protein composing the inner
225 rod of the *Bordetella* T3SS) was not detected in the bacterial cells (Fig. 5). These results
226 suggest that Bcr4 contributes to the establishment of the T3SS in *B. bronchiseptica* by
227 stabilizing BscI (Fig. 6).

228 To date, chaperones for needles, translocators and effectors have been reported in
229 bacteria that retain the T3SS, such as *Yersinia* (28-31). However, a chaperone for the inner
230 rod protein has not been reported, and the stability of the inner rod in the bacterial cell and
231 the transport of inner rods to the T3SS remain unclear.

232 In the present study, it was suggested that the 58–173 amino acids region of Bcr4 is
233 required for the binding of Bcr4 to BscI (Fig. 2B). To investigate whether the 1–57 amino
234 acids region of Bcr4 is also required for the binding of Bcr4 to BscI, we attempted to purify
235 Bcr4 lacking the N-terminal 1–57 amino acids region (Bcr4Δ1-57-Strep). However, the
236 amount of Bcr4Δ1-57-Strep produced in *E. coli* was extremely low (data not shown), so a
237 pull-down assay could not be performed. Figure 2C suggests that the 159–163 amino acids
238 region of Bcr4 is required for the function of the T3SS. These results suggest that the
239 binding of Bcr4 to BscI is necessary for the T3SS function, because the region of Bcr4
240 required for the T3SS function (aa 159–163) is contained in the region where Bcr4 binds to

241 BscI (aa 58–173) (Fig. 2).

242 Since the *bscI* and *bopD* genes are located in the *bsc* locus (Fig. 1A) and transcribed
243 from the same promoter, the mRNA levels of the *bscI* and *bopD* genes in the bacterial cells
244 are likely to be the same. In this study, the BopD signal was detected in the WCL of the *B.*
245 *bronchiseptica* wild-type strain, but the BscI signal was not detected (Fig. 3). To test the
246 anti-BscI antibody sensitivity, purified recombinant BscI and the Sup sample containing
247 BopD were subjected to SDS-PAGE. The amounts of BscI and BopD in the samples were
248 adjusted to the same weight when we loaded the samples on the gel. A Western blotting
249 analysis was then performed using antibodies against BscI or BopD. The results showed
250 that the BscI and BopD signals were detected with almost the same intensity, indicating that
251 the sensitivity of the anti-BscI antibody for antigen detection was not low. Therefore, the
252 amount of BscI present in *B. bronchiseptica* was considered to be lower than that of BopD.
253 The reason for this is unknown, but it could be due to the low efficiency of translation of the
254 *bscI* gene.

255 BspR is a negative regulator that represses the transcription of genes on the *bsc* locus
256 (14). In strains that are unable to secrete type III secreted proteins, such as Bcr4-deficient
257 strains (ΔBcr4), the repression of gene transcriptions on the *bsc* locus by BspR is enhanced
258 (24), and thus the BscJ signal in the WCL sample of ΔBcr4 was weaker than that of the
259 wild-type (Fig. 5). In this study, we attempted to detect the BscI signal using a
260 BspR-deficient strain (ΔBspR), in which the gene transcriptions on the *bsc* locus are

261 promoted (Fig. 5), because the BscI signal was not detected in the WCL sample of the *B.*
262 *bronchiseptica* wild-type (Fig. 3). The *bscJ* gene is located downstream of the *bscI* gene
263 (Fig. 1A), and the BscJ protein is predicted to function as an inner membrane ring, a
264 component of the T3SS. The signal intensity of BscJ was not decreased in the WCL sample
265 of the BspR/Bcr4 double-deficient strain (ΔBspRΔBcr4) compared to that of ΔBspR (Fig. 5),
266 suggesting that Bcr4 is not involved in the stability of BscJ. Therefore, it is suggested that
267 Bcr4 specifically stabilizes BscI. In addition, when BscI was overexpressed in ΔBcr4, a
268 small amount of extracellular secretion of type III secreted proteins was observed (data not
269 shown), suggesting that Bcr4 is not a component of the T3SS but a chaperone of BscI.
270 Previous studies have shown that overexpression of Bcr4 promotes the production and
271 secretion of type III secreted proteins in *B. bronchiseptica* (24). It is also known that Bcr4 is
272 not secreted out of the *B. bronchiseptica* bacterial cell (24). *Pseudomonas aeruginosa* Pcr4
273 is a homologue of Bcr4 (identity: 26.6%). Since there has been no report on overexpression
274 of Pcr4 in *Pseudomonas*, it is unclear whether Pcr4 overexpression enhances the
275 production and secretion of type III secreted proteins (32). In addition, since Pcr4 has been
276 reported to be an extracellularly secreted protein in *Pseudomonas*, it is quite possible that
277 Bcr4 functions differently from Pcr4. The hypothetical protein (NH44784_014631) of
278 *Achromobacter xylosoxidans*, which like *Bordetella*, belongs to the family Alcaligenaceae
279 has 49.1% identity with Bcr4. This identity is higher than the identity between Pcr4 and Bcr4.
280 Therefore, it is possible that NH44784_014631 and Bcr4 have the same function. However,

281 there has been no report describing how NH44784_014631 functions in *Achromobacter*.
282 Further functional analysis of Bcr4 homologues in other bacteria will provide more
283 information on the function of Bcr4.

284 Homologues of BscI include YscI of *Yersinia pseudotuberculosis*, but the identity
285 between BscI and YscI is low at 18.3%. In addition, no gene encoding a homologue of Bcr4
286 has been found in the genome of *Yersinia*. Therefore, in bacteria such as *Yersinia* and other
287 Enterobacteriaceae, for which there are numerous reports on the T3SS, the inner rod may
288 be stabilized by a system different from that in *Bordetella*. It is also unknown why inner rod
289 production is low in *B. bronchiseptica* (Fig. 3). Furthermore, it is still unclear how Bcr4
290 antagonizes the BspR negative regulation activity, and how overexpression of Bcr4
291 promotes the production and secretion of type III secreted proteins in *B. bronchiseptica*.
292 Further analysis is needed to elucidate the detailed molecular mechanism by which Bcr4
293 contributes to the establishment of the T3SS.

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301 **Materials and Methods**

302 **Bacterial strains and cell culture.**

303 The strains used in this study are listed in Table 1. *B. bronchiceptica* S798 (6) was used
304 as the wild-type strain. ΔBscN (a BscN-deficient strain), ΔBcr4 (a Bcr4-deficient strain),
305 ΔBspR (a BspR-deficient strain) and ΔBspRΔBcr4 (a BspR and Bcr4-deficient strain) have
306 been described elsewhere (6, 14, 24). *Escherichia coli* DH10B, BL21 (33) and Sm10λ*pir*
307 were used for DNA cloning, recombinant protein expression and conjugation, respectively
308 (Table 1). *B. bronchiceptica* was grown on Bordet-Gengou agar plates at 37ºC for 2 days.
309 Fresh colonies of *B. bronchiceptica* were cultured in Stainer-Scholt (SS) medium (34) with a
310 starting A_{600} of 0.23 under vigorous shaking conditions at 37ºC. L2 cells, a rat lung epithelial
311 cell line (ATCC CCL-149), were maintained in F-12K (Invitrogen). The cell culture medium
312 was supplemented with 10% fetal calf serum (FCS). L2 cells were grown at 37°C under a
313 5% CO_2 atmosphere.

314

315 **Pull-down assay.**

316 The primers used in this study are listed in Table 2. In order to express *bscI*, *bscK* or *bcr4*
317 tagged with a V5 sequence at the respective C-terminus (*bscI*-V5, *bscK*-V5 or *bcr4*-V5), we
318 amplified DNA fragments encoding the *bscI*, *bscK* or *bcr4* genes with the primer sets of
319 B1-bscI-V5 and B2-bscI-V5, B1-bscK-V5 and B2-bscK-V5 or B1-bcr4-comp and
320 B2-bcr4-V5, respectively, using *B. bronchiseptica* S798 genomic DNA as the template.

321 Each resulting PCR product was cloned into pDONR201 to obtain pMGKU404, pMGKU405
322 or pMGKU406, respectively, by means of adapter PCR and site-specific recombination
323 techniques using the Gateway cloning system (Invitrogen). Each plasmid was mixed with
324 an expression vector such as p99*ccdB*-V5 (35) to obtain pMGKU407, pMGKU408 or
325 pMGKU409 using the Gateway cloning system.

326 BL21 cells carrying pMGKU407, pMGKU408 or pMGKU409 were cultured overnight at
327 37ºC with shaking, and then diluted 1:40 in LB liquid medium containing 50 μL/mL ampicillin
328 and incubated for 2 h at 37ºC with shaking. Each bacterial culture was further incubated for
329 5 h at 30ºC in the presence of isopropyl-beta-thiogalactopyranoside (IPTG) at the final
330 concentration of 1 mM. Bacteria were collected by centrifugation at 2,600×*g* for 15 min, and
331 suspended in cold TBS containing protease inhibitor cocktail, cOmplete (Roche). Each
332 bacterial suspension was sonicated, and each supernatant was used for the pull-down
333 assay.

334 Next, in order to purify Bcr4 or BscI tagged with six histidine residues (6×His) at the
335 respective N-terminus and Strep at the respective C-terminus, we amplified DNA fragments
336 encoding *bcr4* or *bscI* with the primer sets of 5-HindIII-bcr4 and 3-bcr4-Strep, or
337 5-HindIII-bscI and 3-bscI-Strep using *B. bronchiseptica* S798 genomic DNA as the template.
338 Each amplified DNA fragment was used as a template for 2nd PCR with a primer set
339 consisting of the upper primer used in the 1st PCR and 3-Strep-HindIII to add a 24 bp
340 sequence encoding the Strep tag. Each resulting PCR product was cloned into the HindIII

341 recognition sites of pColdII to obtain pMGKU410 or pMGKU411 by the In-Fusion Cloning
342 System (Clontech), respectively. In order to purify Bcr4 lacking the amino acid regions 58–
343 109 or 110–173, we amplified the DNA fragment with the primer sets of
344 5-bcr4-58-109-Strep-IF and 3-IF-bcr4-58-109-Strep, or 5-bcr4-110-173-Strep-IF and
345 3-IF-bcr4-110-173-Strep using pColdII-*bcr4*-Strep as the template. Each amplified fragment
346 was self-ligated by the In-Fusion Cloning System, respectively, and then designated
347 pMGKU412 or pMGKU413.

348 BL21 cells carrying pMGKU410, pMGKU411, pColdII-*bteA*-N-Strep (36), pMGKU412 or
349 pMGKU413 were cultured overnight at 37ºC with shaking, and then diluted 1:100 in LB
350 liquid medium containing 50 μL/mL ampicillin and incubated for 2 h at 37ºC with shaking,
351 respectively. Each bacterial culture was further incubated overnight at 15ºC in the presence
352 of IPTG at the final concentration of 0.05 mM. Bacteria were collected by centrifugation at
353 2,600×*g* for 20 min, and suspended in cold TBS containing cOmplete. Each bacterial
354 suspension was sonicated and each supernatant except for that of pMGKU411 was
355 subjected to purification using Ni-NTA agarose (Qiagen) according to the manufacturer's
356 instructions. The purified proteins were dialyzed with TBS. As for pMGKU411, the bacterial
357 suspension was sonicated and the pellet was suspended in Inclusion Body Solubilization
358 Reagent (Thermo) according to the manufacturer's instructions. The BscI-Strep in
359 solubilized solution was refolded as described previously (37). Briefly, the solubilized
360 solution was diluted with 5-fold larger refolding buffer (20 mM Tris-HCl, pH-8, 150 mM NaCl,

361 10% glycerol) overnight at 4°C on a rotator, and then centrifugated at 20,000×*g* for 15 min.
362 The supernatant was dialyzed with dialysis buffer (20 mM Tris-HCl, pH-8, 150 mM NaCl,
363 10% glycerol), and then centrifugated at 20,000×*g* for 15 min. The supernatant was used
364 as purified protein for the pull-down assay.

365 We mixed the dual-tagged protein (6×His and Strep) and 30 μl Strep-Tactin resin (IBA) in
366 an Eppendorf tube and rotated the tube at 4°C for 1 h. Then, we washed the beads with
367 TBS three times. Next, the V5-tagged protein-containing *E. coli* lysate was added to the
368 tube and rotated at 4°C for 3 h. We transferred 30 μL supernatant to new Eppendorf tube
369 and added 30 μL 2×SDS-PAGE sample buffer to prepare the Sup. Then we washed the
370 beads with TBS (Fig. 1B) or TBS containing 0.1% Triton X-100 (Fig. 1C) three times and
371 added 30 μL 2×SDS-PAGE sample buffer to prepare the Pellet samples.

372

373 **Construction of a *bscI* gene-disrupted strain.**

374 To construct the *bscI* mutant, a 2.4 kb DNA fragment encoding *bscI* and its flanking
375 regions was amplified by PCR with primers B1-bscI and B2-bscI using *B. bronchiceptica*
376 S798 genomic DNA as the template. The resulting PCR product was cloned into
377 pDONR201 to obtain pMGKU414 using the Gateway cloning system. An inverse PCR was
378 carried out with the primers R1-bscI and R2-bscI using circular pMGKU414 as the template.
379 The resulting PCR product was self-ligated using the In-Fusion Cloning System to obtain
380 pMGKU415. This plasmid contained a 369-bp in-frame deletion from 30 bp downstream of

381 the 5' end of the *bscI* gene to 30 bp upstream of the 3' end of the gene. This plasmid,
382 pMGKU415, was mixed with a positive suicide vector such as pABB-CRS2 (38) to obtain
383 pMGKU416 using the Gateway cloning system. The pMGKU416 plasmid in turn was
384 introduced into *E. coli* Sm10λ*pir*, and transconjugated into the S798 wild-type as described
385 previously (39). The resulting mutant strain was designated ΔBscI.
386
387 **Construction of plasmids used for producing Bcr4 derivatives and BscI**
388 **complementation**
389 In order to produce full-length Bcr4 tagged with a FLAG sequence at the C-terminus, we
390 performed an inverse PCR with primers of 5-bcr4-FLAG-IF and 3-IF-bcr4-FLAG using
391 pDONR-*bcr4* (24) as the template. The resulting PCR product was self-ligated to obtain
392 pMGKU417 by the In-Fusion Cloning System. To produce Bcr4 lacking the amino acid
393 regions 159–173, 164–173 or 169–173, inverse PCR was carried out with the primer sets of
394 5-*bcr4*-FLAG-IF and 3-IF-bcr4Δ169-173-FLAG, 5-*bcr4*-FLAG-IF and
395 3-IF-*bcr4*Δ164-173-FLAG, or 5-*bcr4*-FLAG-IF and 3-IF-*bcr4*Δ159-173-FLAG using
396 pMGKU417 as the template. Each amplified fragment was self-ligated by the In-Fusion
397 Cloning System to obtain pMGKU418, pMGKU419 or pMGKU420, respectively. For BscI
398 complementation, a PCR was carried out with the primers of B1-bscI-V5 and
399 3-B2-bscI-comp using *B. bronchiceptica* S798 genomic DNA as the template. The resulting
400 PCR product was cloned into pDONR201 to obtain pMGKU421 using the Gateway cloning

401 system. These plasmids were mixed with pRK-R4-R3-F, pDONR-*fha*P and pDONR-*rrnB*
402 (10) to obtain pMGKU422, pMGKU423, pMGKU424, pMGKU425 and pMGKU426 using
403 the Gateway cloning system.
404
405 **Preparation of proteins from culture supernatants and whole bacterial cell lysates.**
406 Proteins secreted into bacterial culture supernatants and whole bacterial cell lysates
407 were prepared as described previously (40). The loaded sample amounts were adjusted by
408 the A_{600} of each bacterial culture to load samples prepared from the same amounts of
409 bacteria. The protein samples were separated by SDS-PAGE and analyzed by Western
410 blotting.
411
412 **Antibodies.**
413 Anti-BteA, anti-BopD, anti-Bsp22 and anti-Bcr4 antibodies were purified from rabbit
414 serum in our previous study (7, 10, 24, 41). Mouse anti-V5 monoclonal antibody was
415 purchased from Santa Cruz Biotechnology. To detect filamentous hemagglutinin (FHA)
416 signals, we used mouse anti-FHA serum kindly gifted from K. Kamachi (National Institute of
417 Infectious Diseases). To prepare the anti-BscI and anti-BscJ antibodies, the peptides
418 corresponding to the C-terminus regions of BscI (CKAIGRATQNVDTLARMS) and BscJ
419 (CRGEGRGGAGAGATEGAGHD) were conjugated with hemocyanin from keyhole limpets
420 (Sigma), respectively, by using 3-maleimidobenzoic acid N-hydroxysuccinimide ester

421 (Sigma). These cross-linked peptides were used to immunize rabbits, and the resulting
422 anti-sera were incubated with the same peptides immobilized on epoxy-activated
423 sepharose 6B (Amersham) to obtain specific Ig-fractions.
424
425 **Immunofluorescence staining of L2 cells infected with *B. bronchiseptica.***
426 Immunofluorescent staining assay was performed as previously described (40), with
427 slight modifications. Briefly, L2 cells were seeded on coverslips in 6-well plates and
428 incubated overnight. In order to detect adequate amounts of Bsp22 signals, we infected the
429 L2 cells with *B. bronchiseptica* cultured in iron-depleted SS medium at an MOI of 2000. To
430 avoid excessive killing of L2 cells with bacteria, the plates were not centrifuged. After being
431 incubated for 1 h at 37°C under a 5% CO_2 atmosphere, the infected L2 cells were
432 immunostained. Average numbers of Bsp22 signals on single L2 cells were measured by a
433 fluorescence microscope.
434
435 **LDH Assays.**
436 LDH assays were performed as previously described (40). Briefly, to examine whether
437 lactate dehydrogenase (LDH) is released from *B. bronchiseptica*-infected cells, 5.0×10^4
438 cells/well of L2 cells seeded in 24-well plates were infected with bacteria at an MOI of 200.
439 The plates were centrifuged at 900×*g* for 5 min and incubated for 3 h at 37ºC under a 5%
440 CO_2 atmosphere. The amounts of LDH were measured spectrophotometrically using a

441 Cyto-Tox 96 Non-radioactive Cytotoxicity Assay kit (Promega). The LDH (%) level is shown
442 as a ratio, with the value obtained from the well treated with 0.1% Triton X-100 set as
443 100%.
444
445 **Statistical analyses**
446 The statistical analyses were performed using the nonparametric unpaired *t*-test with a
447 one-tailed p-value with Prism ver. 5.0f software (Graphpad, La Jolla, CA). Values of $p<0.05$
448 were considered significant.
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481 **References**

482 1. Goodnow RA. 1980. Biology of *Bordetella bronchiseptica*. Microbiol Rev
483 44:722-738.

484 2. Mattoo S, Cherry JD. 2005. Molecular pathogenesis, epidemiology, and clinical
485 manifestations of respiratory infections due to *Bordetella* pertussis and other
486 *Bordetella* subspecies. Clin Microbiol Rev 18:326-382.

487 3. Cornelis GR, Van Gijsegem F. 2000. Assembly and function of type III secretory
488 systems. Annu Rev Microbiol 54:735-774.

489 4. Büttner D. 2012. Protein export according to schedule: architecture, assembly, and
490 regulation of type III secretion systems from plant- and animal-pathogenic bacteria.
491 Microbiol Mol Biol Rev 76:262-310.

492 5. Deng W, Marshall NC, Rowland JL, McCoy JM, Worrall LJ, Santos AS, Strynadka
493 NCJ, Finlay BB. 2017. Assembly, structure, function and regulation of type III
494 secretion systems. Nat Rev Microbiol 15:323-337.

495 6. Kuwae A, Ohishi M, Watanabe M, Nagai M, Abe A. 2003. BopB is a type III secreted
496 protein in *Bordetella bronchiseptica* and is required for cytotoxicity against cultured
497 mammalian cells. Cell Microbiol 5:973-983.

498 7. Nogawa H, Kuwae A, Matsuzawa T, Abe A. 2004. The type III secreted protein BopD
499 in *Bordetella bronchiseptica* is complexed with BopB for pore formation on the host
500 plasma membrane. J Bacteriol 186:3806-3813.

501 8. Yuk MH, Harvill ET, Cotter PA, Miller JF. 2000. Modulation of host immune
502 responses, induction of apoptosis and inhibition of NF-*kappa*B activation by the
503 *Bordetella* type III secretion system. Mol Microbiol 35:991-1004.
504 9. Panina EM, Mattoo S, Griffith N, Kozak NA, Yuk MH, Miller JF. 2005. A genome-wide
505 screen identifies a *Bordetella* type III secretion effector and candidate effectors in
506 other species. Mol Microbiol 58:267-279.
507 10. Kuwae A, Matsuzawa T, Ishikawa N, Abe H, Nonaka T, Fukuda H, Imajoh-Ohmi S,
508 Abe A. 2006. BopC is a novel type III effector secreted by *Bordetella bronchiseptica*
509 and has a critical role in type III-dependent necrotic cell death. J Biol Chem
510 281:6589-6600.
511 11. Nagamatsu K, Kuwae A, Konaka T, Nagai S, Yoshida S, Eguchi M, Watanabe M,
512 Mimuro H, Koyasu S, Abe A. 2009. *Bordetella* evades the host immune system by
513 inducing IL-10 through a type III effector, BopN. J Exp Med 206:3073-3088.
514 12. Abe A, Nishimura R, Kuwae A. 2017. *Bordetella* effector BopN is translocated into
515 host cells via its N-terminal residues. Microbiol Immunol 61:206-214.
516 13. Ahuja U, Liu M, Tomida S, Park J, Souda P, Whitelegge J, Li H, Harvill ET, Parkhill J,
517 Miller JF. 2012. Phenotypic and genomic analysis of hypervirulent human-associated
518 *Bordetella bronchiseptica*. BMC Microbiol 12:167.
519 14. Kurushima J, Kuwae A, Abe A. 2012. The type III secreted protein BspR regulates
520 the virulence genes in *Bordetella bronchiseptica*. PLoS One 7:e38925.

521 15. Medhekar B, Shrivastava R, Mattoo S, Gingery M, Miller JF. 2009. *Bordetella* Bsp22
522 forms a filamentous type III secretion system tip complex and is immunoprotective in
523 vitro and in vivo. Mol Microbiol 71:492-504.
524 16. Ahuja U, Shokeen B, Cheng N, Cho Y, Blum C, Coppola G, Miller JF. 2016.
525 Differential regulation of type III secretion and virulence genes in *Bordetella*
526 *pertussis* and *Bordetella bronchiseptica* by a secreted anti-sigma factor. Proc Natl
527 Acad Sci U S A 113:2341-2348.
528 17. Feldman MF, Cornelis GR. 2003. The multitalented type III chaperones: all you can
529 do with 15 kDa. FEMS Microbiol Lett 219:151-158.
530 18. Stebbins CE, Galán JE. 2001. Maintenance of an unfolded polypeptide by a cognate
531 chaperone in bacterial type III secretion. Nature 414:77-81.
532 19. Lloyd SA, Norman M, Rosqvist R, Wolf-Watz H. 2001. *Yersinia* YopE is targeted for
533 type III secretion by N-terminal, not mRNA, signals. Mol Microbiol 39:520-531.
534 20. Lee SH, Galán JE. 2004. *Salmonella* type III secretion-associated chaperones
535 confer secretion-pathway specificity. Mol Microbiol 51:483-495.
536 21. Quinaud M, Chabert J, Faudry E, Neumann E, Lemaire D, Pastor A, Elsen S,
537 Dessen A, Attree I. 2005. The PscE-PscF-PscG complex controls type III secretion
538 needle biogenesis in *Pseudomonas aeruginosa*. J Biol Chem 280:36293-36300.
539 22. Wood SE, Jin J, Lloyd SA. 2008. YscP and YscU switch the substrate specificity of
540 the *Yersinia* type III secretion system by regulating export of the inner rod protein

541 YscI. J Bacteriol 190:4252-4262.

542 23. Monlezun L, Liebl D, Fenel D, Grandjean T, Berry A, Schoehn G, Dessein R, Faudry
543 E, Attree I. 2015. PscI is a type III secretion needle anchoring protein with in vitro
544 polymerization capacities. Mol Microbiol 96:419-436.

545 24. Nishimura R, Abe A, Sakuma Y, Kuwae A. 2018. *Bordetella bronchiseptica* Bcr4
546 antagonizes the negative regulatory function of BspR via its role in type III secretion.
547 Microbiol Immunol 62:743-754.

548 25. Wattiau P, Bernier B, Deslée P, Michiels T, Cornelis GR. 1994. Individual chaperones
549 required for Yop secretion by *Yersinia*. Proc Natl Acad Sci U S A 91:10493-10497.

550 26. Kamanova J. 2020. *Bordetella* Type III Secretion Injectosome and Effector Proteins.
551 Front Cell Infect Microbiol 10:466.

552 27. Kurushima J, Kuwae A, Abe A. 2012. Iron starvation regulates the type III secretion
553 system in *Bordetella bronchiseptica*. Microbiol Immunol 56:356-362.

554 28. Sun P, Tropea JE, Austin BP, Cherry S, Waugh DS. 2008. Structural characterization
555 of the *Yersinia pestis* type III secretion system needle protein YscF in complex with
556 its heterodimeric chaperone YscE/YscG. J Mol Biol 377:819-830.

557 29. Day JB, Guller I, Plano GV. 2000. *Yersinia pestis* YscG protein is a Syc-like
558 chaperone that directly binds yscE. Infect Immun 68:6466-6471.

559 30. Francis MS, Aili M, Wiklund ML, Wolf-Watz H. 2000. A study of the YopD-lcrH
560 interaction from *Yersinia pseudotuberculosis* reveals a role for hydrophobic residues

561 within the amphipathic domain of YopD. Mol Microbiol 38:85-102.

562 31. Woestyn S, Sory MP, Boland A, Lequenne O, Cornelis GR. 1996. The cytosolic SycE
563 and SycH chaperones of *Yersinia* protect the region of YopE and YopH involved in
564 translocation across eukaryotic cell membranes. Mol Microbiol 20:1261-1271.

565 32. Yang H, Shan Z, Kim J, Wu W, Lian W, Zeng L, Xing L, Jin S. 2007. Regulatory role
566 of PopN and its interacting partners in type III secretion of *Pseudomonas aeruginosa*.
567 J Bacteriol 189:2599-2609.

568 33. Studier FW, Moffatt BA. 1986. Use of bacteriophage T7 RNA polymerase to direct
569 selective high-level expression of cloned genes. J Mol Biol 189:113-130.

570 34. Stainer DW, Scholte MJ. 1970. A simple chemically defined medium for the
571 production of phase I *Bordetella pertussis*. J Gen Microbiol 63:211-220.

572 35. Ogino T, Ohno R, Sekiya K, Kuwae A, Matsuzawa T, Nonaka T, Fukuda H,
573 Imajoh-Ohmi S, Abe A. 2006. Assembly of the type III secretion apparatus of
574 enteropathogenic *Escherichia coli*. J Bacteriol 188:2801-2811.

575 36. Kuwae A, Momose F, Nagamatsu K, Suyama Y, Abe A. 2016. BteA Secreted from
576 the *Bordetella bronchiseptica* Type III Secetion System Induces Necrosis through an
577 Actin Cytoskeleton Signaling Pathway and Inhibits Phagocytosis by Macrophages.
578 PLoS One 11:e0148387.

579 37. Halder PK, Roy C, Datta S. 2019. Structural and functional characterization of type
580 three secretion system ATPase PscN and its regulator PscL from *Pseudomonas*

581 *aeruginosa*. Proteins 87:276-288.

582 38. Sekiya K, Ohishi M, Ogino T, Tamano K, Sasakawa C, Abe A. 2001. Supermolecular
583 structure of the enteropathogenic *Escherichia coli* type III secretion system and its
584 direct interaction with the EspA-sheath-like structure. Proc Natl Acad Sci U S A
585 98:11638-11643.

586 39. Donnenberg MS, Kaper JB. 1991. Construction of an *eae* deletion mutant of
587 enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. Infect
588 Immun 59:4310-4317.

589 40. Goto M, Hanawa T, Abe A, Kuwae A. 2020. Transcriptional Downregulation of a Type
590 III Secretion System under Reducing Conditions in *Bordetella pertussis*. J Bacteriol
591 202.

592 41. Kurushima J, Kuwae A, Abe A. 2012. Btc22 chaperone is required for secretion and
593 stability of the type III secreted protein Bsp22 in *Bordetella bronchiseptica*. FEMS
594 Microbiol Lett 331:144-151.

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601 **Figure legends**

602 **Fig. 1. The interaction of Bcr4 with inner rod protein BscI.** (A) The *bcr4* gene and its
603 peripheral genes localized in the T3SS apparatus locus (*bsc* locus) on the *B.*
604 *bronchiseptica* S798 chromosome are depicted. (B) The purified Bcr4 (Bcr4-Strep) was
605 loaded on Strep-Tactin beads. Then, the beads were mixed with the lysates prepared from
606 *E. coli* BL21 producing BscI or BscK tagged with V5 (BscI-V5 or BscK-V5), respectively.
607 After 3 h incubation at 4°C, the supernatant fraction (Sup) and pellet fraction (Pellet)
608 samples were prepared. The Sup and Pellet samples were separated by SDS-PAGE and
609 analyzed by Western blotting (WB) with anti-V5 antibody (top). Pellet samples were also
610 stained with Coomassie Brilliant Blue (CBB, bottom). (C) The purified BscI (BscI-Strep) or
611 N-terminal moiety of BteA (amino acid region 1–312, BteA-N-Strep) were loaded on
612 Strep-Tactin beads. Then, the beads were mixed with the lysate prepared from *E. coli* BL21
613 to produce Bcr4 tagged with V5 (Bcr4-V5) at 4°C for 3 h. The prepared Sup and Pellet
614 samples were separated by SDS-PAGE and analyzed by WB with anti-V5 antibody (top).
615 Pellet samples were also stained with CBB (bottom). Experiments were performed at least
616 three times, and representative data are shown.

617

618 **Fig. 2. The Bcr4 domain is required for interaction with BscI and T3SS activity.** (A)
619 Bcr4 derivatives used for pull-down assay in panel B and the analysis of T3SS activity in
620 panel C are depicted. The yellow part shows the Bcr4 domain required for T3SS activity. (B)

621 The purified Bcr4-FL, Bcr4Δ58-109 or Bcr4Δ110-173 (Bcr4-FL-Strep, Bcr4Δ58-109-Strep or
622 Bcr4Δ110-17-Strep) was loaded on Strep-Tactin beads, and then the beads were mixed
623 with the lysate prepared from *E. coli* BL21, producing BscI-V5, respectively. After 3 h
624 incubation at 4°C, the supernatant fraction (Sup) and pellet fraction (Pellet) samples were
625 prepared. The Sup and Pellet samples were separated by SDS-PAGE and analyzed by
626 Western blotting (WB) with anti-V5 antibody (top), and stained with Coomassie Brilliant
627 Blue (CBB, bottom). (C) The *B. bronchiseptica* wild-type strain, Bcr4-deficient strain (ΔBcr4),
628 and ΔBcr4 producing Bcr4-FL-FLAG (ΔBcr4+Bcr4-FL-FLAG), Bcr4Δ169-173-FLAG
629 (ΔBcr4+Bcr4Δ169-173-FLAG), Bcr4Δ164-173-FLAG (ΔBcr4+Bcr4Δ164-173-FLAG), and
630 Bcr4Δ159-173-FLAG (ΔBcr4+Bcr4Δ159-173-FLAG) were cultured in SS medium. Whole
631 cell lysates (WCL) and supernatant fraction (Sup) samples were separated by SDS-PAGE
632 and analyzed by Western blotting using antibodies against Bcr4, FLAG, BteA or BopD. AP
633 indicates the antigen peptide used for anti-Bcr4 antibody generation. WCL and Sup
634 samples were prepared from equal volumes of bacterial culture. When we performed
635 western blotting using anti-BteA or BopD antibodies, we loaded a 100- or 50-fold smaller
636 amount of ΔBcr4+Bcr4-FL-FLAG, ΔBcr4+Bcr4Δ169-173-FLAG and
637 ΔBcr4+Bcr4Δ164-173-FLAG Sup samples on the SDS-PAGE gel than on the wild type,
638 ΔBcr4 and ΔBcr4+Bcr4Δ159-173-FLAG Sup samples to avoid obtaining excess signal
639 intensities. Experiments were performed at least three times, and representative data are
640 shown.

641

642 **Fig. 3. The effect of BscI deletion on T3SS activity in *B. bronchiseptica*.** Whole cell
643 lysates (WCL) and supernatant fraction (Sup) samples were prepared from the wild-type
644 strain, ΔBscI (BscI-deficient strain), ΔBscI+BscI (BscI-complemented strain) and ΔBscN
645 (T3SS-inactive strain) cultured in SS medium. WCL and Sup samples were separated by
646 SDS-PAGE and analyzed by Western blotting using antibodies against BscI, BteA, BopD or
647 FHA. BscI tagged with Strep (BscI-Strep) was loaded as a control. *BscI-Strep signal; **NS
648 (nonspecific signals); ***A faint signal of BscI. Loaded WCL and Sup samples were
649 prepared from equal volumes of bacterial culture. Experiments were performed at least
650 three times, and representative data are shown.

651

652 **Fig. 4. Immunofluorescent staining of Bsp22 on L2 cells infected with *B.*
653 ***bronchiseptica* and the results of the LDH assay.** (A) L2 cells were infected with the
654 wild-type strain, ΔBscI (BscI-deficient strain), ΔBscI+BscI (BscI-complemented strain) or
655 ΔBscN (T3SS-inactive strain) as described in the Materials and Methods. After fixation,
656 cells were stained with anti-Bsp22 antibody (green) and rhodamine phalloidin (red).
657 Arrowheads indicate the Bsp22 (green) signals. (B) Bsp22 signals per one cell were
658 counted under a fluorescent microscope. At least 120 cells were randomly chosen. (C) The
659 results of LDH assay at an MOI of 200 are shown as a histogram. Error bars indicate the
660 SEMs from triplicate experiments. *P<0.05. Experiments were performed at least three

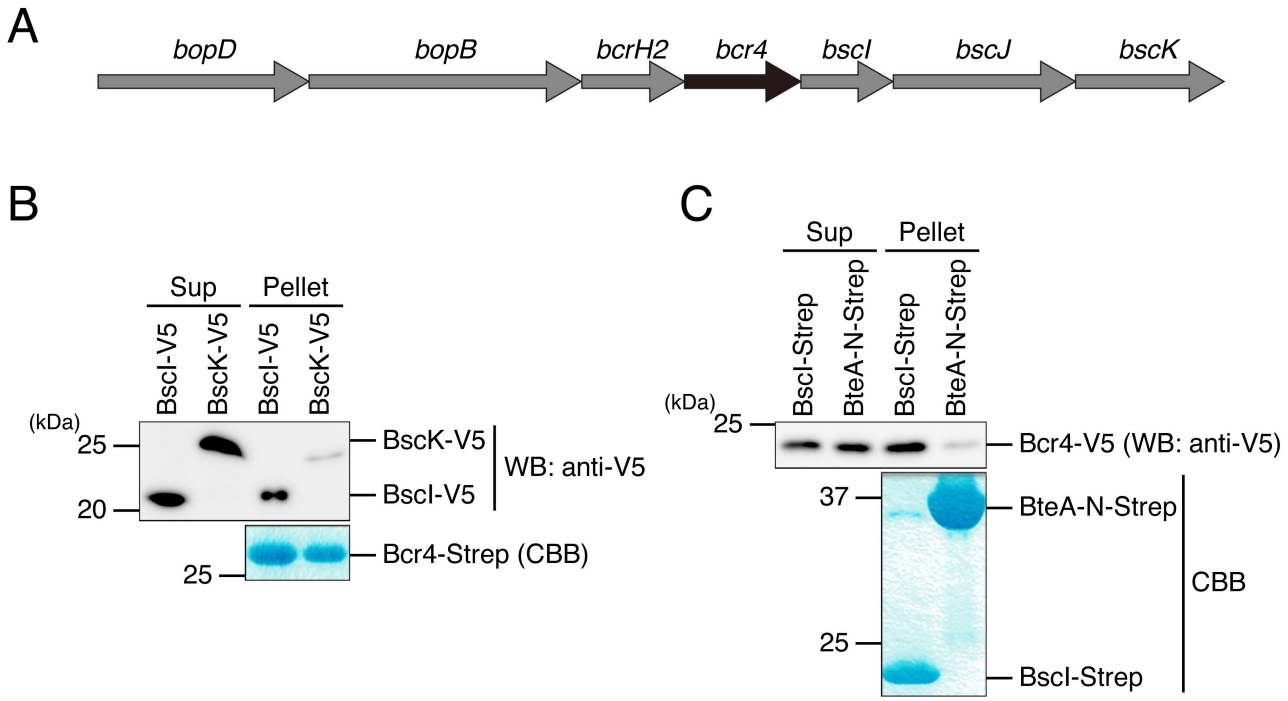
661 times, and representative data are shown.

662

663 **Fig. 5. The effect of Bcr4 on BscI stability in *B. bronchiseptica*.** The whole cell lysates
664 (WCL) were prepared from wild-type strain, ΔBcr4 (Bcr4-deficient strain), ΔBspR
665 (BspR-deficient strain) or ΔBspRΔBcr4 (BspR- and Bcr4-deficient strain) cultured in SS
666 medium. WCL were separated by SDS-PAGE and analyzed by Western blotting with
667 antibodies against BscI (inner-rod protein), BscJ (inner-ring protein) and Bcr4. The samples
668 were prepared from equal volumes of bacterial culture. When we carried out western
669 blotting using anti-BscJ or Bcr4 antibodies, we loaded a 10-fold-smaller amount of ΔBspR
670 or ΔBspRΔBcr4 WCL samples on the SDS-PAGE than WT or ΔBcr4 WCL samples to avoid
671 obtaining excess signal intensities. The production of type III secreted proteins and the
672 secretion level of each strain are shown as native (+), high (+++) or null (–). Experiments
673 were performed at least three times, and representative data are shown.

674

675 **Fig. 6. Schematic depiction of the Bcr4 and BscI functions.** In the *B. bronchiseptica*
676 bacterial cytosol, Bcr4 (blue) stabilizes BscI (orange). Then, BscI is presumed to be
677 localized to the T3SS machinery (gray) and form the inner rod. BscI is essential for T3SS
678 activity and secreted from the bacterial cell. BscJ (pink) is predicted to function as an inner
679 membrane ring. Bsp22 (green) forms a filament-like structure. OM and IM indicate the outer
680 membrane (white) and inner membrane (white), respectively.



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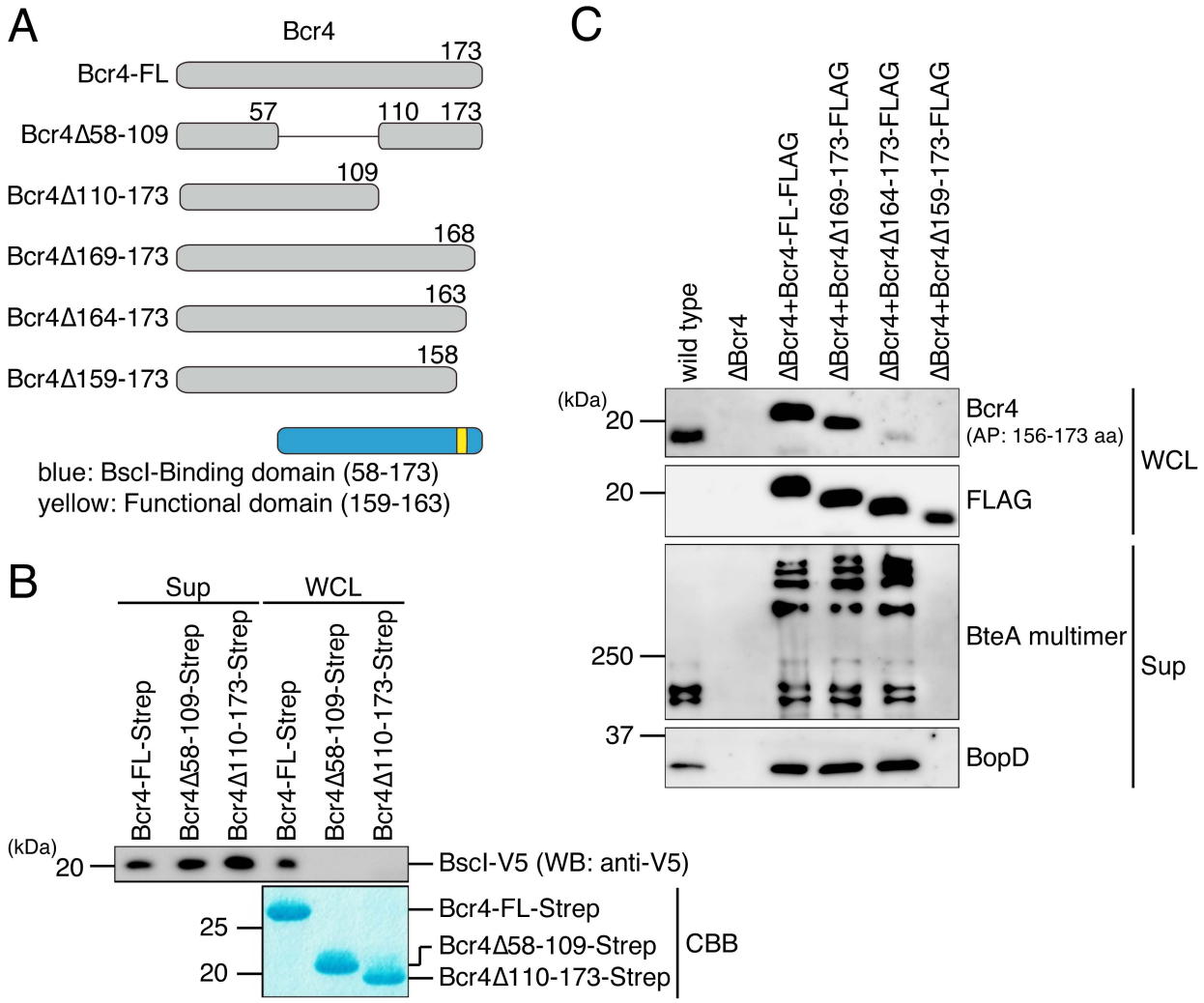

Fig. 2. The Bcr4 domain is required for interaction with BscI and T3SS activity. (A) Bcr4 derivatives used for pull-down assay in panel B and the analysis of T3SS activity in panel C are depicted. The yellow part shows the Bcr4 domain required for T3SS activity. (B) The purified Bcr4-FL, Bcr4Δ58-109 or Bcr4Δ110-173 (Bcr4-FL-Strep, Bcr4Δ58-109-Strep or Bcr4Δ110-17-Strep) was loaded on Strep-Tactin beads, and then the beads were mixed with the lysate prepared from *E. coli* BL21, producing BscI-V5, respectively. After 3 h incubation at 4°C, the supernatant fraction (Sup) and pellet fraction (Pellet) samples were prepared. The Sup and Pellet samples were separated by SDS-PAGE and analyzed by Western blotting (WB) with anti-V5 antibody (top), and stained with Coomassie Brilliant Blue (CBB, bottom). (C) The *B. bronchiseptica* wild-type strain, Bcr4-deficient strain (ΔBcr4), and ΔBcr4 producing Bcr4-FL-FLAG (ΔBcr4+Bcr4-FL-FLAG), Bcr4Δ169-173-FLAG (ΔBcr4+Bcr4Δ169-173-FLAG), Bcr4Δ164-173-FLAG (ΔBcr4+Bcr4Δ164-173-FLAG), and Bcr4Δ159-173-FLAG (ΔBcr4+Bcr4Δ159-173-FLAG) were cultured in SS medium. Whole cell lysates (WCL) and supernatant fraction (Sup) samples were separated by SDS-PAGE and analyzed by Western blotting using antibodies against Bcr4, FLAG, BteA or BopD. AP indicates the antigen peptide used for anti-Bcr4 antibody generation. WCL and Sup samples were prepared from equal volumes of bacterial culture. When we performed western blotting using anti-BteA or BopD antibodies, we loaded a 100- or 50-fold smaller amount of ΔBcr4+Bcr4-FL-FLAG, ΔBcr4+Bcr4Δ169-173-FLAG and ΔBcr4+Bcr4Δ164-173-FLAG Sup samples on the SDS-PAGE gel than on the wild type, ΔBcr4 and ΔBcr4+Bcr4Δ159-173-FLAG Sup samples to avoid obtaining excess signal intensities. Experiments were performed at least three times, and representative data are shown.

Fig. 2

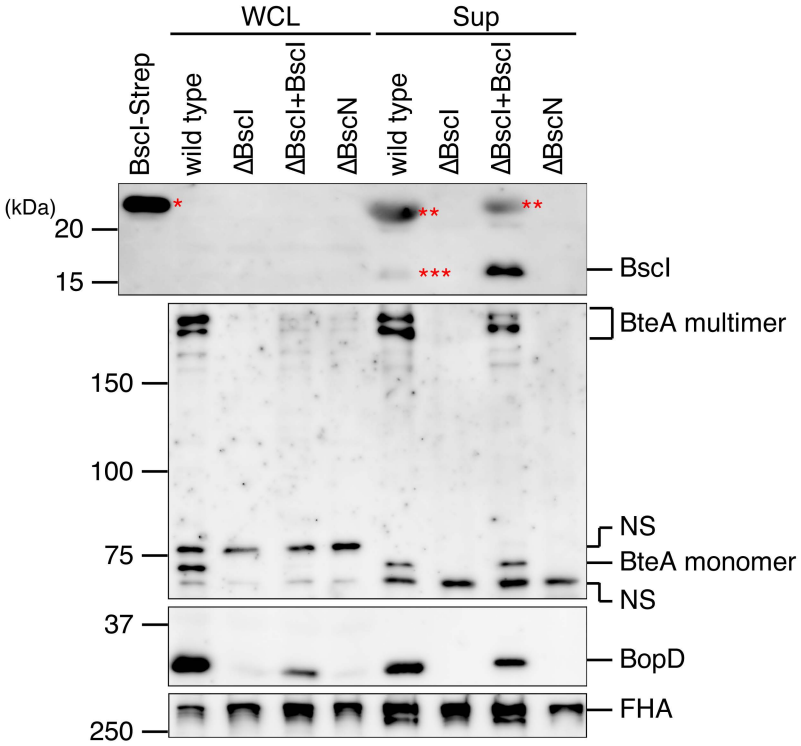

Fig. 3. The effect of BsclI deletion on T3SS activity in *B. bronchiseptica*. Whole cell lysates (WCL) and supernatant fraction (Sup) samples were prepared from the wild-type strain, ΔBsclI (BsclI-deficient strain), ΔBsclI+BsclI (BsclI-complemented strain) and ΔBscN (T3SS-inactive strain) cultured in SS medium. WCL and Sup samples were separated by SDS-PAGE and analyzed by Western blotting using antibodies against BsclI, BteA, BopD or FHA. BsclI tagged with Strep (BsclI-Strep) was loaded as a control. *BsclI-Strep signal; **NS (nonspecific signals); ***A faint signal of BsclI. Loaded WCL and Sup samples were prepared from equal volumes of bacterial culture. Experiments were performed at least three times, and representative data are shown.

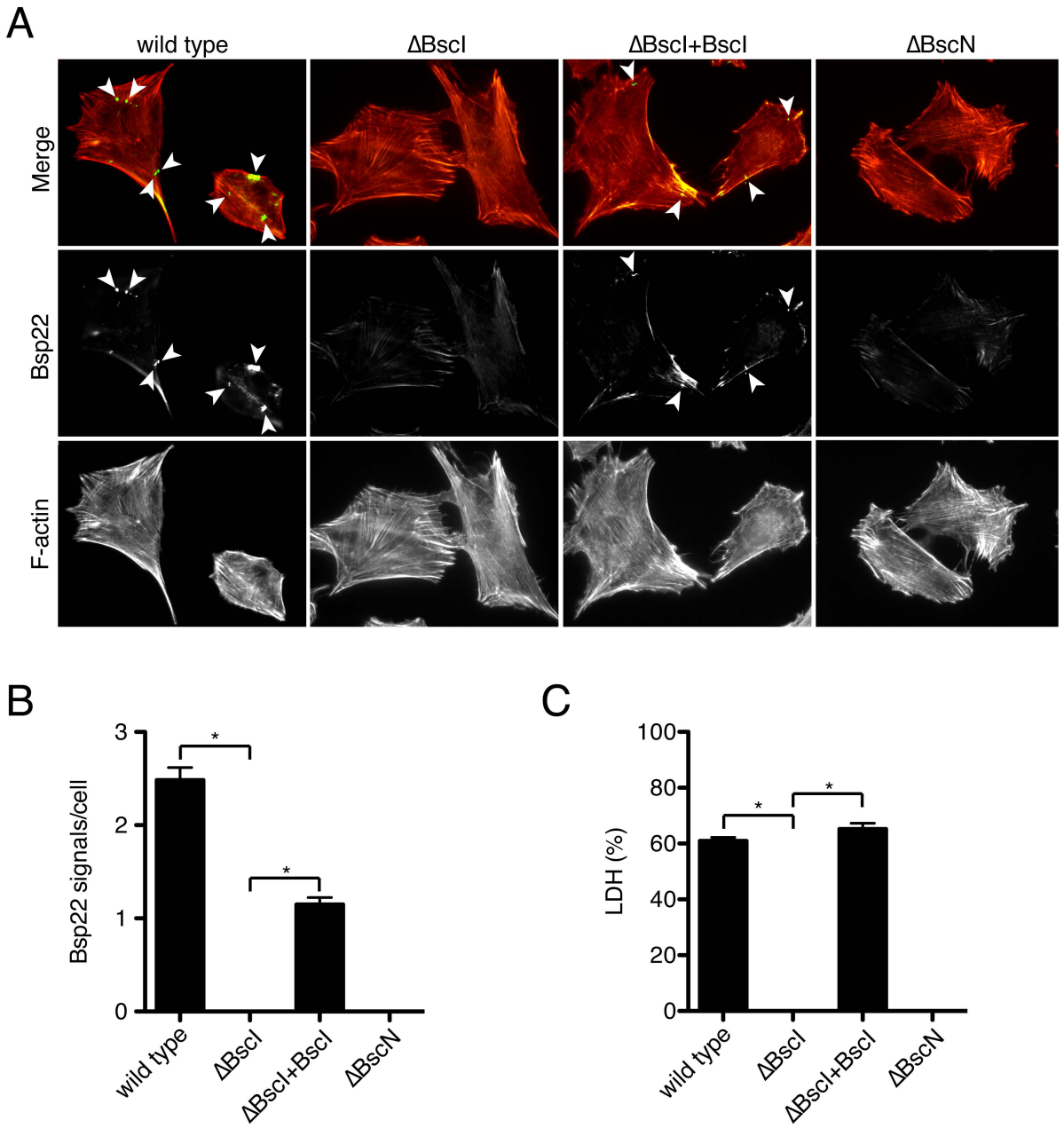

Fig. 4. Immunofluorescent staining of Bsp22 on L2 cells infected with *B. bronchiseptica* and the results of the LDH assay. (A) L2 cells were infected with the wild-type strain, ΔBscI (BscI-deficient strain), ΔBscI+BscI (BscI-complemented strain) or ΔBscN (T3SS-inactive strain) as described in the Materials and Methods. After fixation, cells were stained with anti-Bsp22 antibody (green) and rhodamine phalloidin (red). Arrowheads indicate the Bsp22 (green) signals. (B) Bsp22 signals per one cell were counted under a fluorescent microscope. At least 120 cells were randomly chosen. (C) The results of LDH assay at an MOI of 200 are shown as a histogram. Error bars indicate the SEMs from triplicate experiments. *$P<0.05$. Experiments were performed at least three times, and representative data are shown.

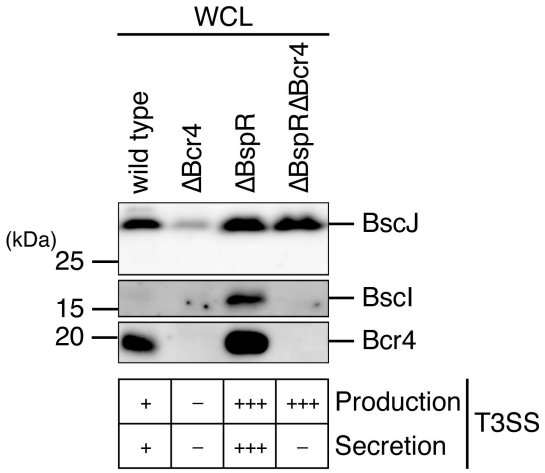

Fig. 5. The effect of Bcr4 on BscI stability in *B. bronchiseptica*. The whole cell lysates (WCL) were prepared from wild-type strain, ΔBcr4 (Bcr4-deficient strain), ΔBspR (BspR-deficient strain) or ΔBspRΔBcr4 (BspR- and Bcr4-deficient strain) cultured in SS medium. WCL were separated by SDS-PAGE and analyzed by Western blotting with antibodies against BscI (inner-rod protein), BscJ (inner-ring protein) and Bcr4. The samples were prepared from equal volumes of bacterial culture. When we carried out western blotting using anti-BscJ or Bcr4 antibodies, we loaded a 10-fold-smaller amount of ΔBspR or ΔBspRΔBcr4 WCL samples on the SDS-PAGE than WT or ΔBcr4 WCL samples to avoid obtaining excess signal intensities. The production of type III secreted proteins and the secretion level of each strain are shown as native (+), high (+++) or null (–). Experiments were performed at least three times, and representative data are shown.

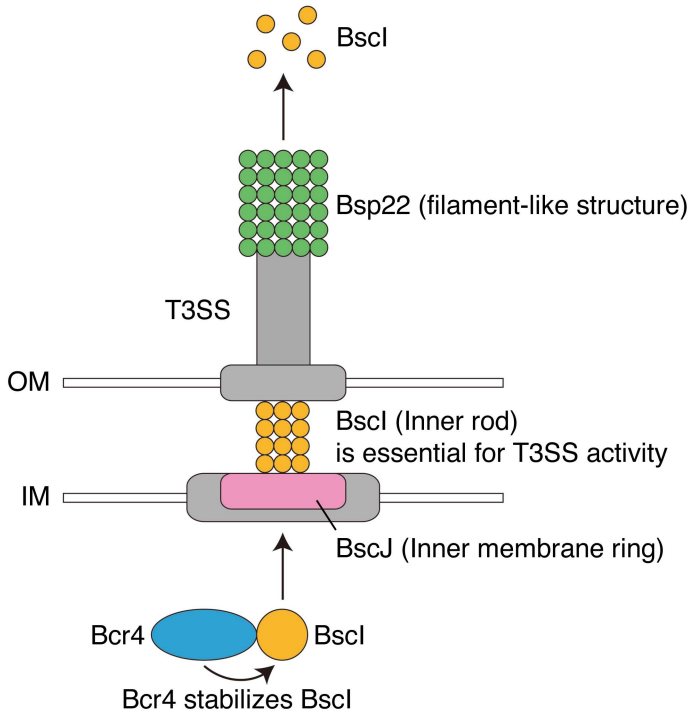

Fig. 6. Schematic depiction of the Bcr4 and BscI functions. In the *B. bronchiseptica* bacterial cytosol, Bcr4 (blue) stabilizes BscI (orange). Then, BscI is presumed to be localized to the T3SS machinery (gray) and form the inner rod. BscI is essential for T3SS activity and secreted from the bacterial cell. BscJ (pink) is predicted to function as an inner membrane ring. Bsp22 (green) forms a filament-like structure. OM and IM indicate the outer membrane (white) and inner membrane (white), respectively.

Table 1. Bacterial strains and plasmids used in the study

Strain or plasmid	Relevant genotype or description	Reference or source
Strains		
B. bronchiseptica		
S798	wild-type strain	6
ΔBscI	BscI-deficient strain	This study
△BscN	BscN-deficient strain	6
△Bcr4	Bcr4-deficient strain	24
△BspR	BspR-deficient strain	14
△BspR△Bcr4	BspR and Bcr4-deficient strain	24
E. coli		
DH10B	Host strain for pDONR201, p99-*ccdB*-V5 and pRK415-R4-R3-F	Invitrogen
BL21	Host strain for pColdII	33
Sm10λ*pir*	Host strain for pABB-CRS2	6
Plasmids		
pDONR201	DNA cloning vector, Km^r	Invitrogen
pMGKU404	pDONR201, *bscI* gene tagged with V5 sequence at the C terminus	This study
pMGKU405	pDONR201, *bscK* gene tagged with V5 sequence at the C terminus	This study
pMGKU406	pDONR201, *bsc4* gene tagged with V5 sequence at the C terminus	This study
p99*ccdB*-V5	Expression vector for V5 tagged gene, Amp^r	35
pMGKU407	p99-*ccdB*-V5, *bscI* gene tagged with V5 sequence at the C terminus	This study
pMGKU408	p99-*ccdB*-V5, *bscK* gene tagged with V5 sequence at the C terminus	This study
pMGKU409	p99-*ccdB*-V5, *bsc4* gene tagged with V5 sequence at the C terminus	This study
pColdII	Expression vector for His tagged gene, Amp^r	TAKARA
pMGKU410	pColdII, *bcr4* gene tagged with Strep sequence at the C terminus	This study
pMGKU411	pColdII, *bscI* gene tagged with Strep sequence at the C terminus	This study
pMGKU412	pColdII, *bcr4* gene lacking amino acids region 58-109 tagged with Strep sequence at the C terminus	This study
pMGKU413	pColdII, *bcr4* gene lacking amino acids region 110-173 tagged with Strep sequence at the C terminus	This study
pColdII-*bteA*-N-Strep	pColdII, *bteA* gene coding amino acids region 1-312 tagged with Strep sequence at the C terminus	36
pMGKU414	pDONR201, *bscI* gene	This study
pMGKU415	pDONR201, *bscI* gene containing internal sequence-deletion and its flanking region	This study
pABB-CRS2	Suicide vector for conjugation, Amp^r	38
pMGKU416	pABB-CRS2, bscI gene containing internal sequence-deletion and its flanking region	This study
pDONR-*bcr4*	pDONR201, *bcr4* gene for complementation	24
pMGKU417	pDONR201, *bcr4* gene tagged with FLAG sequence at the C terminus for complementation	This study
pMGKU418	pDONR201, *bcr4* gene lacking amino acids region 169-173 tagged with FLAG sequence at the C terminus	This study
pMGKU419	pDONR201, *bcr4* gene lacking amino acids region 164-173 tagged with FLAG sequence at the C terminus	This study
pMGKU420	pDONR201, *bcr4* gene lacking amino acids region 159-173 tagged with FLAG sequence at the C terminus	This study
pMGKU421	pDONR201, *bscI* gene for complementation	This study
pRK415-R4-R3-F	pRK415, recombination sites for MultiSite Gateway, Tet^r	10
pDONR-*fhaP*	pDONR-P4-P1R, *fha* promoter	10
pDONR-*rrnB*	pDONR-P2R-P3, *rrnB* terminator	10
pMGKU422	pRK415-R4-R3-F, *bcr4* gene tagged with FLAG sequence at the C terminus for complementation	This study
pMGKU423	pRK415-R4-R3-F, *bcr4* gene lacking amino acids region 169-173 tagged with FLAG sequence at the C terminus	This study
pMGKU424	pRK415-R4-R3-F, *bcr4* gene lacking amino acids region 164-173 tagged with FLAG sequence at the C terminus	This study
pMGKU425	pRK415-R4-R3-F, *bcr4* gene lacking amino acids region 159-173 tagged with FLAG sequence at the C terminus	This study
pMGKU426	pRK415-R4-R3-F, *bscI* gene	This study

Table 2. Primers used in the study

Name	Sequence (5'→3')
B1-bscI-V5	AAAAAGCAGGCTTGTTGGACAGCGGACCCGCC
B2-bscI-V5	AGAAAGCTGGGTTTGACATTCTCGCCAGCGTGTC
B1-bscK-V5	AAAAAGCAGGCTTGGCGCACGCGCTGGTCCCG
B2-bscK-V5	AGAAAGCTGGGTTGGCTTGGGCGGGGAACGAGG
B1-bcr4-comp	AAAAAGCAGGCTGCCAGGTCCGGTCTCGCACCG
B2-bcr4-V5	AGAAAGCTGGGTTTCCAGGAGCTCCAGGTAATG
5-HindIII-bcr4	ATCCGAATTCAAGCTTCATTCAGACTCAGGTTCAGATTC
3-bcr4-Strep	CGGGTGGCTCCATCCAGGAGCTCCAGGTAATG
5-HindIII-bscI	ATCCGAATTCAAGCTTAATTTGGATCTGACGGCGATC
3-bscI-Strep	CGGGTGGCTCCATGACATTCTCGCCAGCGTGTC
3-Strep-HindIII	GCAGGTCGACAAGCTTTCATTTTTCGAACTGCGGGTGGCTCCA
5-bcr4-58-109-Strep-IF	GCCGTCAGCGATCTGTCGGACTGGACGCCTGGTG
3-IF-bcr4-58-109-Strep	CAGATCGCTGACGGCCGCCG
5-bcr4-110-173-Strep-IF	TGGAGCCACCCGCAGTTCGA
3-IF-bcr4-110-173-Strep	CTGCGGGTGGCTCCAGCGGTCCTCCGCCTGTGCCG
B1-bscI	AAAAAGCAGGCTTCATGACTGTTCACGACGAC
B2-bscI	AGAAAGCTGGGTGCACAAGATCCAGCGCGACC
R1-bscI	CGACGTTGGCGTTGATCGCCGTCAGATC
R2-bscI	TCAACGCCAACGTCGACACGCTGGCGAG
5-bcr4-FLAG-IF	CCACCCGCAGTTCGAAAAATGAATTTGGATCTACCCAGCTTTCTTG
3-IF-bcr4-FLAG	TCGAACTGCGGGTGGCTCCATCCAGGACCTCCAGGTAATGGCTCG
3-IF-bcr4△169-173-FLAG	GTCATCCTTGTAGTCTAATGGCTCGGACTGCAACG
3-IF-bcr4△164-173-FLAG	GTCATCCTTGTAGTCCAACGGCGCGGGCCGCATCATG
3-IF-bcr4△159-173-FLAG	GTCATCCTTGTAGTCCATCATGGCGGGTCCGCTGTC
3-B2-bscI-comp	AGAAAGCTGGGTTTGACATTCTCGCCAGCGTGTC