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 Bscl, 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 Bscl and to activate the T3SS. Moreover, the deletion of Bscl 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 Bscl is unstable in the absence of Bcr4. These results suggest that Bcr4 supports the construction of the T3SS machinery by stabilizing Bscl. This is the first demonstration of a chaperone for the T3SS inner rod protein among the virulence bacteria possessing the T3SS.

Importance

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

Introduction

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

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

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

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

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

Results

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

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

Next, to reconfirm that Bcr4 binds to Bscl, a pull-down assay was performed by adding *E. 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 (Bcr4 Δ 58-109-Strep), and Bcr4 lacking amino acids region 110–173 (Bcr4 Δ 110-173-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 Bcr4 Δ 58-109-Strep, or Bcr4 Δ 110-173-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 Bcr4 Δ 58-109-Strep and Bcr4 Δ 110-173-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 (Bcr4 Δ 169-173-FLAG), Bcr4 with the 10 C-terminal amino acids deleted
142 (Bcr4 Δ 164-173-FLAG), and Bcr4 with the 15 C-terminal amino acids deleted
143 (Bcr4 Δ 159-173-FLAG) (Fig. 2A) within a Bcr4-deficient strain (Δ Bcr4)
144 (Δ Bcr4+Bcr4-FL-FLAG, Δ Bcr4+Bcr4 Δ 169-173-FLAG, Δ Bcr4+Bcr4 Δ 164-173-FLAG and
145 Δ Bcr4+Bcr4 Δ 159-173-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, Δ Bcr4+Bcr4-FL-FLAG
154 and Δ Bcr4+Bcr4 Δ 169-173-FLAG, but almost no signals were detected in
155 Δ Bcr4+Bcr4 Δ 164-173-FLAG and Δ Bcr4+Bcr4 Δ 159-173-FLAG (Fig. 2C). On the other hand,
156 when anti-FLAG antibody was used, signals were detected in Δ Bcr4+Bcr4 Δ 164-173-FLAG
157 and Δ Bcr4+Bcr4 Δ 159-173-FLAG (Fig. 2C). These results confirmed that the Bcr4 partial
158 deletion mutant proteins were produced in the Δ Bcr4 strain. When anti-BteA or anti-BopD
159 antibodies were used against the Sup samples, signals were detected in the wild-type,
160 Δ Bcr4+Bcr4-FL-FLAG, Δ Bcr4+Bcr4 Δ 169-173-FLAG and Δ Bcr4+Bcr4 Δ 164-173-FLAG,

respectively, but not in $\Delta Bcr4$ or $Bcr4\Delta 159-173$ -FLAG (Fig. 2C). These results suggest that the region required for the T3SS function is located in the 159–163 amino acids region of Bcr4.

Bscl is required for the function of the T3SS in *B. bronchiseptica*.

Bscl is a homologue of Yscl, an inner rod of the *Yersinia* T3SS, and Yscl is essential for the function of the T3SS (Wood, JB, 2008). To confirm that Bscl is required for the function of the *B. bronchiseptica* T3SS, the *B. bronchiseptica* S798 wild-type, Bscl-deficient strain ($\Delta Bscl$), Bscl-complemented strain ($\Delta Bscl+Bscl$), and BscN-deficient strain ($\Delta BscN$) were cultured in SS medium. WCL and Sup samples were prepared, and these samples were separated by SDS-PAGE, followed by Western blotting using antibodies against Bscl, BteA, BopD or FHA (filamentous hemagglutinin, an adhesion factor) (Fig. 3). As a result, no signals of Bscl were detected in the WCL samples of all strains used here (Fig. 3). The Bscl signal was detected in the Sup of $\Delta Bscl+Bscl$, and a faint Bscl signal was detected in the Sup of the wild-type (Fig. 3). The BteA and BopD signals were detected in the Sup of the wild-type and $\Delta Bscl+Bscl$, but not in those of $\Delta Bscl$ and $\Delta BscN$ (Fig. 3). As expected, the FHA signals were detected in the WCL and Sup samples of all strains used here (Fig. 3). These results suggest that in *B. bronchiseptica*, Bscl is required for the function of the T3SS and is secreted out of the bacterial cell.

181 **Bscl plays an important role in the function of the *B. bronchiseptica* T3SS during**
 182 **infection of cultured mammalian cells.**

183 To further investigate whether Bscl 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, Δ Bscl, Δ Bscl+Bscl or Δ BscN and measured the number of Bsp22 (a translocator,
 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 Δ Bscl+Bscl, but not on the L2 cells infected with Δ Bscl or Δ BscN (Fig. 4A, B).
 194 We also infected L2 cells with the wild-type, Δ Bscl, Δ Bscl+Bscl or Δ BscN 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 Δ Bscl+Bscl, but not in the medium of L2 cells infected with
 198 Δ Bscl or Δ BscN (Fig. 4C). These results suggest that Bscl is required for the construction of
 199 the T3SS and induction of T3SS-dependent death of mammalian cells in *B. bronchiseptica*.

200

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

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

Discussion

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

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

In the present study, it was suggested that the 58–173 amino acids region of Bcr4 is required for the binding of Bcr4 to BscI (Fig. 2B). To investigate whether the 1–57 amino acids region of Bcr4 is also required for the binding of Bcr4 to BscI, we attempted to purify Bcr4 lacking the N-terminal 1–57 amino acids region (Bcr4 Δ 1-57-Strep). However, the amount of Bcr4 Δ 1-57-Strep produced in *E. coli* was extremely low (data not shown), so a pull-down assay could not be performed. Figure 2C suggests that the 159–163 amino acids region of Bcr4 is required for the function of the T3SS. These results suggest that the binding of Bcr4 to BscI is necessary for the T3SS function, because the region of Bcr4 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 (Δ Bcr4), 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 Δ Bcr4 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 (Δ BspR), 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 (Δ BspR Δ Bcr4) compared to that of Δ BspR (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 Δ Bcr4, 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 Bscl include Yscl of *Yersinia pseudotuberculosis*, but the identity
 285 between Bscl and Yscl 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. Δ BscN (a BscN-deficient strain), Δ Bcr4 (a Bcr4-deficient strain),
305 Δ BspR (a BspR-deficient strain) and Δ BspR Δ Bcr4 (a BspR and Bcr4-deficient strain) have
306 been described elsewhere (6, 14, 24). *Escherichia coli* DH10B, BL21 (33) and Sm10 λ 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 37°C for 2 days.
309 Fresh colonies of *B. bronchiceptica* were cultured in Stainer-Scholt (SS) medium (34) with a
310 starting A₆₀₀ of 0.23 under vigorous shaking conditions at 37°C. 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% CO₂ atmosphere.

314

315 **Pull-down assay.**

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

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

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

Next, in order to purify Bcr4 or Bscl tagged with six histidine residues (6×His) at the respective N-terminus and Strep at the respective C-terminus, we amplified DNA fragments encoding *bcr4* or *bscl* with the primer sets of 5-HindIII-*bcr4* and 3-*bcr4*-Strep, or 5-HindIII-*bscl* and 3-*bscl*-Strep using *B. bronchiseptica* S798 genomic DNA as the template. Each amplified DNA fragment was used as a template for 2nd PCR with a primer set consisting of the upper primer used in the 1st PCR and 3-Strep-HindIII to add a 24 bp 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 37°C with shaking, and then diluted 1:100 in LB
 350 liquid medium containing 50 μ L/mL ampicillin and incubated for 2 h at 37°C with shaking,
 351 respectively. Each bacterial culture was further incubated overnight at 15°C in the presence
 352 of IPTG at the final concentration of 0.05 mM. Bacteria were collected by centrifugation at
 353 2,600 $\times$ *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,

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

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

Construction of a *bscI* gene-disrupted strain.

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

the 5' end of the *bscI* gene to 30 bp upstream of the 3' end of the gene. This plasmid, pMGKU415, was mixed with a positive suicide vector such as pABB-CRS2 (38) to obtain pMGKU416 using the Gateway cloning system. The pMGKU416 plasmid in turn was introduced into *E. coli* Sm10 λ *pir*, and transconjugated into the S798 wild-type as described previously (39). The resulting mutant strain was designated Δ Bscl.

Construction of plasmids used for producing Bcr4 derivatives and Bscl complementation

In order to produce full-length Bcr4 tagged with a FLAG sequence at the C-terminus, we performed an inverse PCR with primers of 5-bcr4-FLAG-IF and 3-IF-bcr4-FLAG using pDONR-*bcr4* (24) as the template. The resulting PCR product was self-ligated to obtain pMGKU417 by the In-Fusion Cloning System. To produce Bcr4 lacking the amino acid regions 159–173, 164–173 or 169–173, inverse PCR was carried out with the primer sets of 5-*bcr4*-FLAG-IF and 3-IF-bcr4 Δ 169-173-FLAG, 5-*bcr4*-FLAG-IF and 3-IF-bcr4 Δ 164-173-FLAG, or 5-*bcr4*-FLAG-IF and 3-IF-bcr4 Δ 159-173-FLAG using pMGKU417 as the template. Each amplified fragment was self-ligated by the In-Fusion Cloning System to obtain pMGKU418, pMGKU419 or pMGKU420, respectively. For Bscl complementation, a PCR was carried out with the primers of B1-bscl-V5 and 3-B2-bscl-comp using *B. bronchiceptica* S798 genomic DNA as the template. The resulting PCR product was cloned into pDONR201 to obtain pMGKU421 using the Gateway cloning

system. These plasmids were mixed with pRK-R4-R3-F, pDONR-*fhaP* and pDONR-*rrnB* (10) to obtain pMGKU422, pMGKU423, pMGKU424, pMGKU425 and pMGKU426 using the Gateway cloning system.

Preparation of proteins from culture supernatants and whole bacterial cell lysates.

Proteins secreted into bacterial culture supernatants and whole bacterial cell lysates were prepared as described previously (40). The loaded sample amounts were adjusted by the A_{600} of each bacterial culture to load samples prepared from the same amounts of bacteria. The protein samples were separated by SDS-PAGE and analyzed by Western blotting.

Antibodies.

Anti-BteA, anti-BopD, anti-Bsp22 and anti-Bcr4 antibodies were purified from rabbit serum in our previous study (7, 10, 24, 41). Mouse anti-V5 monoclonal antibody was purchased from Santa Cruz Biotechnology. To detect filamentous hemagglutinin (FHA) signals, we used mouse anti-FHA serum kindly gifted from K. Kamachi (National Institute of Infectious Diseases). To prepare the anti-BscI and anti-BscJ antibodies, the peptides corresponding to the C-terminus regions of BscI (CKAIGRATQNVDTLARMS) and BscJ (CRGEGRGGAGAGATEGAGHD) were conjugated with hemocyanin from keyhole limpets (Sigma), respectively, by using 3-maleimidobenzoic acid N-hydroxysuccinimide ester

(Sigma). These cross-linked peptides were used to immunize rabbits, and the resulting anti-sera were incubated with the same peptides immobilized on epoxy-activated sepharose 6B (Amersham) to obtain specific Ig-fractions.

Immunofluorescence staining of L2 cells infected with *B. bronchiseptica*.

Immunofluorescent staining assay was performed as previously described (40), with slight modifications. Briefly, L2 cells were seeded on coverslips in 6-well plates and incubated overnight. In order to detect adequate amounts of Bsp22 signals, we infected the L2 cells with *B. bronchiseptica* cultured in iron-depleted SS medium at an MOI of 2000. To avoid excessive killing of L2 cells with bacteria, the plates were not centrifuged. After being incubated for 1 h at 37°C under a 5% CO₂ atmosphere, the infected L2 cells were immunostained. Average numbers of Bsp22 signals on single L2 cells were measured by a fluorescence microscope.

LDH Assays.

LDH assays were performed as previously described (40). Briefly, to examine whether lactate dehydrogenase (LDH) is released from *B. bronchiseptica*-infected cells, 5.0×10⁴ cells/well of L2 cells seeded in 24-well plates were infected with bacteria at an MOI of 200. The plates were centrifuged at 900×g for 5 min and incubated for 3 h at 37°C under a 5% CO₂ atmosphere. The amounts of LDH were measured spectrophotometrically using a

Cyto-Tox 96 Non-radioactive Cytotoxicity Assay kit (Promega). The LDH (%) level is shown as a ratio, with the value obtained from the well treated with 0.1% Triton X-100 set as 100%.

Statistical analyses

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

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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, Bcr4 Δ 58-109 or Bcr4 Δ 110-173 (Bcr4-FL-Strep, Bcr4 Δ 58-109-Strep or
622 Bcr4 Δ 110-17-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 (Δ Bcr4),
628 and Δ Bcr4 producing Bcr4-FL-FLAG (Δ Bcr4+Bcr4-FL-FLAG), Bcr4 Δ 169-173-FLAG
629 (Δ Bcr4+Bcr4 Δ 169-173-FLAG), Bcr4 Δ 164-173-FLAG (Δ Bcr4+Bcr4 Δ 164-173-FLAG), and
630 Bcr4 Δ 159-173-FLAG (Δ Bcr4+Bcr4 Δ 159-173-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 Δ Bcr4+Bcr4-FL-FLAG, Δ Bcr4+Bcr4 Δ 169-173-FLAG and
637 Δ Bcr4+Bcr4 Δ 164-173-FLAG Sup samples on the SDS-PAGE gel than on the wild type,
638 Δ Bcr4 and Δ Bcr4+Bcr4 Δ 159-173-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, Δ BscI (BscI-deficient strain), Δ BscI+BscI (BscI-complemented strain) and Δ BscN
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, Δ BscI (BscI-deficient strain), Δ BscI+BscI (BscI-complemented strain) or
655 Δ BscN (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, Δ Bcr4 (Bcr4-deficient strain), Δ BspR
665 (BspR-deficient strain) or Δ BspR Δ Bcr4 (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 Δ BspR
670 or Δ BspR Δ Bcr4 WCL samples on the SDS-PAGE than WT or Δ Bcr4 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. 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.

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Table 1. Bacterial strains and plasmids used in the study

Strain or plasmid	Relevant genotype or description	Reference or source
Strains		
<i>B. bronchiseptica</i>		
S798	wild-type strain	6
ΔBscI	BscI-deficient strain	This study
ΔBscN	BscN-deficient strain	6
ΔBcr4	Bcr4-deficient strain	24
ΔBspR	BspR-deficient strain	14
ΔBspRΔBcr4	BspR and Bcr4-deficient strain	24
<i>E. coli</i>		
DH10B	Host strain for pDONR201, p99- <i>ccdB</i> -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, <i>bscI</i> gene tagged with V5 sequence at the C terminus	This study
pMGKU405	pDONR201, <i>bscK</i> gene tagged with V5 sequence at the C terminus	This study
pMGKU406	pDONR201, <i>bsc4</i> gene tagged with V5 sequence at the C terminus	This study
p99- <i>ccdB</i> -V5	Expression vector for V5 tagged gene, Amp ^r	35
pMGKU407	p99- <i>ccdB</i> -V5, <i>bscI</i> gene tagged with V5 sequence at the C terminus	This study
pMGKU408	p99- <i>ccdB</i> -V5, <i>bscK</i> gene tagged with V5 sequence at the C terminus	This study
pMGKU409	p99- <i>ccdB</i> -V5, <i>bsc4</i> gene tagged with V5 sequence at the C terminus	This study
pColdII	Expression vector for His tagged gene, Amp ^r	TAKARA
pMGKU410	pColdII, <i>bcr4</i> gene tagged with Strep sequence at the C terminus	This study
pMGKU411	pColdII, <i>bscI</i> gene tagged with Strep sequence at the C terminus	This study
pMGKU412	pColdII, <i>bcr4</i> gene lacking amino acids region 58-109 tagged with Strep sequence at the C terminus	This study
pMGKU413	pColdII, <i>bcr4</i> gene lacking amino acids region 110-173 tagged with Strep sequence at the C terminus	This study
pColdII- <i>bteA</i> -N-Strep	pColdII, <i>bteA</i> gene coding amino acids region 1-312 tagged with Strep sequence at the C terminus	36
pMGKU414	pDONR201, <i>bscI</i> gene	This study
pMGKU415	pDONR201, <i>bscI</i> gene containing internal sequence-deletion and its flanking region	This study
pABB-CRS2	Suicide vector for conjugation, Amp ^r	38
pMGKU416	pABB-CRS2, <i>bscI</i> gene containing internal sequence-deletion and its flanking region	This study
pDONR- <i>bcr4</i>	pDONR201, <i>bcr4</i> gene for complementation	24
pMGKU417	pDONR201, <i>bcr4</i> gene tagged with FLAG sequence at the C terminus for complementation	This study
pMGKU418	pDONR201, <i>bcr4</i> gene lacking amino acids region 169-173 tagged with FLAG sequence at the C terminus	This study
pMGKU419	pDONR201, <i>bcr4</i> gene lacking amino acids region 164-173 tagged with FLAG sequence at the C terminus	This study
pMGKU420	pDONR201, <i>bcr4</i> gene lacking amino acids region 159-173 tagged with FLAG sequence at the C terminus	This study
pMGKU421	pDONR201, <i>bscI</i> gene for complementation	This study
pRK415-R4-R3-F	pRK415, recombination sites for MultiSite Gateway, Tet ^r	10
pDONR- <i>fhaP</i>	pDONR-P4-P1R, <i>fha</i> promoter	10
pDONR- <i>rmB</i>	pDONR-P2R-P3, <i>rmB</i> terminator	10
pMGKU422	pRK415-R4-R3-F, <i>bcr4</i> gene tagged with FLAG sequence at the C terminus for complementation	This study
pMGKU423	pRK415-R4-R3-F, <i>bcr4</i> gene lacking amino acids region 169-173 tagged with FLAG sequence at the C terminus	This study
pMGKU424	pRK415-R4-R3-F, <i>bcr4</i> gene lacking amino acids region 164-173 tagged with FLAG sequence at the C terminus	This study
pMGKU425	pRK415-R4-R3-F, <i>bcr4</i> gene lacking amino acids region 159-173 tagged with FLAG sequence at the C terminus	This study
pMGKU426	pRK415-R4-R3-F, <i>bscI</i> gene	This study

Table 2. Primers used in the study

Name	Sequence (5'→3')
B1-bscl-V5	AAAAAGCAGGCTTGTGGACAGCGGACCCGCC
B2-bscl-V5	AGAAAGCTGGGTTTGACATTCTCGCCAGCGTGTC
B1-bscK-V5	AAAAAGCAGGCTTGGCGCACGCGCTGGTCCCG
B2-bscK-V5	AGAAAGCTGGGTTGGCTTGGGCGGGGAACGAGG
B1-bcr4-comp	AAAAAGCAGGCTGCCAGGTCCGGTCTCGCACCG
B2-bcr4-V5	AGAAAGCTGGGTTTCCAGGAGCTCCAGGTAATG
5-HindIII-bcr4	ATCCGAATTCAAGCTTCATTCAAGCTCAGGTTTCAGATTC
3-bcr4-Strep	CGGGTGGCTCCATCCAGGAGCTCCAGGTAATG
5-HindIII-bscl	ATCCGAATTCAAGCTTAATTTGGATCTGACGGCGATC
3-bscl-Strep	CGGGTGGCTCCATGACATTCTCGCCAGCGTGTC
3-Strep-HindIII	GCAGGTGCGACAAGCTTTTCATTTTTCGAAGTGCAGGTTGGCTCCA
5-bcr4-58-109-Strep-IF	GCCGTCAGCGATCTGTGCGACTGGACGCCTGGTG
3-IF-bcr4-58-109-Strep	CAGATCGCTGACGGCCGCCG
5-bcr4-110-173-Strep-IF	TGGAGCCACCCGCAGTTTCA
3-IF-bcr4-110-173-Strep	CTGCGGGTGGCTCCAGCGGTCTCCGCCTGTGCCG
B1-bscl	AAAAAGCAGGCTTCATGACTGTTACGACGAC
B2-bscl	AGAAAGCTGGGTGCACAAGATCCAGCGCGACC
R1-bscl	CGACGTTGGCGTTGATCGCCGTCAGATC
R2-bscl	TCAACGCCAACGTCGACACGCTGGCGAG
5-bcr4-FLAG-IF	CCACCCGCAGTTTCGAAAAATGAATTTGGATCTACCCAGCTTTCTTG
3-IF-bcr4-FLAG	TCGAAGTGCAGGTTGGCTCCATCCAGGACCTCCAGGTAATGGCTCG
3-IF-bcr4 Δ 169-173-FLAG	GTCATCCTTGTAAGTCTAATGGCTCGGACTGCAACG
3-IF-bcr4 Δ 164-173-FLAG	GTCATCCTTGTAAGTCCAAACGGCGCGGGCCGCATCATG
3-IF-bcr4 Δ 159-173-FLAG	GTCATCCTTGTAAGTCCATCATGGCGGGTCCGCTGTC
3-B2-bscl-comp	AGAAAGCTGGGTTTGACATTCTCGCCAGCGTGTC