Type IV pili-associated secretion of a biofilm matrix protein from *Clostridium perfringens* that forms intermolecular isopeptide bonds

Running title: Isopeptide bonds link *C. perfringens* biofilm proteins

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Abstract

Clostridium perfringens is a Gram-positive anaerobic spore-forming bacterial pathogen of humans and animals. *C. perfringens* also produces type IV pili (T4P) and has two complete sets of T4P-associated genes, one of which has been shown to produce surface pili needed for cell adherence. One hypothesis about the role of the other set of T4P genes is that they could comprise a system analogous to the type II secretion systems (TTSS) found in Gram-negative bacteria, which is used to export folded proteins from the periplasm through the outer membrane to the extracellular environment. Gram-positive bacteria have a similar secretion barrier in the thick peptidoglycan (PG) layer, which blocks secretion of folded proteins >25 kD. To determine if the T4P-associated genes comprise a Gram-positive TTSS, the secretome of mutants lacking type IV pilins were examined and a single protein, a von Willebrand A domain containing protein BsaC (CPE0517) was identified as being dependent on PilA3 for secretion. BsaC is in an operon with a signal peptidase and two putative biofilm matrix proteins with homology to *Bacillus subtilis* TasA. One of these proteins, BsaA, was shown by another group to produce high mol wt oligomers. We analyzed BsaA monomer interactions with *de novo* modeling, which projected that the monomers formed isopeptide bonds as part of a donor strand exchange process. Mutations in residues predicted to form the isopeptide bonds led to loss of oligomerization, supporting the predicted bond formation process. Phylogenetic analysis showed the BsaA family of proteins are widespread among bacteria and archaea but only a subset are predicted to form isopeptide bonds.

Importance

For bacteria to secrete folded proteins to the environment, they have to overcome the physical barriers of an outer membrane in Gram-negative bacteria and the thick peptidoglycan layer in

72 Gram-positive bacteria. One mechanism to do this is the use of a Type II secretion system in
73 Gram-negative bacteria, which has a similar structure as type IV pili and is modeled to act as a
74 piston that pumps folded proteins through the outer membrane to the environment. *Clostridium*
75 *perfringens*, like all or most all of the clostridia, has type IV pili and, in fact, has two sets of
76 pilus-associated genes. Here we present evidence that *C. perfringens* uses one set of pilus genes
77 to secrete a biofilm associated protein and may be responsible for secreting the main biofilm
78 protein BsaA. We show that BsaA monomers are, unlike most other biofilm matrix proteins,
79 linked by intermolecular isopeptide bonds, enhancing the physical strength of BsaA fibers.

Introduction

Bacterial protein secretion to the exterior environment involves transporting the protein across several physical barriers. For Gram-negative bacteria these include the cytoplasmic membrane (CM), a relatively thin peptidoglycan (PG) cell wall and the outer membrane. Gram-negative bacteria have evolved several different mechanisms to achieve this, including Type II secretion systems (TTSS), which have many proteins similar to those found in Type IV pili (T4P) (Fig. S1 and (1, 2)). TTSS are modeled to function as pistons that use a short pseudopilus to pump folded periplasmic proteins through a protein channel (secretin) in the outer membrane (Fig. S1 and (3, 4)). Protein secretion in Gram-positive bacteria involves transport across the CM and a usually thick PG layer. There are many protein secretion mechanisms for transiting the CM known for Gram-positive bacteria, including the Sec system, Twin arginine translocation (Tat), Type IV secretion systems, Esat-6 or type VII secretion system, flagella export apparatus (FEA), fimbrilin (pilus) protein exporter (FPE), and holin-dependent translocation. The Type IV, Type VII and FEA are able to translocate proteins across the PG layer (5, 6). However, proteins secreted through the CM via the Sec system, used for the majority of proteins, need to fold and, once they do, the PG layer of Gram-positive bacteria acts as a barrier to secretion to the environment because the mesh size in the PG layer is too small to allow diffusion of globular proteins >25 kD, as determined by measurements of the PG from the Gram-positive bacterium *Bacillus subtilis* (7).

One possible mechanism for secretion of folded proteins from the space between the CM and the PG layer in Gram-positive bacteria would be a system analogous to TTSS in Gram-negative bacteria. Our discovery that T4P systems are ubiquitous and perhaps universal in all of the Clostridia (8) provided a means for a potential TTSS to exist in the Clostridia. We hypothesized in a review (9) that in Clostridial species with two separate T4P systems, one of them could function

103 as a TTSS to transport proteins through the PG layer. Multiple species of Clostridia do carry
104 multiple copies of complete T4P systems, including *C. perfringens* (Fig. 1) and Clostridioides
105 difficile (9). The core proteins needed for functional T4P usually include pili, an assembly ATPase
106 (PilB), an inner membrane core protein (PilC), and associated assembly proteins PilM, PilN, and
107 PilO (9). *C. perfringens* carries two homologs of PilB, two homologs of PilC and single copies of
108 PilM, PilN, and PilO (Fig.1), making it plausible that one of these systems could function as a
109 TTSS. To test this hypothesis, we examined the secretome of strains with in-frame deletions in
110 genes encoding Type 4 pilins; in *C. perfringens* these are designated as PilA1-PilA4 (Fig. 1). We
111 noticed a protein of ~70 kD was present in the WT secretome but absent in the secretome of one
112 deletion strain, *pilA3* (Fig. 2A). Mass spectrometry identified the 70 kD protein as CPE0517,
113 which was encoded by a gene in an operon with three other genes, including a SipW signal
114 peptidase homolog (Fig. 2C). We expressed the entire operon in *C. perfringens* and found that it
115 produced large amounts of biofilm matrix proteins (Fig. S2B), and that the biofilm matrix protein
116 (CPE0515) was present as a long oligomer and was resistant to boiling in SDS-PAGE buffer (Fig.
117 S2D). We also noted that CPE0515 had some predicted structural homology to the TasA protein of
118 *B. subtilis*, which is also a biofilm matrix protein that uses a SipW signal peptidase for secretion
119 (10-12). At around this same time, a paper by Obana et al (13) was published in which they
120 examined many features of the same operon, which they designated as *sipW-bsaA-bsaB-bsaC*.
121 While the majority of their work concerned the regulation of matrix protein synthesis, they
122 discovered some important physical features of the biofilm matrix protein (BsaA, Fig. 2C): it is
123 the main component of the biofilm matrix; it required SipW for signal processing and secretion;
124 expression in *E. coli* of a form of BsaA lacking a signal sequence led to spontaneous

125 oligomerization of BsaA, which was resistant to denaturation by SDS or formic acid, indicting the
126 oligomers were held together by very strong intermolecular interactions (13).
127 In this report we describe the process of identifying T4P proteins involved in secreting BsaC, the
128 70 kD protein described above, as well as pilin proteins involved in secreting the BsaA oligomers.
129 We also identified isopeptide bond formation as the likely mechanism accounting for the stability
130 of the BsaA oligomer and we characterized the nature of the bonds and isopeptide bond formation
131 phylogeny in the BsaA/TasA family of proteins. Identifying a potential TTSS-like system in a
132 Gram-positive bacterium and the novel finding of isopeptide bond formation in a well-studied
133 family of proteins will lead to significant advances in both of these emerging fields of microbial
134 cell biology.
135

136 **Results**

137 **Secretome analysis of a *pilA3* mutant indicates a 70 kD protein with a Von Willebrand**
138 **domain A (CPE0517, BsaC) is absent.** We screened the secretome of in-frame deletion mutants
139 in four pilin-encoding genes, *pilA1*, *pilA2*, *pilA3*, *pilA4* for missing proteins, indicating they may
140 be dependent on the pilin for secretion. Only the *pilA3* mutant showed evidence of a distinct
141 missing band in the secretome and this band was restored by complementation of a WT copy of
142 the *pilA3* gene (Fig. 2A). The band was excised from a gel and subjected to trypsin digestion and
143 analysis by mass spectrometry. Peptide fragments matched those of the CPE0517 (BsaC) protein
144 from the *C. perfringens* strain 13 genome. The protein has an unknown function but does contain
145 a von Willebrand A domain, which can potentially function in protein-protein interactions with
146 host cells. This gene is the fourth gene in a four gene operon containing a type I signal peptidase
147 (CPE0514, SipW) and two genes (*CPE0515-CPE0516*, *bsaA-bsaB*) encoding proteins with

148 similarity to the TasA protein in *B. subtilis* (Fig. 2C). Western blots using anti-BsaC antibodies
149 showed a similar loss of CPE0517 secretion by the *pilA3* mutant and complementation by
150 expression of a wild-type copy of the *pilA3* gene (Fig. 2B).
151 To determine if other Type IV pilus (TFP) related genes were needed for secretion of BsaC, we
152 expressed the gene in a bank of in-frame deletion mutants encompassing each gene related to
153 synthesis and assembly of TFP (Fig. 1) using a vector we developed containing a lactose-inducible
154 promoter (14). We noted variation in day to day results with the WT strain and, since these were
155 western blots on TCA precipitated protein from culture supernatants, we normalized all of the data
156 from mutant strains to that of the WT strain on the same blot (Fig. 3). Using quantitative
157 densitometry on western blots of the secreted oligomers, we measured statistically significant
158 decreased secretion of BsaC in the following mutants: *pilA3*, *pilB2*, *pilC1*, *pilD*, *pilM*, *pilN* and
159 *pilO* (Fig. 3). With the exception of *pilC1*, these results suggest efficient secretion of BsaC requires
160 components of the TFP system present in the large *pilD-CPE2277* operon in *C. perfringens* (Fig.
161 1 and (8, 9)). Whether these form a type IV pilus with PilA3 as the pilin or a Gram-positive TTSS
162 remains to be determined. However, a major difference in the structures of T4P and TTSS is the
163 type IV pilus extends beyond the outer membrane (Gram-negative) or PG layer (Gram-positive,
164 (8)) but in the TTSS in Gram negative bacteria the pilus does not (2). Our model for a TTSS in
165 Gram-positive bacteria is that the pilus (i.e., PilA3) does not extend past the PG layer (Fig. S1).
166 To test this model, we exposed *C. perfringens* cells to rabbit anti-PilA3 antibodies and a
167 fluorescently tagged goat anti-rabbit secondary antibody and found that we could detect binding
168 localized to the poles of the cells (Fig. S2). This does refine the location of the pili, just that they
169 are extend far enough out past the PGY layer to be exposed to antibodies.

170 To determine if there are generalized secretion defects in TFP mutants, we examined
171 whether each mutant in the TFP-associated genes were able to secrete another protein, the
172 phospholipase-sphingomyelinase, PLC. Secretion of PLC can be easily detected by a ring of
173 precipitation around a colony using egg yolk agar and we found that none of the TFP mutants
174 showed a detectable decrease in PLC secretion (Fig. S3), suggesting the secretion defects seen
175 with BsaC were not due to generalized secretion defects.

176 **Expression of the *bsa* operon leads to synthesis of extracellular biofilm matrix material.** Since
177 the *bsa* operon encodes proteins with similar synteny and predicted structural homology to the
178 *sipW-tasA-tapA* biofilm matrix operon in *B. subtilis* (12), we placed the entire operon (*sipW-bsaA-*
179 *bsaB-bsaC*) under control of the lactose inducible promoter for high level expression. The cells
180 produced large quantities of an extracellular gel like material (Fig. S4B), which, after staining with
181 crystal violet, appeared as amorphous material between the bacterial cells (Fig. S4C). A His_6 tag
182 was added to the C-terminus of the BsaA protein and, when the operon was expressed, a distinct
183 high molecular weight ladder of BsaA was visible in anti- His_6 antibody western blots after running
184 on SDS-PAGE gels using samples boiled in SDS-PAGE buffer (Fig. S4D). This suggested BsaA
185 was in very heat-stable oligomers. This was confirmed by the results of Obana et al who found
186 that BsaA was the major component of the biofilm matrix and that BsaA oligomers were stable
187 after boiling in SDS-PAGE buffer and resistant to dissociation by high levels of SDS and formic
188 acid (13). These results suggested BsaA oligomers were in an extremely stable conformation which
189 may include covalent bonds. However, these stable oligomers were not due to the activity of the
190 sortase-encoding gene *srtB* (Fig. 2B), since a mutant lacking that gene still produced highly stable
191 oligomers of BsaA (13).

192 **Efficient secretion of the BsaA oligomer is dependent on some TFP-associated genes.** Since
193 it was possible that, like BsaC, BsaA in its oligomer form was secreted with the aid of TFP-
194 associated proteins, we expressed a clone containing just the *sipW* and *bsaA* genes in which a
195 FLAG-tag was added to the C-terminus of BsaA (pSK4) for quantification in western blots using
196 densitometry. An example of a typical blot is shown in Fig. S5A. The WT strain showed significant
197 levels of secretion of the BsaA oligomer (Fig. S5B) but this varied from day to day experiments.
198 Therefore, as described for westerns with BsaC, above, we normalized each western blot by setting
199 the WT level at 1.0 for each individual western blot experiment. Using this method, we found that
200 the *pilA1*, *pilB1*, *pilB1/pilB2*, *pilN* and *pilO* mutants exhibited decreased levels of BsaA oligomers
201 in the supernatants (Fig. S5B). However, since this profile of T4P-associated genes lacked an inner
202 membrane core protein (*pilC*) component, which is essential for TFP functions (15) the results
203 were not definitive. Therefore, we used a quantitative slot blot method as an alternative. In this
204 experiment, culture supernatants were serially diluted in medium and passed through a membrane,
205 and probed with an anti-FLAG antibody (Fig. S6A). The densitometry readings were then plotted
206 to identify an amount of signal that was in the linear range for quantification (Fig. S6B). However,
207 using this method only the *pilA1*, *pilA2* (higher), and *cpe1841* (higher) mutants showed a statistical
208 difference from the WT strain (Fig. S6C), which does not comprise a full T4P system.
209 **Models of BsaA oligomers predicts an isopeptide bond is formed linking adjacent monomers.**
210 BsaA forms very heat and SDS resistant oligomers ((13) and Figs. S4D and S5A). To investigate
211 the structural nature of the interactions between monomers of BsaA that leads to this stability, a
212 structure would need to be developed of a homomultimer. Due to the difficulty of solving the
213 structure of long, thin chains by experimental methods, computational modelling was used. Models
214 were initially created using Phyre2 (16), with the top model being an x-ray crystal structure of

215 TasA from *B. subtilis* (17). That structure, and homology models from that structure, were
216 monomeric, and not useful for determining intermolecular interactions between monomers.
217 AlphaFold Multimer was then used to create a multimeric model to determine inter-chain
218 interactions. Using ColabFold 1.3.0 (18), which is based on AlphaFold 2.2.0 (19), a homotrimeric
219 structure of BsaA was created (Fig. 4A). The SipW signal sequences (residues 1-27) were included
220 in the initial model, but only formed disordered loops which do not have any notable interactions.
221 Removing the signal sequences did not change the overall structure of the monomers so this was
222 done for subsequent analyses.

223 In monomeric structures, residues 28-38 would extend out as disordered loops (Fig. 4A,
224 monomer 1). In the multimeric structures, these residues incorporated themselves into the beta
225 sheet structure of adjacent monomers (Fig. 4A). Being fully incorporated into a nearby structure
226 indicates that residues 28-38 provide specific interactions with the receiving monomer, providing
227 the basis of a mechanism for which oligomers of BsaA can be created. For example, V33 and F37
228 of the interdigitated strand become part of the hydrophobic core of the receiving monomer (Fig.
229 4B). The interactions between specific side chains of residues from the interdigitated strand and
230 the receiving monomer were investigated further for less standard interactions.

231 The *de novo* created structures indicate a bond between two side chains: N35 of the
232 interdigitated strand and K74 of the receiving monomer (Fig. 4C). At first, this was thought to be
233 an error in AlphaFold's model generation, since the chemistry would not be possible. The bond
234 was created between the oxygen of N35 and the nitrogen of K74, resulting in three bonds in the
235 oxygen. However, this bond was created in nearly every model created, so it was investigated
236 further. A crosslink between asparagine and lysine is a type of isopeptide bond, which has been
237 characterized through a few different mechanisms and can be found as an intermolecular crosslink,

238 as in ubiquitylation (20), or as an intramolecular crosslink found in Gram-positive pili (21). In
239 Gram-positive pili, this bond is formed between an asparagine and a lysine, autocatalyzed by a
240 glutamate within the hydrophobic core of the protein. Residues equivalent to these are found
241 surrounding the bond in BsaA (Fig. 4C). The hydrophobic region surrounding the N35-K74 pair
242 (Fig. 4B, brown colored residues) is believed to be necessary to help the catalytic cross-linking
243 reaction (22).
244 It is unclear why this bond was created by AlphaFold. In the documentation of AlphaFold Multimer
245 (23), there are no mentions of predicting crosslinks. One potential reason is that it generated the
246 side chains so close to each other there was a steric clash, but the rest of the model was so favorable
247 the structure was created regardless. Models created in ColabFold have an option to perform
248 energy minimization using Amber. For these models, this was not performed. Recreating this
249 model while enabling energy minimization eliminates the bond every time. This is expected given
250 the incorrect chemistry of the bond. AlphaFold3 (24) does not include a minimization step, and,
251 like AlphaFold2, creates the isopeptide bond.
252 This amyloid-like fold and interdigitation is seen in other Gram-positive bacteria, such as
253 TasA in *B. subtilis*. A cryo-EM structure has been created for a chain of TasA (10), which
254 demonstrated how hydrophobic interactions between donor and acceptor monomers drive
255 interdigitation. No crosslinks were found or mentioned, and the required triad of N-E-K residues
256 is not present in the structure (PDB 8aur). Therefore, the isopeptide bonds in BsaA oligomers
257 would be the first example of an intermolecular bond of this mechanism found in this family of
258 proteins.
259 **Mutations in isopeptide bond forming residues abolish oligomerization of BsaA.** The
260 AlphaFold Multimer models predicted an isopeptide bond was formed between N35 and K74 from

261 adjacent residues in a BsaA oligomer. To test this computational prediction, we made point mutant
262 changes of each residue to alanine, forming N35A and K74A mutants, along with a double
263 N35A/K74A mutant. His-tagged versions of these mutant forms were expressed along with *sipW*
264 and the cell pellets and culture supernatants were examined for the presence of the mutant proteins
265 in oligomeric form. Mutating N35 or K74 to alanine residues resulted in a loss of oligomers in the
266 supernatants and the appearance of monomers of the K74A mutant but not of the N35A mutant,
267 suggesting the N35A version was not effectively released by the cell (Fig. 5A). Monomeric forms
268 of both mutants appeared in the cell pellet of some samples (Fig. 5A). In some experiments, small
269 amounts of dimers and higher mol wt forms of BsaA appeared in the cell pellet, but the large
270 majority were in monomeric form, which was absent in the WT form of BsaA (Fig. 5A), perhaps
271 due to the stability of oligomers even in the absence of isopeptide bond. To test this, we suspended
272 cell pellet extracts from BsaA-His_6 and N35A-His_6 in SDS-PAGE buffer and heated at 95 C for 0,
273 10, 20 and 40 min before running the samples on SDS-PAGE gels and performing western blots.
274 The N35A-His mutant maintained stable oligomers in SDS-PAGE buffer and being run through
275 an SDS-PAGE gel in the absence of heating, but heating the samples for 10-40 min led to
276 dissociation of the oligomers, in contrast to the WT BsaA protein, which was not affected by
277 heating (Fig. S5). This suggests the monomer-monomer interactions are stable even in the presence
278 of 2% SDS (the concentration in the SDS-PAGE buffer), which is in contrast to oligomers formed
279 by the *B. subtilis* TasA protein, which were dissociated by SDS (11).

280 We also tested the N35A/K74A mutant and observed that it showed a similar loss of
281 oligomerization as did the N35A mutant (Fig. 5B, left four lanes). These experiments were all done
282 in strain HN13 (WT) background where there was an intact *bsaA* gene. To determine if this
283 potential source of BsaA, even though it was not His-tagged, affected the results we observed with

284 the mutants, we expressed the N35A and N35A/K74A mutants in a strain with an in-frame deletion
285 of the *bsaA* gene, but did not observe any differences to the pattern seen in the strain with an intact
286 *bsaA* gene (Fig. 7B, right four lanes), suggesting the chromosomal source of BsaA was not forming
287 oligomers in combination with the N35A and N35A/K74A mutant forms of BsaA.

288 **The Glu56 residue of BsaA is essential for efficient isopeptide bond formation.** Isopeptide
289 bond formation is catalyzed by an acidic Glu or Asp residue located within the hydrophobic pocket
290 where the Asn and Lys residues are located (22). In AlphaFold 3 BsaA models of dimer interfaces,
291 Glu56 is predicted to be in the precise location to catalyze bond formation (Fig. 4B). To test this
292 was actually occurring, we constructed an E56A substitution in the BsaA protein and examined its
293 ability to oligomerize by using western blotting on cell extracts and concentrated supernatants
294 from the mutant and WT strain (Fig. 5C). The E56A mutant showed a clear lack of oligomer
295 formation and an accumulation of the monomeric form of the protein which was not observed with
296 the WT form of BsaA (Fig. 5C), providing strong evidence that Glu56 is responsible for catalyzing
297 isopeptide bond formation in BsaA.

298 **A FAST fusion to BsaA displays surface exposed localization.** To investigate the cellular
299 location of the BsaA oligomers, a FAST gene fusion to the *bsaA* gene was constructed and, along
300 with the *sipW* gene, was placed under control of a lactose-inducible promoter. The FAST protein
301 binds a soluble dye which, upon binding, exhibits a large increase in fluorescence (25-27) allowing
302 localization of the fusion proteins. We expressed the *sipW-bsaA*-FAST fusion genes and exposed
303 the cells to two different dyes, Coral, which is membrane permeable, and Amber-NP which is
304 impermeable to membranes (both obtained from The Twinkle Factory). To test if the Coral and
305 Amber-NP permeability profiles were working in *C. perfringens* membranes, we expressed a *pilT*-
306 FAST gene fusion, which should be located only in the cytoplasm, and exposed them to the Coral

307 and Amber-NP dyes. Consistent with the predicted cytoplasmic location of the *pilT*-FAST fusion,
308 only the Coral dye showed significant fluorescence after treatment (Fig. 6A). However, when the
309 *bsaA*-FAST fusion was expressed along with *sipW*, both dyes were able to bind and fluoresce (Fig.
310 6B), indicating BsaA-FAST fusion was competent for secretion in *C. perfringens* and was located
311 on the surface of the bacteria.

312 **BsaA oligomers are anchored to the membrane and not the peptidoglycan in strain HN13.**
313 Because the BsaA-FAST fusion experiments indicated BsaA oligomers were located on the cell
314 surface and SEM images of the BsaA matrix proteins suggested they were in the form of fibrils
315 associated with the bacterial surface (13), we wanted to determine if the oligomers were embedded
316 in the cytoplasmic membrane or covalently linked to the PG layer. To test this, we expressed *sipW*-
317 *bsaA*-His_6 constructs for the WT and each mutant in our lactose-inducible promoter system and
318 then boiled the cells in 10% SDS for 30 min, followed by digestion of the PG matrix by lysozyme.
319 Our experimental approach was based on the hypothesis that if BsaA was anchored in the
320 membrane, boiling in SDS would release it from the cell but if it was covalently anchored to the
321 PG, it would be freed only after digestion with lysozyme. We tested the WT, N35A, K74A and
322 N35/K74 mutants and found that all of the long wild-type oligomers were released after the SDS
323 boiling step, while the cells appeared to retain a small number of monomers and dimers of BsaA
324 (Fig. S4). However, after treatment with lysozyme, the monomers and dimers were no longer
325 detected. For the mutants that can't oligomerize, the monomer/dimers were retained after boiling
326 in SDS, but not after lysozyme treatment.. We interpret these findings as indicating the oligomers
327 are anchored to the membrane but after SDS boiling they are released. A small number of
328 monomers and dimers remain trapped inside the sacculi produced by boiling in SDS but lysozyme
329 treatment releases these from the sacculi but they are then too dilute to detect on western blots.

330 This is consistent with an analysis of the *bsaA* and *bsaB* protein sequences, both of which lack a
331 recognizable LPXTG motif, despite the presence of a sortase gene, *srtB*, adjacent to the operon
332 (Fig. 1C).

333 **BsaB, an ortholog of BsaA, forms dimers but not long oligomers.** Previous results by Obana et
334 al (13) showed a *bsaB* mutant still made significant amounts of the BsaA oligomers but they did
335 not test to see if BsaB was incorporated into BsaA oligomers. Therefore, we added an HA-tag to
336 the BsaB protein and expressed the *sipW*-*bsaA*-*bsaB*-HA-*bsaC* genes in our inducible system and
337 examined the culture supernatants for BsaB-HA using western blots. Based on the predicted mol
338 wt of the BsaB protein, we detected BsaB-HA only in a monomeric and a higher molecular weight
339 form which could be either a homo-dimer or a BsaA-BsaB heterodimer (Fig. S6), but it is not
340 covalently attached to the longer BsaA oligomers.

341 **Phylogeny of isopeptide bond formation in the TasA family.** Since a BlastP search with BsaA
342 only identifies homologs in closely related Clostridial species, an iterative search with profile
343 hidden Markov models was employed. This analysis resulted in the identification of 2,172 proteins,
344 including the previously described TasA from *B. subtilis* and CalY from *Bacillus cereus* (Fig. 7).
345 Of these, 632 hits belong to the "peptidase M73, camelysin" (InterPro: IPR022121) family; so-
346 called because of the likely mis-identification of CalY as a peptidase. In addition to the use of
347 probabilistic models, the relatedness of these proteins is evident at the structural level. Like other
348 family members, BsaA is predicted to adopt a β-sheet–rich Ig-fold-like structure and is predicted
349 to participate in donor-strand exchange (Fig. 4), as described for TasA (10). These analyses suggest
350 that while the structures of these proteins and homopolymer assembly are highly conserved,
351 sequences are highly divergent.

352 In addition to BsaA, we identified three other uncharacterized proteins in *C. perfringens*
353 (BsaB, CPE2262 and CPE0554), but these paralogs are not expected to form an intermolecular
354 isopeptide bond, because the N-E-K triad is missing. Nevertheless, over 100 BsaA-like proteins in
355 other species were identified with the N-E-K triad, suggesting conservation of the donor-strand-
356 exchange-and-lock mechanism. These proteins are largely from Clostridia with some homologs
357 from *Coprobacillaceae*, *Enterococcaceae*, and *Thermococcus* (Fig. 7).

358 Based on the putative functions of gene neighbors, the BsaA/TasA family members appear
359 to be involved in the assembly of surface filament structures (Fig. 7C). They are often in putative
360 operons with genes encoding proteins containing the Gram-positive cell wall anchor motif LPxTG,
361 fibronectin domains, SpaA domains, choice-of-anchor A domains, as well as signal peptidase and
362 sortase domains. Multiple BsaA/TasA paralogs can be found in a single putative operon (Fig. 7C),
363 suggesting the possibility of donor-strand exchange assembly of heteropolymers.

364

365 **Discussion**

366 The original goal of this work to determine if Gram-positive bacteria have the functional
367 equivalent of TTSS that are found in Gram-negative bacteria. Since TTSS have a similar overall
368 architecture and mechanism as T4P (Fig. S1), it made sense to test a Gram-positive bacterium
369 known to have T4P. With a few notable exceptions (e.g., *Streptococcus sanguinis* (28-30))
370 amongst Gram-positive bacteria, T4P are confined within the clostridia (9) so *C. perfringens* was
371 chosen as representing a typical clostridial species. The overall concept was that the thick Gram-
372 positive PG layer represents a similar barrier to protein secretion to the environment as the Gram-
373 negative outer membrane. Since TTSS depend upon a pseudopilus to function, we tested mutants
374 with deletions in each of the four main pilin-encoding genes in *C. perfringens* for changes in the

375 secretome compared to a WT strain. We identified one protein BsaC (CPE0517) that was depleted
376 in the secretome of a *pilA3* deletion strain (Fig. 2A-B). We then examined a bank of in-frame
377 deletion mutants in all of pilin-associated genes (Fig. 1) and found that, for BsaC secretion, a
378 homolog of each of the essential proteins needed for a type IV pilus assembly, PilA3 (pilin), PilB2
379 (assembly ATPase), PilC1 (inner membrane core protein), PilD (prepilin peptidase), and PilM-N-
380 O, (inner membrane accessory proteins) (Fig. 1) were required for efficient secretion (Fig. 3). This
381 appears to be specific for BsaC secretion, since the secretion of the toxin PLC was not affected
382 by mutations in any of the T4P-associated genes (Fig. S2). While this may indicate the presence
383 of a TTSS like apparatus in *C. perfringens*, the lines separating T4P- and T2SS-dependent
384 secretion have been blurred by the discoveries that *Pseudomonas aeruginosa* and *Dichelobacter*
385 *nodosus*, Gram-negative bacteria, have T4P systems capable of mediating protein secretion (31,
386 32). However, further analysis of the structure and components of these Gram-negative pilus-
387 dependent secretion systems have not been published. PilA3 pilin was detected o the surface of C.
388 perfringens so it is possible the PilA3-dependent secretion system acts more like a T4P.

389 BsaC monomer secretion may appear to be independent of BsaA oligomers, but it is
390 possible that they are being secreted simultaneously, since we did not track BsaA and BsaC
391 secretion at the same time. The biological role that BsaC plays in biofilm matrix functions is
392 unknown, but it is intriguing that the protein contains a VWA domain, which are widespread in
393 eukaryotes, bacteria and archaea and is associated with protein-protein interactions (33).
394 Therefore, the role BsaC plays in biofilm functions is also currently under investigation in the
395 laboratory.

396 BsaC is in an operon with genes encoding a signal peptidase, SipW and two proteins (BsaA
397 and BsaB) with homology to the main biofilm protein of *B. subtilis*, TasA (Fig. 2C). Because this

398 gene synteny is similar to the *sipW-tasA-tasB* biofilm operon in *B. subtilis*, we expressed the entire
399 operon to see if a biofilm matrix was produced and noted that high levels of a gel like matrix
400 material was formed in the culture supernatant (Fig. S3B-C). A high mol wt oligomer of BsaA
401 was seen in SDS-PAGE gels (Fig. S3D), which was similar to that seen by Obana et al in their
402 analysis of the same operon (13). Because BsaC was dependent on T4P-associated proteins for
403 secretion, it was logical to determine if secretion of the BsaA oligomer was as well. Two different
404 methods were tried, using densitometry of western blots of the BsaA oligomers in culture
405 supernatants and densitometry to measure the amount of BsaA in the culture supernatants using a
406 slot blot device. Although we saw significantly decreased levels of secretion in mutants using both
407 methods (Fig. S4 and S5), neither one gave conclusive evidence that a full T4P system was needed
408 for secretion. Both experimental approaches were hindered by relatively high amounts of
409 experimental variability in trying to measure an oligomer in solution; this can be seen by the high
410 range of the SEM determined for most of the samples (Fig. S4 and S5) despite multiple repetitions
411 of each experiments. However, the fact that several genes in each type of experiment was shown
412 to be necessary for efficient secretion, does strongly suggest that T4P protein play a role in the
413 process.

414 Although we observed that a *pilA1* deletion strain did not secrete the biofilm matrix
415 oligomers efficiently (Fig. S4 and S5), we did not observe this phenotype when screening stained
416 protein gels in our initial screening for proteins absent in the secretome, as shown for PilA3 in Fig.
417 2A-B. We think this is likely due to the fact that our growth conditions for the experiment,
418 overnight culture in BHI liquid medium at 37 ºC, is not optimum for production of the biofilm
419 matrix proteins, as described by Obana et al (13). These findings point out the limitations for
420 screening for T4P-dependent secretion: using only a single type of environmental condition may

421 not be the one in which a protein is made and secreted so it would not be discovered. Even 2-D
422 gels would be unlikely to have uncovered the presence of the BsaA oligomers due to their
423 extremely high mol weight. Therefore, it seems protein based mass spectrometry on samples from
424 a wide variety of environmental conditions would be optimal for detecting T4P-dependent
425 secretion substrates.

426 Since our results and that of Obana et al both showed that oligomers of BsaA were
427 extremely resistant to heat and other denaturants (Fig, S3D and (13)), this suggested that covalent
428 bonding might be involved. Subsequent analysis using AlphaFold 3 indicated there were likely
429 isopeptide bonds being formed by donor strand exchange involving an N35 of one monomer and
430 a K74 of another, catalyzed by an E56 residue in the recipient monomer (Fig. 4). This model was
431 confirmed by subsequent substitution of each residue involved with an Ala, which led to a
432 complete lack of oligomerization (Fig. 5). However, isopeptide bonds are formed relatively slowly
433 (22) so we hypothesized that the donor strand exchange resulted in formation of a temporally stable
434 interaction that gave the isopeptide bonds time to form. This does seem to be the case because
435 even in the N35A mutant, which cannot form an isopeptide bond, the oligomers were resistant to
436 denaturation in SDS-PAGE buffer and being run through an SDS-PAGE gel and only heating in
437 the SDS-PAGE buffer at 95 ºC led to complete depolymerization of the oligomer (Fig. S6). We
438 further characterized the formation of the oligomer and showed, using FAST fluorescent protein
439 fusions, that the oligomers were anchored to the surface of the bacteria (Fig. 6) but via the
440 cytoplasmic membrane and not directly to the PG layer (Fig. S7).

441 BsaB, the other biofilm protein encoded in the operon, did not form oligomers, did not
442 become covalently linked to BsaA oligomers (Fig. S8) and both BsaA and BsaB have no apparent
443 LPXTG motif present in their protein sequence (data not shown). These structural features seem

444 somewhat at odds with the presence of a sortase gene (*srtB*) adjacent to, but oriented in the opposite
445 direction, of the *sipW* gene (Fig. 2C). Obana et al (13) tested the premise that SrtB was responsible
446 for oligomerization of BsaA, but found that a *srtB* mutant had normal levels of oligomer present
447 (13). However, examination of the *bsa* operon in other strains of *C. perfringens* reveals that a
448 different gene synteny is in place, where *sipW* is followed by a *bsaA* homolog and two *bsaB*-like
449 genes, the first of which carries an identifiable LPXTG motif (Fig. 2D). The BsaB sequence from
450 strain 13, when subjected to a BLAST analysis (34) against other *C. perfringens* strains, shows
451 proteins with high levels of homology to either the N-terminal half or C-terminal half of the strain
452 13 BsaB, but not both. This suggests strain 13 underwent a genetic rearrangement event in which
453 the two *bsaB* homologs were recombined into a single gene with the loss of the LPXTG motif
454 found in the first gene (Fig. 2D). This brings up the distinct possibility that the SrtB sortase could
455 catalyze the cross-linking of the first BsaB ortholog to the PG layer in strains other than strain 13.
456 What role BsaB plays in BsaA oligomerization is still unknown, since a *bsaB* mutant still formed
457 similar amounts of oligomers of BsaA as the wild-type strain (13). However, based on the
458 differences in composition of BsaB homologs between strain 13 and other strains (Fig. 2C-D), it
459 is possible that BsaB is involved in anchoring the BsaA oligomers to the PG layer via the activity
460 of SrtB and the BsaB homolog with an LPXTG motif, but this is speculative.

461 Taking all of these results into consideration, we have constructed a model showing the
462 mechanisms underlying the assembly of BsaA into cross-linked oligomers (Fig. 8). In this model,
463 BsaA monomers are secreted via the Sec pathway, fold on the outer surface of the CM but are
464 anchored in the membrane by their N-terminal signal sequence. An oligomer of BsaA with the
465 most recent monomer having its N-terminal disordered region exposed, contacts the newly
466 secreted monomer and undergoes a donor strand exchange in which the N-terminal disordered

467 region binds to the exposed beta-sheet fold and interdigitates as a newly formed beta sheet (Fig.
468 4A). This is followed by cleavage of the signal sequence by the signal peptidase SipW. We believe
469 the SipW acts after oligomer binding to the newly secreted monomer because the BsaA oligomers
470 appear to be anchored to the membrane (Fig. S7) and the oligomers are in a relatively stable
471 assembly even before the isopeptide bonds are formed (Fig. S6). After SipW cleavage, the
472 oligomer is released and can then be used to add another monomer or be secreted through the PG
473 layer to the external medium. The presence of significant amounts of oligomers attached to the
474 bacterial surface (Fig. 6) suggests the monomers assemble in the space between the CM and PG
475 rather than being secreted as monomers for oligomerization in the environment. The mechanism
476 for export of the oligomers through the PG layer is not complete but there is evidence that T4P
477 play at least some role in the process (Fig. S4 and S5).

478 The phylogeny of BsaA-like proteins shows they are widespread amongst Gram-positive
479 bacteria but also present in some Gram-negative bacteria and archaea (Fig. 7). However, the
480 homologs that are predicted to form isopeptide bonds are more narrowly focused in the Clostridia
481 and Enterococcus genera (Fig. 7). This brings up the question as to what advantage isopeptide
482 bond formation bestows on the biofilm matrix properties of the bacteria that form them. Formation
483 of isopeptide bonds can greatly increase the mechanical resistance to distension and separation of
484 the BsaA fibers. As an example, the intramolecular isopeptide bonds formed in sortase dependent
485 pili made by Gram-positive bacteria have been shown to greatly increase the amount of force that
486 needs to be applied to distend the individual subunits, thereby strengthening the entire fiber (35).
487 To trap macrophages, neutrophils or other phagocytic cells, *C. perfringens* and other species may
488 have evolved a mechanism to form isopeptide bonds to increase the mechanical strength of the
489 fibers, since macrophages, neutrophils and other phagocytic cells can impart a large locomotive

490 force (1.9 to 10.7 nN) during their motility (36). It is also worth considering that *C. perfringens* is
491 an extremely ubiquitous bacterium in nature, found in soil, freshwater sediments and even extreme
492 environments like Antarctica (37, 38) and likely forms biofilms in these natural environments. The
493 BsaA biofilm matrix fibers could protect them from killing by amoeba, protozoa and other
494 predators found in these environments. Analyzing the relative mechanical strength of fibers with
495 and without isopeptide bonds will help answer some of these questions.
496

497 **Materials and Methods**

498 **Bacterial strains and growth conditions.** Bacterial strains, plasmids, and primers used in this
499 study are listed in Table 1. *Escherichia coli* strain DH10B was grown in Luria Bertani broth at
500 37°C for all transformations. When necessary, kanamycin and chloramphenicol were added to the
501 media at a concentration of 100 μg/ml and 20 μg/ml, respectively. *Clostridium perfringens* strain
502 HN13, a Δ*galKT* derivative of strain 13, was used as the wild type strain in this study. *C.*
503 *perfringens* strains were grown anaerobically in PGY (30 g proteose peptone #3, 20 g glucose, 10
504 g yeast extract, 1 g sodium thioglycolate per liter), brain-heart infusion (BHI) (Thermo Fisher) in
505 an anaerobic chamber (Coy Laboratory Products, Inc.). Anaerobic egg yolk agar medium was
506 prepared as previously described (https://www.fda.gov/food/laboratory-methods-food/bam-
507 media-m12-anaerobic-egg-yolk-agar#).
508 **Construction of in-frame deletions of TFP-associated genes.** In-frame deletions of TFP-
509 associated genes were made using the method of Nariya et al. (39), modified as described in
510 Hendrick et al (40). The primers used to amplify the flanking DNA for each gene are listed in
511 Table S1. All deletions were confirmed by PCR across the deleted region. Complementation of
512 the in-frame *pilA3* mutant (Fig. 2A-B) was done using the lactose-inducible promoter vector

513 pKRAH1 (14) to express the *pilA3* gene. The construct was made by PCR using the primers listed
514 in Table S8.

515 **SDS-PAGE and staining of secretome proteins.** Cultures of strain HN13 were grown for 16 h
516 in BHI medium (i.e., into stationary phase) and the bacteria were removed by centrifugation in a
517 table top centrifuge. The culture supernatants were removed and passed through a 0.45 μM filter.
518 Proteins in the culture medium were precipitated using trichloroacetic acid (TCA) precipitation
519 and washed twice in acetone. After drying, sample pellets were resuspended in water and then 4X
520 SDS-PAGE sample buffer (41) was added to a final concentration of 1 X. Secretome samples
521 were separated on an SDS-PAGE gel and then stained with the fluorescent protein stain, Sypro-
522 Ruby according to the manufacturer's instructions (Thermo Fisher). Stained gels were imaged
523 with a Gel-Doc Imaging system (Bio-Rad, Inc).

524 **Cloning the *bsa* operon into the lactose-inducible expression vector, pKRAH1.** Because the
525 SipW signal peptidase is likely needed for processing the secretion signal on BsaC (13), to analyze
526 secretion of BsaC in TFP gene associated mutants, the entire *sipW-basA-bsaB-bcaC* operon was
527 expressed under control of the lactose inducible promoter in plasmid pKRAH1 (14) using primers
528 OHL145 (PstI site) and OHL146 (BamHI site) by PCR (Table 1 and Table S3). The PCR fragment
529 was ligated to the PCR cloning vector pGEM-T to make pHLL64. pHLL64 and pKRAH1 were
530 digested with PstI and BamHI and ligated to make pHLL65.

531 **Construction of a FAST protein fusion to BsaA.** The gene fusion was constructed using
532 overlapping PCR of the FAST coding region on plasmid pAG104 FAST (25) to the *bsaA* gene
533 linked to *sipW* (i.e., the *bsaB* and *bsaC* genes were absent). The FAST protein was fused to the C-
534 terminal end of BsaA because of the processing of the signal sequence on the N-terminal end. The
535 primers used to construct the gene fusion are listed in Table S5.

536 **Production of antibodies to BsaC, PilA3 and immunofluorescence microscopy.** Rabbit
537 polyclonal antibodies against the BsaC peptide sequence N-CNIEGDKISWDRGEN-C were
538 produced by GenScript USA, Inc. along with pre-immune serum as a negative control. Rabbit
539 polyclonal antibodies against the PilA3 peptide sequence N-CIKIDNSKDSIDLTR-C were
540 produced by Yenzyme Antibodies, LLC. along with pre-immune serum as a negative control. To
541 test for surface exposure of PilA3 pilins, the bacteria were fixed with 2.5% paraformaldehyde in
542 Dulbecco's phosphate buffer saline (DPBS), incubated with rabbit serum with anti-PilA3
543 antibodies, washed with DPBS and incubated with goat anti-rabbit antibodies fluorescently tagged
544 with Alexafluor 647 (Invitrogen). Stained cells were imaged on an Olympus IX71 fluorescent
545 inverted microscope equipped with a CoolSnap HQ2 CCD camera and DeltaVision deconvolution
546 and image analysis software.
547 **Quantitative immunoblotting of secreted BsaC.** Overnight cultures were grown in BHI
548 supplemented with chloramphenicol, and diluted 1:50 into fresh BHI medium pre-incubated at
549 37ºC. The cultures were grown for one hour then induced by adding a 500 mM lactose stock
550 solution, to a final concentration of 10 mM, to the growth media every 30 min for 3 h. Following
551 induction, 1 mL of culture supernatant was collected by centrifugation, and proteins precipitated
552 using TCA, as described above. Resulting precipitates were resuspended in 50 uL of SDS-PAGE
553 loading buffer with 100 mM DTT, the pH was adjusted using one µL of 1.5 M Tris-HCl, pH 9, if
554 necessary. Five µL of sample was loaded per well, and SDS-PAGE was performed. Following
555 SDS-PAGE, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane utilizing
556 the iBlot system (Bio-Rad, Inc), and developed with serum containing anti-BsaC antibodies at
557 1:250 dilution, followed by a DyLight 550 labeled fluorescent secondary goat anti-rabbit antibody

558 (Thermo-Fisher). Imaging was performed on a Typhoon gel scanner, and mean pixel intensity
559 was measured for each band utilizing the Amersham Typhoon densitometry control software.
560 **Purification of BsaC.** The *bsaC* gene with a C-terminal His_6 tag was cloned into the expression
561 vector pET24a by PCR using the primers listed in Table S4. An overnight culture of *E. coli*
562 LOBSTR pET24a::BsaC-His_6 was used to inoculate 2 L of 37ºC pre-warmed terrific broth in a 4
563 L beveled flask. The culture was allowed to grow to 0.6 OD_{600} where it was induced with 1 mM
564 IPTG. After 6 hours of induction, the culture was centrifuged to remove cells (BsaC-His_6 is
565 secreted by *E. coli*), and the resulting supernatant was precipitated with 90% ammonium sulfate.
566 Precipitated protein was solubilized in PBS, and used to load on to an ÄKTA purifier FPLC for
567 Ni-NTA chromatography with PBS with 10 mM imidazole as loading buffer, and PBS with 1M
568 imidazole as elution buffer. Eluted fractions were concentrated with an Amicon Ultra 2 mL 10 K
569 cutoff centrifugation columns. Concentrated elution fractions were then further processed using a
570 Superose 6 gel filtration column.
571 **Immunoblotting of BsaA oligomers.** BsaA oligomer secretion by *C. perfringens* was analyzed
572 using western blots and slot blots. For the immunoblot experiments, the proteins were blotted onto
573 a PVDF membrane while submerged in cold buffer which contained 3 mM Na_2CO_3, 10 mM
574 $NaHCO_3$ and 20% methanol.
575 **Immunoblotting Method One.** The membranes were placed in a Snap i.d. 2.0 system
576 (Millipore Sigma) which uses a vacuum system to pull solutions through the membrane (referred
577 to a swashing steps below). Once set up, a solution containing Tris-buffered saline (TBS) (pH 7.4),
578 0.1% Tween 20 (TBST), and 1% bovine serum albumin (BSA) was added as a blocker. Both
579 primary and secondary antibodies were added to separate aliquots of the solution containing TBST
580 and 1% BSA. In the first step, the primary antibody solution was incubated for 10 minutes on the

581 membrane, followed by three washings with TBST. Then the secondary antibody solution was left
582 to sit for 10 minutes on the membrane, followed by three washings with TBST. The primary
583 antibody utilized was OctA-probe H-5 (1:250 dilution) used to detect FLAG (Santa Cruz
584 Biotechnology). The secondary antibody utilized for these experiments was StarBright700 goat
585 anti-mouse (Bio-Rad) (1:10,000 dilution). The fluorescently labeled immunoblots were imaged
586 using a ChemiDoc MP imaging system (Bio-Rad), and the intensity of the fluorescence was
587 measured using the ChemiDoc MP imaging software for densitometry measurements.

588 **Immunoblotting method 2**. Membranes were transferred to a container and blocked with
589 EveryBlot blocking Buffer (Bio-Rad). Antibodies were diluted with EveryBlot blocking buffer and
590 applied to membranes, followed by a minimum of five washes with TBST.

591 The primary antibodies utilized included OctA-probe H-5 (1:227 dilution) used to detect
592 FLAG, and HA-Tag F-7 (1:227 dilution) used to detect HA (both from Santa Cruz Biotechnology,
593 Santa Cruz, CA). Membranes submerged in EveryBlot blocking buffer with primary antibody were
594 incubated at 4°C overnight. The secondary antibody utilized for these experiments was
595 StarBright700 goat anti-mouse (Bio-Rad) (1:13,333 dilution), incubated for one hour at room
596 temperature. Images of the immunoblots were taken using a ChemiDoc MP imaging system (Bio-
597 Rad), to measure the intensity of the fluorescence.

598 **Slot blots to detect secretion of BsaA oligomers**. For slot blots, supernatants samples were
599 prepared by diluting supernatants with sterile BHI. For initial experiments, samples of 100% pure
600 culture supernatant, 50% culture supernatant and 50% blank media, 25% culture supernatant and
601 75% blank media, 10% culture supernatant and 90% blank media, 1% culture supernatant and 99%
602 blank media, and 100% blank media were prepared. For later experiments, samples of 50% culture
603 supernatant and 50% blank media, 20% culture supernatant and 80% blank media, 10% culture

604 supernatant and 90% blank media, 5% culture supernatant and 95% blank media, 1% culture
605 supernatant and 99% blank media, and 100% blank media were prepared. The 10% culture
606 supernatant samples were most often used to collect the raw intensity data for quantitative results.
607 A PVDF membrane was soaked in TBST prior to being loaded into the slot blot device
608 while still damp. The slot blot was loaded with TBST in each slot for 10 minutes. The TBST was
609 pulled through by vacuum, and then the diluted supernatants for the slot blots were loaded into the
610 individual slots of the device, and allowed to sit for 10 minutes. The supernatants were pulled
611 through by vacuum, and then the slot blot were loaded with TBST in each slot for another 10
612 minutes. The TBST was vacuumed off as well, and the membrane was developed using
613 immunoblotting method 2.
614 **Western blots on *bsaA* mutants.** Western blots to detect oligomerization of BsaA mutants were
615 performed by expressing the *sipW-bsaA-His*$_6$ genes under control of the lactose-inducible
616 promoter. Supernatants were separated form cell pellets by centrifugation. The proteins in the
617 supernatant were concentrated by TCA precipitation (as described above), suspended in SDS-
618 PAGE loading buffer with 100 mM DTT and heated at 95 °C for 20 min. The bacteria in the cell
619 pellet were suspended in PBS and disrupted by bead beating with 0.1 mm zirconia-silica beads.
620 The beads were then removed by centrifugation and the cell extract brought to 1 X SDS-PAGE
621 buffer with 100 mM DTT. After running on SDS-PAGE gels, the proteins were transferred to a
622 PVDF membrane and developed using immunoblotting method 2 above, except the primary
623 antibody was mouse monoclonal anti-His6 antibody His.H8 (Santa Cruz Biotechnology).
624 **Modeling BsaA Structure.** BsaA from *Clostridium perfringens* (accession number
625 WP_011009871) was created as a homotrimer using ColabFold v1.3.0 (18) based on AlphaFold
626 v2.2.0 (19). Amber minimization was not enabled. In the top ranked model based on pLDDT

PyMOL v2.5.0 was used to analyze side chains of interdigitated beta strands for potential sidechain crosslinks. Images were created using ChimeraX v1.2. (42, 43) The same trimer of BsaA was created in AlphaFold 3 (24) to observe if the new version creates crosslinks, and all trimer models made did contain the crosslinks. Structures of BsaC and BsaA were created with AlphaFold 3 (24).

Phylogeny of BsaA-like proteins. BsaA-like protein sequences were identified with 3 iterations of jackhmmer (44) (HmmerWeb version 2.41.2) (45) using BsaA (UniProt:Q8XN23) from *C. perfringens* as the seed. The protein sequence similarity network of proteins identified either with jackhmmer or as a match to IPR022121 was constructed using the EFI-EST tool (https://efi.igb.illinois.edu/efi-est/) (46, 47) with an alignment score of 10. Nodes were collapsed at a sequence identity of 100%. The network was visualized with Cytoscape (https://www.cytoscape.org) (48) using the Prefuse Force Directed OpenCL Layout. Gene neighborhoods were retrieved using the EFI-GNT tool (https://efi.igb.illinois.edu/efi-gnt/) (47). The presence of a N-E-K triad was determined by manual inspection of the multiple sequence alignment. For phylogenetic reconstruction, sequences were aligned with MAFFT on XSEDE using CIPRES (49, 50), edited in Jalview (51), and analyzed with IQ-Tree (52-54). Maximum likelihood trees were constructed using the LG+F+G4 substitution model and ultrafast bootstrap (1000 replicates). Consensus trees were visualized, rooted at the midpoint, and annotated with iTOL (55). Data files are available in Supplemental File Data Set 1.

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848

849 **Figure legends**

850 **Figure 1.** Diagram showing the type IV pili-associated genes in *C. perfringens* strain 13. Figure
851 derived from reference (9).

852

853 **Figure 2. (A).** SDS-PAGE gel showing the proteins in the secretome of the respective strains. The
854 gel was stained with the fluorescent protein dye Sypro-Ruby, the colors inverted to make the
855 protein bands in the samples appear dark but the pre-stained molecular weight markers appear as
856 bright bands. **(B).** Western blot showing the levels of secretion of the BsaC protein in different
857 strains. The secondary antibody was fluorescently tagged for visualization. Purified BsaC was
858 added in lane 5 as a positive control for detecting BsaC. **(C).** The gene synteny of the *bsa* operon
859 and adjacent sortase-encoding gene in *C. perfringens* strain 13. **(D).** The gene synteny of the *bsa*
860 operon and adjacent sortase-encoding gene in *C. perfringens* strain ATCC 13124 and most other
861 strains of *C. perfringens* that were observed. The sections of the *bsaB* gene from strain 13 that
862 were homologous to those in strain ATCC 13124 are labeled, along with the location of an LPXTG
863 sortase dependent sequence in one of the *bsaB*-encoding genes.

864

865 **Figure 3.** Results from densitometry analysis of western blots from the secretome of strains with
866 in-frame deletions of T4P-encoding genes. Each strain contained plasmid pGC1 for regulated
867 expression of the *bsa* operon with a His_6 tag added to the *bsaC* gene. The role of each gene product
868 in pilus function is shown in Fig. 1. For each experiment, the relative fluorescence intensity of the
869 wild-type strain (HN13) was set at 1.0 and the relative fluorescence intensity for each other strain
870 in the same experiment was then set relative to that value. Each strain was tested at least three
871 times with triplicate samples for each experiment. All of the experimental runs were combined and

872 one sample t and Wilcoxon tests were run to determine if the mutant strains were statistically
873 different from 1 and asterisks indicate strains that were different ($P < 0.05$) from strain HN13.
874
875 **Figure 4. (A)** AlphaFold model of a homotrimer of BsaA in cartoon representation. Each
876 independent monomer is colored separately. Residues 28-38 of a monomer incorporate themselves
877 into the beta strands of an adjacent monomer. **(B)** Close-up view of interdigitated beta strands from
878 two monomers in cartoon representation, showing relevant side chains as sticks colored by
879 element. N35 and the giving chain are colored purple, E56, K74, and the receiving monomer are
880 colored cyan. Side chains forming the hydrophobic pocket are colored brown. The bond created
881 by AlphaFold between N35 and K74 is between an oxygen and nitrogen due to limitations in
882 AlphaFold; in reality, an amide bond would form between the side chains with ammonia as the
883 leaving group. **(C)** Close-up view of the N35-E56-K74 catalytic triad, showing the close proximity
884 of the catalytic E56 residue to the bond forming N35 and K74. All images were created with
885 ChimeraX v1.2. (42, 43).
886
887 **Figure 5.** Western blots showing loss of oligomer formation in BsaA with mutations in residues
888 involved in isopeptide bond formation**. (A)** Oligomer formation seen in TCA precipitated culture
889 supernatants or in the disrupted whole cell extracts of strains producing BsaA with single
890 mutations. **(B)** Comparison of BsaA oligomer formation in the N35A and N35A/K74A mutants in
891 a wild-type background (left side) and one in which the chromosomal copy of the *bsaA* gene was
892 deleted. Note that the chromosomal *bsaA* mutation did not change the oligomerization levels of
893 the mutants. **(C)** Western blot showing mutation of the E56 residue to Ala abolished
894 oligomerization of BsaA. P, disrupted cell pellet; S, TCA precipitate of culture supernatant.

895

896 **Figure 6.** BsaA oligomers are found on the bacterial surface. **(A)** Expression of a FAST protein
897 fusion to the cytoplasmic PilT protein (pSM413) only shows fluorescence with the membrane
898 permeable Coral dye. **(B)** A BsaA-FAST fusion protein (pSM412) fluoresces in the presence of
899 both a membrane permeable dye (Coral) and a membrane impermeable dye (Amber-NP),
900 indicating it is surface exposed to these dyes.

901

902 **Figure 7.** BsaA phylogeny**.** (**A**) A consensus tree of representative BsaA-like proteins. Leaf labels
903 are colored by taxonomy according to the color key. BsaA, BsaB, and two other BsaA-like proteins
904 identified in *C. perfringens* are labeled, as are TasA from *B. subtilis* and CalY from *B. cereus*.
905 Bootstrap values are represented as scaled circles according to the inset key. (**B**) Sequence
906 similarity network of identified BsaA-like proteins. Nodes representing labelled *Clostridium* and
907 *Bacillus* proteins in panel A are labeled with an arrow. Nodes are colored by taxonomy according
908 to the color key. Proteins with conserved residues that would be expected to be involved in
909 formation of a spontaneous intermolecular isopeptide bond are represented by triangle nodes with
910 a red outline. (**C**) Example gene neighborhoods of genes encoding BsaA-like proteins and genes
911 encoding putative surface filament structures. The complete matrix tree data set is included in a
912 Supplemental Information file.

913

914 **Figure 8.** Model of BsaA oligomerization. BsaA monomers (purple ovals) are oligomerized by
915 isopeptide bond formation after secretion via the Sec system in *C. perfringens*. SipW is the signal
916 peptidase for BsaA secretion (Fig. 2C and (13)). Secretion of the oligomers past the PG layer may
917 be due to the action of T4P-associated proteins (Fig. S4).

918

919 **Table 1.** Strains and plasmids used in this report.

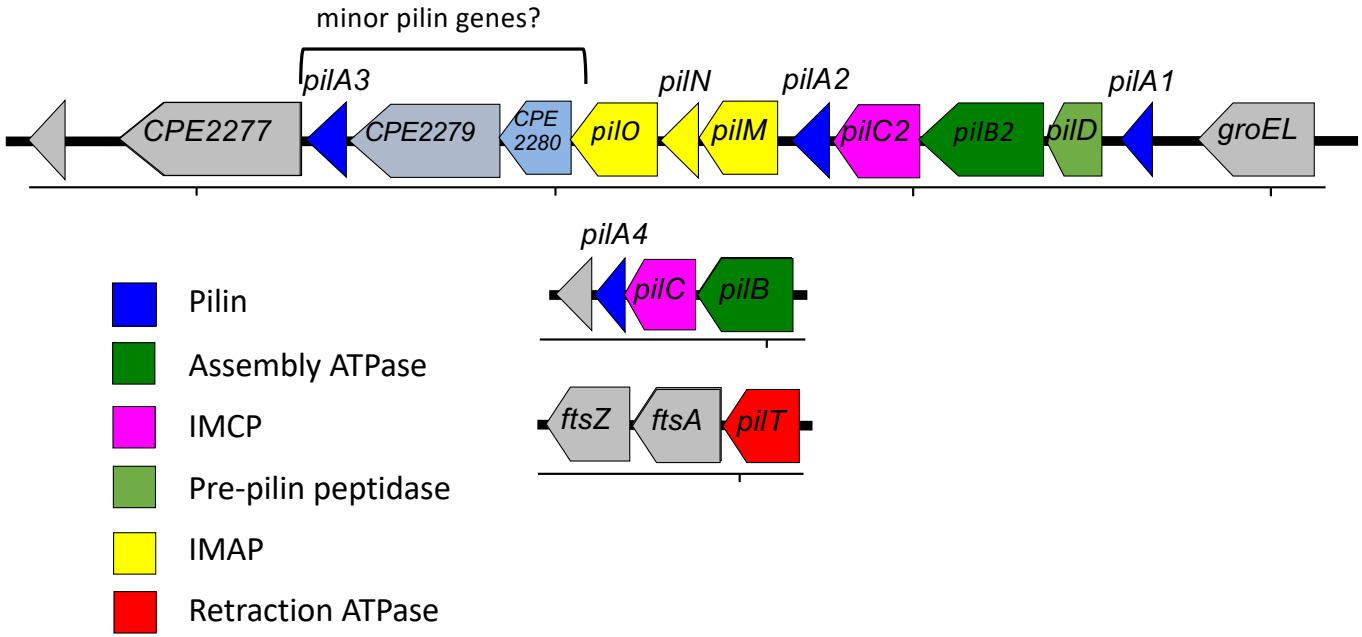
Strain or plasmid	Relevant characteristics	Reference or Source
Strains		
Clostridium perfringens type A strain HN13	*ΔgalKT*	(39)
Escherichia coli strain DH10B	F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L *nup*G λ–	(56)
Escherichia coli strain LOBSTR	Derivative of strain BL21, designed for low background binding to Ni-affinity columns	(57)
Plasmids		
pKRAH1	Cm^r; Lactose-inducible expression system plasmid for use in *C. perfringens*	(14)
pET24a	Protein expression vector	Novagen
pCRBluntIITOPO	PCR cloning vector	Invitrogen
pBAD30	Amp^r; Arabinose-inducible expression system plasmid for use in *E. coli*	(58)
pKRAH1-*pilA3*	Expression of *pilA3* for complementation	This study
pHLL65	The *bsa* operon in pKRAH1	This study
pGC1	pHLL65 with a His_6 tag added to the C-terminus of *bsaC*	This Study
pGC8	Vector based on pET24-a; used to express and purify VWA-His6 in *E. coli*	This Study
pSK1	The *bsa* operon in pKRAH1 with a FLAG-tag added to the C-terminus of *bsaA*	This Study
pSK2	Same as pSK1 with an HA tag at the C-terminus of *bsaB*	This Study
pSK4	The *sipW* and *bsaA* genes in pKRAH1 with a FLAG tag at the C-terminus of *bsaA*.	This Study

pSM407	The *sipW* and *bsaA* genes in pKRAH1 with a His_6 tag at the C-terminus of *bsaA*.	This Study
pSM408	pSM407 with a N35A mutation in *bsaA*	This Study
pSM409	pSM407 with a K74A mutation in *bsaA*	This Study
pSM410	pSM407 with N35A/K74A mutations in *bsaA*	This Study
pSM411	pSM407 with E56A mutations in *bsaA*	This Study
pSM412	pSM407 with a FAST gene fusion at the C-terminus of *bsaA* in place of the His_6 tag	This Study
pSM413	A *pilT*-FAST gene fusion in pKRAH1	This Study

920

921

Figure 1


minor pilin genes?
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pilN
pilA2
pilA1
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CPE2279
CPE 2280
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pilB2
pilD
groEL
pilA4
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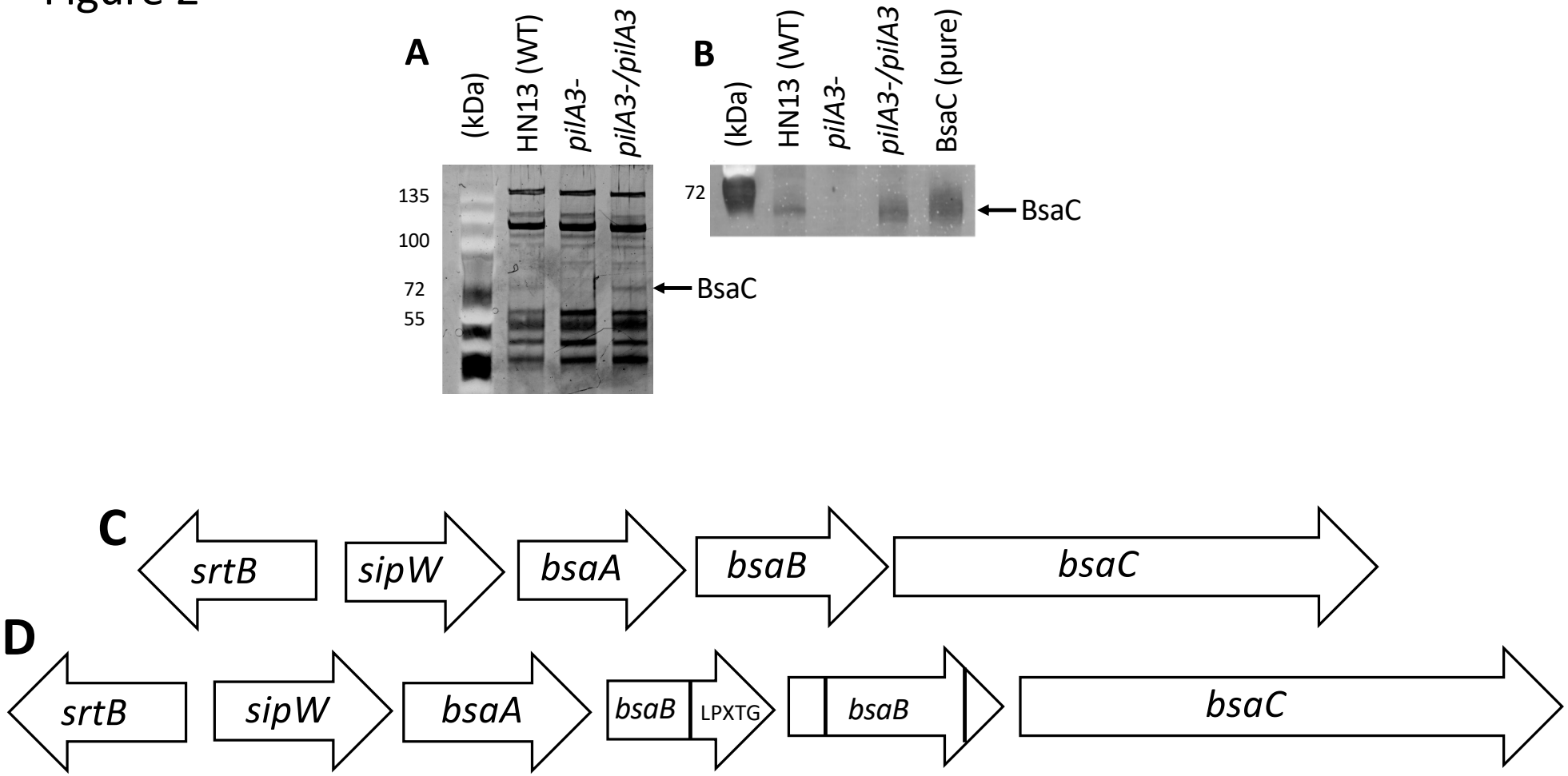
Pre-pilin peptidase
IMAP
Retraction ATPase

Figure 2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(kDa)
HN13 (WT)
pilA3-
pilA3-/pilA3
135
100
72
55
BsaC
B
(kDa)
HN13 (WT)
pilA3-
pilA3-/pilA3
BsaC (pure)
72
BsaC
C
srtB
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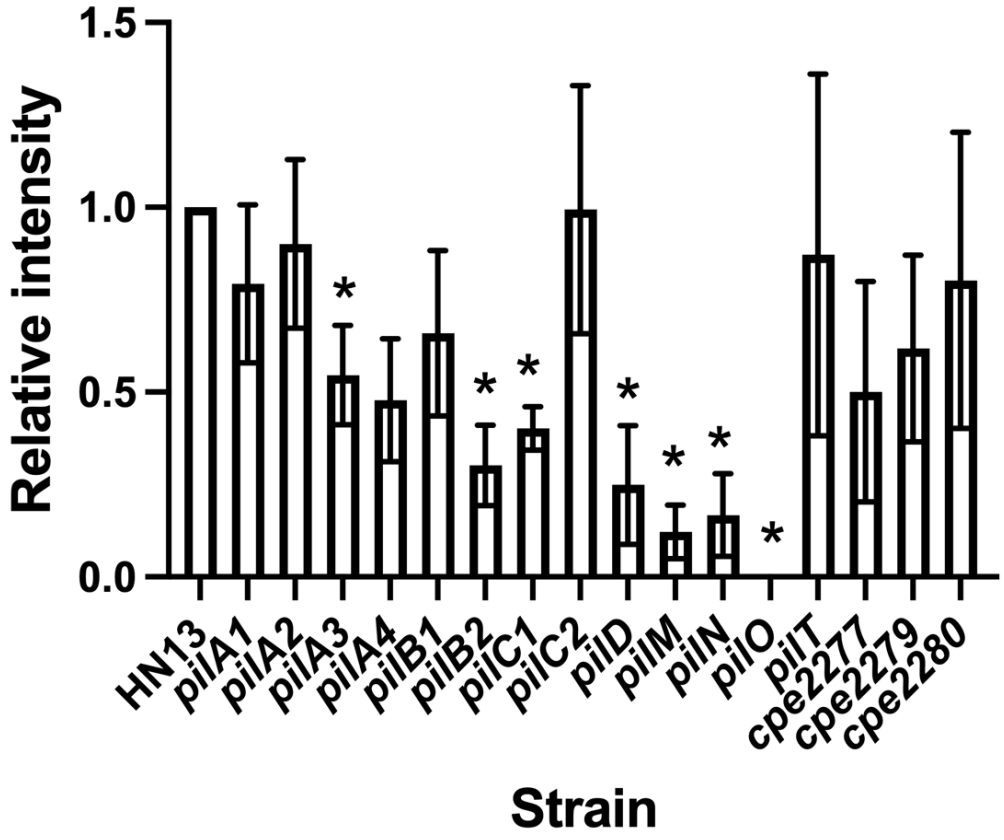
bsaB
bsaC

Figure 3


1.5
1.0
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Relative intensity
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HN13
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pilA4
pilB1
pilB2
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Strain

Figure 4

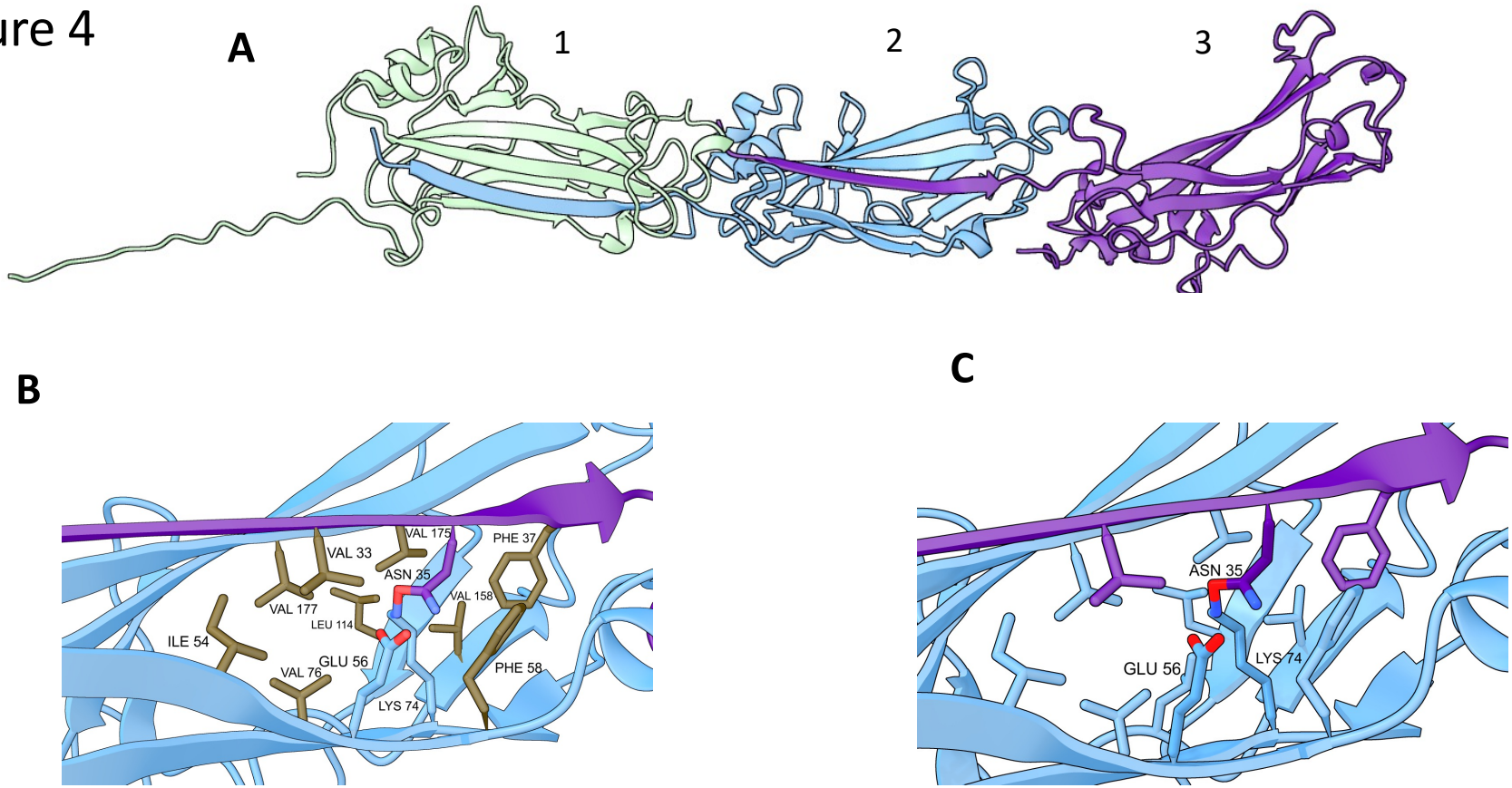


Figure 1: **(A)** Alphafold model of a homotrimer of BsaA in cartoon representation. Each independent monomer is colored separately. The signal sequence (residues 1-27) are hidden in this image, though were included in the Alphafold model. Residues 28-38 of a monomer incorporate themselves into the beta strands of an adjacent monomer. (B) Close-up view of interdigitated beta strands from two monomers in cartoon representation, showing relevant side chains as sticks colored by element. N35 and the giving chain are colored purple, E56, K74, and the receiving monomer are colored cyan. Side chains forming the hydrophobic pocket are colored brown. The bond created by Alphafold between N35 and K74 is between an oxygen and nitrogen due to limitations in Alphafold; in reality, an amide bond would form between the side chains with ammonia as the leaving group.

Figure 5

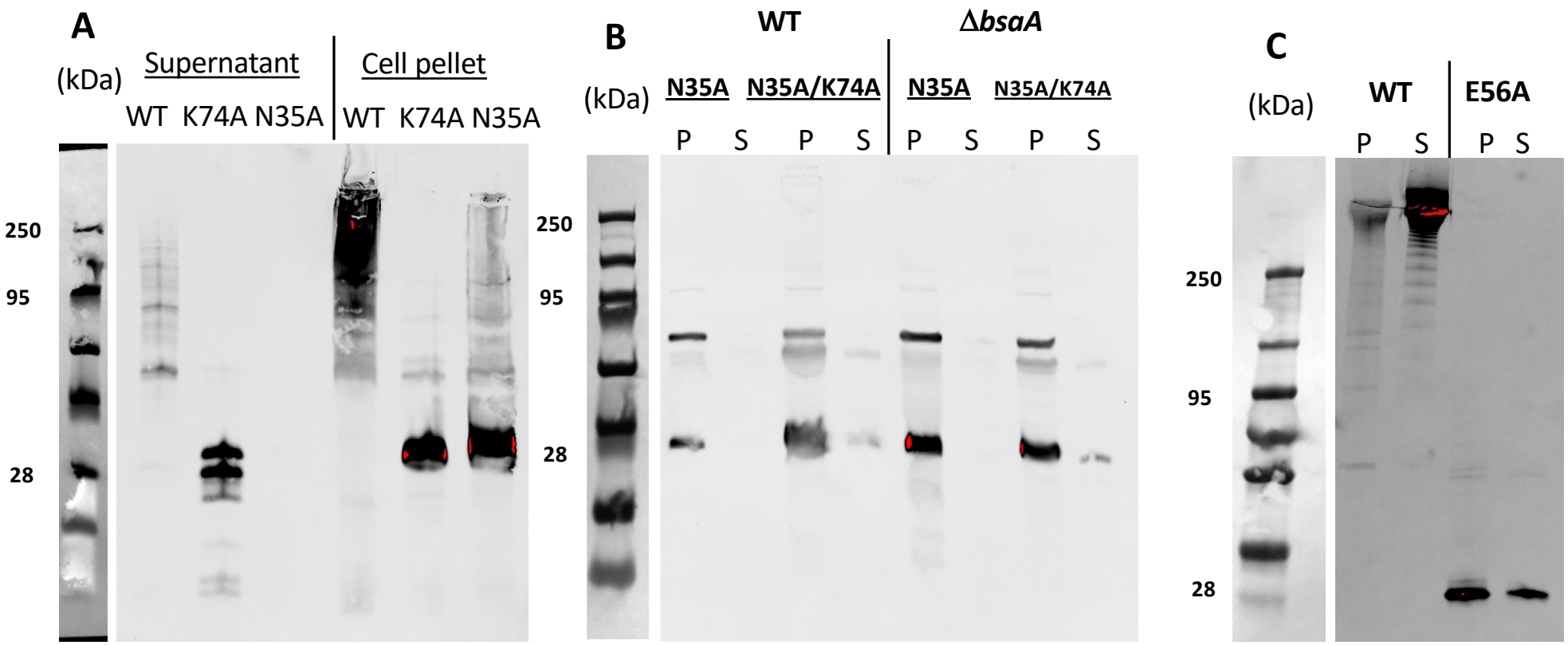
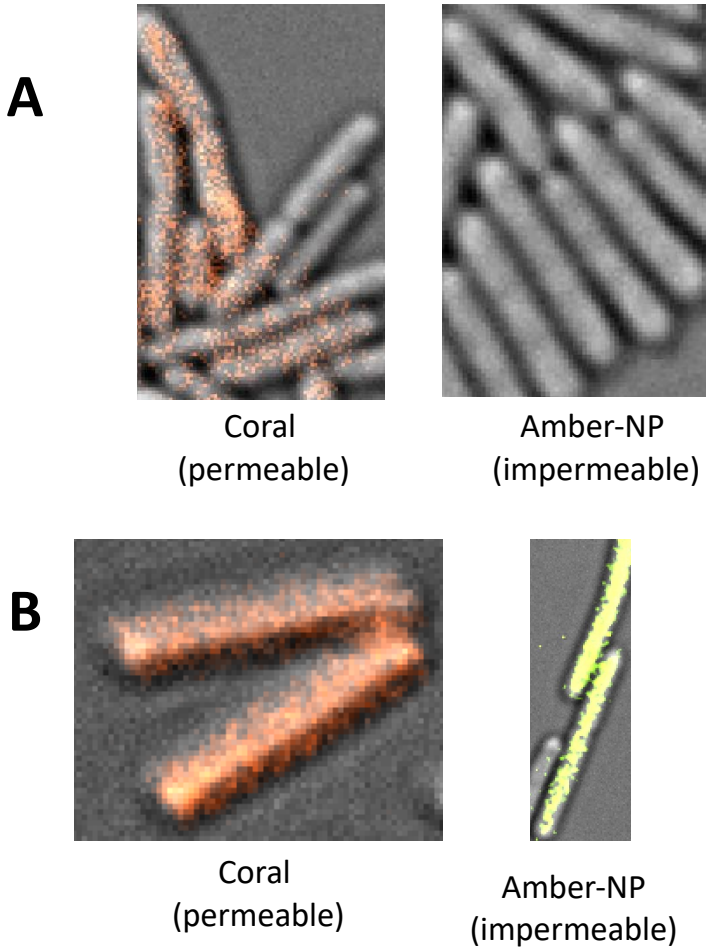

A
(kDa)
Supernatant
Cell pellet
WT K74A N35A
WT K74A N35A
250
95
28
B
(kDa)
WT
ΔbsaA
N35A
N35A/K74A
N35A
N35A/K74A
P S P S P S P S
250
95
28
C
(kDa)
WT
E56A
P S P S
250
95
28

Figure 6


A
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Amber-NP
(impermeable)
B
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(permeable)
Amber-NP
(impermeable)

Figure 7

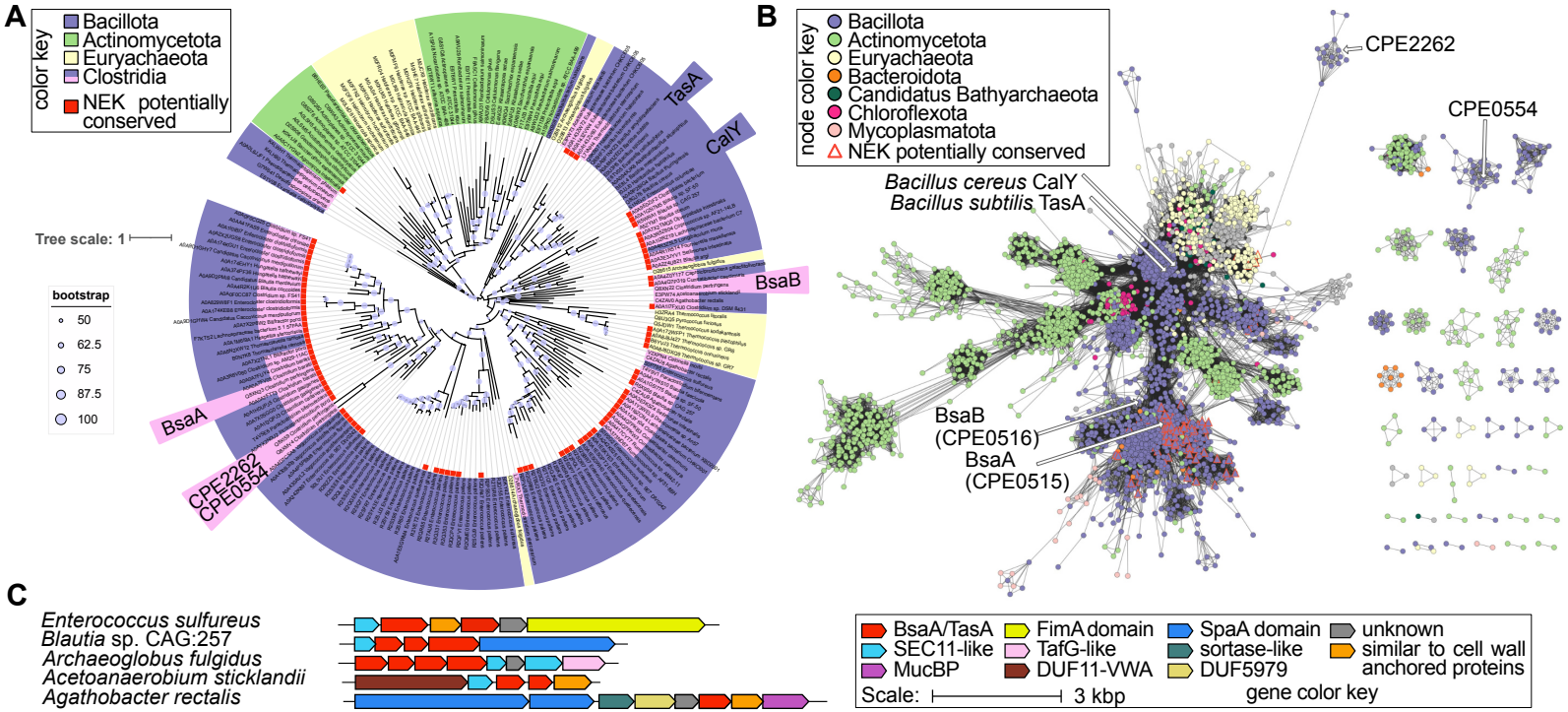
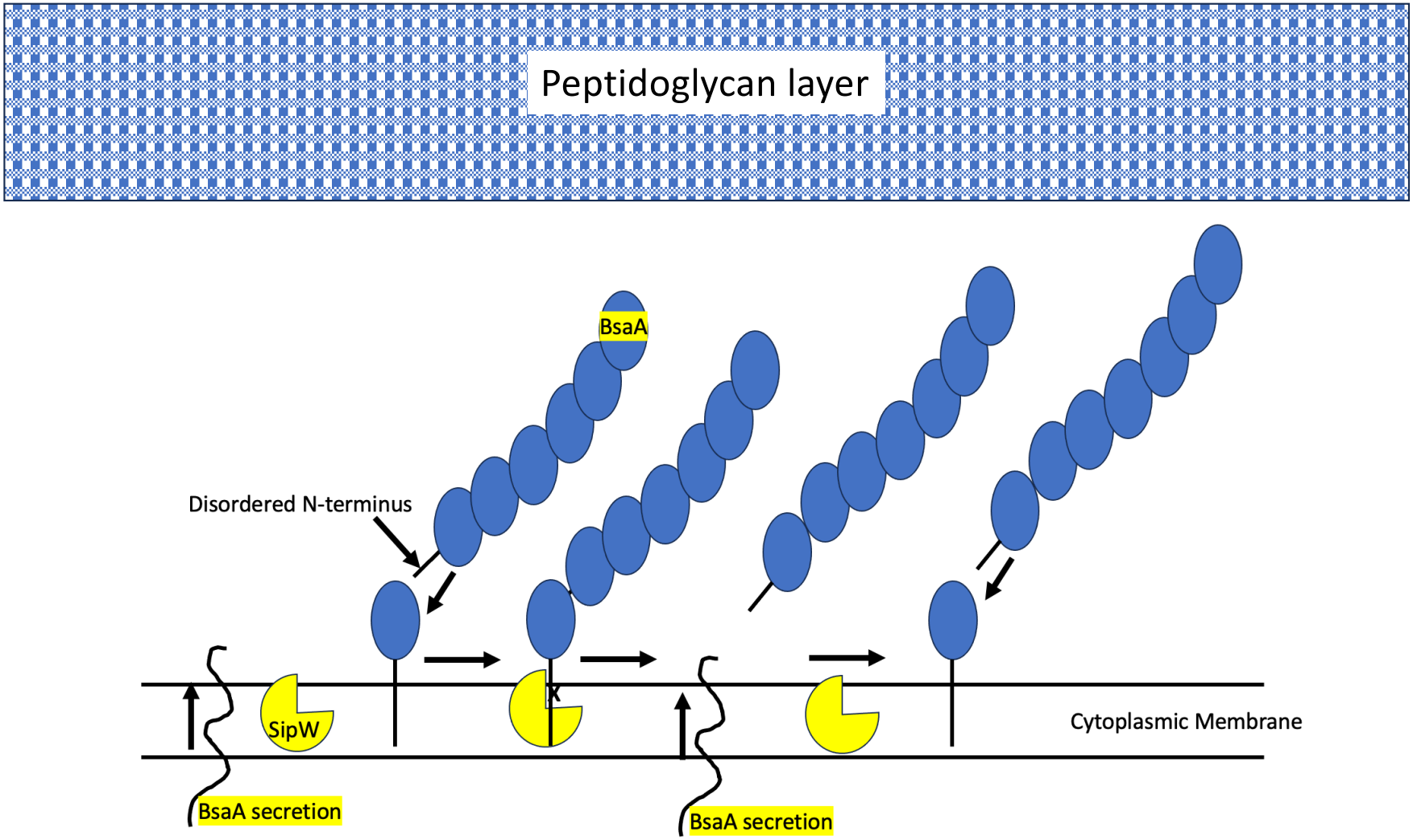


Figure BsaA and friends phylogeny. A, consensus tree of representative BsaA-like proteins. Leaf labels are colored by taxonomy according to the color key. BsaA, BsaB, and two other BsaA-like proteins identified in *C. perfringens* are labeled, as are TasA from *B. subtilis* and CalY from *B. cereus*. Bootstrap values are represented as scaled circles according to the inset key. **B**, sequence similarity network of identified BsaA-like proteins. Nodes representing labelled Clostridium and Bacillus proteins in panel A are labeled with an arrow. Nodes are colored by taxonomy according to the color key. Proteins with conserved residues that would be expected to be involved in formation of a spontaneous intermolecular isopeptide bond are represented by triangle nodes with a red outline. **C**, example gene neighborhoods of genes encoding BsaA-like proteins and genes encoding putative surface filament structures.

Figure 8


Peptidoglycan layer
BsaA
Disordered N-terminus
SipW
Cytoplasmic Membrane
BsaA secretion
BsaA secretion