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3 **Title: The type IX secretion system is required for virulence of the fish pathogen**
4 ***Flavobacterium psychrophilum*.**

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6

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32 Running Title: Type IX secretion and *F. psychrophilum* virulence

33

34 **ABSTRACT:** *Flavobacterium psychrophilum* causes bacterial cold-water disease in wild
35 and aquaculture-reared fish, and is a major problem for salmonid aquaculture. The mechanisms
36 responsible for cold-water disease are not known. It was recently demonstrated that the related
37 fish pathogen, *Flavobacterium columnare*, requires a functional type IX protein secretion system
38 (T9SS) to cause disease. T9SSs secrete cell-surface adhesins, gliding motility proteins,
39 peptidases, and other enzymes, any of which may be virulence factors. The *F. psychrophilum*
40 genome has genes predicted to encode components of a T9SS. Here, we used a SacB-mediated
41 gene deletion technique recently adapted for use in the *Bacteroidetes* to delete a core *F.*
42 *psychrophilum* T9SS gene, *gldN*. The Δ *gldN* mutant cells were deficient for secretion of many
43 proteins in comparison to wild-type cells. Complementation of the mutant with wild-type *gldN*
44 on a plasmid restored secretion. Compared to wild-type and complemented strains, the Δ *gldN*
45 mutant was deficient in adhesion, gliding motility, and in extracellular proteolytic and hemolytic
46 activities. The Δ *gldN* mutant exhibited reduced virulence in rainbow trout and complementation
47 restored virulence, suggesting that the T9SS plays an important role in the disease.

48 **IMPORTANCE:** Bacterial cold-water disease, caused by *F. psychrophilum*, is a major
49 problem for salmonid aquaculture. Little is known regarding the virulence factors involved in
50 this disease, and control measures are inadequate. A targeted gene deletion method was adapted
51 to *F. psychrophilum* and used to demonstrate the importance of the T9SS in virulence. Proteins
52 secreted by this system are likely virulence factors, and targets for the development of control
53 measures.

54 The fish pathogen *Flavobacterium psychrophilum* is a major cause of disease and
55 mortality for aquaculture-reared salmonids, such as rainbow trout (*Oncorhynchus mykiss*) (1-3).
56 Ayu (*Plecoglossus altivelis*), European eel (*Anguilla anguilla*), and other fish are also impacted
57 by this pathogen (2-5). *F. psychrophilum* infections cause bacterial cold-water disease (BCWD),
58 which often results in erosion of the tail, destruction of tissues near the dorsal fin (saddleback
59 lesion), and systemic spread to internal organs such as the spleen and kidney. In young fish
60 mortality may occur rapidly and without obvious surface lesions, a condition sometimes referred
61 to as rainbow trout fry syndrome. *F. psychrophilum* infections are a challenge to sustainable
62 aquaculture of salmonids, and result in large economic losses (6). The mechanisms used by *F.*
63 *psychrophilum* to cause disease are poorly understood and methods to prevent or control
64 outbreaks are inadequate.

65 Antibiotic treatments have been the primary method to control outbreaks of BCWD (2,
66 7). The expense of such treatments, the danger of development of resistant strains, and the
67 potential spread of antibiotic resistance genes to other bacteria make this a poor solution.
68 Vaccine development has been complicated by the need to protect young fry that may not have
69 fully developed immune systems, uncertainties regarding the most protective antigens, and the
70 need to efficiently administer the vaccine to large numbers of fish (6). Bacteriophages are
71 promising tools to control outbreaks, but additional studies are needed to determine if this
72 approach will be broadly successful (8). Rainbow trout lines resistant to *F. psychrophilum* have
73 also been developed and used to control the disease (9-11). Improved understanding of the
74 mechanisms used by *F. psychrophilum* to cause disease could aid the continued development of
75 these and other measures to prevent or control BCWD outbreaks.

76 Previous studies identified *F. psychrophilum* genes and proteins that may be involved in
77 disease. Peptidases, and other secreted enzymes, and cell-surface adhesins are suspected to be
78 involved (1, 2, 12, 13). *F. psychrophilum* gliding motility, which allows cells to crawl over
79 surfaces, has also been suggested as a potential virulence factor (7, 14). Genome analyses suggest
80 that *F. psychrophilum* uses the type IX secretion system (T9SS) to secrete many proteins to the
81 cell surface and beyond (15, 16). T9SSs are common in, but apparently confined to, members of
82 the phylum *Bacteroidetes* (16-20). T9SSs secrete cell-surface adhesins, and soluble or cell-
83 associated peptidases, nucleases, and other hydrolytic enzymes. T9SSs also secrete motility
84 adhesins to the cell surface and are thus required for gliding motility (21-23). These adhesins are
85 propelled along the cell surface by the rest of the gliding motility machinery, resulting in cell
86 movement (24). Core components of the T9SS include the cytoplasmic membrane proteins GldL
87 and GldM, the periplasmic protein GldN, the lipoprotein GldK, and the outer membrane protein
88 SprA (25-29). These proteins are thought to form an envelope spanning complex that secretes
89 proteins through the outer membrane SprA channel (22, 25, 27). GldL and GldM have been
90 proposed to harvest the proton gradient across the cytoplasmic membrane to power both secretion
91 and gliding (22, 27). Additional proteins involved in secretion are associated with these core
92 components (18-20).

93 Proteins secreted by the T9SS have N-terminal signal peptides that facilitate their export
94 across the cytoplasmic membrane by the Sec system, and C-terminal domains (CTDs) that target
95 them to the T9SS for secretion across the outer membrane (18-20). Most characterized T9SS
96 CTDs belong to either the TIGRFAM protein domain family TIGR04183 (type A CTDs) or to
97 TIGR04131/pfam13585 (type B CTDs) (30, 31). Type A CTDs are typically removed during or
98 after secretion (32), but removal of type B CTDs has not been carefully examined. Some proteins

99 secreted by T9SS are secreted in soluble form, whereas others become covalently attached to the
100 cell surface (17, 33-35). Genome analyses can be used to identify genes predicted to encode
101 proteins with type A CTDs or type B CTDs, thus facilitating discovery of proteins secreted by
102 T9SSs (30, 34). Many *Bacteroidetes* are predicted to secrete dozens to hundreds of proteins using
103 this system, and T9SS-mediated secretion of many of these proteins has been biochemically
104 verified (34, 36, 37).

105 Recent experiments demonstrate that T9SSs are required for virulence of the human
106 pathogens *Porphyromonas gingivalis* and *Prevotella melanogenica*, the fish pathogen
107 *Flavobacterium columnare*, and the bird pathogen *Riemerella anatipestifer* (17, 21, 38-41). In
108 each case, disruption of a gene encoding a core component of the T9SS resulted in secretion
109 defects and loss of virulence. Proteins secreted by the T9SSs of each of these organisms are thus
110 potential virulence factors. The role of core components of the *F. psychrophilum* T9SS in
111 virulence has not been directly explored, but mutations in *gldD* and *gldG* that disrupted gliding
112 motility and virulence also impacted the T9SS (14).

113 Methods to genetically manipulate *F. psychrophilum* have been developed (42, 43). These
114 include replicative plasmids, transposons, and methods to make site directed insertions or
115 deletions by homologous recombination. Genetic manipulations remain challenging however, at
116 least in part because of the low frequencies of gene transfer into *F. psychrophilum* (42). This is
117 especially challenging for the gene deletion approach described above, which requires multiple
118 rounds of gene transfer (43). Another method to construct gene deletions was recently developed
119 for *F. columnare* (44), and modified for use in many other *Bacteroidetes* (45). This method
120 involves cloning regions upstream and downstream of the target gene into a plasmid that cannot
121 replicate in members of the *Bacteroidetes*. The plasmid is introduced into the bacterium and gene

122 deletion occurs as a result of two recombination events between the regions on the plasmid and
123 the identical sequences on the chromosome. The plasmid carries an antibiotic resistance gene that
124 allows selection for the first recombination (integrating the plasmid into the chromosome) and
125 *sacB*, which confers sensitivity to sucrose, allowing selection for the second recombination (45,
126 46). Here we describe the use of this system to construct an *F. psychrophilum gldN* deletion
127 mutant, and demonstrate the importance of the T9SS for *F. psychrophilum* virulence.

128

129 **RESULTS**

130

131 **The *F. psychrophilum* T9SS and predicted T9SS-secreted proteins.** Analysis of the *F.*
132 *psychrophilum* strain OSU THCO2-90 genome (47) revealed each of the T9SS components
133 (Table S1), as previously reported for other *F. psychrophilum* strains (15-17). Most known
134 proteins that are secreted by T9SSs have CTDs that belong to either TIGRFAM protein family
135 TIGR04183 (type A CTDs) or TIGR04131/pfam13585 (type B CTDs). We examined the 2344
136 predicted *F. psychrophilum* strain OSU THCO2-90 proteins and identified 49 proteins predicted
137 to be secreted by the T9SS. Thirty-nine of these had type A CTDs (Table 1) and ten had type B
138 CTDs (Table 2). This probably underestimates the number of T9SS-secreted proteins, because
139 some proteins secreted by T9SSs have novel CTDs that are not easily assigned to either the type
140 A or type B CTD families (33). Included in the 49 predicted secreted proteins are nine predicted
141 peptidases, two predicted nucleases, one predicted lipase, one predicted glycoside hydrolase, and
142 28 potential adhesins, any of which may contribute to virulence. The 28 potential adhesins
143 include 17 that contain leucine-rich repeats (LRR) and are encoded by tandemly organized genes
144 (Table 1). These LRR proteins are similar to *Bacteroides forsythus* BspA and *Treponema*

145 *denticola* LrrA, which are proposed to be involved in attachment to host cells (48, 49). One of
146 the non-LRR adhesins, SprB, is involved in gliding motility in related bacteria (50).
147 *Flavobacterium johnsoniae* SprB is propelled along the cell surface, resulting in cell movement
148 (24, 50). SprB-mediated gliding of *F. psychrophilum* cells over and through fish mucus and
149 tissues may be important for virulence. The 49 predicted T9SS-secreted proteins are highly
150 conserved in other *F. psychrophilum* strains. Orthologs of each protein were present in the three
151 additional *F. psychrophilum* strains that we examined, JIP 02/86, CSF-259-93, and the type
152 strain ATCC 49418^T (12, 13, 51).

153 Previous studies of *F. johnsoniae* revealed general features of T9SS-secreted proteins
154 (30) that we examined here for *F. psychrophilum*. As with *F. johnsoniae*, *F. psychrophilum*
155 proteins with type B CTDs were typically much larger than those with type A CTDs (Tables 1
156 and 2). The median sizes of proteins with type A CTDs and type B CTDs were 46.0 kDa and
157 179.5 kDa respectively, and the largest proteins with type A CTDs and type B CTDs were 142.1
158 kDa and 366.8 kDa respectively. As for *F. johnsoniae*, many *F. psychrophilum* proteins with
159 type A CTDs are predicted or known to have enzymatic functions, whereas this was not the case
160 for any proteins with type B CTDs (Tables 1 and 2). Instead, many of the type B CTD proteins
161 are suspected to be cell surface adhesins, at least one of which (SprB) is likely involved in
162 gliding motility.

163 *F. johnsoniae* proteins with type B CTDs require the assistance of additional outer
164 membrane proteins that belong to the PorP/SprF protein domain family TIGR03519 for secretion
165 by the T9SS (30, 52). In many cases these PorP/SprF-like proteins are specific for individual
166 secreted proteins. *F. johnsoniae* SprB, for example, requires co-expression with SprF for its
167 secretion. PorP/SprF-like proteins are often encoded by the genes immediately downstream of

168 those encoding their cognate type B CTD-containing secreted proteins. *F. psychrophilum* strain
169 OSU THCO2-90 has seven *porP/sprF* like genes (Tables S1 and S2), and six of these lie
170 immediately downstream of genes encoding type B CTD proteins. This synteny suggests that, as
171 for *F. johnsoniae*, these six *F. psychrophilum* type B CTD proteins may each require their
172 cognate PorP/SprF-like proteins for secretion. The remaining four type B CTD proteins may use
173 the ‘orphan’ PorP/SprF protein, THC0290_0614, although other possibilities exist. Three of the
174 *F. psychrophilum porP/sprF* genes lie immediately upstream of, and are transcribed in the same
175 direction as, genes encoding predicted peptidoglycan-binding proteins related to *P. gingivalis*
176 PG1058, which is required for T9SS function (53). These proteins share similarity to the C-
177 terminal peptidoglycan-binding domain of *Escherichia coli* OmpA (54, 55). The secreted type B
178 CTD proteins, PorP/SprF-like proteins, and PG1058-like proteins may form cell-surface
179 complexes, as predicted for similar *F. johnsoniae* proteins (30).

180 ***sacB*-mediated deletion of the *F. psychrophilum* T9SS gene *gldN*.** To investigate the
181 role of the T9SS in *F. psychrophilum* virulence, we deleted *gldN*, which is essential for T9SS
182 function in related bacteria (17, 21, 56). Regions upstream and downstream of *gldN* of
183 approximately three-kbp in size were amplified by PCR, inserted into the *sacB*-containing
184 suicide vector pYT313 (45) and introduced into *F. psychrophilum* strain OSU THCO2-90 by
185 conjugation. Selection for erythromycin resistance resulted in 123 colonies. These had the
186 plasmid inserted in the genome by homologous recombination either upstream or downstream of
187 *gldN*. Three colonies were streaked for isolation, and then grown in broth culture without
188 selection to allow a second recombination event, resulting in loss of the inserted plasmid. These
189 cultures were selected for sucrose resistance, and hundreds of colonies lacking the plasmid were
190 obtained. Plasmid loss was expected to result in approximately equal numbers of wild-type

191 colonies and *gldN* deletion mutant colonies, depending on whether the second recombination
192 occurred on the same side of *gldN* as the first recombination or on the opposite side, respectively.
193 PCR analyses using primers flanking *gldN* were used to identify deletion mutants. Of eleven
194 independent sucrose resistant colonies examined, seven had *gldN* deleted, and the remaining four
195 had *gldN* intact and were thus wild type. One representative mutant colony (FpT13), which we
196 refer to as Δ *gldN*, was selected for further analysis.

197 **GldN is required for gliding motility and formation of spreading colonies.** GldN is
198 required for gliding motility in related bacteria (21, 56-58). Cells of wild-type *F. psychrophilum*
199 moved over agar by gliding, whereas cells of the Δ *gldN* mutant did not (Fig. 1, and Movie S1).
200 Complementation of the Δ *gldN* mutant by introduction of pBFp4, which carries *gldN*, restored
201 gliding motility. As a result of the gliding movements, cells of the wild-type and complemented
202 strains formed thin spreading colonies on 5% TYES medium (TYES diluted 20-fold) that was
203 solidified with agar (10 g/l), whereas cells of the Δ *gldN* mutant formed nonspreading colonies
204 (Fig. 2). In our experiments, the wild type formed spreading colonies on TYES agar media in
205 which all components except the agar had been diluted at least 2-fold, and all of the strains
206 formed nonspreading colonies on full-strength TYES agar (data not shown). This is consistent
207 with previous results that demonstrated that spreading *F. psychrophilum* colonies were only
208 observed on nutrient-poor media or media prepared with decreased amounts of agar (59).

209 **The *gldN* deletion mutant appears to be deficient in protein secretion.** Cell-free
210 culture fluid from wild-type, mutant and complemented strains were examined by SDS-PAGE
211 for soluble secreted proteins. The Δ *gldN* mutant cells released much less protein than did wild-
212 type or complemented cells (Fig. 3), suggesting a protein secretion defect. Samples of cell-free
213 culture fluid were examined by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

214 The number of spectral counts of predicted secreted proteins detected are shown in Table 3. In
215 most cases, the $\Delta gldN$ mutant had far fewer spectral counts than did the wild-type or
216 complemented strains, corroborating a secretion defect in the mutant. The exceptions were
217 primarily the secreted leucine-rich repeat (LRR) proteins. All predicted LRR proteins had type A
218 T9SS CTDs, and requirement of GldN for their secretion was expected. However, LRR proteins
219 were detected in the cell-free spent culture fluid of both wild-type and mutant cells, and in some
220 cases higher amounts were detected for the culture fluid of the $\Delta gldN$ mutant. We do not know
221 the reason for this observation, but the $\Delta gldN$ mutant did not appear to have a general
222 permeability defect, as revealed by Fig. 3 and by the full LC-MS/MS data set that includes all
223 proteins detected in the cell-free spent medium (Dataset S1), which had very few spectra
224 corresponding to ribosomal proteins or other abundant cytoplasmic proteins.

225 **Deletion of *gldN* results in decreased extracellular proteolytic activity. *F.***

226 *psychrophilum* secretes many peptidases (14). We examined wild-type, $\Delta gldN$ mutant, and
227 complemented strains of *F. psychrophilum* for extracellular proteolytic activity on TYESG agar
228 supplemented with casein (Fig. 4A). Wild-type and complemented strains formed clearing zones
229 around the cell growth, indicating digestion of casein, whereas the $\Delta gldN$ mutant did not.
230 Extracellular proteolytic activity in cell-free spent culture medium from each strain was
231 quantified using an azocasein-based assay (Fig. 4B). For wild-type and complemented strains,
232 high levels of soluble extracellular proteolytic activities that increased over time through late
233 exponential and early stationary phases of growth were detected. In contrast, extracellular
234 proteolytic activities for the $\Delta gldN$ mutant were low and failed to increase over time. *In vitro* cell
235 growth kinetics in TYESG broth cultures were not affected by *gldN* deletion (Fig. 4B). TYESG
236 contains tryptone (casein that has been digested by trypsin) and yeast extract, which may explain

237 the lack of requirement for high levels of secreted peptidases for growth in this medium. In
238 contrast, the lack of peptidase secretion may impair growth of $\Delta gldN$ mutant cells within the
239 host. Wild-type *F. psychrophilum* produces peptidases (some apparently strain dependent) that
240 digest collagen, fibrinogen, elastin and fish muscle tissue, and fish with BCWD often present
241 signs of tissue erosion (60, 61).

242 **The *gldN* deletion mutant is deficient in hemolytic activity and hemoglobin**
243 **utilization.** Hemoglobin is the most abundant reservoir of iron in vertebrates, and represents an
244 attractive nutrient source for pathogenic bacteria (62). *F. psychrophilum* infections in young
245 rainbow trout result in hemorrhagic septicemia. This bacterium lyses rainbow trout erythrocytes
246 and uses hemoglobin as an iron source for growth (63, 64). Wild-type cells grown on TYESG
247 agar supplemented with rainbow trout blood exhibited hemolytic activity whereas cells of the
248 $\Delta gldN$ mutant did not (Fig. 5). Hemolysis by the wild-type strain was accompanied by brown
249 coloration of the colonies, which was not observed for the $\Delta gldN$ mutant. The introduction of
250 *gldN* on pBFp4 into the $\Delta gldN$ mutant restored the phenotypic properties associated with the
251 wild-type strain, indicating that hemolysis and the brown coloration are dependent on a
252 functional T9SS. Dark colony pigmentation on blood agar occurs for other pathogenic bacteria
253 and may result from binding of hemoglobin or heme to the surface of bacterial cells. In *P.*
254 *gingivalis*, hemoglobin released following lysis of erythrocytes is digested by gingipain proteases
255 that are secreted by the T9SS. The released heme accounts for the black pigmentation of *P.*
256 *gingivalis* colonies and contributes to oxidative stress resistance (65). To evaluate the role of the
257 *F. psychrophilum* T9SS in hemoglobin utilization, wild-type and mutant strains were grown to
258 stationary phase in TYESG broth supplemented with 0.5 μ M hemoglobin. Residual free heme
259 and hemoglobin were measured using a porphyrin detection method that relies on growth

260 stimulation of a heme-auxotrophic bacterial reporter strain (66). Stationary phase wild-type
261 cultures resulted in minimal growth stimulation of the reporter, indicating little if any residual
262 porphyrin in the medium (Fig. 6). In contrast, uninoculated medium, or stationary phase cultures
263 of the $\Delta gldN$ mutant, stimulated growth of the reporter, indicating that little if any heme
264 porphyrin was used by the $\Delta gldN$ mutant cells. Hemoglobin consumption was restored to the
265 $\Delta gldN$ mutant by introduction of *gldN* on pBFp4. The results indicate that a functional T9SS is
266 required for efficient hemoglobin utilization by *F. psychrophilum*. This may in part involve
267 secretion of peptidases that cleave hemoglobin, releasing heme. The brown color of wild-type
268 colonies (Fig. 5) suggests that released heme was captured by the cells. Heme was likely
269 imported into wild-type *F. psychrophilum* cells, because it was absent in the medium after
270 growth.

271 **The *gldN* deletion mutant is deficient in adhesion, biofilm formation, and cell**
272 **sedimentation.** Wild-type *F. psychrophilum* cells adhere readily to polystyrene (67). Cells of the
273 $\Delta gldN$ mutant were partially deficient in adhesion to polystyrene compared to wild-type cells or
274 to cells of the complemented mutant (Fig. 7A). Adhesion of cells to a surface is the first step in
275 biofilm formation, and *F. psychrophilum* is known to form biofilms on polystyrene (68, 69). The
276 $\Delta gldN$ mutant was deficient in biofilm formation compared to the wild-type and complemented
277 strains (Fig. 7B). *F. psychrophilum* cells also interact with each other, causing them to sediment
278 from suspension (14). Wild-type and complemented $\Delta gldN$ mutant cells readily sedimented from
279 suspension, whereas the $\Delta gldN$ mutant cells did not (Fig. 7C). The results indicate that the T9SS
280 is involved in efficient adhesion, biofilm formation, and sedimentation, as was also suggested by
281 analysis of *F. psychrophilum gldD* and *gldG* mutants (14). The many predicted adhesins

282 demonstrated to be secreted by the T9SS (Tables 1-3) may explain some of the defects in these
283 processes, which each require adhesion, in the $\Delta gldN$ mutant cells.

284 **GldN is required for virulence in rainbow trout.** Wild-type, $\Delta gldN$ mutant, and
285 complemented strains were examined for virulence using a rainbow trout injection challenge
286 (70). At the dose examined (2.8×10^6 CFU/fish) intramuscular injection of the $\Delta gldN$ mutant
287 caused no mortalities, whereas exposure to similar doses of the wild-type and complemented
288 strains resulted in greater than 60% mortalities within 10 d of challenge (Fig. 8). The
289 complemented strain was less virulent than the wild type. *F. psychrophilum* was isolated from all
290 mortalities examined: wild-type (n=13/13 fish), and $\Delta gldN$ complemented with pBFp4 (n=11/11
291 fish).

292 The effect of *gldN* deletion on the virulence of *F. psychrophilum* was also investigated
293 using an immersion model that more closely mimics the natural route of infection. Fish were
294 bathed for 24 h in water containing wild-type, $\Delta gldN$ mutant or complemented strains at final
295 concentrations of $\sim 2 \times 10^7$ CFU/ml (Fig. 9A). Virulence was compared between the three strains
296 by recording the mortality rates and by determining the *F. psychrophilum* bacterial loads in
297 external (gills) and internal (spleen) organs. Five days after the immersion challenge, 93% of fish
298 infected with the wild-type strain had died (Fig. 9B). In contrast, the cumulative mortality of fish
299 exposed to the $\Delta gldN$ mutant was less than 2%, and was similar to that of the non-infected
300 control group. Complementation with pBFp4 restored virulence to the $\Delta gldN$ mutant, resulting in
301 cumulative mortality similar to that caused by the wild type. The increased time-to-death
302 observed for fish exposed to the complemented strain compared to the wild-type strain may be a
303 result of the increased copy number of *gldN* or of an effect of the *gldN* deletion on expression of
304 nearby genes. Six of each group of 42 fish were arbitrarily sampled for the presence of *F.*

305 *psychrophilum* 6 h and 30 h after the end of the immersion challenge. At 30 h, *F. psychrophilum*
306 was detected at high levels in the gills (average, 6×10^3 CFU per gill arch) and spleen (4×10^4
307 CFU per spleen) of all fish exposed to the wild-type strain (Fig. 9C). Strikingly, the bacterium
308 was detected in the organs of only five out of 24 fish exposed to the $\Delta gldN$ mutant, and bacterial
309 loads were significantly lower compared to fish exposed to the wild type. Bacterial loads in the
310 spleen and gills of fish exposed to the complemented mutant were similar to those of fish
311 exposed to the wild type. Together, the results indicate that a functional T9SS is required for full
312 virulence of *F. psychrophilum* in rainbow trout.

313 **DISCUSSION**

314 *F. psychrophilum* is an important pathogen of aquaculture-reared salmonids and ayu and
315 results in large economic losses for the aquaculture industry worldwide. Genetic manipulation to
316 identify *F. psychrophilum* virulence factors has been hampered by the lack of efficient genetic
317 tools. Here we demonstrate the usefulness of a *sacB*-mediated gene deletion system to construct
318 mutations in *F. psychrophilum*, and we use this approach to demonstrate that the T9SS is
319 required for virulence.

320 A gene deletion procedure was previously developed for *F. psychrophilum* strain OSU
321 THCO2-90 (43). This double recombination procedure was similar to the one described here, but
322 relied on an introduced restriction enzyme site for counter-selection against strains carrying the
323 integrated plasmid. While the procedure allowed mutant construction, it was inefficient,
324 presumably because of the multiple rounds of gene transfer required to introduce the integrative
325 plasmid and later introduce the restriction enzyme-encoding plasmid. Here we used a procedure
326 that was demonstrated to allow efficient targeted mutagenesis of other members of the genus
327 *Flavobacterium* and phylum *Bacteroidetes* (44, 45). This procedure, adapted to *F.*

328 *psychrophilum*, required a single gene transfer event, relied on *sacB*-mediated sucrose sensitivity
329 for counterselection, and resulted in many *F. psychrophilum* gene deletion colonies. This
330 approach allows efficient targeted mutagenesis of *F. psychrophilum* strain OSU THCO2-90.
331 However, many other *F. psychrophilum* strains have resisted gene transfer (42), and additional
332 research is needed before similar studies will be possible for a diverse collection of *F.*
333 *psychrophilum* strains.

334 Genome analysis revealed *F. psychrophilum* OSU THCO2-90 genes encoding T9SS
335 components. Forty-nine proteins predicted to be secreted by this system were also identified,
336 including predicted peptidases, nucleases, adhesins, and motility proteins, any of which could
337 contribute to virulence. Each of the predicted secreted proteins were conserved across the
338 genomes of the four *F. psychrophilum* strains analyzed (OSU THCO2-90, ATCC 49418^T, JIP
339 02/86, and CSF-259-93), and many of them were identified as secreted proteins by proteomic
340 analyses (Table 3). Many of the proteins secreted by the *F. psychrophilum* T9SS were previously
341 identified as predicted virulence factors in the initial genome analysis of strain JIP 02/86 (12). Of
342 the 13 peptidases identified as potential virulence factors in that study, seven have type A CTDs
343 (FP0082, FP0086, FP0231, FP0232, FP0280, FP0281, and FP1763). Two additional peptidase-
344 encoding genes appear disrupted in strain JIP 02/86 (FP1776/FP1777 and FP1024/FP1023),
345 whereas strain OSU THCO2-90 has full length orthologous genes (THC0290_0931 and
346 THC0290_1343, respectively) encoding predicted peptidases with type A CTDs (Tables 1 and
347 3). Duchaud et al (12) also identified genes encoding 27 predicted adhesins, including 15
348 tandemly arrayed genes that encode proteins with LRRs that were similar to *B. forsythus* BspA
349 and *T. denticola* LrrA (48, 49). *F. psychrophilum* OSU THCO2-90 has 17 tandemly arrayed
350 genes encoding LRR proteins that are similar to those of strain JIP 02/86 (Table 1). Each of these

351 have type A CTDs (as do the strain JIP 02/86 LRR proteins), and are thus predicted to be
352 secreted by the T9SS. Other previously predicted T9SS-secreted adhesins of strain JIP 02/86
353 (12), with strain OSU THCO2-90 orthologs indicated in parentheses, include the type A CTD
354 protein FP1830 (THC0290_0878), and the type B CTD proteins FP2413 (THC0290_2338),
355 FP0016 (THC0290_0025; SprB), and FP0595 (THC0290_1616).

356 GldN was originally identified as an *F. johnsoniae* gliding motility protein (57), and was
357 later shown to be a core component of the *F. johnsoniae* and *P. gingivalis* T9SSs (17, 56). *gldN*
358 is required for T9SS-mediated secretion in *F. johnsoniae*, *F. columnare*, and *P. gingivalis* (17,
359 21, 56). Similarly, as shown here, *F. psychrophilum gldN* was required for both gliding motility
360 and T9SS function. The *gldN* mutant was nonmotile, and appeared to be deficient in secretion of
361 many proteins, including predicted peptidases, hemolysins, and adhesins. Deletion of *gldN*
362 greatly reduced the extracellular proteolytic and hemolytic activities of cells, and also reduced
363 their adhesiveness and ability to form biofilms. Motility, adhesion, proteolysis, hemolysis, and
364 ability to form biofilms have all been suggested to play roles in BCWD (1, 2, 7, 12, 13, 71). The
365 only correlation observed between virulence (estimated by LD₅₀ using intramuscular injection
366 challenge) and these phenotypic traits in a recent study of 26 *F. psychrophilum* isolates was that
367 the avirulent strain studied was deficient in colony spreading and exoproteolytic activity (7),
368 suggesting a probable T9SS deficiency. Adhesins may allow initial attachment, and motility
369 could facilitate penetration of tissues and spread of the infection. Secreted peptidases and
370 hemolysins may digest host tissues and proteins, providing nutrients to the bacterium and/or
371 destroying host defenses. The $\Delta gldN$ mutant was completely deficient in virulence in rainbow
372 trout infected by injection or immersion. An *in vivo* adhesion defect of the $\Delta gldN$ mutant is
373 suggested by the reduced recovery of *F. psychrophilum* cells from gill tissue 6 h after exposure

374 by immersion (Fig. 9C). Loss of secretion of enzymes, adhesins, and motility proteins, may
375 together result in the avirulent phenotype of the mutant. The T9SS was also recently
376 demonstrated to be critical for *F. columnare* to cause columnaris disease (21), suggesting
377 similarities between at least some aspects of the virulence strategies employed by these related
378 fish pathogens.

379 The results reported here are consistent with those obtained by Pérez-Pascual et al. (14)
380 who examined transposon-induced motility mutants of *F. psychrophilum* for virulence. In that
381 study, mutations in the motility genes *gldD* and *gldG* were linked to T9SS defects, resulting in
382 reduction in extracellular proteolytic activity, adhesiveness, and virulence (14). GldD and GldG
383 are not core T9SS components, and are absent from nonmotile bacteria that have T9SSs, such as
384 *P. gingivalis* (16). However, recent studies of *F. johnsoniae* indicate that the gliding motility and
385 T9SS machineries are intertwined (23), and suggest an explanation for the phenotypes of the *F.*
386 *psychrophilum gldD* and *gldG* mutants. In *F. johnsoniae*, absence of motility protein GldJ results
387 in apparent instability of the T9SS protein GldK, and thus in loss of protein secretion (23).
388 Further, mutations in some other motility genes (including *gldD* and *gldG*) result in apparent
389 instability of GldJ protein (72), and thus in loss of GldK and of T9SS function (23). The apparent
390 interconnections between the motility and T9SS machineries may explain how mutations in the
391 *F. psychrophilum* motility genes *gldD* and *gldG* perturb T9SS function.

392 Our results may also help to explain the lack of virulence of rifampicin resistant *F.*
393 *psychrophilum* mutants that have been isolated and examined as vaccine candidates (73). The
394 most attenuated of these strains were deficient in motility (74). Given the link between gliding
395 motility and the T9SS described above, the mutants may have also been compromised for protein
396 secretion, which could have contributed to the attenuated phenotype.

397 The proteomic analysis of spent cell-free culture fluid confirmed our predictions
398 regarding secretion of many proteins that carried type A or type B CTDs, but also revealed some
399 unexpected results. Numerous LRR proteins, each of which had type A CTDs, were predicted to
400 be secreted by the T9SS, but surprisingly, many of these were found at similar, or for some
401 higher, levels in the cell-free culture fluid of the $\Delta gldN$ mutant. The LRR proteins were predicted
402 to be cell-surface proteins (12). We do not know why similar amounts of LRR proteins were
403 found in the cell-free spent culture fluids of wild-type and $\Delta gldN$ mutant cells. Perhaps the LRR
404 proteins were not actually secreted in soluble form, but instead were present on the surface of, or
405 inside of, extracellular outer membrane vesicles released by wild type and mutant cells
406 respectively. Our proteomic results for secretion of LRR proteins by wild type and $\Delta gldN$ mutant
407 cells differ from those obtained in a similar study of wild type, *gldD*, and *gldG* mutant cells (14).
408 The *gldD* and *gldG* mutants secreted much smaller amounts of LRR proteins than did wild type
409 cells. We do not know the reasons for this difference. As discussed above, GldD and GldG, are
410 not thought to be components of the T9SS, but they impact its function. Mutations in *gldD* and
411 *gldG* may thus affect the functioning of the T9SS differently than mutations in *gldN*, which
412 encodes a core component of the system.

413 The 49 proteins with type A and type B CTDs that are predicted to be secreted by the *F.*
414 *psychrophilum* T9SS probably underestimate the actual number of proteins secreted by this
415 system, since some T9SS-secreted proteins from related bacteria have novel CTDs (33).
416 Supporting this, deletion of *gldN* appeared to disrupt secretion of many proteins that lacked
417 obvious T9SS CTDs (Dataset S1). Included among these proteins are 14 predicted peptidases
418 that may contribute to virulence. Analysis of the C-terminal regions of seven proteins that lacked
419 obvious type A or type B CTDs but that appeared to be secreted by the T9SS as indicated by

420 Dataset S1, and by the results of a previous study (14) revealed two putative adhesins
421 (THC0290_0754 and THC0290_2201) and one probable peptidase (THC0290_0500) that exhibit
422 some similarity to T9SS CTDs (Fig. S1). These may represent a novel T9SS CTD type, or may
423 indicate that the sequence constraints delineating CTD domains may need to be relaxed to avoid
424 false negatives. Identification of additional proteins secreted by the T9SS will not be surprising
425 given our current limited understanding of the functioning of this system. An alternative
426 potential explanation for the apparent T9SS-mediated secretion of some non-CTD proteins is that
427 deletion of *gldN*, which is expected to result in the failure of numerous cell-surface proteins to
428 reach their destination, could have pleiotropic effects on the cell surface that perturb other protein
429 secretion systems. *F. psychrophilum* has multiple predicted type I secretion systems (12) that
430 could have been affected in this way.

431 Our analyses of *F. psychrophilum* motility proteins SprC and SprD identified some
432 unexpected features that were not immediately obvious from earlier studies. *F. johnsoniae* SprC
433 was not previously recognized as a potential T9SS-secreted protein (30, 52). *F. psychrophilum*
434 SprC also fell below the trusted cutoff for inclusion in type B CTD family TIGR04131, but it had
435 a conserved region (pfam13585; Table 2) that is found in all type B CTDs. The regions
436 recognized by TIGR04131 and pfam13585 are nearly identical (19). Further, *sprD*, which is
437 immediately downstream of *sprC*, encodes a protein with a PorP/SprF-like region (TIGR03519;
438 Table S2), suggesting that it may interact with SprC and facilitate its secretion by the T9SS.
439 Similar conserved regions are found in SprC (pfam13585) and SprD (TIGR03519) proteins of *F.*
440 *columnare* and other related bacteria. *sprC*, *sprD*, *sprB*, and *sprF* appear to form an operon in *F.*
441 *psychrophilum* and in many other *Bacteroidetes*, and SprC and SprD support SprB-mediated
442 gliding motility (30, 52). As mentioned above, SprB also requires its cognate PorP/SprF-like

443 protein (SprF) for its secretion (30). The results presented here enhance our understanding of the
444 possible interactions between these four common *Bacteroidetes* gliding motility proteins, and
445 their relationships to the T9SS.

446 The *F. psychrophilum* T9SS is important for virulence, but which of the many secreted
447 proteins contribute to BCWD remains unclear. The roles of the individual secreted proteins can
448 now be examined systematically using the gene deletion approach described above. Since this
449 deletion technique does not leave antibiotic resistance genes or other foreign DNA in the mutant,
450 it can be used iteratively to generate strains lacking many genes. This is important given the
451 many potential secreted virulence factors, and should allow questions of redundancy to be
452 addressed. Construction of mutants lacking one or more critical secreted virulence factors may
453 reveal the most important mechanisms employed by *F. psychrophilum* to cause disease, and
454 could suggest control measures. Such mutants may function as attenuated vaccine strains that
455 interact with fish and generate a protective immune response, but fail to cause disease. Since
456 T9SSs are involved in diseases caused by many related animal and human pathogens (17, 21, 38-
457 41), improved understanding of *F. psychrophilum* virulence may have impacts beyond fish
458 health and sustainable aquaculture.

459 MATERIALS AND METHODS

460 **Bacterial strains, plasmids, and growth conditions.** *F. psychrophilum* strain OSU
461 THCO2-90 (47, 75), was the wild-type strain used in this study. *F. psychrophilum* cultures were
462 grown at 18°C in tryptone yeast extract salts (TYES) medium (76, 77), which contains per l, 4 g
463 tryptone, 0.4 g yeast extract, 0.5 g MgSO₄·7H₂O, and 0.5 g CaCl₂·2H₂O, pH adjusted to 7.2. For
464 some experiments TYESG, which contains per l, 4 g tryptone, 0.4 g yeast extract, 0.5 g
465 MgSO₄·7H₂O, 0.2 g CaCl₂·2H₂O, and 0.5 g D-glucose, pH adjusted to 7.2, was used. TYESG

466 differs from TYES only by the addition of glucose and by the reduced amount of CaCl₂. For
467 solid media, agar was used at 15 g/l unless indicated otherwise. For most experiments, *F.*
468 *psychrophilum* was recovered from -80°C freezer tubes onto TYES agar and incubated 72 hours
469 at 18°C. Strains were streaked on fresh agar, incubated 72 hours at 18°C, and then used to
470 inoculate 20 ml TYES broth cultures, which were incubated for 24 to 48 h at 18°C with shaking
471 at 200 rpm. Extracellular proteolytic activity on solid medium was visualized using TYESG agar
472 supplemented with 7.5 g casein (Sigma-Aldrich Ref. C8654) per l or 10 g hemoglobin (Hb;
473 Sigma-Aldrich Ref. 08449) per l. Hemolysis and colony pigmentation were visualized on
474 TYESG agar supplemented with 50 ml rainbow trout blood per l. Blood was collected by caudal
475 venipuncture of anesthetized rainbow trout and immediately mixed with heparin at a final
476 concentration of 250 IU/ml (Heparine Choay® 25000 IU/5 ml injectable solution, Sanofi
477 Aventis) at 4°C. Colony spreading was examined on 5% TYES (TYES with all components
478 diluted 20-fold) solidified with 10 g agar per l.

479 *Bacteroides thetaiotaomicron* VPI-5482^T (78) was grown in Difco™ M17 medium
480 #218561 (Becton Dickinson, Sparks, MD), supplemented with (per l) 5 g glucose, 0.5 g cysteine,
481 and 6 g agar, and is referred to here as M17-glu. *E. coli* strains were grown in lysogeny broth
482 (LB) at 37°C (79). Strains and plasmids used in this study are listed in Table 4 and primers are
483 listed in Table 5. Antibiotics were used at the following concentrations when needed: ampicillin,
484 100 µg/ml; erythromycin, 10 µg/ml.

485 **Conjugative transfer of plasmids into *F. psychrophilum*.** Plasmids were transferred
486 from *E. coli* S17-1 λ -*pir* into *F. psychrophilum* strains by conjugation. Briefly, *E. coli* strains
487 were incubated overnight with shaking in LB at 37°C. *F. psychrophilum* strains were incubated
488 30 h with shaking in TYES broth at 18°C. Cells were collected by centrifugation at 3220 × g for

489 25 min, washed twice with 1 ml TYES and suspended in 500 μ l TYES. The suspensions of *E.*
490 *coli* and *F. psychrophilum* cells were mixed, spotted on TYES agar and incubated at 18°C for 48
491 hours. The cells were removed from the plate with a scraper and suspended in 2 ml TYES. 100-
492 μ l aliquots were spread on TYES agar containing 10 μ g erythromycin per ml and incubated at
493 18°C for 5 to 7 days.

494 **Construction of the *gldN* deletion mutant.** To delete *F. psychrophilum gldN*
495 (THC0290_0743), a 2,858-bp fragment spanning the last 387 bp of *gldK* (THC0290_0740), *gldL*
496 (THC0290_0741), *gldM* (THC0290_0742) and including the first 141 bp of *gldN* was amplified
497 using Phusion DNA polymerase (New England Biolabs, Ipswich, MA) and primers 2060
498 (introducing a BamHI site) and 2061 (introducing a SalI site). The fragment was digested with
499 BamHI and SalI and ligated into the suicide vector pYT313, which had been digested with the
500 same enzymes, to generate pBFp0. A 3,132-bp fragment spanning the final 153 bp of *gldN*,
501 THC0290_0744, THC0290_0745 and the first 1,220 bp of THC0290_0746 was amplified with
502 primers 2062 (introducing a SalI site) and 2063 (introducing an SphI site). The fragment was
503 digested with SalI and SphI and inserted into pBFp0 that had been digested with the same
504 enzymes, to generate the deletion construct pBFp1. pBFp1 was transferred by conjugation into *F.*
505 *psychrophilum* OSU THCO2-90, and colonies that had the plasmid integrated into the
506 chromosome by recombination were obtained by selecting for erythromycin resistance. Resistant
507 colonies were streaked for isolation on TYES agar containing erythromycin. An isolated colony
508 was used to inoculate 10 ml TYES broth without erythromycin, which was incubated for 30 h at
509 18°C to allow loss of the integrated plasmid. Recombinants that had lost the plasmid were
510 selected by plating on TYES containing 50 g/l sucrose, and incubating at 18°C. Isolated sucrose-
511 resistant colonies were picked and streaked for isolation on TYES agar containing sucrose to

512 eliminate background cells that had not lost the plasmid. Colonies were screened by PCR using
513 primers 2076 and 2077, which flank *gldN* to identify the *gldN* deletion mutant. Streaking
514 colonies for isolation on selective media at both the plasmid integration (erythromycin selection)
515 and plasmid loss (sucrose selection) steps was critical to eliminate nonselected cells.

516 **Complementation of the *gldN* deletion mutant.** A plasmid carrying *F. psychrophilum*
517 *gldN* was constructed using shuttle vector pCP11 (80). Primers 2076 (introducing a KpnI site)
518 and 2077 (introducing an SphI site) were used to amplify a 1,161 bp product spanning *gldN*. The
519 product was digested with KpnI and SphI and ligated into pCP11, which had been digested with
520 the same enzymes, to generate pBFp4, which was transferred into *F. psychrophilum* by
521 conjugation.

522 **Analysis of colony spreading and cell motility.** *F. psychrophilum* wild-type, $\Delta gldN$
523 mutant, and complemented strains were serially diluted and plated on 5% TYES (TYES diluted
524 20-fold) solidified with 10 g agar per l. The plates were incubated for 32 h at 18°C to obtain
525 isolated colonies. Colonies were examined using a Photometrics Cool-SNAP_{cf}² camera mounted
526 on an Olympus IMT-2 phase-contrast microscope. Colonies were also obtained by spotting 5 μ l
527 of cells at OD₆₀₀ of 1.5 on 5% TYES solidified with 10 g agar per l and incubating for 12 d at
528 18°C, and these were examined macroscopically using a Nikon D7200 camera. Motility of
529 individual cells on agar was examined by spotting cells on a pad of full-strength TYES solidified
530 with 10 g agar per l on a glass slide, allowing the spot to dry briefly, and covering it with an O₂-
531 permeable Teflon membrane (Yellow Springs Instrument Co., Yellow Springs, OH) that
532 prevented dehydration and served as a coverslip. Cell movements over agar were observed using
533 an Olympus BH-2 phase-contrast microscope. Images were recorded using a Photometrics Cool-
534 SNAP_{cf}² camera and analyzed using MetaMorph software (Molecular Devices, Downingtown,

535 PA). Rainbow traces of cell movements were made using ImageJ version 1.45s
536 (<http://rsb.info.nih.gov/ij/>) and macro Color FootPrint (24).

537 **Analysis of secreted proteins by SDS-PAGE and LC-MS/MS.** *F. psychrophilum* wild-
538 type, Δ *gldN* mutant, and complemented mutant were grown in TYES broth at 18°C for
539 approximately 48 h. Growth was monitored with a Klett-Summerson colorimeter (Klett
540 Manufacturing Co., Inc. Long Island City, New York) and cultures were harvested in early
541 stationary phase when the Klett readings reached 160. Cultures were centrifuged at $16,873 \times g$
542 for 10 min at 4°C. The fluid was filtered with 0.45 μ m pore size HT Tuffryn filters (PALL Life
543 Sciences). Proteins were precipitated by mixing 1 volume of trichloroacetic acid (TCA) with 9
544 volumes of cell-free spent culture fluid, incubating at -20°C for 1 h, and centrifuging for 45 min
545 at $12,210 \times g$ at 4°C. The pellet was washed with acetone (0°C) and centrifuged for 10 min at
546 $12,210 \times g$ to collect the pellet, which was dried for 10 min at room temp. Proteins were
547 suspended in SDS-PAGE loading buffer, boiled for 10 min, and were separated by SDS-PAGE
548 (81) using a 12% polyacrylamide gel, and detected using the BioRad (Hercules, CA) silver stain
549 kit. Protein pellets (TCA-precipitated from cell-free spent culture fluid and acetone-washed as
550 above) were also analyzed directly by enzymatic digestion and nano-LC-MS/MS at the
551 University of WI-Madison Mass Spectrometry Facility as outlined on the website
552 (<https://www.biotech.wisc.edu/services/massspec>), and as described previously (21) except that
553 LC-MS/MS data were searched against proteins encoded by the *F. psychrophilum* strain OSU
554 THCO2-90 genome (47).

555 **Analysis of proteolytic activity.** Proteolytic activity was quantified using azocasein as a
556 substrate as previously described (82). Briefly, *F. psychrophilum* strains were grown in 25 ml
557 TYESG broth at 18°C and 200 rpm for 2 days. Two ml of cultures at OD₆₀₀ of 0.5, 1.0 and 2.0

558 were centrifuged, the supernatants filtered using 0.22 μm Millipore filters and stored at 4°C for
559 24 h. For protease activity assay, a solution of 20 g azocasein per l (Sigma-Aldrich) was prepared
560 in 0.1 M Tris-HCl pH 7.4. The cell-free supernatant (150 μl) was mixed with 150 μl azocasein
561 solution and incubated for 4 h at 25°C. The reaction was stopped by adding 750 μl of TCA (50
562 g/l). The reaction mix was centrifuged at 20,000 \times g for 30 min and 150 μl of the supernatant
563 was mixed in a 96-well microplate well containing 150 μl of 1 M NaCl. The OD_{440nm} was
564 measured using a Tecan Microplate Reader (Infinite 200 PRO) and the negative control OD_{440nm}
565 value was subtracted from this. One unit of proteolytic activity was defined as an increase in
566 absorbance of 0.001 under the conditions of the assay. All cultures were performed in triplicate
567 and an average of the three experiments was used as the measure of proteolytic activity over the
568 growth curve.

569 **Hemoglobin utilization.** The ability to use hemoglobin as a heme source was
570 investigated by measuring the residual porphyrin (heme, hemoglobin or protoporphyrin IX)
571 remaining after growth of *F. psychrophilum* to stationary phase in TYESG broth supplemented
572 with 0.5 μM hemoglobin (Sigma-Aldrich Ref. H2500), using the heme-protoporphyrin IX-screen
573 method (66). This method is a bacterial-growth-based assay that exploits the heme auxotrophy of
574 *B. thetaiotaomicron*. Briefly, a vertical gel set-up with 0.8-cm spacers was filled with M17-glu
575 medium containing 8×10^5 *B. thetaiotaomicron* CFU/ml. Stationary phase *F. psychrophilum*
576 cultures in TYESG broth (with or without 0.5 μM hemoglobin) were heat-inactivated by
577 incubation at 37°C for 3 h. The cultures (100 μl volumes) were loaded in wells of the *B.*
578 *thetaiotaomicron*-containing gel and sterile TYESG broth containing 0.5 μM hemoglobin was
579 used as a positive control. The gel was overlaid with a 5 ml agar plug (12 g/l). *B.*
580 *thetaiotaomicron* growth stimulation was visualized as dense growth around wells after

581 overnight incubation at 37°C. The surface area of growth stimulation, which correlates with
582 heme concentration in the sample, was determined using ImageJ (V1.45 s; Wayne Rasband,
583 National Institute of Health, USA). The results corresponded to three independent *F.*
584 *psychrophilum* cultures.

585 **Adhesion, biofilm, and sedimentation assays.** Adhesion to polystyrene, biofilm
586 formation on polystyrene, and cell sedimentation were determined as previously described (14)
587 except that cells were grown in TYES instead of TYESG for the adhesion assay, and in half-
588 strength TYES instead of half-strength TYESG for the biofilm and sedimentation assays, as
589 outlined briefly below.

590 For the adhesion assay, cells were grown in TYES broth to $OD_{600} = 0.7$. One ml of each
591 culture was centrifuged at $11,000 \times g$ for 5 min, the supernatant was removed, and cell pellets
592 were suspended in 1 ml sterile distilled water. One hundred microliters of each strain suspension
593 was added to a 96-well microtiter polystyrene plate with flat bottom (Nunclon™ Delta surface,
594 ThermoFisher Scientific, Waltham, MA) and sterile water was used as a negative control in non-
595 inoculated wells. The plate was incubated at 18°C for 3 h without shaking. Subsequently, wells
596 were washed twice with sterile distilled water and the adherent cells were stained with 100 µl of
597 crystal violet (10 g/l) for 30 min at room temperature. The wells were washed four times with
598 sterile distilled water, and 100 µl of absolute ethanol was added to each well to solubilize the
599 remaining crystal violet. Cell adhesion was determined by measuring OD_{595} using a CLARIOstar
600 Microplate Reader (BMG Labtech, Ortenberg, Germany). The level of adhesion observed for
601 each strain was compared with the adhesion of the wild-type strain, which was set as 100. All
602 assays were performed in quadruplicate and repeated at least two times. The absorbance of the
603 negative control was subtracted from the absorbance of each strain.

604 For biofilm formation, cells were grown in half-strength TYES broth to the mid-
605 exponential phase. The cultures were diluted 1:100 in half-strength TYES broth and 150 μ l of
606 each diluted bacterial culture was deposited in wells of 96-well flat bottom polystyrene
607 microtiter plates. The plates were covered with aluminum foil and incubated in a humid
608 environment at 18°C under static conditions for 120 h. Biofilm development was evaluated in
609 four wells per strain, and wells containing sterile non-inoculated medium were used as negative
610 controls. The culture fluid was discarded, the wells were washed twice with 200 μ l of sterile
611 distilled water. 150 μ l of crystal violet (10 g/l) was added to each well and incubated at room
612 temperature for 30 min. Unbound stain was removed by washing the wells four times with 200
613 μ l of sterile distilled water. Stain bound to biofilm cells was solubilized in 100 μ l of ethanol and
614 the absorbance (OD_{595 nm}) was determined.

615 To measure bacterial cell sedimentation, cells were grown in half-strength TYES broth at
616 18°C, 200 rpm for 96 h. Tubes were allowed to stand static for 10 min before being
617 photographed.

618 **Rainbow trout challenges.** Two experimental infection models differing by the infection
619 route (injection or immersion), size of fish, and lines of fish were used. For the intramuscular
620 injection model, fish (27.5 g average mass) of the ARS-Fp-C line were used, which are
621 intermediate in resistance (83). Four groups of 20 fish (n=80) were anesthetized and challenged
622 with each *F. psychrophilum* strain by intramuscular injection at a point midway between the
623 insertion of the dorsal fin and the lateral line. Injection of 25 μ l was performed using a 26 g
624 needle attached to an Eppendorf repeating syringe. All challenge fish were maintained using
625 flow-through spring water. Two groups of 20 fish were injected with sterile PBS, as a negative
626 control. CFU counts (triplicate dilution series) for wild type were $2.6 (\pm 0.8) \times 10^6$, Δ *gldN*

627 mutant $2.7 (\pm 1.3) \times 10^6$ and $\Delta gldN$ complemented mutant $2.8 (\pm 1.5) \times 10^6$ CFU/fish. The water
628 temperature during challenge was 13.2°C. Mortalities were recorded daily and examined for
629 clinical signs of disease and for the presence of *F. psychrophilum*.

630 For the bath immersion challenge the rainbow trout (*O. mykiss*) homozygous line A36
631 was used as previously described (84, 85). The uniformity of genetic background in isogenic
632 lines and the high susceptibility of line A36 to *F. psychrophilum* infection makes this line useful
633 to examine bacterial virulence (14). Fish were reared at 10°C in dechlorinated recirculated water
634 until they reached 1.4 g, and were then transferred to continuous flow aquaria for infection
635 experiments. Bacteria used for infections were prepared as follows: wild type, $\Delta gldN$, and $\Delta gldN$
636 complemented with pBFp4 were grown in TYESG broth at 200 rpm and 18°C until late-
637 exponential phase ($OD_{600nm} = 1.3$). The bacterial cultures were directly diluted (200 fold) into 15
638 l of aquarium water. Bacteria were maintained in contact with fish ($n = 42$ per group) for 24 h by
639 stopping the water flow, and were subsequently removed by restoring flow. Sterile TYESG broth
640 was used for the control group. *F. psychrophilum* bacterial counts were determined at the
641 beginning and at the end of the immersion challenge by plating serial dilutions of water samples
642 on TYESG agar. Water was maintained at 10°C under continuous aeration during the
643 experiment. Virulence was evaluated according to i) fish mortality during twelve days post-
644 infection and ii) bacterial loads in organs. Six of each group of 42 fish were randomly chosen
645 and sacrificed 6 h and 30 h after the end of the immersion challenge to evaluate the *F.*
646 *psychrophilum* bacterial load in the spleen (whole organ) and gills (one gill arch). Organs were
647 mechanically disrupted in Lysing Matrix tubes containing 400 μ l of peptone (10 g/l water) and 1
648 mm ceramic beads (Mineralex SAS, Lyon, France). Samples were homogenized at 6.0 m s⁻¹ for
649 two cycles of 20 s on a FastPrep-24 instrument (ThermoFisher Scientific, Waltham, MA). Serial

650 dilutions of the homogenized solution were plated on TYESG agar supplemented with fetal calf
651 serum (50 ml/l) and incubated for 3 d at 18°C. Two independent experiments were performed.
652 Statistical differences of bacterial loads between groups were analyzed using the Mann-Whitney
653 test; the Kaplan-Meier method was used to draw survival curves that were compared using the
654 Gehan-Breslow-Wilcoxon test with GraphPad Prism 8.1.2 (GraphPad Software, San Diego, CA,
655 USA).

656 **Bioinformatic analyses.** Genome sequences were analyzed for T9SS genes encoding
657 proteins that belong to appropriate TIGRFAM multiple-sequence alignment families (86). This
658 was accomplished using the Integrated Microbial Genomes (IMG version 4.0.1
659 [<https://img.jgi.doe.gov/>]) Function Profile Tool to examine the genomes for sequences predicted
660 to encode orthologs of GldK (TIGR03525), GldL (TIGR03513), GldM (TIGR03517), GldN
661 (TIGR03523), and SprA (TIGR04189). The genomes were also examined for genes encoding
662 proteins with type A CTDs (TIGR04183), type B CTDs (TIGR04131 and pfam13585) (19), and
663 for genes encoding PorP/SprF-like proteins (TIGR03519) in the same way. In each case, the
664 trusted cutoffs assigned by The J. Craig Venter Institute (JCVI) that allow identification of the
665 vast majority of family members with vanishingly few false positives (86) were used. Other
666 potential *F. psychrophilum* T9SS proteins listed in Table S1 (PorU, PorV, SprE, SprT, Plug,
667 PorQ, PorZ, PorX, PorY, and PG1058) were identified by BLASTP analysis using the
668 appropriate *F. johnsoniae* or *P. gingivalis* protein as query.

669 **Data Availability**

670 All data associated with this work is included either in the manuscript or in the online
671 supplemental materials.

672 **Ethics statements**

673 The immersion challenges were performed in accordance with the European Directive
674 2010/2063/UE regarding animal experiments, and were approved by the institutional review
675 ethics committee, COMETHEA, of the INRAE Center in Jouy-en-Josas, France. Authorizations
676 were approved by the Direction of the Veterinary Services of Versailles (authorization number
677 15-58). Injection challenge was performed under the guidelines of NCCCWA Institutional
678 Animal Care and Use Committee Protocol #132.

679

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689 004-03S. The views contained in this document are those of the authors and should not be
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977
978

979 **Table 1. Predicted T9SS type A CTD-containing proteins of *F. psychrophilum* strain OSU THC02-90¹**
980

Locus tag	Protein name	AA ²	Mol. Mass (kDa) ³	Domains ⁴	Description/ known or predicted function
THC0290_0086		642	70.9	M1_APN_like (cd09603) Peptidase_M1 (pfam01433)	Probable peptidase precursor (M1 family metalloprotease)
THC0290_0091		1139	122.8	Sortilin-Vps10 super family (cl25791) Peptidase_M6 super family (cl29544)	Probable glycoside hydrolase precursor
THC0290_0128		113	12.8		Putative outer membrane protein precursor
THC0290_0129		324	33.6		Probable cell surface protein (Leucine-rich repeat protein) precursor
THC0290_0170		398	41.7	LRR_5 (pfam13306)	Probable cell surface protein precursor (Leucine-rich repeat protein) precursor
THC0290_0171		421	44.5	LRR_5 (pfam13306)	Probable cell surface protein precursor (Leucine-rich repeat protein) precursor
THC0290_0172		329	35.2	LRR_5 (pfam13306)	Probable cell surface protein (Leucine-rich repeat protein)
THC0290_0173		444	47.2	LRR_5 (pfam13306)	Probable cell surface protein (Leucine-rich repeat protein) precursor
THC0290_0174		422	44.5	LRR_5 (pfam13306)	Probable cell surface protein (Leucine-rich repeat protein) precursor
THC0290_0175		330	35.3	LRR_5 (pfam13306)	Probable cell surface protein (Leucine-rich repeat protein) precursor
THC0290_0176		375	40.1	LRR_5 (pfam13306)	Probable cell surface protein (Leucine-rich repeat protein) precursor
THC0290_0177		536	56.0	LRR_5 (pfam13306)	Probable cell surface protein (Leucine-rich repeat protein) precursor
THC0290_0178		307	33.1	LRR_5 (pfam13306)	Probable cell surface protein (Leucine-rich repeat protein) precursor
THC0290_0179		421	44.3	LRR_5 (pfam13306)	Probable cell surface protein (Leucine-rich repeat protein) precursor
THC0290_0180		398	42.1	LRR_5 (pfam13306)	Probable cell surface protein (Leucine-rich repeat protein) precursor
THC0290_0181		467	50.1	LRR_5 (pfam13306)	Probable cell surface protein (Leucine-rich repeat protein) precursor
THC0290_0182		329	35.0	LRR_5 (pfam13306)	Probable cell surface protein (Leucine-rich repeat protein) precursor
THC0290_0183		306	33.2	LRR_5 (pfam13306)	Probable cell surface protein (Leucine-rich repeat protein) precursor
THC0290_0184		351	37.0	LRR_5 (pfam13306)	Probable cell surface protein (Leucine-rich repeat protein)

THC0290_0185		329	35.3	LRR_5 (pfam13306)	precursor Probable cell surface protein (Leucine-rich repeat protein)
THC0290_0186		328	35.3	LRR_5 (pfam13306)	precursor Probable cell surface protein (Leucine-rich repeat protein)
THC0290_0237	Fpp1	1138	119.6	ZnMc super family (cl00064) FN3 super family (cl21522) P_protein super family (cl27557)	Peptidase precursor (Psychrophilic metalloprotease Fpp1)
THC0290_0238	Fpp2	942	100.3	ZnMc_pappalysin_like (cd04275) Cleaved_Adhesin super family (cl06636)	Peptidase precursor (Psychrophilic metalloprotease Fpp2)
THC0290_0299		914	100.1	Peptidase_M36 (pfam02128) PA_subtilisin_1 (cd04818)	Probable peptidase precursor (M36 fungalysin family metalloprotease)
THC0290_0300		919	99.8	Peptidase_M36 (pfam02128) PA_subtilisin_1 (cd04818)	Probable peptidase precursor (M36 fungalysin family metalloprotease)
THC0290_0878		747	78.2	FN3 (cd00063)	Putative adhesin precursor
THC0290_0931		960	103.3	ZnMc super family (cl00064)	Peptidase precursor (Collagenase; Zn-dependent metalloprotease)
THC0290_0944		544	58.5	Peptidases_S8_9 (cd07493)	Probable peptidase precursor (S8 subtilisin family serine endopeptidase)
THC0290_0977		585	64.9	Abhydrolase super family (cl21494)	Esterase/lipase/thioesterase family protein precursor
THC0290_1054		408	46.0	SGNH_hydrolase super family (cl01053) PKD repeat (COG3291)	
THC0290_1343		1218	134.1	ZnMc super family (cl00064) PKD (COG3291, 3 copies) PCC super family (cl28216)	Probable cell surface protein precursor; putative metallopeptidase, metzincin clan
THC0290_1494		657	71.5	HNHc super family (cl00083)	Probable ribonuclease
THC0290_1520		613	66.2	Endonuclease_1 (pfam04231) FN3 (cd00063) COG2374 super family (cl28586)	Probable endonuclease precursor
THC0290_1595		628	67.9		Putative adhesin precursor
THC0290_1797		263	29.4	DOMON_DOH (cd09631)	
THC0290_2029		301	32.9		
THC0290_2146	PorU	1278	142.1	Peptidase_C25_N (cd02258)	Peptidase precursor (T9SS CTD signal peptidase, PorU)
THC0290_2157		166	18.7		
THC0290_2385		456	49.2	ATS1 super family (cl34932) RCC1 (pfam00415)	RCC1 (Regulator of Chromosome Condensation) repeat domain protein precursor
Average		550	59.3		
Median		422	46.0		

Range	113	12.8
	to	to
	1278	142.1

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¹The T9SS type A CTD (TIGR04183) is a C-terminal domain that targets a protein for secretion by the T9SS. TIGR04183 is described as 'Por secretion system C-terminal sorting domain' on the J. Craig Venter Institute TIGR website (<http://www.jcvi.org/cgi-bin/tigrfams/index.cgi>). Each of these proteins contains an N-terminal signal peptide for export across the cytoplasmic membrane as determined using SignalP 5.0 with a cutoff of 0.8, and a T9SS type A C-terminal domain (CTD) for secretion across the outer membrane by the T9SS as determined using the Integrated Microbial Genomes (IMG version 4.0.1 [<https://img.jgi.doe.gov/>]) tools and trusted cutoffs.

²Length of full-length protein in amino acids before removal of signal peptide or CTD.

³Molecular mass (kDa) of full-length protein in amino acids before removal of signal peptide or CTD.

⁴Domains other than signal peptide and CTDs, with pfam, cl, cd, or COG numbers indicated.

994 **Table 2. Predicted T9SS type B CTD-containing proteins of *F. psychrophilum* strain OSU THC02-90¹**
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Locus tag	Name	AA ²	Mol. Mass (kDa) ³	Domains ⁴	Description/Function or predicted function
THC0290_0023	SprC	477	51.2	FlgD_ig super family (cl21544)	Gliding motility protein precursor, SprC
THC0290_0025	SprB	3521	366.8	SprB repeat (pfam13573, 3 copies) FlgD Ig-like domain (cl21544)	Gliding motility adhesin precursor, SprB
THC0290_0332		2653	269.2	ATS1 super family (cl34932) Calx-beta domain (cl02522) Herpes_BLLF1 super family (cl37540)	Putative adhesin precursor
THC0290_1047		798	87.8		Putative adhesin precursor
THC0290_1048		757	82.6		Putative adhesin precursor
THC0290_1527		2229	233.6	Ig_2 (pfam13895)	Putative adhesin precursor
THC0290_1616		1253	129.6	YjdB (COG5492, 2 copies) Big_2 (cl02708)	Putative adhesin precursor
THC0290_1818		1452	158.9		Putative adhesin precursor
THC0290_1932		2001	200.0	DUF11 (pfam01345) PRK08026 super family (cl35635)	Putative adhesin precursor
THC0290_2338		2008	208.3	fn3 (pfam00041, 2 copies) FN3 (cd00063, 2 copies) CUB (cd00041)	Putative adhesin precursor
Average		1715	178.8		
Median		1727	179.5		
Range		477 to 3521	51.2 to 366.8		

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 997 ¹The T9SS type B CTD (TIGR04131/pfam13585) is a C-terminal domain that targets a protein for secretion by the T9SS. TIGR04131
 998 is described as 'gliding motility-associated C-terminal domain' on the J. Craig Venter Institute TIGR website (<http://www.jcvi.org/cgi-bin/tigrfams/index.cgi>). Each of these proteins contains an N-terminal signal peptide for export across the cytoplasmic membrane as
 999 determined using SignalP 5.0 with a cutoff of 0.7, and a T9SS type B CTD for secretion across the outer membrane by the T9SS as
 1000 determined using the IMG tools and trusted cutoffs. THC0290_0023 (SprC) matched only pfam13585, whereas each of the other
 1001 proteins matched both TIGR04131 and pfam13585.
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 1003

1004 ²Length of full-length protein in amino acids before removal of signal peptide or CTD.
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1006 ³Molecular mass (kDa) of full-length protein in amino acids before removal of signal peptide or CTD.
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1008 ⁴Domains other than signal peptide and CTDs, with pfam, cl, cd, or COG numbers indicated.
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1010 **Table 3. *F. psychrophilum* T9SS type A CTD and type B CTD proteins identified by LC-MS/MS analysis of cell-free spent**
 1011 **culture medium^a**
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Locus tag/Protein name	Mol. Mass (kDa) ^b	T9SS CTD ^c	Predicted protein function or description	Spectral counts from culture fluid		
				Wild type	$\Delta gldN$	$\Delta gldN$ + pBfp4
T9SS-CTD proteins other than leucine-rich repeat proteins						
THC0290_0238	100.3	type A	Peptidase	813	72	903
/Fpp2						
THC0290_0025	366.8	type B	Gliding motility adhesin SprB	268	59	315
/SprB						
THC0290_0931	103.3	type A	Peptidase (collagenase)	170	22	143
THC0290_2338	208.3	type B	Adhesin	84	32	94
THC0290_1932	200.0	type B	Adhesin	48	17	36
THC0290_1520	66.2	type A	Endonuclease	37	0	40
THC0290_2029	32.9	type A		34	19	45
THC0290_1797	29.4	type A		32	1	23
THC0290_1616	129.6	type B	Adhesin	27	8	53
THC0290_1595	67.9	type A	Adhesin	24	8	16

THC0290_0944	58.5	type A	Peptidase	21	0	23
THC0290_2146	142.1	type A	T9SS-specific CTD peptidase	20	17	32
/PorU						
THC0290_1494	71.5	type A	Ribonuclease	19	0	24
THC0290_0332	269.2	type B	Adhesin	15	4	16
THC0290_1527	233.6	type B	Adhesin	13	5	20
THC0290_0023	51.2	type B	Gliding motility protein SprC	9	4	9
/SprC						
THC0290_1047	87.8	type B	Adhesin	8	1	15
THC0290_1343	134.1	type A	Peptidase	6	0	6
THC0290_0086	70.9	type A	Peptidase	6	0	9
THC0290_0237	119.6	type A	Peptidase	6	0	5
/Fpp1						
THC0290_0129	33.6	type A		6	1	9
THC0290_1818	158.9	type B	Adhesin	6	0	6
THC0290_0091	122.8	type A	Glycoside hydrolase	5	2	4
THC0290_1048	82.6	type B	Adhesin	3	0	7
THC0290_0299	100.1	type A	Peptidase	1	16	6
THC0290_1054	46.0	type A		0	0	3
THC0290_0128	12.8	type A		0	2	0

Leucine-rich repeat proteins ^d						
THC0290_0183	33.2	type A	leucine-rich repeat protein	51	22	46
THC0290_0174	44.5	type A	leucine-rich repeat protein	23	13	22
THC0290_0171	44.5	type A	leucine-rich repeat protein	22	18	32
THC0290_0170	41.7	type A	leucine-rich repeat protein	20	40	24
THC0290_0181	50.1	type A	leucine-rich repeat protein	17	18	18
THC0290_0184	37.0	type A	leucine-rich repeat protein	15	25	23
THC0290_0186	35.3	type A	leucine-rich repeat protein	12	15	13
THC0290_0182	35.0	type A	leucine-rich repeat protein	9	17	10
THC0290_0175	35.3	type A	leucine-rich repeat protein	7	16	12
THC0290_0185	35.3	type A	leucine-rich repeat protein	7	9	10
THC0290_0172	35.2	type A	leucine-rich repeat protein	7	19	11
THC0290_0180	42.1	type A	leucine-rich repeat protein	0	20	0
THC0290_0177	56.0	type A	leucine-rich repeat protein	0	15	0

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1015 ^a Proteins in cell-free culture fluid from wild-type *F. psychrophilum*, Δ *gldN* mutant, and Δ *gldN* complemented with pBFp4, were
 1016 analyzed by LC-MS/MS. Total/unweighted spectrum counts corresponding to total number of spectra associated to a single protein
 1017 and indicative of relative abundance of that protein are indicated for each strain. Only proteins with predicted T9SS CTDs are shown.
 1018 For the full dataset, which includes additional proteins, see Dataset S1. The following type A CTD proteins were not detected:
 1019 THC0290_0173, THC0290_0176, THC0290_0178, THC0290_0179, THC0290_0300, THC0290_0878, THC0290_0977,
 1020 THC0290_2157, THC0290_2385. However, the LRR proteins THC0290_0173, THC0290_0176, THC0290_0178, THC0290_0179,

1021 may have been present, because they are similar in sequence to the other LRR proteins, resulting in ambiguity regarding protein
1022 assignment for some peptides (Dataset S1). Similarly, THC0290_300 may have been missed because of similarity in sequence to
1023 THC0290_0299.

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1025 ^b Molecular mass as calculated for full-length protein before removal of signal peptide.

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1027 ^c Type A CTDs belong to TIGRFAM protein domain family TIGR04183. Type B CTDs belong to TIGRFAM protein domain family
1028 TIGR04131 and/or pfam13585.

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1030 ^d These proteins have paralogs in the genome that complicate assignment of peptides.

1031 **Table 4. Strains and plasmids used in this study**
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Strain or plasmid	Description ^a	Source or reference
<i>Bacteroides thetaiotaomicron</i> strain VPI-5482 ^T	Used to measure porphyrin (heme, hemoglobin or protoporphyrin IX)	(78)
<i>E. coli</i> DH5 α MCR	Strain used for general cloning	Life Technologies (Grand Island, NY)
<i>E. coli</i> S17-1 λ pir	Strain used for conjugation	(87)
<i>F. psychrophilum</i> strain OSU THCO2-90	Wild type	(47, 75)
<i>F. psychrophilum</i> strain FpT13	Δ <i>gldN</i> in strain OSU THCO2-90	This study
Plasmids		
pCP11	<i>E. coli-Flavobacterium</i> shuttle plasmid; Ap ^r (Em) ^f	(80)
pYT313	Suicide vector carrying <i>sacB</i> ; Ap ^r (Em) ^f	(45)
pBFp0	2,858 bp region upstream of <i>gldN</i> amplified with primers 2060 and 2061 and cloned into BamHI and Sall sites of pYT313; Ap ^r (Em) ^f	This study
pBFp1	Construct used to delete <i>gldN</i> ; 3,132 bp region downstream of <i>gldN</i> amplified with primers 2062 and 2063 and cloned into Sall and SphI sites of pBFp0; Ap ^r (Em) ^f	This study
pBFp4	Plasmid for complementation of <i>gldN</i> ; <i>gldN</i> was amplified with primers 2076 and 2077 and cloned into KpnI and SphI sites of pCP11; Ap ^r (Em) ^f	This study

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1034 ^aAntibiotic resistance phenotypes: ampicillin, Ap^r; erythromycin, Em^r. Unless indicated

1035 otherwise, the antibiotic resistance phenotypes are those expressed in *E. coli*. The antibiotic

1036 resistance phenotypes given in parentheses are those expressed in *F. psychrophilum* but not in *E.*

1037 *coli*.

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1039 **Table 5. Primers used in this study**

Primers	Sequence ^a
2060	5' GCTAGGGATCCGCTAGAAATGATAGAGGTTGTTATT 3'
2061	5' GCTAGGTCGACGTCATTATCTTTTAGTTGTTGTGCT 3'
2062	5' GCTAGGTCGACCGTTTTAATGCACTGACTTATAAAG 3'
2063	5' GCTAGGCATGCGTAATTCGCCATCTAGATATTCT 3'
2076	5' GCTAGGGTACCAGCTAGCTTCTCTGGAATAG 3'
2077	5' GCTAGGCATGCATTTGGTAAGAGTTTTTTAA 3'

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1041 ^aUnderlined sequences indicate introduced restriction enzyme sites.

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1045 **Figure 1. Gliding of wild-type and $\Delta gldN$ mutant cells on agar**

1046 Cells were grown in TYES at 18°C for 24 h to late exponential phase, (OD approximately 1.3).
1047 Ten microliters of cultures were spotted on TYES solidified with agar (10 g/l) and observed for
1048 motility using an Olympus BH-2 phase-contrast microscope. Wild-type (WT) *F. psychrophilum*,
1049 $\Delta gldN$ mutant, and $\Delta gldN$ complemented with wild-type *gldN* on pBFp4 are shown. In each case
1050 a series of images were taken using a Photometrics Cool-SNAP_{cf}² camera. Individual frames
1051 were colored from red (time 0) to yellow, green, cyan, and finally blue (132 s) and integrated
1052 into one image, resulting in 'rainbow traces' of gliding cells. The top row shows the first frame
1053 for each strain, and the bottom row shows the corresponding 132 sec rainbow trace. White cells
1054 in the bottom panel correspond to cells that exhibited little if any net movement. Bar at lower
1055 right (20 μ m) applies to all panels. The rainbow traces correspond to the sequences in Movie S1.

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1058 **Figure 2. Colonies of wild-type, $\Delta gldN$ mutant, and complemented strains of *F.***

1059 *psychrophilum*

1060 (A-C) Micro-colonies of wild type (WT), $\Delta gldN$ mutant, and $\Delta gldN$ mutant complemented with
1061 wild-type *gldN* on pBFp4. Colonies arose from cells incubated for 32 h at 18°C on 5% TYES
1062 solidified with agar (10 g/l). Photomicrographs were taken with a Photometrics Cool-SNAP_{cf}²
1063 camera mounted on an Olympus IMT-2 phase contrast microscope. Bar in panel C (0.5 mm)
1064 applies to panels A, B, and C. (D) Macro-colonies of wild type, $\Delta gldN$ mutant, and
1065 complemented mutant. Cells were spotted on 5% TYES solidified with agar (10 g/l), incubated at
1066 18°C for 12 d, and photographed using a Nikon D7200 camera. Bar in panel D indicates 10 mm.

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1070 **Figure 3. Soluble extracellular proteins of wild-type and $\Delta gldN$ mutant cells**1071 Cells of wild-type *F. psychrophilum*, $\Delta gldN$ mutant, and $\Delta gldN$ mutant complemented with wild-1072 type *gldN* on pBFp4, were grown in TYES medium at 18°C with shaking until cells reached

1073 early stationary phase of growth (Klett readings of 160). Cells were removed by centrifugation

1074 followed by filtration (0.45 μ m). Proteins from equal amounts of cell-free spent media of wild-

1075 type, mutant, and complemented cells were precipitated with TCA, solubilized in loading buffer,

1076 and separated by SDS-PAGE. Proteins were detected by silver staining. Lane labeled 'TYES'

1077 contained an equivalent amount of TYES growth medium that had not been inoculated with *F.*1078 *psychrophilum*, indicating bands that correspond to components of the growth medium.

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1080 **Figure 4. Proteolytic activity of wild-type and $\Delta gldN$ mutant cells**

1081 Exoproteolytic activity of *F. psychrophilum* wild type (WT), $\Delta gldN$ mutant, and $\Delta gldN$
1082 complemented with pBFp4. A) stationary phase cultures were spotted on TYESG agar
1083 supplemented with casein (7.5 g/l) and proteolytic activity was visualized as clearing zones
1084 around the bacterial growth after incubation at 18°C for 4 days. B) proteolytic activity measured
1085 using azocasein as a substrate. Cells were grown in TYESG broth at 18°C with shaking.
1086 Proteolytic activity was measured in cell-free supernatants from each strain at different stages of
1087 growth. The results of three experiments are presented (values and error bars are means and
1088 standard deviations, respectively). Solid lines: OD₆₀₀ indicating growth. Dashed lines: proteolytic
1089 activity units. Color code: blue (WT), red ($\Delta gldN$), green ($\Delta gldN$ complemented with pBFp4).
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1096 **Figure 5. Hemolytic activity of wild-type and $\Delta gldN$ mutant cells**1097 Hemolytic activities of cells of *F. psychrophilum* wild type (WT), $\Delta gldN$ mutant, and $\Delta gldN$

1098 complemented with pBFp4, incubated for 4 days at 18°C on TYESG agar supplemented with

1099 rainbow trout blood (50 ml/l). The results are representative of three independent experiments.

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1105 **Figure 6. Hemoglobin utilization by wild-type and $\Delta gldN$ mutant cells**1106 Detection of residual heme after growth of *F. psychrophilum* wild type (WT), $\Delta gldN$ mutant, and1107 $\Delta gldN$ complemented with pBFp4 to stationary phase in TYESG broth supplemented with 0.51108 μM hemoglobin. The heme-protoporphyrin IX-screen is a bacterial-growth-based assay that1109 exploits *Bacteroides thetaiotaomicron* heme auxotrophy. The stimulation area (cm^2) is

1110 proportional to the amount of porphyrin (heme, hemoglobin or protoporphyrin IX) in the sample.

1111 TYESG Hb and TYESG \emptyset : sterile medium with (Hb) or without (\emptyset) addition of hemoglobin to1112 0.5 μM . The results correspond to three independent bacterial cultures (mean and standard

1113 deviation are presented).

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1119 **Figure 7. Adhesion, biofilm formation, and sedimentation of wild-type and $\Delta gldN$ mutant**1120 **cells**1121 (A) Adhesion of wild-type *F. psychrophilum* (blue), $\Delta gldN$ mutant (red), and $\Delta gldN$ mutant

1122 complemented with pBFp4 (green) to polystyrene after 3 h of incubation at 18°C without shaking

1123 as determined by staining with crystal violet and measuring absorbance at 595 nm. Adhesion

1124 shown in relation to the wild-type strain, which was set as 100. (B) Biofilm formation on

1125 polystyrene by the same strains (identical color code) grown in half-strength TYES broth for 120

1126 h at 18°C without shaking. (C) Cell sedimentation of the same strains grown in half-strength

1127 TYES broth for 96 h at 18°C with shaking at 200 rpm.

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1133 **Figure 8. Analysis of virulence of wild-type and $\Delta gldN$ mutant cells toward rainbow trout**1134 **following challenge by injection.** Kaplan-Meier survival curves of rainbow trout after1135 intramuscular injection challenge. Four groups of 20 fish (n=80) were challenged with each *F.*1136 *psychrophilum* strain at doses (CFU/fish) of 2.6×10^6 (wild type), 2.7×10^6 ($\Delta gldN$ mutant) and1137 2.8×10^6 ($\Delta gldN$ mutant complemented with pBFp4). Survival curves for fish inoculated with1138 PBS (Control) and for fish inoculated with the $\Delta gldN$ mutant were identical. Survival curves for1139 fish inoculated with either the wild type or the $\Delta gldN$ mutant complemented with pBFp4 are1140 significantly different from the $\Delta gldN$ mutant (Mantel-Cox Log-rank test pvalues <0.0001). The1141 survival curve of the $\Delta gldN$ mutant complemented with pBFp4 was modestly higher than the

1142 wild type (pvalue = 0.0012).

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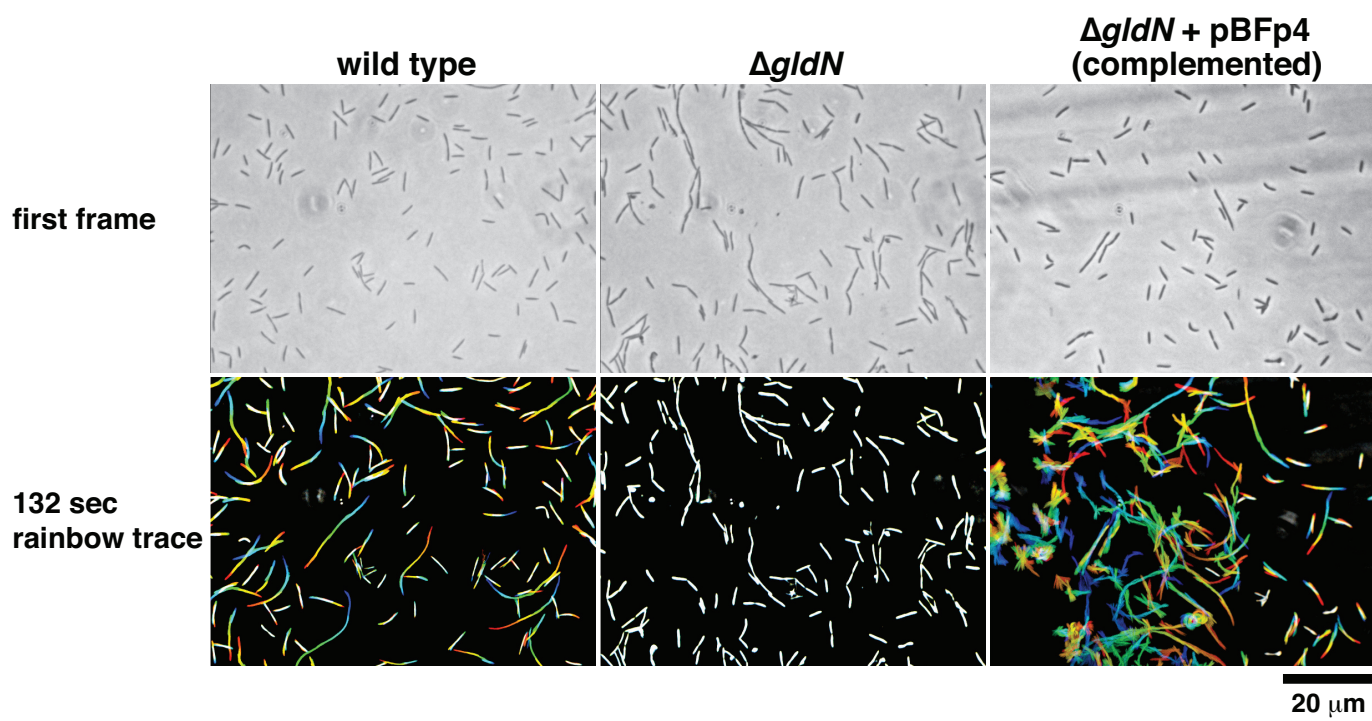
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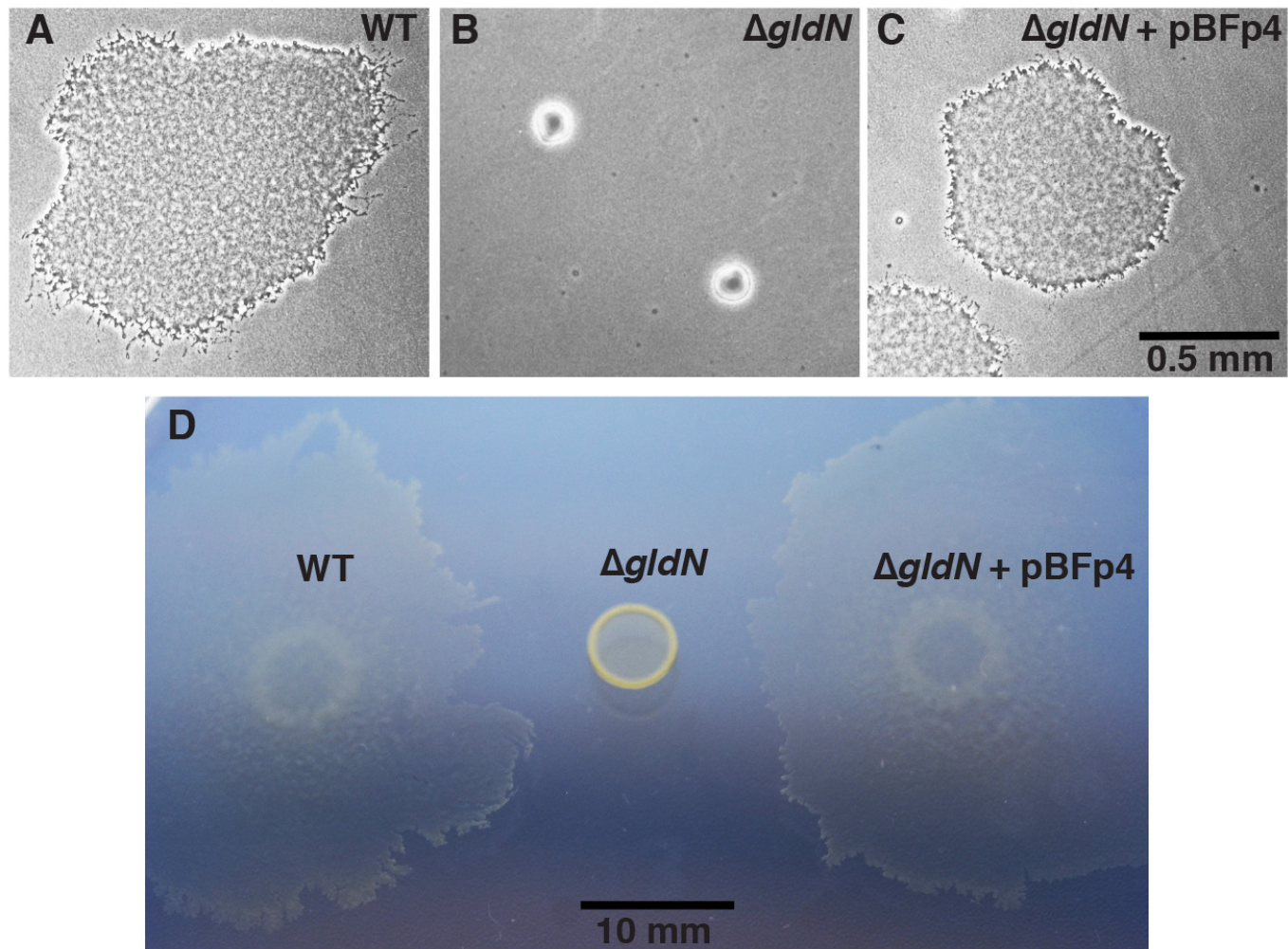
1145 **Figure 9. Analysis of virulence of wild-type and $\Delta gldN$ mutant cells toward rainbow trout**1146 **following immersion challenge**

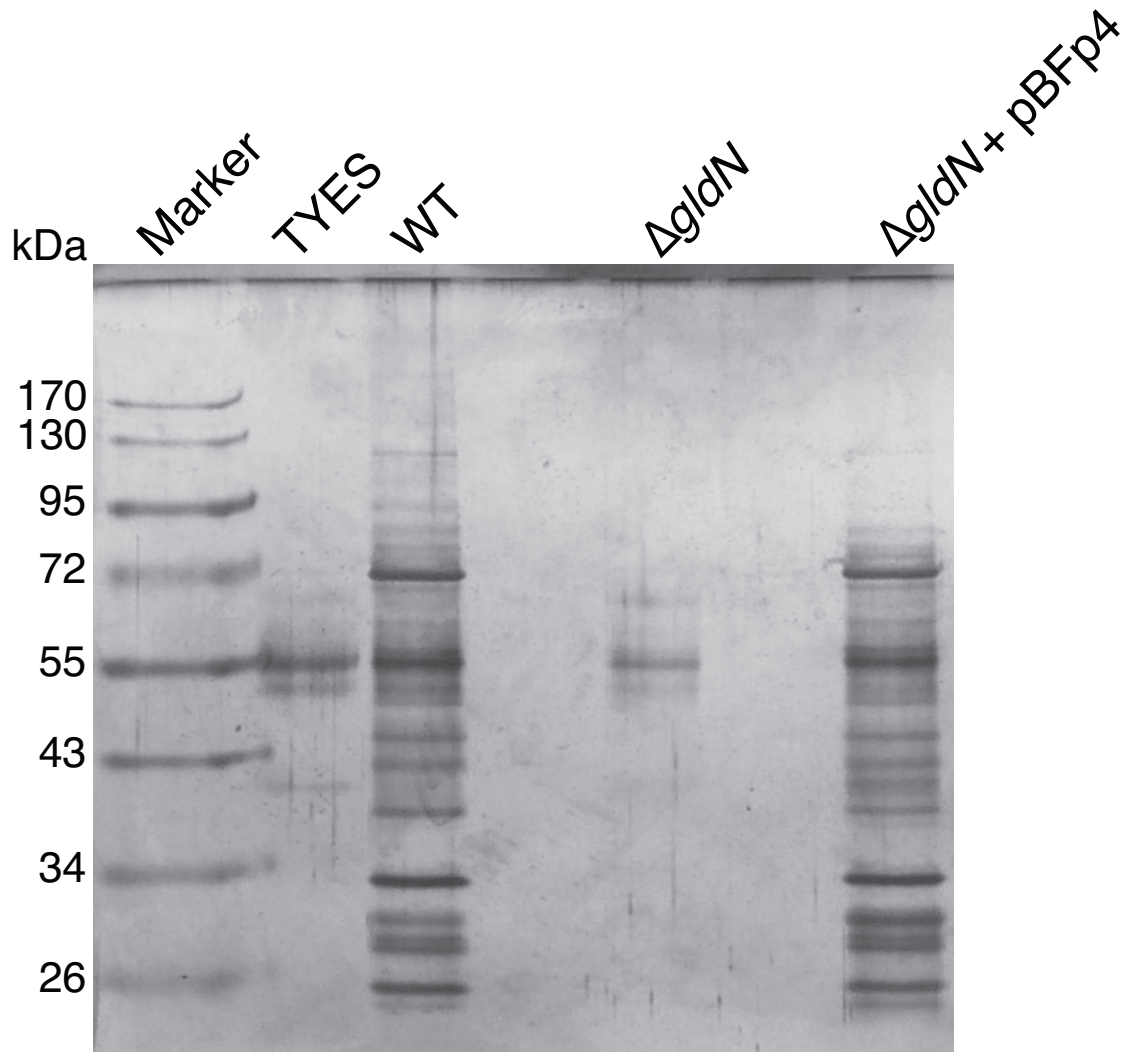
1147 Groups of 42 fish were infected by immersion with *F. psychrophilum* wild type (WT), $\Delta gldN$,
1148 and $\Delta gldN$ complemented with pBFp4. The results of two independent experiments are
1149 presented. A) *F. psychrophilum* bacterial loads in aquarium water during fish infection by
1150 immersion. Average of bacterial quantification determined at the beginning (0 h) and end (24 h)
1151 of fish infection challenge. B) Kaplan-Meier survival curves of rainbow trout after immersion
1152 challenge (each group composed of 30 fish). Survival curves for fish challenged with the $\Delta gldN$
1153 mutant, or with either the wild type or the $\Delta gldN$ mutant complemented with pBFp4 are
1154 significantly different (Mantel-Cox Log-rank test pvalues <0.0001). Colored shaded areas
1155 indicate 95% confidence intervals. C) Bacterial loads in organs of rainbow trout after immersion
1156 challenge. Six fish were sacrificed at 6 h and 30 h post-infection for each group. Serial dilutions
1157 of homogenized organs were incubated on TYESG agar supplemented with fetal calf serum (50
1158 ml/l) to determine the CFU. Bacterial loads of spleen (left panel) and gills (right panel) are
1159 shown. Horizontal dashed line indicates the detection threshold. Values are significantly lower
1160 for $\Delta gldN$ relative to wild-type and complemented strains (Mann-Whitney pvalues < 0.0001).

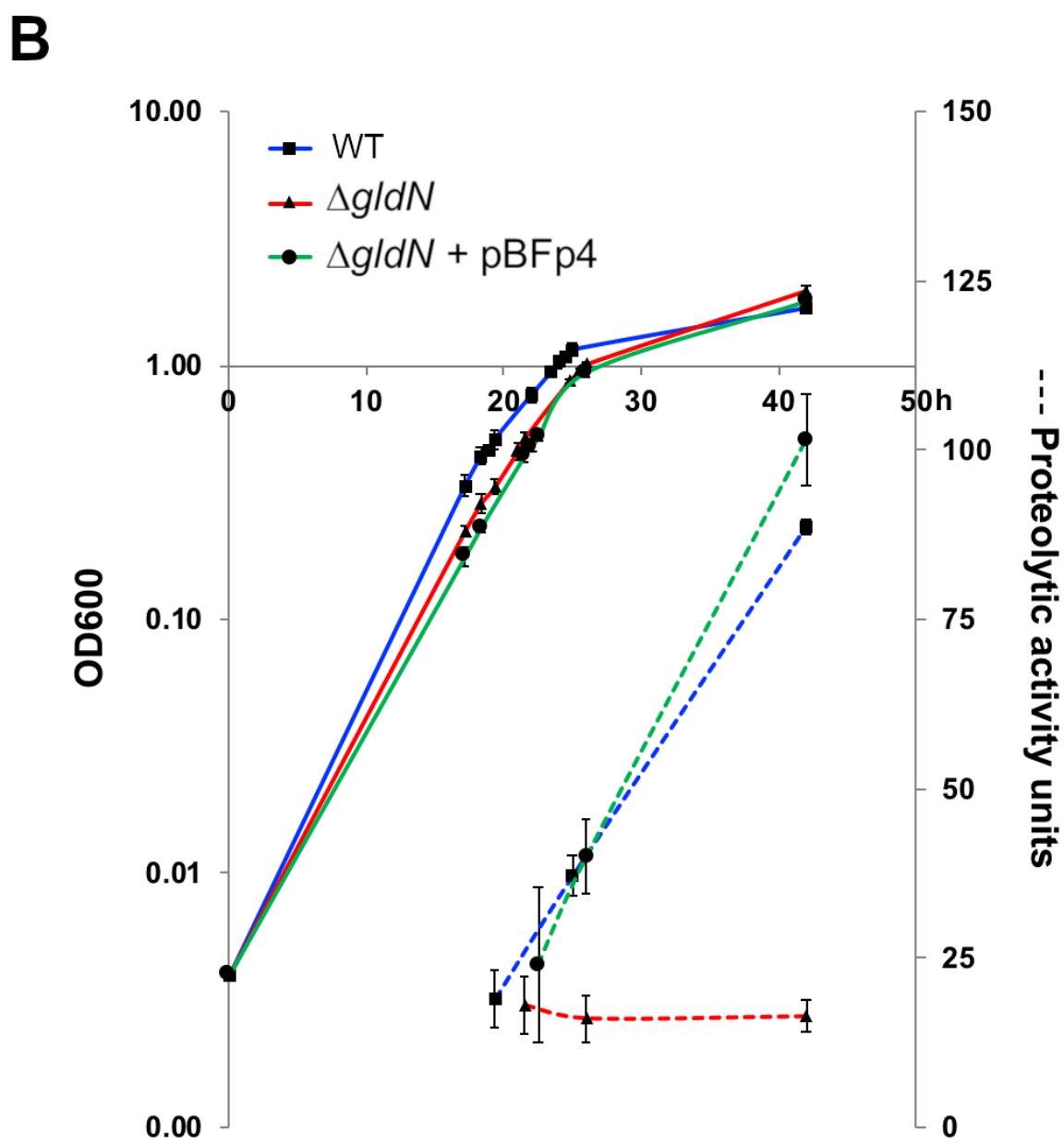
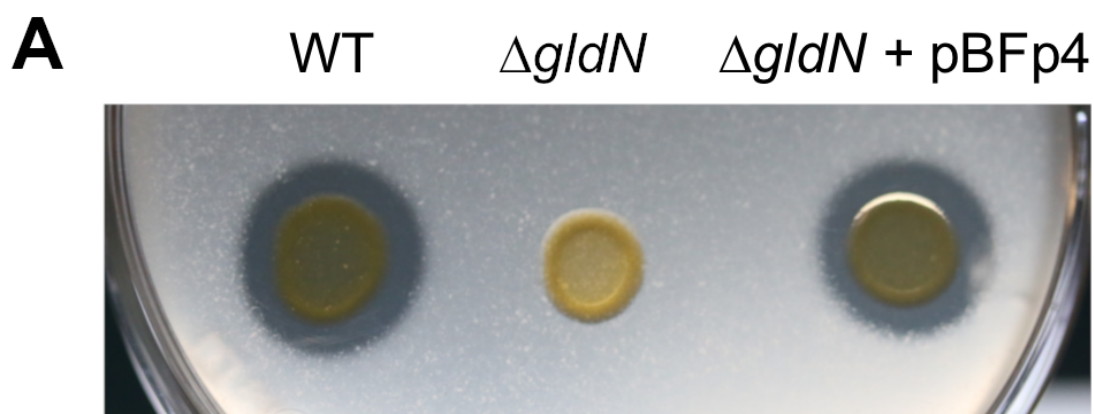
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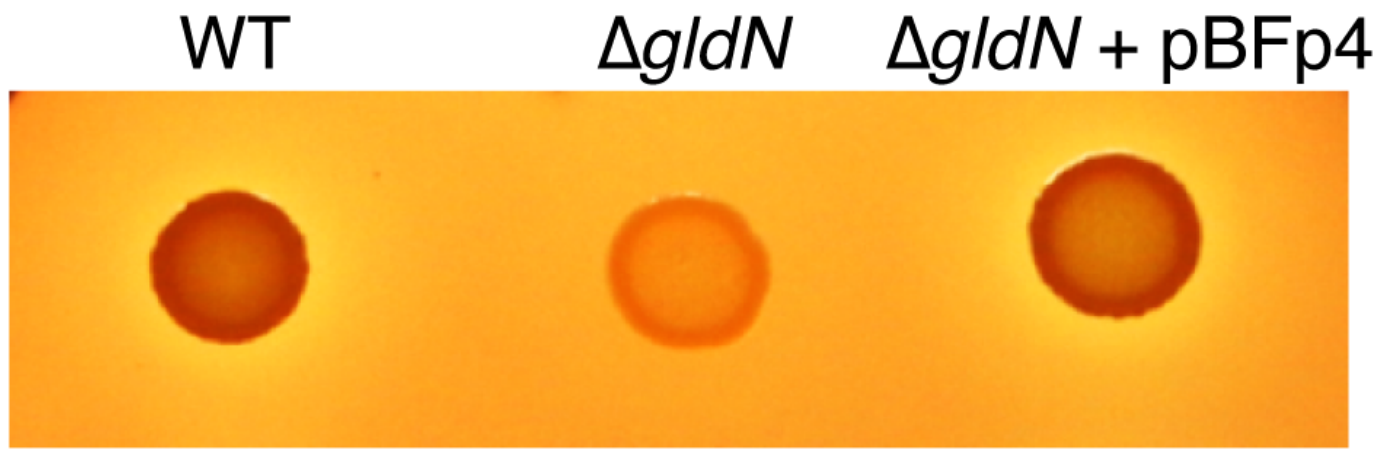
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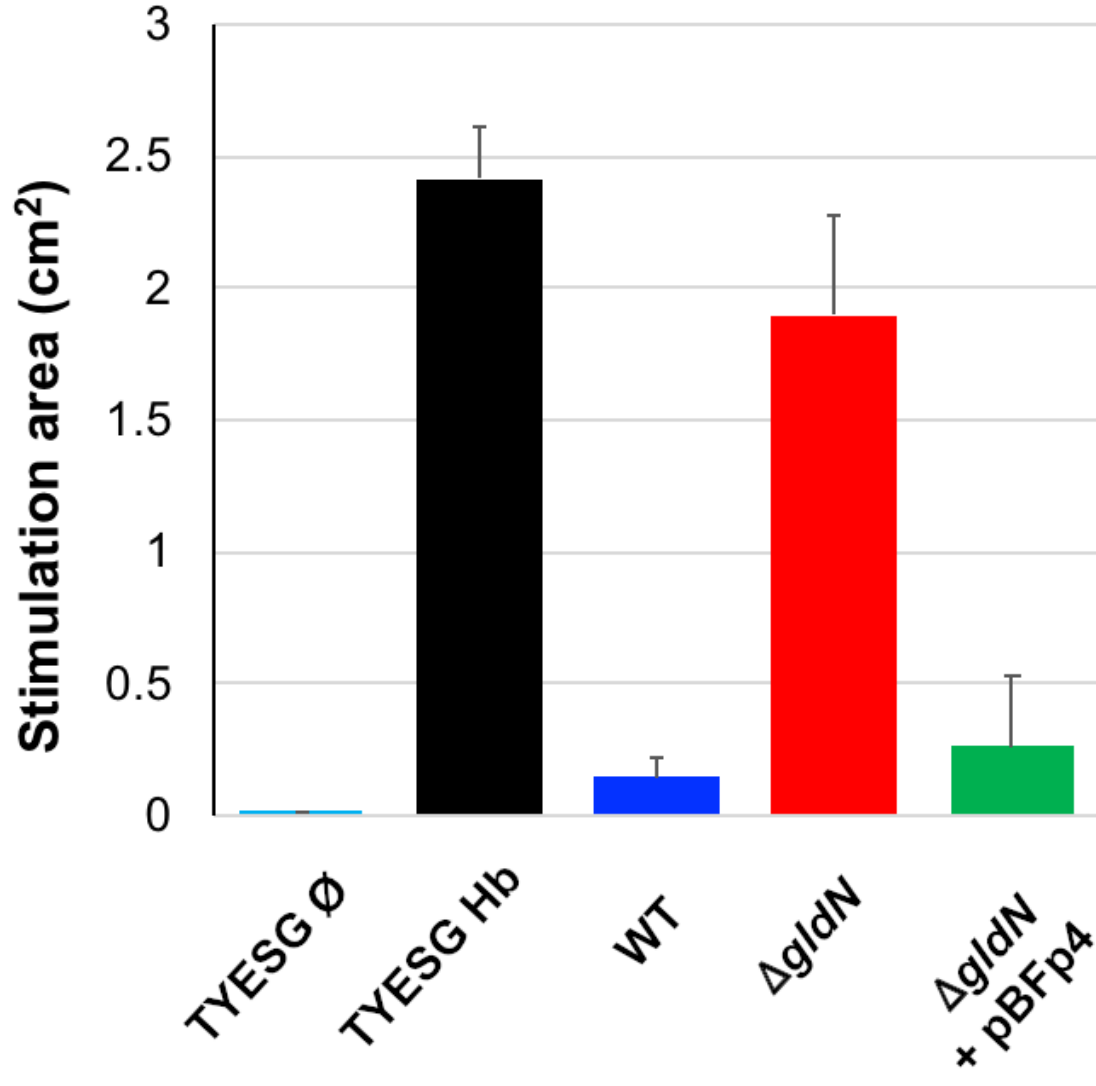


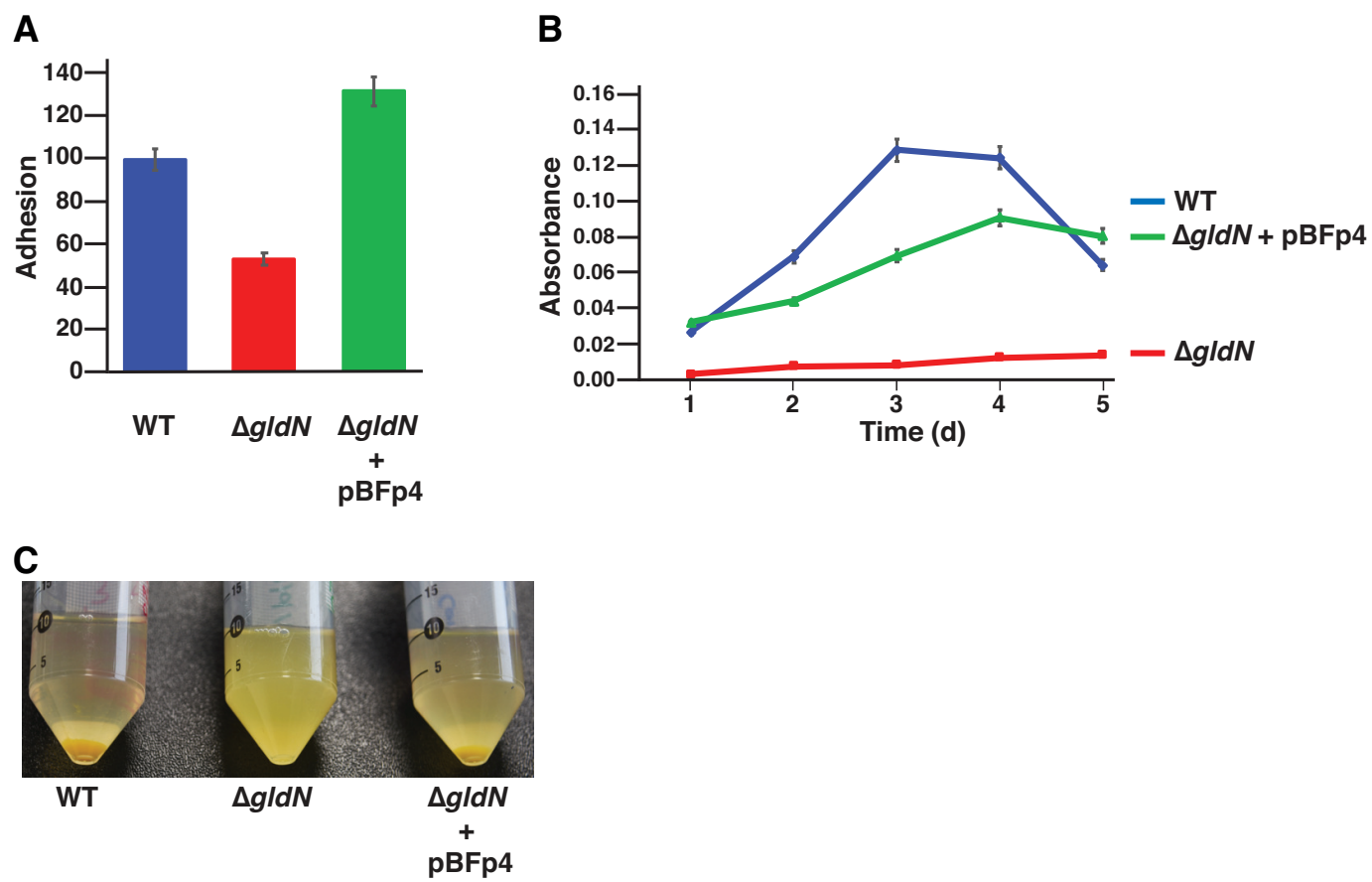


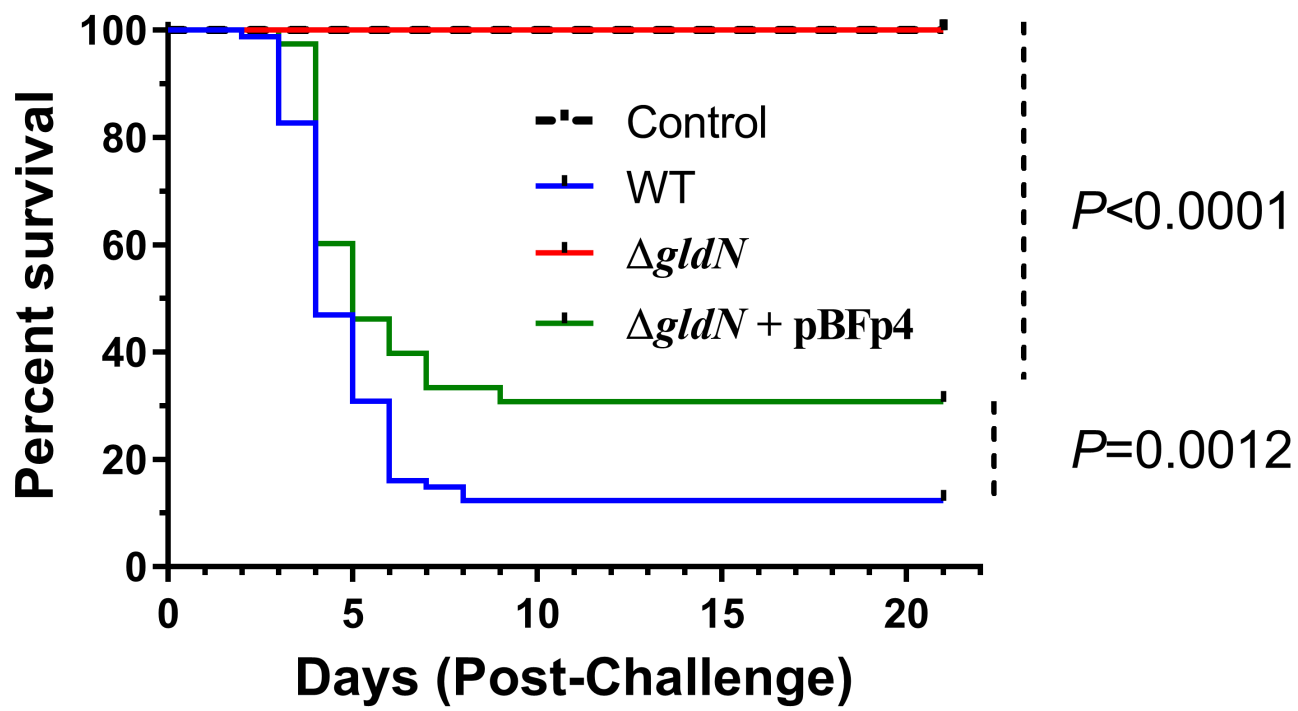








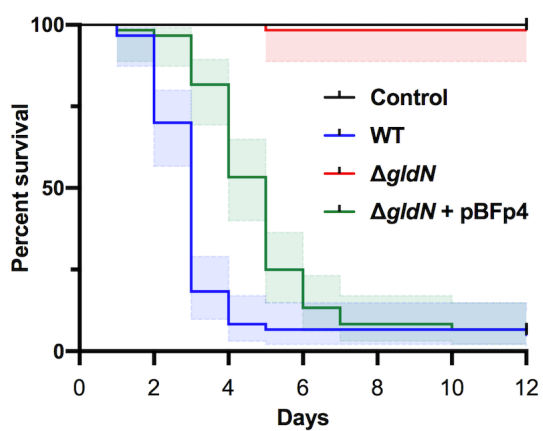




A

Strain	CFU/ml	
	Time (hours)	
	0	24
Wild-type	2.4×10^7	2.0×10^7
$\Delta glbN$	2.0×10^7	1.2×10^7
$\Delta glbN + pBFp4$	2.0×10^7	4.5×10^7

B



C

