1 DegP initiates regulated processing of filamentous hemagglutinin in *Bordetella bronchiseptica*

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15 **Abstract**

16 Filamentous hemagglutinin (FhaB) is a critical virulence factor for both *Bordetella pertussis*, the
 17 causal agent of whooping cough, and the closely related species *Bordetella bronchiseptica*. FhaB
 18 is an adhesin, suppresses inflammatory cytokine production, and protects against phagocytic cell
 19 clearance during infection. Regulated degradation of the FhaB C-terminal prodomain is required to
 20 establish a persistent infection in mice. Two proteases, CtpA in the periplasm and SphB1 on the
 21 bacterial surface, are known to mediate FhaB processing, and we recently determined that CtpA
 22 functions before, and controls the FhaB cleavage site of, SphB1. However, the data indicate that
 23 another periplasmic protease must initiate degradation of the prodomain by removing a portion of
 24 the FhaB C terminus that inhibits CtpA-mediated degradation. Using a candidate approach, we
 25 identified DegP as the initiating protease. Deletion of *degP* or substitution of its predicted catalytic
 26 residue resulted in reduced creation of FHA', the main product of FhaB processing, and an
 27 accumulation of full-length FhaB in whole cell lysates. Also, FHA' was no longer released into
 28 culture supernatants in *degP* mutants. Alterations of the FhaB C terminus that relieve inhibition of
 29 CtpA abrogate the need for DegP, consistent with DegP functioning prior to CtpA in the
 30 processing pathway. DegP is not required for secretion of FhaB through FhaC or for adherence of
 31 the bacteria to host cells, indicating that DegP acts primarily as a protease and not a chaperone
 32 for FhaB in *B. bronchiseptica*. Our results highlight a role for HtrA family proteases in activation of
 33 virulence factors in pathogenic bacteria.

34

35 **Importance**

36 Two-partner secretion (TPS) systems are broadly distributed among Gram-negative bacteria and
 37 play important roles in bacterial pathogenesis. FhaB-FhaC is the prototypical member of the TPS
 38 family and here we identified the protease that initiates a processing cascade that controls FhaB
 39 function. Our results are significant because they provide insight into the molecular mechanism

underlying the ability of *Bordetella* species to prevent clearance by phagocytic cells, which is critical for bacterial persistence in the lower respiratory tract. Our findings also highlight an underappreciated role for HtrA family proteases in processing specific bacterial virulence factors.

Introduction

Gram-negative bacteria from the genus *Bordetella* cause highly contagious respiratory infections in mammals that have adverse impacts on public health and industrial agriculture(1). The human-specific pathogen *Bordetella pertussis* causes pertussis, also known as whooping cough, and is responsible for infecting an estimated 24 million children annually, causing 160,000 deaths(2). A majority of pertussis cases are from areas of the world with low (<85%) vaccine coverage. However, pertussis incidence has been increasing over the last 30 years even in areas where >90% of the population has received three doses of the dTap/Tdap vaccines, including the United States(3). The resurgence of pertussis correlates with the switch from whole-cell (wP) vaccines to acellular (aP) vaccines, which are composed of up to four purified *B. pertussis* proteins. While the increased incidence of pertussis is in part due to abiologic factors, such as improved diagnosis and case reporting, studies using baboons indicate that although wP and aP vaccines protect against disease, neither prevents *B. pertussis* colonization, and the aP vaccine also failed to prevent transmission(4).

The most widely used aP vaccine contains pertussis toxin, pertactin, fimbriae subunits, and a proteolytically processed version of filamentous hemagglutinin (FhaB) called FHA. FhaB is produced by all *Bordetella* species that infect mammals and is required for bacterial adherence to eukaryotic cells(5–7). Infection of small rodents with *Bordetella bronchiseptica*, a species closely related to *B. pertussis* that produces a nearly identical set of virulence factors, indicate that FhaB is functionally interchangeable between *B. bronchiseptica* and *B. pertussis* and that FhaB is required for persistent infection in the mammalian lower respiratory tract (LRT)(8–11). The

65 mechanism by which FhaB mediates protection from inflammatory clearance is not
66 understood(11).

67 FhaB is a member of the broadly distributed two-partner secretion (TPS, also known as Type
68 Vb) family of bacterial secretion systems(12–14). It is synthesized as a 379 kDa preproprotein that
69 possesses an extended N-terminal signal sequence that mediates Sec-dependent delivery to the
70 periplasm(15, 16). Following removal of the signal sequence, the conserved N-terminal TPS
71 domain of FhaB interacts with the periplasmic POTRA domains of its cognate transporter FhaC to
72 begin translocation of FhaB across the outer membrane (OM)(17–19). While the FhaB N terminus
73 remains anchored to FhaC, two-thirds of FhaB is secreted through FhaC in the N- to C-terminal
74 direction, emerging on the bacterial surface as a hairpin consisting of a β -helical shaft that is
75 topped by the globular mature C-terminal domain(20) (MCD) (Figure 1A). The remaining C-
76 terminal third of FhaB (~1,200 amino acids), called the prodomain (PD), is retained in the
77 periplasm by the prodomain N terminus (PNT), which prevents further secretion through
78 FhaC(20). FhaB undergoes complex regulated processing by multiple proteases. In *B.*
79 *bronchiseptica* the tail-specific family member CtpA mediates degradation of the prodomain,
80 resulting in the generation of the 250 kDa product FHA', which stays membrane-associated
81 temporarily but is ultimately released from the bacterial surface (Fig 1A). Under certain conditions
82 (i.e., overnight growth in rich media) FHA' can be cleaved by the surface-anchored exoprotease
83 SphB1 to produce the 243 kDa product FHA (Fig 1A, dashed arrow), which is immediately
84 released. The biological significance of SphB1-dependent cleavage is unclear. Although initially
85 identified as a protease mediating release of FHA(21), it has subsequently been shown that FhaB
86 polypeptides lacking the prodomain, such as FHA', are efficiently released by both wild-type and
87 SphB1-deficient strains of *B. bronchiseptica*(12, 22). The fully processed protein (i.e., FHA) was
88 long considered the functional molecule, as the PD is thoroughly degraded and not detectable as

a standalone protein(11). However, full-length FhaB plays a vital role during infection, as mutants lacking portions of the PD are rapidly cleared from the murine LRT, despite being able to adhere to host cells as effectively as wild-type *B. bronchiseptica*¹¹. These findings suggest that full-length FhaB is required specifically for defense against inflammation-mediated clearance from the LRT, and we hypothesize that this activity requires properly regulated degradation of the FhaB PD.

Previous work from our laboratory determined that the last 98 amino acids of FhaB, called the ‘extreme C-terminus’ (ECT), negatively regulate CtpA-dependent FhaB processing(11). While characterizing CtpA, we identified a novel polypeptide intermediate called the “FhaB clipped product” (FhaB^{CP}), which lacks ~90 amino acids (i.e., most of the ECT) from the C terminus(22). FhaB^{CP} is stabilized in whole-cell lysates (WCLs) harvested from *B. bronchiseptica* $\Delta ctpA$ cultures, consistent with the discovery that the native FhaB C terminus is a poor substrate for CtpA(22) and the hypothesis that FhaB^{CP} is the preferred substrate for CtpA. The presence of FhaB^{CP} in $\Delta ctpA$ WCLs indicates that yet another protease, that we are calling the initiating protease, is responsible for converting FhaB into FhaB^{CP} through removal of the ECT. According to our model, CtpA degrades FhaB^{CP} to FHA’, which is either released from the bacterial surface or, if the bacteria are grown for extended periods of time, is cleaved by SphB1 to produce FHA, which is then immediately released (Figure 1A). We hypothesize that cleavage of the PD to form FhaB^{CP} is the critical control point as it initiates PD degradation. The goal of this study was to identify the initiating protease and to determine its contribution to FhaB processing and function.

Results

BB3749 (*degP*) appears to encode the initiating protease. To identify candidates for the initiating protease we searched the genome of wild-type *B. bronchiseptica* strain RB50 for ORFs predicted to encode proteases containing N-terminal Sec or TAT signal peptides. The 11 ORFs

114 identified include those encoding both of the previously characterized proteases involved in
115 processing FhaB, *sphB1*(21) and *ctpA*(22), as well as nine predicted to encode endopeptidases
116 and carboxy-terminal acting peptidases (Table 1). We generated *B. bronchiseptica* strains
117 containing either an in-frame deletion (Δ BB0299, Δ BB3068, Δ BB2112) or an integrated plasmid
118 disruption (Ω BB1398, Ω BB1721, Ω BB2462, Ω BB3749, Ω BB3990, Ω BB4867) in one of the nine
119 ORFs and examined FhaB/FHA' in whole cell lysates (WCL) using α -MCD specific

120 antisera (Figure 1C). We included the strain carrying an in-frame deletion of *ctpA* (Δ *ctpA*) that
121 has been previously shown to be required for normal FhaB processing as a control (Figure 1C).
122 Instead of processing FhaB to the 250kDa FHA' product, the Δ *ctpA* strain converts FhaB to the
123 larger FHA²⁷⁵ polypeptide, likely due to aberrant processing by as yet unidentified proteases(22).
124 Two of the mutants displayed altered FhaB processing compared to the wild-type strain. BB0299
125 (*envC*) is located directly 5' to, and is co-transcribed with, *ctpA*(22) and is predicted to encode an
126 M23 family peptidase with a degenerate LytM (dLytM) domain. dLytM homologs in other species,
127 such as EnvC in *E. coli*, serve as accessory factors that allosterically activate cell-wall cleaving
128 hydrolases(24, 25). The phenotype of the Δ *envC*_{Bb} mutant is similar, but not identical, to that of the
129 Δ *ctpA* strain, suggesting that EnvC_{Bb} may function as an accessory factor promoting CtpA activity
130 (Figure 1C). As *envC* is in an operon with *ctpA*, it is also possible that the in-frame deletion
131 mutation in *envC* could alter expression of *ctpA*. Characterizing the precise role of EnvC_{Bb} in FhaB
132 processing is beyond the scope of the current work and will be addressed with future studies.

133 The strain containing a disruption in BB3749 (Ω BB3749) displayed the expected phenotype for
134 loss of initiating protease activity; a majority of the FhaB protein remained as the full-length ~371
135 kDa form (Figure 1C). We observed partially degraded FhaB polypeptides in Ω BB3749 WCLs that
136 are not present in lysates of wild-type bacteria, suggesting that in the absence of the BB3749
137 gene product, unidentified proteases inefficiently degrade FhaB following overnight growth.

BB3749 is predicted to encode a high-temperature requirement A (HtrA) family serine protease. Prokaryotic HtrA proteases are extra-cytoplasmic enzymes initially identified as factors necessary for bacterial survival at elevated temperatures(26–28). They contain trypsin-like serine protease domains at their N termini, and either one or two protein-binding PDZ domains at their C termini. The protein encoded by BB3749 is predicted to contain a serine protease domain with a conserved catalytic triad (a GNSGG motif and nearby histidine and aspartic acid residues), as well as two PDZ domains (Fig. 2A). Although annotated as a homolog of *mucD*, which encodes a protease responsible for regulating mucoidy (due to alginate overproduction) in *Pseudomonas aeruginosa* (Fig. S1), BB3749 is not associated with mucoidy in *B. bronchiseptica*. Moreover, the amino acid sequence of the protein encoded by BB3749 is 99.8% identical to that of *B. pertussis* DegP(29). Hence, we will hereafter refer to BB3749 as *degP* (or *degP_{Bb}* when distinguishing it from homologs is necessary) and its protein product as DegP (or DegP_{Bb}).

To determine if the FhaB processing defect displayed by the Ω *degP* strain was due to the loss of DegP (and not polar effects of the integrated plasmid), we constructed a strain in which codons 6-491 of *degP* were deleted (Δ *degP*). We also created a derivative of Δ *degP* in which a wild-type copy, driven by the constitutive S12 promoter, was inserted at the *attTn7* site (Δ *degP* + *degP*). We grew the bacteria overnight in media containing 20mM MgSO₄ (which inactivates the BvgAS regulatory system that activates expression of *fhaB* and other virulence factor-encoding genes) and then outgrew the bacteria in media without MgSO₄ (BvgAS-activating conditions) for four hours to induce production of FhaB before collecting WCLs as previously described(22). As with the Ω *degP* strain, nearly all of the FhaB protein in the Δ *degP* strain was the full-length ~371 kDa form, and normal processing was restored in the complemented strain (Fig. 2B and 2C). The intermediate FhaB products that were apparent in WCLs from the Ω BB3749 overnight cultures, were less prominent in the WCLs from Δ *degP* after four hours of growth under BvgAS-activating conditions, indicating that these aberrant products accumulate only after extended periods of

163 growth (Fig 2B). For the remainder of this study we examined FhaB processing following four
164 hours of growth under BvgAS-activating conditions.

165 To determine if the catalytic activity of DegP is required for FhaB processing, we constructed a
166 strain (iDegP) in which codon 237 was changed to encode alanine instead of the predicted
167 catalytic serine. The FhaB/FHA' profile of the iDegP mutant was nearly identical to that of the
168 $\Delta degP$ mutant, indicating that Ser237, and most likely the proteolytic activity, of DegP is required
169 for efficient FhaB processing (Figure 2B and 2C).

170

171 **DegP is required for *B. bronchiseptica* growth at elevated temperatures.** DegP homologs
172 from other bacterial species, including *B. pertussis*(29), are required for bacterial survival and
173 growth at elevated temperatures. To determine if *B. bronchiseptica* also requires DegP to grow
174 under heat stress, we inoculated BG blood agar plates with wild-type, $\Delta degP$, iDegP,
175 and $\Delta degP + degP$ strains and incubated them at either 37°C or 42°C for 72 hours. Both
176 the $\Delta degP$ and iDegP mutant strains were attenuated for growth at 42°C but not at 37°C (Fig. S2).
177 Failure of the $\Delta degP$ strain to grow at 42°C was restored by expression of a wild-type copy
178 of *degP* in trans ($\Delta degP + degP$, Fig. S2). These data indicate that DegP, and its catalytic
179 activity, are required for *B. bronchiseptica* to grow at 42°C. BvgAS activates (either directly or
180 indirectly) the expression of all genes encoding *Bordetella*'s known protein virulence factors,
181 including *fhaB*(30). Many of these factors are surface-localized or secreted into the extracellular
182 environment and most, if not all, are produced at a high level when BvgAS is active. The severe
183 growth defect displayed by *degP* mutants in response to elevated temperature was greatly
184 alleviated in bacteria grown in the presence of 50 mM $MgSO_4$, which inactivates
185 BvgAS. Magnesium ions (Mg^{2+}) are crucial for envelope stability(31), so we also grew all four
186 strains on plates containing 50mM $MgCl_2$. Unlike $MgSO_4$, which rescued the growth of the mutant
187 strains, addition of $MgCl_2$ had no effect on bacterial growth at 42°C. These data suggest that the

188 requirement for DegP under heat stress conditions is primarily due to its proteolytic activity on one
189 or more BvgAS-activated virulence factors. (29)(32)

190

191 **DegP acts first in stepwise processing of the prodomain.** Our current model for FhaB
192 processing involves three proteases: two periplasmic enzymes, CtpA and the initiating protease,
193 and one exoprotease, SphB1 (Figure 1A). We previously determined that CtpA and SphB1
194 function in series, with CtpA acting prior to, and determining the cleavage site of, SphB1(22). In
195 fact, our data suggest that SphB1-dependent processing only occurs after extended time in
196 culture and may have no functional relevance under normal (*in vivo*) circumstances. We have also
197 shown previously that the ECT inhibits CtpA from degrading the PD, as deletion of the ECT
198 (Δ ECT) causes rapid and thorough conversion of FhaB to FHA' (which we call hyperprocessing) in
199 a CtpA-dependent manner(11, 22). We hypothesized that the initiating protease is responsible for
200 removing the ECT to convert full-length FhaB into the CtpA substrate FhaB^{CP}. To test this
201 hypothesis, we first showed (as we have done previously(22)) that addition of an HA epitope N-
202 terminal to the PRR (Fig. 3A, strain FhaB_{HA-PRR}) has no effect on FhaB processing (Fig. 3B, both
203 full-length FhaB and FHA' are present in WCL of the FhaB_{HA-PRR} strain). Deletion of *degP* in this
204 strain resulted in an accumulation of full-length FhaB_{HA-PRR} and a reduced amount of FHA', just as
205 it did in wild-type bacteria (Fig. 2), and complementation with wild-type *degP* restored FhaB
206 processing (Fig. 3B). Deletion of *ctpA* does not alter the abundance of full-length FhaB in WCL,
207 and results in the formation of FHA²⁷⁵ (Fig. 3B and(22)). (11, 22)FHA²⁷⁵ is a ~275 kDa polypeptide
208 containing an incompletely degraded PD. We next examined if *degP* is required for the
209 hyperprocessing of FhaB that occurs in the absence of the ECT. In wild-type bacteria producing
210 FhaB lacking the ECT (FhaB _{Δ ECT}, Fig. 3A), full-length FhaB is barely detectable (Fig. 3C). The
211 same was true for the Δ *degP* strain (Fig. 3C), indicating that DegP does not contribute to the
212 hyperprocessing of FhaB lacking the ECT. By contrast, FhaB _{Δ ECT} was abundant in the Δ *ctpA*

strain (Fig. 3C, yellow band as this strain also contains an HA epitope immediately N-terminal to the PRR), showing that the hyperprocessing of FhaB_{ΔECT} is CtpA-dependent. These data indicate that DegP acts prior to CtpA and is required for removal of the ECT to allow degradation of the prodomain by CtpA.

Similar to deleting the ECT, and in contrast to inserting an HA epitope N-terminal to the PRR, adding an HA epitope to the C terminus of FhaB (FhaB_{CT-HA}) results in CtpA-dependent hyperprocessing (Fig. 3D and(22)), i.e., this epitope somehow converts the FhaB C terminus into a substrate for CtpA. We reasoned that addition of the HA epitope to the C terminus could impact FhaB processing in two ways: 1) The HA epitope is itself could be a substrate for the tail-specific protease CtpA, and/or 2) Addition of the HA epitope could disrupt proper folding of the ECT causing it to be degraded by DegP. Deletion of *degP* did not prevent hyperprocessing of FhaB_{CT-HA}, indicating that DegP is not required for CtpA-dependent degradation of the full-length FhaB_{CT-HA} and that CtpA proteolytic activity is not dependent on DegP. Consistent with CtpA-dependent degradation of FhaB_{CT-HA}, we observed an accumulation of the full-length polypeptide (371 kDa yellow band) in WCLs from the $\Delta ctpA$ strain. We also detected the FhaB^{CP} in $\Delta ctpA$ WCLs, which we believe is the product formed following removal of the ECT by the initiating protease (~362 kDa green band). The presence of the FhaB^{CP} in $\Delta ctpA$ FhaB_{CT-HA} and not in $\Delta ctpA$ FhaB_{HA-PRR} WCLs indicates that the addition of the C-terminal HA epitope promotes removal of the ECT-HA by the initiating protease (DegP). Taken together these data indicate that DegP acts first in stepwise processing of the PD.

DegP is required for release of FhaB-derived polypeptides from the bacterial surface. *B. bronchiseptica* efficiently releases FhaB-derived polypeptides into culture supernatants and the specific polypeptide released depends on the age of the culture and the proteases produced by the bacterial strain (Fig. 4B). Wild-type *B. bronchiseptica* primarily releases FHA' during the first 6

hours in culture under BvgAS-activating conditions, before SphB1 begins to cleave accumulated FHA' to cause the formation and immediate release of FHA(22) (Supp. Fig 3). In the absence of CtpA, FHA' is not produced, and instead FhaB is converted to the aberrant FHA²⁷⁵ product, which is retained on the cell surface. FHA²⁷⁵ cannot be released due to the presence of the complete PNT. However, FhaB or FHA²⁷⁵ can be cleaved by SphB1 promiscuously at non-preferred sites to form the smaller polypeptides FHA_{1/2}, which are immediately released(20, 22)(Fig. 4A and 4B). None of the processed polypeptides (FHA', FHA, or FHA_{1/2}) were detected in significant amounts in the $\Delta degP$ mutant, demonstrating that DegP is critically important for release of FhaB-derived polypeptides.

As shown in Fig. 4B and in our previous publication(22), CtpA or SphB1 is required for release of FhaB-derived polypeptides. In the absence of DegP, however, neither CtpA nor SphB1 is sufficient; FHA' or FHA_{1/2} were barely detectable in supernatants of any strain containing the $\Delta degP$ mutation (Fig. 4B). These results reinforce the conclusion that DegP-mediated cleavage of FhaB occurs first and is required for subsequent degradation by CtpA and then cleavage by SphB1. When considered in conjunction with the WCL data (Fig. 4C), these data are also consistent with our model(12) (Fig. 1 and depicted in greater detail in Supplemental Fig 3.), which states that the polypeptide formed in the absence of CtpA (FHA²⁷⁵) cannot be released, but it can serve as a poor substrate for SphB1, cleavage by which generates FHA_{1/2} (because the PNT prevents movement through the FhaC channel that would allow the preferred SphB1 cleavage site to be exposed on the surface), which is immediately released (Fig. 4A and 4B). It is important to note that the supernatant samples have been concentrated substantially compared to the WCLs. Therefore, while levels of FHA_{1/2} in supernatants of the $\Delta ctpA$ strain appear to be similar to levels of FHA' in supernatants of wild-type bacteria, no FHA_{1/2} was detected in WCLs of the $\Delta ctpA$ mutant and a substantial amount of FHA' was present in WCLs of wild-type bacteria, indicating that in the absence of CtpA, generation of FHA_{1/2} by SphB1 is less efficient than generation of

263 FHA' by wild-type bacteria. In the absence of DegP, FHA²⁷⁵ is not formed and only a small amount
264 of FHA' is generated which cannot be detected in culture supernatants (Fig 4B).

265

266 **DegP is not required for translocation of FhaB through FhaC.** DegP could be indirectly
267 required for FhaB processing by promoting FhaB translocation through FhaC. To determine if loss
268 of DegP prevents efficient translocation of FhaB to the cell surface, we used a dot blot assay to
269 compare relative amounts of FhaB on the surface of intact bacteria following four hours of growth
270 under BvgAS-activating conditions (Figure 5). In this assay, only surface-exposed epitopes are
271 detectable by antibodies when cells are intact. When the bacteria are boiled to disrupt the
272 membranes, internal epitopes can additionally be detected by antibodies. The α -MCD antibody
273 (green) detected the MCD in unboiled bacteria, indicating that the MCD was located external to
274 the outer membrane. However, the HA-tag was only detected after boiling the bacteria, indicating
275 that the PD remains localized in the periplasm. As expected, neither the MCD nor the HA-tag was
276 detected in the $\Delta fhaB$ strain. The protease mutants, $\Delta ctpA$ and $\Delta degP$, were similar to wild-type
277 bacteria in that the MCD was detected regardless of boiling while the HA-tag was only detected
278 post-boiling. These results indicate that neither CtpA nor DegP is required for FhaB to be secreted
279 through the outer membrane or for the PD to remain within the periplasm. Therefore, the reduction
280 in FhaB processing that occurs in $degP$ mutant strains is not due to mislocalization or instability of
281 FhaB.

282

283 **DegP is not required for FhaB-mediated adherence to host cells *in vitro*.** While the dot blot
284 analysis indicates that DegP is not required for translocation of FhaB to the bacterial surface, it
285 cannot distinguish between active or inactive conformations of FhaB. We performed *in vitro*
286 adherence assays to determine if the FhaB on the surface of $\Delta degP$ bacteria is capable of
287 promoting bacterial adherence to human epithelial cells *in vitro*. Adherence of the $\Delta degP$ strain

288 was similar to that of wild-type *B. bronchiseptica*, indicating that DegP is not required for
289 translocation of FhaB to the bacterial surface or for the MCD to fold into an adherence-competent
290 conformation (Figure 6). These data, along with the finding that strains that produce catalytically
291 inactive DegP are defective for FhaB processing, are consistent with the primary function of DegP
292 being that of a protease, rather than a chaperone, at least with regard to FhaB.

293

294 **DegP does not signal through the RseA-RpoE pathway to control FhaB processing**
295 **indirectly**. Some HtrA proteins, such as *E. coli* DegS (DegS_{Ec}), are not degradative proteases.
296 Instead, they transduce signals from the periplasm to cytoplasmic effectors, controlling the
297 expression of genes encoding stress response factors indirectly. In the *E. coli* RseA-RpoE signal
298 transduction system, the anti-sigma factor RseA, which is embedded in the inner membrane,
299 binds and sequesters the RpoE sigma factor (σ E) in the cytoplasm, preventing σ E from binding to
300 and directing RNA polymerase to activate expression of target genes(33). In *E. coli* undergoing
301 exponential growth under standard laboratory conditions, DegS_{Ec} homotrimers associated with the
302 inner membrane are in an inactive conformation. When *E. coli* is exposed to conditions that
303 disrupt the folding and localization of proteins in the periplasm, misfolded proteins bind DegS_{Ec}'s
304 single PDZ domain, resulting in allosteric activation of DegS_{Ec}'s proteolytic activity(34). When
305 active, DegS_{Ec} cleaves a specific site on a periplasmic region of RseA, initiating a proteolytic
306 cascade that causes the release of σ E and the subsequent activation of stress response
307 genes(33). Collectively this process is referred to as regulated intramembrane proteolysis
308 (RIP)(35). Among the genes activated in the *E. coli* σ E regulon is *degP*(36–39). Although the
309 genomes of the *Bordetella* reference strains, Tohama I (*B. pertussis*) and RB50 (*B.*
310 *bronchiseptica*), encode functional Rse-RpoE systems(40–42), they do not encode DegS
311 homologs, as neither of the two HtrA homologs produced by these strains (DegP and the BB4867
312 gene product) are predicted to include TM domains capable of inserting in the inner membrane.

Therefore, the protease responsible for initiating regulated proteolysis of RseA in *Bordetella* is not known. Unlike *E. coli*, in which *degP* is located distally on the chromosome from *rpoE*, *degP* is in an operon with *rpoE*, *rseA*, and *rseB* in *B. pertussis* and *B. bronchiseptica* (Figure 7A). These observations led us to ask if DegP could regulate FhaB processing indirectly, by cleaving RseA_{Bb} and subsequently increasing σ E-dependent expression of a gene encoding yet another protease. RpoE is thought to be essential in *B. pertussis*(41) but is dispensable for growth of *B. bronchiseptica* under standard laboratory conditions(42). To determine if DegP signals through the RseA-RpoE pathway, we deleted *rpoE* in wild-type, $\Delta degP$, and iDegP *B. bronchiseptica* (Figure 7B). If DegP signals through RseA-RpoE to activate expression of a gene encoding another protease, then loss of RpoE would cause the $\Delta rpoE$ strain to exhibit a similar defect in FhaB/FHA processing seen in WCLs from $\Delta degP$ and iDegP mutants. Instead, we observed that deletion of $\Delta rpoE$ had no effect on FhaB processing by any of the strains under the conditions examined, indicating that DegP is not indirectly regulating degradation of the FhaB PD via RpoE (Figure 7).

326

327 Discussion

Our results indicate that the HtrA family protease DegP initiates degradation of the FhaB prodomain in *B. bronchiseptica*. Three key findings support this conclusion: 1) FhaB processing was dramatically reduced, but not completely abrogated, in strains lacking DegP ($\Delta degP$) or producing DegP in which the predicted catalytic serine was replaced with alanine (S237A, iDegP). The small amount of FHA' detected in WCLs of *degP* mutants was likely due to the prodomain being converted to a CtpA substrate by housekeeping proteases, which is revealed only in the absence of DegP. 2) DegP was dispensable for degradation of FhaB polypeptides with altered C termini (ΔECT and CT-HA) that are efficiently degraded by CtpA (Fig. 3). These data are consistent with our previous studies that determined that the native FhaB C terminus is a poor substrate for CtpA(22), and indicate that, in wild-type bacteria, DegP cleaves the prodomain first,

generating a C terminus that is then efficiently degraded by CtpA. CtpA-dependent degradation of the Δ ECT and CT-HA polypeptides in the absence of DegP also indicate that accumulation of full-length in WCLs from *degP* mutants is not due to the loss of CtpA activity. 3) DegP was not required for translocation of FhaB through FhaC (Fig. 5) or for FhaB to mediate adherence to epithelial cells (Fig. 6), consistent with DegP functioning as a protease and not a chaperone for FhaB. Previous studies determined that if FhaB is stalled in the periplasm (i.e., in *fhaC* mutants), it is degraded(43). The stability of full-length FhaB and the surface-localization of the MCD in *degP* mutants, therefore, indicate that DegP is not required to preserve FhaB stability or to deliver the TPS domain to FhaC in the periplasm. Although it is formally possible that DegP regulates FhaB processing indirectly by activating another protease, the most parsimonious explanation for our findings is that DegP acts on FhaB directly.

FhaB is required to mediate adherence to mammalian cells, prevent bacterial clearance by innate immune effectors, and control inflammatory signaling during infection. While processed FhaB' is sufficient for adherence, full-length FhaB and regulated degradation of the prodomain is required specifically for *B. bronchiseptica* to resist phagocytic cell clearance; mutants with altered prodomain processing are cleared rapidly from the lower respiratory tract but are not altered for adherence *in vitro* or *in vivo* or for suppression of inflammatory cytokines(11). Consistent with these data, the Δ *degP* strain adhered to human A549 epithelial cells as efficiently as wild-type bacteria do (Fig. 6), confirming that regulated prodomain processing is not required for FhaB to function as an adhesin. The fact that FhaB was exported to the cell surface (Fig. 5) and able to mediate adherence in *degP* mutants (Fig. 6) indicates that DegP does not function as a chaperone required to stabilize FhaB in the periplasm or to bring the N terminus of the TPS domain to FhaC. Furthermore, if DegP was an important chaperone for the FhaB prodomain, then lack of DegP would result in hyperprocessing, similar to the Δ ECT strain. However, full-length

362 FhaB was stabilized in *degP* mutants, indicating that DegP does not promote folding or stability of
363 the prodomain.

364 Although *B. bronchiseptica* consistently produces and releases FHA' (and sometimes FHA)
365 from the bacterial surface during *in vitro* culture, neither the mechanism by which release occurs,
366 nor its biological relevance, is understood. Our previous data suggested that either CtpA or SphB1
367 are sufficient for release(22). However, our new data indicate that their sufficiency is dependent
368 on DegP as no FhaB-derived polypeptides are released by *degP* mutants (Fig. 4B). Future studies
369 to determine how DegP and CtpA (and potentially EnvC) cooperate to degrade the prodomain
370 should also reveal the mechanism underlying retention versus release of FhaB-derived
371 polypeptides, another important outstanding question for TPS systems, in general.

372 DegP's proteolytic activity is required for initiating FhaB processing in *B. bronchiseptica*, but
373 it is not known whether the DegP homolog from *B. pertussis* is involved in degradation of the
374 FhaB prodomain in that organism. A previous study that examined DegP in *B. pertussis*
375 determined that this protease may have both catalytic and non-catalytic functions that are required
376 for maintaining envelope integrity and are involved with FhaB secretion(29). However, that study
377 did not examine prodomain degradation(29). Comparison between our study and Baud *et al.*
378 highlights some key differences between *B. pertussis* and *B. bronchiseptica*, namely that *B.*
379 *pertussis degP* mutants are more susceptible to elevated temperature than *B. bronchiseptica*
380 *degP* mutants are. *B. pertussis degP* mutants are attenuated for growth at 37°C(29), while *B.*
381 *bronchiseptica* strains lacking DegP present a growth defect at 42°C but not at 37°C (Supp Fig. 2).

382 Baud *et al.* determined that growth at 37°C of the *degP* mutants could be partially restored
383 by expression of catalytically inactive DegP (S237A) *in trans*, which, combined with examination of
384 binding interactions between DegP and non-native N-terminal FhaB polypeptides *in vitro*, led them
385 to conclude that DegP functions as a chaperone in *B. pertussis*(29). By contrast, we found that
386 DegP's proteolytic activity is required for both FhaB processing and resistance to heat stress. Our

iDegP strains, which produce DegP S237A from the native locus, were as attenuated for growth at 42°C and for FhaB processing as the $\Delta degP$ strain was (Supp Fig. 2 and Fig. 2), consistent with DegP in *B. bronchiseptica* functioning primarily as a protease for both FhaB processing and resistance to envelope stress.

In a follow up study, Baud *et al.* determined that DegP can be both soluble and associated with bacterial membranes as trimers, that membrane associated DegP has a higher affinity for non-native FHA polypeptides (lacking most of the MCD and all of the prodomain), and that membrane associated DegP has proteolytic activity(44). These findings provide insight into how DegP could be regulating degradation of the FhaB prodomain. We hypothesize that membrane associated DegP trimers could form a stable complex with the FhaB prodomain until a signal causes a conformational change in the prodomain such that DegP recognizes the prodomain as a substrate and initiates degradation. The signal that initiates degradation of the prodomain is unknown; however, previous work from our lab and others suggests that FhaB may be part of a novel toxin delivery system. The adenylyl cyclase toxin (ACT) – known to inactivate phagocytic cells(45–47) – binds FhaB on the bacterial surface(48, 49), and we hypothesize that regulated degradation of the FhaB prodomain controls delivery of surface-associated ACT to those cells. Our current model states that ACT forms a stable complex with full-length FhaB on the bacterial surface until these FhaB-ACT complexes interact with the ACT receptor (CR3, CD11b/CD18, Mac1) on phagocytic cells(46, 50). We propose that this interaction between FhaB-ACT and CR3 could generate a signal that is propagated through the FhaB MCD resulting in a conformational change in the prodomain allowing DegP to initiate degradation of the prodomain. Previous data indicates the FhaB ECT – which is 100% identical at the protein level between FhaB homologs in *B. pertussis*, *B. bronchiseptica* and *B. paraptussis* – likely adopts a conformation that stabilizes the prodomain by preventing degradation by CtpA(22). It is interesting to speculate that FhaB-ACT binding the CR3 receptor could trigger the ECT to unfold and therefore become a

substrate for DegP, consistent with the preference for DegP homologs, such as those from *E. coli* and *B. pertussis*, to degrade unfolded proteins(29, 51). It is also possible that the signal that initiates FhaB processing is envelope stress that *B. bronchiseptica* faces during infection, which could induce conformational changes in the FhaB prodomain that cause it to become a substrate for DegP. Future studies will focus on identifying the signal that initiates FhaB processing and determining if regulated processing of FhaB is involved in ACT delivery to specific host cells.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains and plasmids are listed in Supplemental Table 1. In-frame deletions were created by allelic exchange using derivatives of the pSS4245 plasmid(52). For complementation, *degP* was inserted into a derivative of the pUC18 plasmid that contained the S12 promoter and integrated into the chromosome at the *att*:Tn7 site(53). *E. coli* strains were used to amplify vectors (DH5 α) and to transform *B. bronchiseptica* (RHO3). Mutations were confirmed by PCR and/or sequencing. As we have published in the past, our “wild-type” strain for FhaB studies is RBX11, a derivative of RB50 in which *fhaB* is more genetically tractable because the strain lacks *fhaS*, a gene highly homologous to *fhaB* but that plays no role in virulence(43).

Culture media and conditions. *Bordetella bronchiseptica* strains were streaked from -80°C stocks onto Bordet-Gengou agar (BD Biosciences) supplemented with 6% defibrinated sheep blood (HemoStat Laboratories) and grown at 37°C for 2-3 days. Because some combinations of protease deletions, such as $\Delta ctpA \Delta degP$, resulted in growth defects that were minimized if BvgAS was not active, protease dual mutants were streaked onto blood plates supplemented with 50mM MgSO₄. Colonies were picked from these plates and cultured overnight in Stainer-Scholte broth (SS) containing MgSO₄ (50mM) to prevent production of Bvg-induced virulence factors,

including FhaB. Bacteria were pelleted and washed with cold, sterile Dulbecco's Phosphate Buffered Saline (DPBS, Gibco) and then cultured further for 4 hours in fresh Stainer-Scholte broth lacking MgSO_4 to initiate production and processing of FhaB. This culture method is useful to examine early FhaB production and processing and to compare differences found across bacterial mutants that may be hidden after 16 hours of overnight growth(12). For experiments described in Figure 1, bacteria were simply grown overnight in Bvg inducing conditions (SS without MgSO_4). *Escherichia coli* strains were grown at 37 °C in lysogeny broth or on lysogeny broth agar. Where appropriate, media was supplemented with streptomycin (20 $\mu\text{g/mL}$), kanamycin (50 $\mu\text{g/mL}$), and MgSO_4 (50mM). For heat tolerance experiments (supplemental figure 2), the indicated strains were grown in Bvg+ phase liquid cultures (SS broth) overnight, normalized to 1 OD600 and then ten-fold serial dilutions of each culture were spotted (5uL per spot) onto BG blood agar plates with either 0 (Bvg+) or 50mM MgSO_4 (Bvg-) and grown at 42 °C for 72 hours before being imaged.

A549 human epithelial cells were cultured as described by the supplier (ATCC) in Ham's F12 media (Gibco) + 10% FBS (VWR). Cells were grown until they reached 80% confluency in T75 tissue culture treated flasks before passaging and the cells were passaged no more than 15 times out of a freeze and (Corning).

453

Immunoblots. To examine cell-associated proteins by western blot, whole cell lysates were prepared by boiling pelleted cells in Laemmli buffer and shearing by passage through a 26G needle. For released proteins, culture supernatants were filtered through 0.2 μm filters, and proteins were precipitated using 10% trichloroacetic, rinsed with cold acetone, resuspended in 1M Tris-HCl pH 8.8 and Laemmli buffer mixture, and boiled. Proteins were separated by SDS-PAGE using 4% or 5% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes (GE Healthcare), and the membranes were probed with a rabbit polyclonal antibody generated against the FHA mature C-terminal domain (MCD)(10) and a mouse monoclonal antibody generated

462 against an HA epitope (BioLegend). Corresponding α -rabbit and α -mouse IRDye secondary
463 antibodies (LI-COR Biosciences) were used to detect proteins using a LI-COR Odyssey Classic
464 Blot Imager (LI-COR Biosciences). Protein quantification was performed using LI-COR Image
465 Studio version 5 software. *B. bronchiseptica* sample volumes were normalized based on optical
466 density of the cultures.

467 To compare cell surface exoproteins versus internal proteins by dot blot, 100 μ L of
468 0.5OD₆₀₀/mL of bacteria were spotted onto nitrocellulose membranes using a 96-well vacuum
469 manifold. The membranes were probed using anti-MCD and anti-HA antibodies. Signal detected
470 on intact bacteria corresponded to surface-exposed epitopes, and boiling disrupted the membrane
471 to allow additional antibody interaction with otherwise inaccessible internal epitopes.

472

473 **Bacterial adherence assay.** These assays were performed as previously described with minor
474 alterations(5). A549 cells were seeded on sterile coverslips in 12-well plates. Once cells reached a
475 confluency of 50% to 80%, the seeding medium was removed and the cells were inoculated with
476 the indicated *B. bronchiseptica* strains (wt, $\Delta fhaB$, $\Delta degP$, $\Delta degP + degP$) in SS broth at an MOI of
477 100 cfu per epithelial cell (2.5×10^7 CFU/ml of bacteria). One set of wells were treated with SS
478 broth alone (mock infection control). The bacterial density of each inocula were confirmed by
479 plating on BG blood agar. The 12 well plates were centrifuged at 500 x g for 5 minutes at room
480 temperature and then incubated for 15 minutes at 37 °C with 5% CO₂. The inoculum was removed
481 from each well and the cells were washed four times with Hanks' Balanced Salt solution (Difco).
482 Cells were then fixed with 0.5mL of ice-cold methanol for five minutes, after which the methanol
483 was removed, and the coverslips were air dried for 10 minutes. The cells were then stained for 15
484 minutes with 0.5mL of 1:20 diluted Giemsa stain (Sigma Aldrich). The stain was removed, and
485 coverslips were washed twice with water before being air dried and mounted onto slides with
486 permount (Sigma Aldrich). The slides were imaged at 60X magnification (Keyence).

487

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495

496 **Author contributions**

497 RMJ, ZMN and PAC designed the studies, interpreted data and wrote the manuscript. RMJ, ZMN,
 498 MRD and JS acquired and analyzed the data.

499

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644

645 **Figure 1. A screen of FhaB processing in *B. bronchiseptica* strains that carry mutations of**
646 **predicted extra-cytoplasmic proteases identifies BB3749 as encoding a putative FhaB**
647 **protease.** (A) Our current model of regulated FhaB processing by three proteases. An unknown
648 signal (electric bolt) causes the initiating protease to remove the Extreme C-terminus (ECT) that
649 acts as a guard against CtpA. This clipped product (FhaB^{CP}) is recognized by CtpA, which
650 degrades most of the prodomain to form FHA'. Removal of a portion of the PNT by CtpA causes a
651 shift in the molecule that allows the release of FHA' into the supernatant. Under certain conditions
652 this shift also exposes the primary SphB1 cleavage site and allows SphB1 to create FHA (dashed
653 arrow), which is immediately released. (B) Linear schematics of FhaB (without the extended signal
654 sequence) and the main polypeptides that result from processing by each protease. Molecular
655 weights (kDa) are listed on the right. (C) Western blot analysis of whole cell lysates (WCL) from
656 overnight cultures of *B. bronchiseptica* strains with deletions of (Δ) or plasmid disruptions in (Ω)

the indicated ORFs. The antigenic region of the α -MCD polyclonal antibody is indicated by the cartoon of a fluorescent labeled antibody in (B).

Figure 2. BB3749 encodes the HtrA family serine protease DegP, and its catalytic activity is required for efficient FhaB processing. (A) Diagram of DegP_{Bb} with the catalytic triad (HDS) and two PDZ domains indicated. The catalytic serine at position 237 was changed to an alanine to generate the inactive (iDegP) strain. (B) Western blot analysis of WCL from cultures grown for four hours in BvgAS-activating medium (SS) after overnight growth in BvgAS-inactivating medium (SS + 20mM MgSO₄). (C) Ratio of processed FHA' to full-length FhaB in the western blot in (B) and three similar blots. Significance of p<0.05 as determined by unpaired two-tailed T-test indicated by asterisk.

Figure 3. DegP removes the ECT to permit degradation of the prodomain by CtpA. (A) Linear schematic of FhaB_{HA-PRR}, a version of FhaB that contains an HA tag (black wedge) inserted N-terminal to the proline rich region (PRR). The HA tag at this location is not disruptive to normal FhaB processing^{1,2}. FhaB_{ΔECT} lacks the C-terminal 98 amino acids (ECT). FhaB_{CT-HA} has the HA tag located at the C terminus. (B) Western blot analysis of WCLs from strains grown for four hours in BvgAS-activating medium after overnight growth in BvgAS-inactivating medium. Full-length molecules (FhaB_{HA-PRR} and FhaB_{CT-HA} appear yellow due to recognition by both α -MCD (green) and α -HA (red) antibodies. The intermediate polypeptide FhaB^{CP} and final product FHA appear green due to loss of the HA tag. In the absence of CtpA, a 275 kDa polypeptide, FHA²⁷⁵, is created rather than FHA'.

Figure 4. DegP_{Bb} is required for release of FhaB-derived polypeptides. (A) Diagram depicting the C termini of the FhaB-derived polypeptides released by *B. bronchiseptica*. Western blot

analyses of (B) culture supernatants and (C) WCLs of strains grown for four hours in BvgAS-activating medium after overnight growth in BvgAS-inactivating medium. Blots probed with α -MCD antibody.

Figure 5. DegP is not required for FhaB secretion or retention of the prodomain within the periplasm. (A and B) Linear schematic and folded model of FhaB_{HA-PRR} with α -MCD and α -HA antibody binding regions shown. (C) Dot blots of intact and boiled cells. Blots were probed with α -MCD and α -HA antibodies.

Figure 6. Deletion of *degP* does not disrupt FhaB-mediated adherence of *B. bronchiseptica* to epithelial cells *in vitro*. Representative images of Giemsa stained A549 human epithelial cells following a 15-minute inoculation with the indicated strains of *B. bronchiseptica* at an MOI of 100.

Figure 7. RpoE is not required for FhaB processing in *B. bronchiseptica*. (A) Diagram of the BB3753-BB3749 (*degP*) operon in the *B. bronchiseptica* RB50 genome. (B) Western blot analysis of WCL of strains grown for four hours in BvgAS-activating medium following overnight growth in BvgAS-inactivating medium. Blots probed with α -MCD antibody.

Figure S1. BB3749 encodes an HtrA family protease.

(A) Alignment of the amino acid sequences of DegP of *B. bronchiseptica* (Uniport ID A0A0H3M188_BORBR), DegP of *B. pertussis* (Q7VW38_BORPE), MucD of *Pseudomonas aeruginosa* (G3XD20_PSEAE), DegP (DEGP_ECOLI), DegQ (DEGQ_ECOLI) and DegS (DEGS_ECOLI) of *E. coli*, and DegQ of *B. bronchiseptica* (AOA0H3LRV9_BORBR). Alignment was performed with Clustal Omega. (B) Matrix comparing the percent identities between the HtrA amino acid sequences

707
708 **Figure S2. DegP activity is required for *B. bronchiseptica* to grow at 42°C.** Ten-fold serial
709 dilutions of *B. bronchiseptica* were spotted onto BG blood agar plates without magnesium
710 additives (BvgAS-activating) or containing 50mM MgSO₄ (BvgAS-inactivating) or 50mM MgCl₂.
711 Bacteria were grown for 72 hours at 37°C or 42°C as indicated.

712
713 **Figure S3. FhaB processing pathways of *B. bronchiseptica* protease mutants.** Linear
714 diagrams of the model for regulated processing of FhaB in wild-type (A), $\Delta ctpA$ (B), $\Delta degP$ (C),
715 $\Delta sphB1$ (D), $\Delta ctpA \Delta sphB1$ (E), $\Delta ctpA \Delta degP$ (F), and $\Delta degP \Delta sphB1$ (G) strains. Polypeptides
716 detected only in culture supernatants are indicated with a silcrow (§), polypeptides present in both
717 WCL and supernatants are indicated with a psi (ψ), and polypeptides that are present in WCL in
718 *degP* mutant strains, but at greatly reduced levels, are lightly shaded.

719
720 **Table S1. Strains and plasmids used during this study.**

721

Table 1. ORFs encoding initiating protease candidates	
ORF	Superfamily
BB0299 (<i>envC</i>)	M23-dLytM
BB1398	M23
BB1721	Imelysin-like
BB2112	M48_M56
BB2462	M48_M56
BB3068	M14c

BB3749 (<i>degP</i>)	HtrA	722
BB3990	YfgC	723
BB4867	HtrA	724

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735

A

B

C

A

B

C

D

A

B

C

A

B

C

D

Mock

Wt

$\Delta fhaB$

$\Delta degP$

$\Delta degP + degP$

A

B