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Type IX secretion system effectors and virulence of the model *Flavobacterium columnare* strain MS-FC-4

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ABSTRACT:

Flavobacterium columnare causes columnaris disease in wild and cultured freshwater fish and is a major problem for sustainable aquaculture worldwide. The *F. columnare* type IX secretion system (T9SS) secretes many proteins and is required for virulence. The T9SS component GldN is required for secretion and for gliding motility over surfaces. Genetic manipulation of *F. columnare* is inefficient, which has impeded identification of secreted proteins that are critical for virulence. Here we identified a virulent wild-type *F. columnare* strain (MS-FC-4) that is highly amenable to genetic manipulation. This facilitated isolation and characterization of two deletion mutants lacking core components of the T9SS. Deletion of *gldN* disrupted protein secretion and gliding motility and eliminated virulence in zebrafish and rainbow trout. Deletion of *porV* disrupted secretion and virulence but not motility. Both mutants exhibited decreased extracellular proteolytic, hemolytic, and chondroitin sulfate lyase activities. They also exhibited decreased biofilm formation and decreased attachment to fish fins and to other surfaces. Using genomic and proteomic approaches, we identified proteins secreted by the T9SS. We deleted ten genes encoding secreted proteins and characterized the virulence of mutants lacking individual or multiple secreted proteins. A mutant lacking two genes encoding predicted peptidases exhibited reduced virulence in rainbow trout, and mutants lacking a predicted cytolysin showed reduced virulence in zebrafish and rainbow trout. The results establish *F. columnare* strain MS-FC-4 as a genetically amenable model to identify virulence factors. This may aid development of measures to control columnaris disease and impact fish health and sustainable aquaculture.

IMPORTANCE: *Flavobacterium columnare* causes columnaris disease in wild and aquaculture-reared freshwater fish and is a major problem for aquaculture. Little is known regarding the virulence factors involved in this disease and control measures are inadequate. The type IX secretion system (T9SS) secretes many proteins and is required for virulence, but the secreted virulence factors are not known. We identified a strain of *F. columnare* (MS-FC-4) that is well suited for genetic manipulation. The components of the T9SS and the proteins secreted by this system were identified. Deletion of core T9SS genes eliminated virulence. Genes encoding ten secreted proteins were deleted. Deletion of two peptidase-encoding genes resulted in decreased virulence in rainbow trout, and deletion of a cytolysin-encoding gene resulted in decreased virulence in rainbow trout and zebrafish. Secreted peptidases and cytolysins are likely virulence factors and are targets for the development of control measures.

Flavobacterium columnare causes columnaris disease in wild and cultured freshwater fish (1, 2). Most species are susceptible, including rainbow trout (*Oncorhynchus mykiss*), channel catfish (*Ictalurus punctatus*), tilapia (*Oreochromis spp.*), yellow perch (*Perca flavescens*) and many others (3, 4). Improved understanding of columnaris disease, and strategies to prevent or control it are needed.

F. columnare infections are typically characterized by external necrosis of skin tissue behind the dorsal fin (saddleback lesion) and necrosis of gill, jaw, and fins (2). Although columnaris disease is a major problem for the aquaculture industry worldwide, resulting in high mortalities and economic losses, the virulence mechanisms of *F. columnare* are incompletely understood. Secreted peptidases, chondroitin sulfate lyases, and lipopolysaccharides have been suggested as virulence factors (2), but definitive data demonstrating their roles in columnaris disease are lacking.

F. columnare strains are diverse and belong to multiple genetic groups. These were originally classified as multiple genomovars based on evidence such as 16S rRNA gene restriction fragment length polymorphism analyses (5-7). A more recent comparative sequence analysis of 16S rRNA genes and of six housekeeping genes allowed higher resolution and assigned *F. columnare* strains to four phylogenetic groups (8). Genome analyses revealed sufficient differences to suggest that these may define four species, where genetic group-1 would correspond to *F. columnare* (8-10). The four genetic groups appear to have biological relevance (8). Members of genetic group 1 are strongly associated with disease in salmonids and have also been linked to disease in other cool- or cold-water fishes. They are also capable of growth at colder temperatures (11). Genetic groups 2 and 3 are more commonly associated with channel

91 catfish and other warm-water species, and genetic group-4 strains have been isolated primarily
92 from the warm-water fish, tilapia (8).
93 Techniques to genetically manipulate *F. columnare* were developed for several members
94 of genetic groups 1 and 2 (12-14). These include methods to construct site-directed chromosomal
95 deletions and to complement the resulting mutants by introduction of wild-type genes on
96 replicative plasmids. However, it was unclear if these techniques are broadly useful for other
97 members of genetic groups 1 and 2, and they were not examined for their ability to function in
98 members of genetic groups 3 and 4.
99 These genetic techniques were used to demonstrate that the type IX secretion system
100 (T9SS) is required for virulence of strain IA-S-4 (genetic group 1) and strain C#2 (genetic group
101 2) (13). Deletion mutants lacking a core component of the T9SS, GldN, were deficient in
102 secretion and were avirulent. T9SSs are common in, but apparently confined to, members of the
103 phylum *Bacteroidetes*, to which *F. columnare* belongs. Proteins secreted by T9SSs have N-
104 terminal signal peptides that allow export across the cytoplasmic membrane by the Sec system,
105 and C-terminal domains (CTDs) that target them for secretion across the outer membrane by the
106 T9SS (15-17).
107 Most characterized T9SS CTDs belong to the TIGRFAM protein domain families
108 TIGR04183 (type A CTDs) or TIGR04131/pfam13585 (type B CTDs) (18, 19). Most CTDs that
109 have been characterized are removed during or after secretion (16, 17). Some proteins secreted
110 by T9SSs become covalently attached to the cell surface whereas others are released in soluble
111 form (20-23). Analysis of *F. columnare* genomes identified approximately 40 genes in individual
112 strains that are predicted to encode proteins secreted by the T9SS, and proteomic analyses
113 supported secretion of several of these proteins by this system (13). Among the proteins

predicted to be secreted by the *F. columnare* T9SS are peptidases, chondroitin sulfate lyases, nucleases, and adhesins (13), any of which may be virulence factors. Whereas the T9SS is known to be required for virulence, the roles of the individual secreted proteins in this process are not known. Moreover, the roles of the *F. columnare* T9SS in biofilm formation and in attachment of cells to abiotic surfaces and to fish tissues have not been examined. It also is not known if fish exposed to T9SS-deficient mutants are protected from later exposure to wild-type cells.

In addition to secreting enzymes and other proteins to the cell surface and beyond, the T9SS is also required for cell movement over surfaces by gliding motility. Movement of motility adhesins in helical paths along the cell surface results in rotation and translocation of the rod-shaped cell as it crawls over a surface in a tank-like manner. The components of the T9SS are involved in active secretion of the motility adhesins across the outer membrane, and in active movement of these adhesins along the cell surface (20, 24-27). Gliding allows cells to aggregate into the columns that are often observed on infected gills and that gave columnaris disease and *F. columnare* their names. Gliding may thus be important in progression of the disease.

The shared components of the T9SS and gliding machineries make it difficult to separate the roles of secretion and motility in virulence. In *Flavobacterium johnsoniae*, PorV is required for secretion of most proteins targeted to the T9SS, but it is not required for gliding motility (28). Similarly, a *porV* deletion mutant of *F. columnare* genetic group-2 strain C#2 was compromised for secretion and was avirulent, but it retained gliding motility (13). Thus, secretion mediated by the T9SS appears to be critical for virulence of *F. columnare* strain C#2. Similar evidence for genetic group-1 strains is currently lacking (13), since repeated attempts to isolate a *porV* mutant of *F. columnare* strain IA-S-4 failed. The efficiency of plasmid transfer into strain IA-S-4 is low

(this study), which may explain the inability to obtain the *porV* mutant. Inefficient gene transfer also makes comprehensive genetic analysis of the roles of the many secreted proteins in virulence challenging.

Here we screened 39 *F. columnare* strains and identified members of each genetic group that accepted plasmids by conjugation from *Escherichia coli*. One virulent genetic group-1 strain, MS-FC-4, was much more amenable to genetic manipulation than were the others and was developed as a model system to study columnaris disease. Using this strain, we constructed single and multiple gene deletion mutants, and characterized the virulence of T9SS mutants and of mutants lacking individual or multiple secreted proteins. The results suggest that some secreted peptidases and cytolysins may contribute to virulence and provide a path to identify additional secreted virulence factors.

RESULTS

Identification of genetically amenable strains from each *F. columnare* genetic group.

Thirty-nine strains of *F. columnare* were examined for tetracycline-resistant colonies after transfer of pCP23 (29) by conjugation from *E. coli*. Thirteen strains displayed tetracycline-resistant colonies (Table S1), and the presence of pCP23 was verified by plasmid isolation and analysis of restriction fragments. Successful transfer of pCP23 was achieved for at least one member of each of the four *F. columnare* genetic groups, allowing future genetic studies on members of each group. To select the best genetic group-1 strain for genetic manipulation experiments, we compared gene transfer into strain IA-S-4, the genetic group-1 strain that we previously used for genetic studies (13), and strains CSF-298-10 and MS-FC-4. Strain IA-S-4

gave 7-fold and 112-fold fewer transconjugant colonies than did strains CSF-298-10 and MS-FC-4 respectively (Table S2). *F. columnare* strain MS-FC-4 was selected as a model strain to analyze virulence because of this efficiency of gene transfer.

***F. columnare* MS-FC-4 T9SS and predicted T9SS-secreted proteins.** To facilitate the use of strain MS-FC-4 as a model to study *F. columnare* virulence we analyzed its genome (30). The *F. columnare* MS-FC-4 genome encoded each of the components of the T9SS (Table S3 and Table S4), which was previously shown to be required for virulence of strain IA-S-4 (13). We also identified 49 *F. columnare* strain MS-FC-4 proteins predicted to be secreted by the T9SS (Table S5). This included 38 with type A CTDs (TIGR04183) and 8 with type B CTDs (TIGR04131/pfam13585). Three additional predicted secreted proteins have CTDs that belong to protein domain family cl41395 (NCBI family accession number NF033708), which has recently been referred to as T9SS sorting signal type C (type C CTDs) (21, 31). The number of *F. columnare* strain MS-FC-4 proteins predicted to be secreted by the T9SS may be an underestimate because the sequence requirements for secretion by this system are not completely understood (21, 32). The 49 predicted secreted proteins included 16 peptidases, 2 nucleases, 1 glycoside hydrolase, 2 chondroitin sulfate lyases, 1 heme binding protein, and many potential adhesins. Any of these may contribute to virulence. As previously observed for other bacteria that have T9SSs (18, 32) none of the proteins with type B CTDs had predicted enzymatic functions. Moreover, many of the genes encoding proteins with type B CTDs are adjacent to genes encoding proteins linked to T9SS function (*porP*/*sprF*-like genes, and *porE*-like genes; Table S4), as previously described for other bacteria (18, 32-34).

Effect of deletion of the T9SS genes *gldN* and *porV* on gliding motility. To investigate the role of the T9SS in virulence we deleted *gldN*, which in *F. johnsoniae* and other bacteria is

183 essential for T9SS function and for gliding motility (13, 35), and *porV*, which is required for
184 secretion of proteins that have type A or type C CTDs but is not essential for gliding (28). We
185 examined the mutants for motility defects. Wild-type cells moved over glass by gliding, whereas
186 Δ*gldN* mutant cells did not (Fig. 1, and Movie S1). Complementation of the Δ*gldN* mutant by
187 introduction of pLN5, which carries *gldN* expressed from a plasmid promoter (13), restored
188 gliding motility. As a result of the gliding movements, cells of the wild-type and complemented
189 strains formed thin spreading colonies on TYES agar, whereas cells of the Δ*gldN* mutant formed
190 nonspreading colonies (Fig. 2). These results are similar to those previously reported for genetic
191 group-1 *F. columnare* strain IA-S-4 (13). In the previous study (13) we failed to isolate a *porV*
192 mutant of strain IA-S-4, despite repeated attempts. The greater efficiency of gene transfer into
193 strain MS-FC-4 presented a better chance to obtain such a mutant, and many *porV* deletion
194 mutant colonies were obtained on the first attempt. In *F. johnsoniae*, *porV* mutants are motile but
195 are deficient for secretion of many, but not all, proteins by the T9SS (28). This allows partial
196 separation of motility and secretion. The *F. columnare* MS-FC-4 Δ*porV* mutant was motile and
197 produced spreading colonies, although motility and spreading were less than observed for the
198 wild type (Figs. 1 and 2, and Movie S2). Surprisingly, attempted complementation of the Δ*porV*
199 mutant using pYT371, which carries *porV*, resulted in decreased gliding motility (Figs. 1 and 2,
200 and Movie S2). This may indicate that overexpression of *porV* from a multicopy plasmid is
201 detrimental to the cells.
202 To overcome the problems described above associated with use of a multicopy plasmid to
203 complement the *porV* mutant, we developed a strategy to insert a single copy of *porV*, or any
204 other gene of interest, on the chromosome. We used an intergenic region adjacent to a predicted
205 defective integrated phage island in strain MS-FC-4 (30) as a ‘neutral integration site’ (Fig. S1).

We inserted regions immediately upstream and downstream of this site into the nonreplicative plasmid pMS75 (12), which carries genes that confer tetracycline resistance and sucrose sensitivity on *F. columnare*, generating pBFC2 (Fig. S1). pBFC2 carries the *F. johnsoniae ompA* promoter to express the integrated gene. A second plasmid, pBFC5, was constructed to express the gene from its native promoter. *porV* was inserted into both plasmids. Conjugation into the *F. columnare* Δ*porV* mutant, followed by selection for tetracycline resistance, resulted in strains carrying plasmid inserted in the genome by homologous recombination at either the upstream or downstream region. Exposure to sucrose selected for strains in which a second recombination event had resulted in plasmid loss. Approximately half of these colonies retained *porV* inserted at the neutral integration site and these were identified by PCR. We refer to Δ*porV* ectopically complemented with *porV* expressed from the *ompA* promoter at the neutral integration site as Δ$porV_{EC}$, and Δ*porV* ectopically complemented with *porV* expressed from its native promoter as Δ$porV_{EC2}$. The Δ*gldN* mutant was also ectopically complemented (Δ$gldN_{EC}$; expressed from the *ompA* promoter). In each case gliding of individual cells and colony spreading of the ectopically complemented mutants were similar to the wild type (Figs. 1 and 2, and Movie S2). These results illustrate the usefulness of this approach as an alternative to multicopy plasmid complementation. During this analysis, we also assessed the stability of the plasmid pCP23 (the base plasmid used for all replicative plasmid complementation experiments described here) in the absence of antibiotic selection. Most cells with pCP23 retained their tetracycline resistance through at least 3 cycles of dilution into fresh antibiotic-free media followed by incubation for 15 h, at which point cultures were in stationary phase (Fig. S2). pCP23 carrying *gldN* was also stable whereas pCP23 carrying *porV* was somewhat less stable, supporting the suggestion that expression of *porV* from a multicopy plasmid is detrimental to cells.

The Δ*gldN* mutant exhibits defects in protein secretion. Cell-free spent culture fluids from wild-type, mutant, and complemented strains were examined by SDS-PAGE for soluble secreted proteins. The Δ*gldN* mutant cells released less protein than did wild-type or complemented cells (Fig. S3), suggesting a protein secretion defect. Samples of cell-free culture fluid were examined for proteins by liquid chromatography-tandem mass spectrometry (LC-MS/MS; Dataset S1). The Δ*gldN* mutant had far fewer spectral counts than did the wild-type or complemented strains, as expected for a secretion mutant. Of the 49 predicted secreted proteins (Table S5), 39 were detected in this experiment (Dataset S1, sheet 4). In each case there were more spectral counts for these proteins from the wild-type and complemented strains than from the Δ*gldN* mutant. Included among these proteins were 11 peptidases, two chondroitin sulfate lyases, the motility proteins SprB and SprC, and several possible adhesins. Many proteins that did not have obvious T9SS CTDs were also observed at higher levels from the wild-type and complemented strains than from the Δ*gldN* mutant, and some of these may also require the T9SS for their secretion (Dataset S1, sheets 2, 3 and 5). Included among these apparently secreted proteins that lacked obvious T9SS-CTDs were 24 predicted peptidases and two predicted cytolysins. Many of these proteins that lack recognizable T9SS CTDs have type II signal peptides and are thus predicted to be lipoproteins (Dataset S1, sheet 2, column M). Among the 24 predicted peptidases that lacked obvious T9SS CTDs but were detected as T9SS-secreted proteins by LC-MS/MS analysis, 10 were predicted to be lipoproteins (Dataset S1, sheet 5, column M). In contrast, none of the 11 secreted peptidases detected that had T9SS-CTDs were predicted to be lipoproteins. Despite the apparent decreased secretion of many proteins, the Δ*gldN* mutant grew as well as the wild type in TYES broth (Fig. S4). The Δ*porV* mutant was also not affected in growth (Fig. S4).

***F. columnare* T9SS mutants exhibit decreased extracellular proteolytic, chondroitin sulfate lyase, and hemolytic activities.** *F. columnare* secretes many digestive enzymes, including peptidases and chondroitin sulfate lyases (36-39). We examined wild-type, mutant, and complemented strains for extracellular proteolytic and chondroitin sulfate lyase activities. The Δ*gldN* and Δ*porV* mutants exhibited lower levels of extracellular protease and chondroitin sulfate lyase activities than did the wild-type or complemented strains (Fig. 3). Digestion of tissue proteins and chondroitin sulfate by wild-type *F. columnare* may contribute to the tissue erosion that is often associated with columnaris disease. *F. columnare* secretes two predicted cytolysins (C6N29_04500 [CylA] and C6N29_11340 [CylB]) that were apparently secreted by wild-type cells but not by the Δ*gldN* mutant (Dataset S1). Secreted hemolysins and other cell lytic activities are important virulence factors of many bacterial pathogens (40, 41). We examined the ability of cell-free culture fluids from wild-type, mutant and complemented strains to lyse red blood cells. The Δ*gldN* and Δ*porV* mutants exhibited decreased lysis of red blood cells compared to the wild-type and complemented strains (Fig. 3C).

***F. columnare* T9SS mutants are deficient in adhesion, biofilm formation, and cell sedimentation**. The *F. columnare* T9SS appears to secrete many predicted adhesins (Table S5 and Dataset S1). We examined the role of the T9SS in adhesion and biofilm formation. Cells of the Δ*gldN* and Δ*porV* mutants were partially deficient in adhesion to polystyrene compared to wild-type cells or to cells of the complemented mutants (Fig. 4A). Adhesion of cells to a surface is the first step in biofilm formation, and *F. columnare* is known to form biofilms on polystyrene and many other surfaces (42, 43). The Δ*gldN* and Δ*porV* mutants were deficient in biofilm formation compared to the wild-type and complemented strains (Fig. 4B). *F. columnare* cells also adhere to each other (36, 44, 45), sometimes causing them to sediment from suspension.

Wild-type cells sedimented more rapidly from suspension than did the Δ*gldN* and Δ*porV* mutant cells (Fig. 4C). Complementation of the mutants restored sedimentation. Wild-type *F. columnare* cells also attach to and colonize exposed fish tissues, including fins, skin, and gills (46-49). We examined attachment of cells expressing GFP from pNT67 to pectoral fins over a 60 min period. Pectoral fins were used because they exhibited lower levels of autofluorescence than did the other zebrafish fins. Strains were examined at similar concentrations as measured spectrophotometrically and by analysis of fluorescence (Table S6). Fluorescence microscopy verified that all or nearly all bacterial cells were fluorescent (Fig. S5). Hundreds of wild-type cells attached to each of six fins examined, whereas no Δ*gldN* or Δ*porV* mutant cells were observed attached to fins (Fig. 5, Fig. S6). Complementation restored the ability of the mutants to attach to the fins. In total, the results suggest that the T9SS is involved in adhesion of cells to abiotic surfaces, to fish, and to each other, and is needed for efficient biofilm formation. The predicted adhesins (Table S5) that were demonstrated to be secreted by the T9SS (Dataset S1) may be important for each of these processes.

***F. columnare* T9SS mutants are deficient in virulence.** Wild-type, mutant (Δ*gldN* and Δ*porV*) and complemented cells were examined for virulence against adult zebrafish (Fig. 6), and against rainbow trout alevin (sac fry) and fry (Fig. 7). In each case the fish were sensitive to the wild-type strain but were unaffected by the Δ*gldN* and Δ*porV* mutants. Complementation of the Δ*gldN* and Δ*porV* mutants with *gldN* or *porV*, respectively, either on plasmid or ectopically on the chromosome, restored virulence in zebrafish (Fig. 6). In contrast, only ectopic complementation restored virulence in rainbow trout fry (Fig. 7). This is presumably explained by the low number of CFU/ml for the plasmid-containing strains in these experiments. As with the Δ*porV* motility results reported in figures 1 and 2, ectopic complementation appears to be

more reliable than replicative plasmid-based complementation for some experiments. The zebrafish and rainbow trout fry that survived challenge with the Δ*gldN* and Δ*porV* mutants were maintained for 28 days post-exposure (zebrafish) or for approximately 400-degree days (equivalent to 25 days at 16°C) post-exposure (rainbow trout) and examined for resistance to infection by wild-type cells. In each case, the fish were as sensitive as naive fish, indicating that they were not immune to later infection (Figs. 6 and 7).

Deletion of ten genes encoding proteins secreted by the T9SS. To demonstrate the usefulness of high efficiency gene transfer into strain MS-FC-4 we deleted 10 genes (*cslA*, *cslB*, *cylA*, *cylB*, C6N29_11545, C6N29_11550, C6N29_05800, C6N29_07385, C6N29_08610 and C6N29_03400; Table 1) encoding proteins that appeared to be secreted by the T9SS based on LC-MS/MS analyses (Dataset S1, sheet 3, column N). The genes were selected primarily based on numbers of LC-MS/MS spectral counts from the culture fluid of wild-type *F. columnare* strain MS-FC-4 (Dataset S1, sheet 3). They were also selected based on a similar but more limited LC-MS/MS analysis of *F. columnare* genetic group-2 strain C#2 proteins (13). That analysis only identified seven secreted proteins, six of which are orthologs of the *F. columnare* strain MS-FC-4 proteins encoded by the genes that we deleted here. The products of the ten *F. columnare* strain MS-FC-4 genes include a predicted endonuclease and predicted chondroitin sulfate lyases, peptidases, cytolysins, and proteins of unknown function (Table 1).

Some *F. columnare* strain MS-FC-4 mutants lacking genes encoding proteins with predicted functions were examined for these activities. Analysis of the Δ*cslA* and Δ*cslB* mutants and of the Δ*cslA* Δ*cslB* double mutant confirmed the requirement of these genes for digestion of chondroitin sulfate (Fig. 8) as was previously reported for *F. columnare* strain G4 (12). Both *cslA* and *cslB* contributed to chondroitin digestion, with *cslA* appearing to play the dominant role.

The Δ*cslA* Δ*cslB* double mutant failed to digest chondroitin sulfate. Deletion of the two adjacent peptidase encoding genes (C6N29_11545 and C6N29_11550) resulted in partial reduction of secreted proteolytic activity (Fig. 8C). Deletion of the cytolysin-encoding gene *cylB* (C6N29_11340) resulted in decreased hemolytic activity, whereas deletion of *cylA* did not (Fig. 8D). C6N29_07385 encodes a large protein that appears to be abundant and to require the T9SS for its secretion (Dataset S1), but lacks an obvious T9SS-CTD. Since many adhesins are large secreted proteins, we examined the ΔC6N29_07385 mutant for defects in adhesion, biofilm formation and sedimentation. The mutant only showed a slight defect in adhesion to polystyrene and in biofilm formation, and sedimented from suspension similar to the wild type (Fig. 4).

Challenge of germ-free zebrafish larvae to screen *F. columnare* mutants for virulence defects. Germ-free zebrafish larvae were challenged with wild-type, mutant, and complemented strains of *F. columnare* MS-FC-4 essentially as previously described (50) with modifications indicated in Materials and Methods. Most control larvae, and those exposed to Δ*gldN* and Δ*porV* mutants, survived for 10 days of incubation post-exposure, whereas larvae exposed to all other mutants, to the wild type, and to the complemented Δ*gldN* and Δ*porV* mutants died within 3 days post-exposure (Fig. 9). The results for Δ*gldN* and Δ*porV* mutant and complemented strains recapitulated those observed for adult zebrafish (Fig. 6). The results with the other mutants did not indicate obvious effects on virulence, although modest increased time of survival for larvae exposed to some mutants was observed.

Chondroitin sulfate lyases are not required for virulence in adult zebrafish or in rainbow trout. Previous experiments demonstrated that Δ*cslA*, Δ*cslB*, and Δ*cslA* Δ*cslB* mutants of *F. columnare* strain G4 were virulent against grass carp challenged by injection. Because injection probably does not mimic the natural route for *F. columnare* infection, here we

examined the role of chondroitin sulfate lyases in virulence of *F. columnare* strain MS-FC-4 by immersion challenge. Wild type, mutants (Δ*cslA*, Δ*cslB*, and Δ*cslA* Δ*cslB*), and complemented mutants were examined for virulence against zebrafish and rainbow trout (Fig. S7). In each case (and in the larval zebrafish challenges described above) the fish were equally sensitive to the wild-type and mutant cells, indicating that under our challenge conditions chondroitin sulfate lyases were not required for virulence.

Effect of deletion of C6N29_03400, C6N29_07385 and C6N29_08610 on virulence. C6N29_03400 and C6N29_07385 encode a predicted endonuclease and a large protein of unknown function respectively, both of which lack an obvious T9SS CTD. C6N29_08610 encodes a type A CTD protein of unknown function. All three proteins were apparently secreted by wild-type cells, but not by the Δ*gldN* mutant (Dataset S1). Deletion of C6N29_08610 had no effect on virulence against adult zebrafish or against rainbow trout alevin or fry (Fig. S8). Deletion of C6N29_03400 and C6N29_07385 also had no effect on survival of zebrafish but did result in modest increases in survival for rainbow trout fry (Fig. S8).

Deletion of two adjacent peptidase-encoding genes (C6N29_11545 and C6N29_11550) resulted in a partial decrease in virulence against rainbow trout. The mutant FCB54 (Δ3peptidases), which lacks the three peptidase-encoding genes, C6N29_11545, C6N29_11550, and C6N29_05800, exhibited decreased secreted proteolytic activity (Fig. 8) and was examined for virulence against zebrafish and rainbow trout. C6N29_05800 encodes a probable M4 family peptidase (Table 1, Table S5), whereas C6N29_11545 and C6N29_11550, which are adjacent to each other on the genome, encode predicted metallopeptidases (Table 1, Dataset S1, sheet 5) that exhibit 79.4% amino acid identity over their entire sequences. The Δ3peptidases mutant had no obvious defect in virulence in zebrafish (Fig. 9 and Fig. S9), or in

rainbow trout alevin (Fig. 10A) but challenge of rainbow trout fry with the mutant resulted in fewer mortalities than did challenge with wild-type cells (Fig. 10B and Fig. S10). *F. columnare* produces many other secreted peptidases that could contribute to the remaining virulence of the Δ3peptidases mutant. Rainbow trout that survived exposure to the Δ3peptidases mutant were not protected from later exposure to wild-type cells (Fig. S10). Because the Δ3peptidases mutant was partially deficient in virulence in rainbow trout, we also examined a deletion mutant that lacked just C6N29_05800 (Δpeptidase), and another mutant for which the adjacent genes C6N29_11545 and C6N29_11550 were deleted as a single event (Δ2peptidases). The mutant lacking C6N29_05800 was as virulent as wild type, whereas the mutant lacking C6N29_11545 and C6N29_11550 was partially deficient in virulence, equivalent to the Δ3peptidases mutant, which lacks all three genes (Fig. 10B). The results suggest potential roles for the proteases encoded by C6N29_11545 and C6N29_11550 in virulence.

Deletion of the predicted cytolysin-encoding gene *cylA* resulted in reduced virulence. The deletion mutants Δ*cylA*, Δ*cylB* and Δ*cylA* Δ*cylB*, were examined for virulence against zebrafish and rainbow trout. Deletion of these genes had little effect on virulence against zebrafish larvae (Fig. 9), but both the Δ*cylA* and Δ*cylA* Δ*cylB* mutants resulted in fewer mortalities than did the wild type when adult zebrafish were challenged (Fig. 11A). In contrast, the Δ*cylB* mutant behaved like the wild type in adult zebrafish challenges (data not shown). Similar results were obtained for rainbow trout. Rainbow trout alevin were sensitive to each of the strains, whereas fry were sensitive to the wild-type and Δ*cylB* strains but were more resistant to the Δ*cylA* and Δ*cylA* Δ*cylB* mutants (Fig. 11B and Fig. S10). Complementation of the Δ*cylA* Δ*cylB* double mutant with *cylA* on a plasmid restored virulence. Rainbow trout that survived

389 exposure to the Δ*cylA* Δ*cylB* double mutant were not protected from later exposure to wild-type
390 cells (Fig. S10).
391
392 **DISCUSSION**
393 *F. columnare* causes large economic losses worldwide for freshwater aquaculture.
394 Genetic manipulation of several strains of this bacterium was recently demonstrated, but the low
395 efficiencies of gene transfer made large-scale genetic studies difficult. Here we demonstrated
396 plasmid transfer into members of each of the four recognized genetic groups of *F. columnare* and
397 showed that genetic group-1 strain MS-FC-4 was much more efficient at taking up plasmid DNA
398 than were the other strains. We conducted genomic, proteomic, and genetic experiments on this
399 strain to characterize the role of the T9SS and of secreted proteins in virulence.
400 Genome analysis identified the components of the T9SS and 49 proteins predicted to be
401 secreted by this system based on the presence of T9SS CTDs. We previously identified 39
402 predicted secreted proteins of *F. columnare* strain IA-S-4 (13), and strain MS-FC-4 has orthologs
403 to each of these. Strain IA-S-4 also has orthologs of nine of the ten additional strain MS-FC-4
404 secreted proteins that were predicted here, but lacks an obvious ortholog of C6N29_00460. The
405 additional predicted IA-S-4 secreted proteins are not surprising given the improvements that
406 have been made in methods to detect T9SS-secreted proteins, and in the more complete IA-S-4
407 genome sequence (unpublished) used in this analysis. Deletion of the *F. columnare* strain MS-
408 FC-4 T9SS genes *gldN* and *porV* resulted in decreased levels of extracellular peptidase,
409 chondroitin sulfate lyase, and hemolytic activities. *gldN* and *porV* mutants were also deficient in
410 adhesion to zebrafish fins and other surfaces, and in biofilm formation. Proteomic analysis of
411 cell-free culture fluid of wild-type and *gldN* mutant cells suggested that 39 of the predicted 49

412 secreted proteins were produced and secreted, and that *gldN* was required for efficient secretion.
413 Included among these 39 proteins were predicted chondroitin sulfate lyases, nucleases, motility
414 proteins, adhesins, and many peptidases, explaining some of the defects of the T9SS mutants
415 described above.

416 GldN is required for gliding motility and is thought to be a required component of both
417 the T9SS and the gliding motility apparatus (35). The *F. columnare* MS-FC-4 Δ*gldN* mutant was
418 nonmotile, as previously reported for *gldN* mutants of other bacteria (13, 35). PorV is required
419 for secretion of a subset of proteins targeted to the T9SS. PorV is required for secretion of
420 proteins with type A CTDs and for secretion of ChiA, which has a type C CTD (28). PorV is
421 apparently not required for secretion of proteins that have type B CTDs, such as the motility
422 protein SprB (28). The *F. columnare* MS-FC-4 Δ*porV* mutant exhibited gliding motility,
423 suggesting that the type B CTD protein SprB was secreted to the cell surface and that the
424 motility machinery was functional. Although Δ*porV* mutant cells moved, they did not move as
425 well as wild-type cells. The lack of secretion of the many proteins that have type A and type C
426 CTDs could explain the partial defect in gliding. Some of these proteins are expected to localize
427 to the cell surface, and their absence could alter cell-surface properties and could directly or
428 indirectly affect cell gliding. The construction of the Δ*porV* mutant allowed us to partially
429 separate secretion from motility and assess the role of secretion in virulence of our model *F.*
430 *columnare* genetic group-1 strain. The Δ*gldN* and Δ*porV* mutants were both avirulent toward
431 zebrafish and rainbow trout, suggesting that a functional T9SS is required for virulence, as was
432 previously demonstrated for the genetic group-2 strain, C#2 (13). Our analyses of virulence
433 involved larval and adult zebrafish, and rainbow trout alevin and fry. The results with these
434 different fish at different stages of development were similar for the wild type, Δ*gldN*, and

435 Δ*porV* mutants. This indicates that the T9SS is required for virulence against developing and
436 mature fish. The use of larval zebrafish allows high-throughput screening of strains for virulence
437 defects in a multi-well format.

438 In addition to the effect of deletion of *gldN* on secretion of proteins with T9SS CTDs,
439 many proteins that did not have recognizable T9SS CTDs were also detected in larger amounts
440 in the cell-free culture fluid from wild-type cells than from the *gldN* mutant. These included
441 predicted cytolysins and peptidases. These proteins may have novel sequences that target them
442 for secretion by the T9SS, or they may be released by processes that only indirectly involve the
443 T9SS. Many of these proteins were lipoproteins. The T9SS could influence the release of
444 lipoproteins into the cell-free culture fluid without being directly involved in their secretion. For
445 example, lipoproteins might be transported across and inserted into the outer membrane by
446 another system. Subsequent action of peptidases secreted by the T9SS could release peptides
447 from the cell-surface lipoproteins that would then be detected in the culture fluid. Lipoproteins
448 might also be released in outer membrane vesicles, which have been observed for *F. columnare*
449 and are linked to virulence (51-53). This could account for the observed results if wild-type cells
450 release more membrane vesicles than do cells of the Δ*gldN* mutant. It is also possible that the
451 T9SS directly transports these lipoproteins across the outer membrane. Lipoproteins may interact
452 differently with the T9SS than do other secreted proteins and thus may not require a currently
453 recognizable T9SS CTD. Regardless of the explanation, deletion of *gldN* resulted in decreased
454 levels of these non-CTD proteins in the cell-free spent culture fluid.

455 Fish that survived exposure to either the Δ*gldN* or Δ*porV* mutant were not protected from
456 later exposure to the wild-type strain. This was not surprising given the many cell-surface and
457 extracellular proteins that require the T9SS for their secretion. The T9SS mutants failed to

458 interact with zebrafish pectoral fins. Failure to interact with fish tissues could explain the lack of
459 immune protection, although other explanations are also possible. The lack of protection from
460 exposure to the T9SS mutants motivated us to delete genes encoding individual secreted proteins
461 in an attempt to identify critical virulence factors. Cells of such mutants might fail to cause
462 disease but still interact with fish and generate a protective immune response. Such immunity has
463 been reported for fish that had previously survived exposure to wild-type *F. columnare* (54).
464 We deleted ten genes encoding proteins that appeared to require the T9SS for secretion
465 based on our LC-MS/MS results. Deletion of *cslA* and *cslB* eliminated chondroitin sulfate lyase
466 activity but had no effect on virulence in zebrafish and rainbow trout immersion challenges, as
467 was previously demonstrated for injection challenges of grass carp with *F. columnare* strain G4
468 (12). Chondroitin sulfate lyases may still perform important functions for *F. columnare*.
469 Chondroitin sulfate is found in fish tissues (55, 56) and the enzymes may aid growth and survival
470 of *F. columnare* in fish carcasses, resulting in later infection of other fish. A mutant lacking two
471 peptidase-encoding genes exhibited reduced virulence in rainbow trout and mutants lacking the
472 predicted cytolysin CylA exhibited reduced virulence in both rainbow trout and zebrafish.
473 Deletion of *gldN* or *porV* resulted in mutants that were avirulent for zebrafish and
474 rainbow trout, regardless of fish age or challenge system. In contrast, deletion of genes encoding
475 individual or groups of secreted proteins resulted in some mutants that exhibited different levels
476 of virulence depending on the fish species, age of fish, or the challenge system used. There are
477 many possible explanations for these differences. Larval zebrafish and rainbow trout alevin do
478 not have fully developed immune systems but may have other innate or maternal protections
479 against infection (57-59). The larval zebrafish were also germ-free, unlike all other fish tested
480 here. Germ-free zebrafish larvae are more sensitive to *F. columnare* than are larvae with their

normal microbiota (50). Because of this, lower doses of *F. columnare* were used to challenge germ-free zebrafish larvae than to challenge the other fish. Another difference in the challenge systems was the temperature, which was much lower for rainbow trout than it was for zebrafish. Rainbow trout were also maintained in a flow through system after challenge, whereas zebrafish were maintained in static wells (larvae) or static aquaria (adults) after challenge. Any of the differences described above could have affected host-pathogen-environment interactions and contributed to differing challenge results. Since columnaris disease affects many species of freshwater fish, and affects fish of all ages (2), conducting challenges on different species and life-stages and under different conditions may reveal aspects of the bacterial host interactions that would otherwise be missed.

The T9SS is important for virulence, and our results highlight a strategy to identify the most important secreted proteins involved in columnaris disease. The ease of genetic manipulation of strain MS-FC-4 makes it ideally suited to studies of this disease. As demonstrated here, multiple mutations can be easily constructed in a single strain. This may be important to deal with potential redundancies, such as between the many secreted peptidases. Complementation with plasmids or on the chromosome allows one to determine if the mutations constructed are responsible for the phenotypes observed. The avirulent Δ*gldN* and Δ*porV* mutants did not elicit a protective immune response against later exposure to the wild type, but it is possible that elimination of some of the most critical of the secreted proteins may result in avirulent strains that still interact with fish and generate protective immunity. T9SSs are common in members of the phylum *Bacteroidetes* and they are important for virulence of animal and human pathogens (20, 60-63). Improved understanding of the roles of the T9SS and of secreted

proteins in *F. columnare* virulence may thus have impacts beyond fish health and sustainable aquaculture.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *F. columnare* wild-type strain MS-FC-4 and mutants derived from this strain were grown at 28°C for liquid cultures, and at 30°C for agar cultures, in tryptone yeast extract salts (TYES) medium (64). *E. coli* strains were grown at 37°C in lysogeny broth (LB) (65). The strains and plasmids used in this study are listed in Table 2, and primers are listed in Table 3. 100 µg/ml ampicillin was used to select for plasmids in *E. coli*. 5 µg/ml tetracycline (agar culture) or 1 µg/ml tetracycline (liquid cultures) were used to select for plasmids in *F. columnare*. 1 µg/ml tobramycin was used to counter-select against *E. coli* for conjugation experiments, and to facilitate isolation of *F. columnare* from infected fish.

Conjugative transfer of plasmids into *F. columnare.* Plasmids were transferred from *E. coli* S17-1 λ pir into *F. columnare* strain MS-FC-4 by conjugation. *E. coli* was incubated overnight in LB at 37°C in a rotator and *F. columnare* was incubated overnight in TYES at 28°C with shaking. Two ml of *E. coli* overnight culture was inoculated into 8 ml LB with antibiotic and incubated in a rotator at 37°C. At the same time, five ml of *F. columnare* overnight culture was inoculated into 25 ml TYES and incubated with shaking at 28°C until the OD_{600} reached 0.5. Cells were centrifuged at 3,440 × g for 15 min. The pellets of *E. coli* and *F. columnare* were washed with 10 ml of TYES and centrifuged at 3,440 × g for 10 min. *E. coli* and *F. columnare* cells were each suspended in 0.7 ml of TYES. *E. coli* and *F. columnare* suspensions were mixed and centrifuged at 4,600 × g for 3 min. Excess media was removed and the mixture was spotted

526 on TYES agar, dried until no liquid was observed, and incubated for 24 h at 30°C. Then, cells
527 were scraped off the agar and suspended in 1.5 ml of TYES. 100 µl aliquots were spread on
528 TYES agar containing 1 µg/ml tobramycin and 5 µg/ml tetracycline and incubated at 30°C for
529 three to five days. The experiments listed in Table S1 were conducted in a similar way except
530 that Shieh medium (66) was used instead of TYES.
531 **Construction of deletion mutants.** In-frame gene deletion mutants were constructed as
532 previously described (12, 13). These deletions leave upstream and downstream regions unaltered
533 to limit the possibility of polar effects on downstream genes. To delete *gldN*, a 2.9 kbp region
534 upstream of *gldN* was amplified by PCR using Phusion DNA polymerase (New England Biolabs,
535 Ipswich, MA) and primers 1995 (adding a BamHI site) and 1996 (adding a SalI site). The
536 product was digested with BamHI and SalI and ligated into pMS75 that had been digested with
537 the same enzymes, to produce pYT365. A 3.0 kbp region downstream of *gldN* was amplified
538 using primers 1997 (adding a SalI site) and 1998 (adding a SphI site). The product was digested
539 with SalI and SphI and ligated into pYT365 that had been digested with the same enzymes, to
540 generate pYT375. Plasmid pYT375 was transferred to *F. columnare* MS-FC-4 by conjugation,
541 and colonies with the plasmid recombined into the chromosome were obtained by selecting for
542 tetracycline resistance. Colonies were streaked for isolation on TYES containing tetracycline and
543 isolated colonies were grown in liquid without tetracycline to allow for plasmid loss. The cells
544 were plated on TYES media containing 5% sucrose and the mutant was obtained by selecting for
545 sucrose resistance. PCR was performed to confirm the deletion. The other gene deletion mutants
546 were constructed in the same way, using the primers listed in Table 3 to obtain the appropriate
547 upstream and downstream regions to construct the plasmids listed in Table 2.

548 **Plasmid complementation of mutants.** A 1.6 kbp fragment spanning *porV* was
549 amplified used primers 1967 (adding a BamHI site) and 1968 (adding a SphI site). The product
550 was digested with BamHI and SphI and ligated into the shuttle vector pCP23, which was
551 digested with the same enzymes, to produce pYT371. pYT371 was transferred into the *F.*
552 *columnare porV* mutant by conjugation. Plasmid complementation of other mutants was
553 conducted in the same way, using the plasmids listed in Table 2, and the primers listed in Table
554 3. pLN5, which carries *F. columnare* strain IA-S-4 *gldN* (13), was used to complement the *F.*
555 *columnare* strain MS-FC-4 *gldN* mutant. The GldN proteins of these two strains are identical in
556 sequence.
557 **Plasmid stability.** *F. columnare* cultures containing pCP23-based plasmids were
558 inoculated into 50 ml of TYES broth with 1 µg/ml tetracycline and incubated overnight (15 h) at
559 28°C shaking at 200 rpm. 0.5 ml of these starter cultures were inoculated into 50 ml of TYES
560 broth without antibiotic and incubated 15 h at 28°C shaking at 200 rpm, at which point the
561 cultures were in stationary phase. Dilutions of these 'Cycle-1' cultures were plated in triplicate
562 on both TYES agar and TYES agar containing 1 µg/ml tetracycline, incubated for 72 h at 30°C,
563 and colonies were counted. 0.5 ml of Cycle-1 cultures were also inoculated into 50 ml of TYES
564 broth without antibiotic to initiate Cycle-2, which was handled similarly. The process was
565 repeated again for Cycle-3. A wild-type culture was used to verify that 1 µg/ml tetracycline
566 prevented growth of plasmid-less cells on TYES agar and in TYES broth. Plasmid stability after
567 each cycle was determined by comparing colony counts on TYES agar containing tetracycline
568 with those from TYES agar without antibiotic.
569 **Complementation of mutants by ectopic chromosomal insertion.** A 401 bp intergenic
570 region immediately adjacent to a predicted incomplete prophage region previously identified in

571 the *F. columnare* strain MS-FC-4 genome (30) using PHASTER (67) was used as a site for
572 chromosomal insertion of genes for ectopic complementation. The suicide vector pBFC2 (Fig.
573 S1), which allows insertion of genes at this site and expression from the *F. johnsoniae ompA*
574 promoter, was constructed to facilitate the complementation experiments. The 208 bp region
575 spanning the *F. johnsoniae ompA* promoter was amplified using primers 2082 and 2083, and was
576 linked to a 2475 bp region spanning *F. columnare* MS-FC-4 genes C6N29_05040 and
577 C6N29_05045 by overlap PCR using primers 2080, 2081 and 2082. This relied on reverse
578 complementary sequences engineered into primers 2081 and 2082. The product was digested
579 with KpnI and BamHI and inserted into pMS75 that had been digested with the same enzymes to
580 generate pPBR5. The downstream 2241 bp region spanning *F. columnare* C6N29_05050 and
581 C6N29_ 05055 was amplified from genomic DNA using primers 2084 and 2085, digested with
582 PstI and SphI, and inserted into pPBR5 that had been digested with the same enzymes to
583 generate pBFC2. To facilitate the expression of integrated genes by other promoters, pBFC5 was
584 constructed from pBFC2 by removing the *ompA* promoter. The 2475 bp region spanning
585 C6N29_05040 and C6N29_05045 was amplified using primers 2080 and 2120. The product was
586 digested with KpnI and BamHI, and ligated into pBFC2 that had been digested with the same
587 enzymes, to generate pBFC5.
588 Genes to be inserted in the *F. columnare* chromosome upstream of C6N29_05050 are
589 amplified by PCR and inserted into the multiple cloning sites of pBFC2 or pBFC5. The plasmids
590 are introduced into *F. columnare* by conjugation, and tetracycline-resistant colonies arise by
591 plasmid integration into the genome by homologous recombination at either the upstream or
592 downstream region. PCR is used to confirm insertion in the genome. Cells are grown overnight
593 in TYES broth without tetracycline to allow a second recombination event, resulting in loss of

the plasmid from the genome. The cells are plated on TYES agar containing 5% sucrose to select clones lacking the integrated plasmid. The site of the second recombination event (upstream or downstream) determines if the resulting strain is wild type at the intergenic site or has the intended gene inserted there. Typically, approximately half of the sucrose-resistant colonies are wild type, and the other half have the gene of interest inserted in the chromosome. The procedure is similar to that used to construct gene deletions (12, 13), except that the two recombination events result in insertion rather than deletion of a region of DNA at the chosen site.

The Δ*gldN* mutant was ectopically complemented by amplifying *gldN* using primers 1648 and 2116, digesting the product with BamHI and SalI and ligating this into pBFC2 that had been digested with the same enzymes to generate pJS3. pJS3 was introduced into the Δ*gldN* mutant by conjugation and colonies with *gldN* inserted in the chromosome were selected as described above. The Δ*porV* mutant was ectopically complemented in the same way, using wild-type *porV*, amplified with primers 1967 and 2119 and inserted into the BamHI and SalI sites of pBFC2 to generate pJS4. pJS4 expresses *porV* from the *ompA* promoter. The Δ*porV* mutant was also ectopically complemented by *porV* expressed from its native promoter. In this case, wild-type *porV* was inserted into the BamHI and SalI sites of pBFC5 to generate pJS5.

Growth curves. *F. columnare* cultures were streaked from freezer stocks onto TYES agar and incubated for 24 h at 30°C. These cultures were used to inoculate 20 ml of TYES broth and were incubated overnight at 28°C shaking at 200 rpm. Strains containing plasmids were grown with 2.5 µg/ml tetracycline. Overnight cultures were standardized to an OD_{600} of 0.5 and used to inoculate wells in a 48-well polystyrene plate. 40 µl of culture and 960 µl of TYES (without antibiotics) were added to each well. Plates were incubated at 28°C with shaking at 200

rpm and readings were taken at two-hour intervals for 36 h using a CLARIOstar Microplate Reader (BMG Labtech, Ortenberg, Germany).

Analysis of colony spreading and cell motility. *F. columnare* cultures in mid-exponential phase of growth (OD_{600} approximately 0.5) were serially diluted in TYES and plated on ¼ TYES agar (TYES medium diluted 4-fold and solidified with 15 g/l agar). Colonies of wild-type, mutant, and complemented strains were grown for 45 h at 30°C and then examined using a Photometrics Cool-$SNAP_{cf}^2$ camera mounted on an Olympus IMT-2 phase-contrast microscope. Gliding of individual cells was also examined microscopically. Cells were grown with shaking at 28°C in TYES to mid-exponential phase. Tunnel slides were constructed using double-sided tape, glass microscope slides and glass cover slips, as previously described (68). Ten microliters of cultures were introduced into the tunnel slides, incubated for 5 min, and observed for motility using an Olympus BH2 phase-contrast microscope at 25°C. Images were recorded with a Photometrics CoolSNAP$_{cf}^2$ camera and analyzed using MetaMorph software (Molecular Devices, Downingtown, PA). Rainbow traces of cell movements were made using ImageJ version 1.45s (http://rsb.info.nih.gov/ij/) and macro Color FootPrint (25).

Analysis of secreted proteins by SDS-PAGE and LC-MS/MS. *F. columnare* wild type, Δ*gldN* mutant, and mutant complemented with pLN5 were grown in TYES at 28°C for approximately 14 h. Each culture was standardized to OD_{600} of 0.6 and 5 ml was inoculated into 25 ml of fresh TYES media. Growth was monitored with a Klett–Summerson colorimeter (Klett Manufacturing Co., Inc. Long Island City, New York) and cultures were harvested in mid-exponential phase when the Klett readings reached 55. Cell cultures (multiple 1 ml samples) were harvested by centrifugation, the spent culture fluids were passed through 0.45-μm-pore-size filters, and soluble proteins were precipitated with trichloroacetic acid (TCA) as described (32).

Total cell protein was analyzed by SDS-PAGE. Each cell pellet from one ml of culture was suspended in 40 µl SDS-PAGE loading buffer, boiled for 10 min, and 15-µl samples were separated by SDS-PAGE (69) using a 10% polyacrylamide gel and detected by staining with Coomassie blue. Proteins from the cell-free spent media were also analyzed by SDS-PAGE. Precipitated proteins from 1 ml of spent culture fluid were suspended in 50 µl SDS-PAGE loading buffer, boiled for 10 min, and 25-µl samples were separated by SDS-PAGE (69) using a 10% polyacrylamide gel, and detected using the BioRad silver stain kit (Hercules, CA). An equivalent amount of TYES growth medium was precipitated in the same way and analyzed by SDS-PAGE and silver staining. The precipitated proteins from the cell-free spent media were also analyzed by enzymatic digestion and LC-MS/MS at the University of Wisconsin – Madison Mass Spectrometry Facility as described (32) except the LC-MS/MS data were searched against the proteins encoded by the *F. columnare* strain MS-FC-4 genome (30).

Adhesion, biofilm formation and sedimentation assays. Adhesion of *F. columnare* cells to polystyrene, biofilm formation on polystyrene, and cell sedimentation were determined as previously described (32) with modifications. Cells were grown in half-strength TYES for all assays. For the adhesion assay, cells were grown to OD_{600} = 0.7. One ml of each culture was centrifuged at 4,600 × g for 5 min, the supernatant was removed, and cell pellets were suspended in 1 ml sterile Milwaukee municipal tap water. A 100 µl volume of each strain suspension was added to a 96-well microtiter polystyrene plate with flat bottom (Corning 9017, Corning Inc., Kennebunk, ME) and sterile water was used as a negative control in non-inoculated wells. The plate was incubated at 30°C for 3 h without shaking. Subsequently, wells were washed twice with sterile distilled water and the adherent cells were stained with 100 µl of crystal violet (10 g/l) for 30 min at room temperature. The wells were washed four times with sterile distilled

water, and 100 µl of absolute ethanol was added to each well to solubilize the remaining crystal violet. Cell adhesion was determined by measuring OD_{595} using a CLARIOstar Microplate Reader. The level of adhesion observed for each strain was compared with the adhesion of the wild-type strain, which was set as 100. All assays were performed in quadruplicate and repeated at least two times. The absorbance of the negative control was subtracted from the absorbance of each strain.

For biofilm formation, cells were grown in half-strength TYES to mid-exponential phase (OD_{600} = 0.5). The cultures were diluted 1:100 in half-strength TYES and 150 µl of each diluted culture was deposited in wells of 96-well flat bottom polystyrene Corning 9017microtiter plates (Corning, Corning, NY). The plates were covered with aluminum foil and incubated in a humid environment at 30°C under static conditions for 96 h. Biofilm development was evaluated in four wells per strain, and wells containing sterile non-inoculated medium were used as negative controls. The culture fluid was discarded and the wells were washed twice with 200 µl of sterile distilled water. 150 µl of crystal violet (10 g/l) was added to each well and incubated at room temperature for 30 min. Unbound stain was removed by washing the wells four times with 200 µl of sterile distilled water. Stain bound to cells was solubilized in 100 µl of ethanol and the absorbance (OD_{595} nm) was determined.

To measure bacterial cell sedimentation, cells were grown in half-strength TYES at 28°C, 200 rpm for 24 h. Tubes were allowed to stand static at room temperature for 5 h before being photographed.

Attachment of *F. columnare* cells to zebrafish fins was observed using strains carrying pNT67, which expresses GFP. Cells were grown in TYES at 28°C to mid-exponential phase (OD_{600} = 0.5). Culture turbidity (OD_{600}) and fluorescence intensity were measured to ensure that

similar amounts of fluorescent cells were used for each strain (Table S6). Cells were also spotted on a 1% agarose pad prepared in 10 mM Tris, 8 mM $MgSO_4$, pH 7.5 (TM) and examined by fluorescence microscopy to ensure that essentially all cells carried pNT67 and were fluorescent (Fig. S5). Adult Ekkwill zebrafish were immersed in 99.5 ml water to which 0.5 ml *F. columnare* cells (OD_{600} = 0.5) were added. After 60 min at 28°C the fish were transferred to 100 ml of fresh water, incubated for 10 min, transferred again to fresh water and incubated for another 10 min. Fish were euthanized by decapitation with a sharp blade and pectoral fins were surgically removed and examined by phase contrast and fluorescence microscopy using a Nikon Eclipse 50i microscope (Nikon Instruments Inc., Melville, NY) with an ExFo XL120 mercury lamp, a Nikon CFI60 Plan Fluor phase contrast DLL 40X objective lens and an Endow 41017 GFP filter to detect fluorescence (Chroma Technology Corp., Bellows Falls, VT). Images were recorded with a Hamamatsu ORCA-Flash4.0 LT+ camera using NIS-Elements Advanced Research software (Nikon Instruments). Exposure times for all fluorescence images were 500 ms. Overlays were prepared using ImageJ. Three fish were exposed to each strain and six pectoral fins were examined for each strain.

Measurement of chondroitin sulfate lyase activity. Chondroitin sulfate lyase activity was measured as previously described (13). Briefly, cultures were grown in TYES with shaking (200 rpm) at 28°C to mid-log phase (OD_{600} 0.5). Two milliliters of each culture were centrifuged at 4,600 × g for 10 min at 4°C, the supernatants were collected, and cells were removed by passage through a 0.45 µm filter. 20 µl of the cell-free spent culture fluid and 150 µl of 0.2 mg/ml chondroitin sulfate A (Sigma-Aldrich, St. Louis, MO) in 20 mM Tris buffer (pH 7.0) were added to wells in a 96-well plate and incubated at 30°C for 30 min. 20 µl of TYES was used as a control. 30 µl of 0.5% bovine serum albumin (BSA) in 0.45 M acetate buffer (pH 4.0)

was added to each well. The opaque white color that formed was used to determine the amount of undigested chondroitin sulfate A. The optical density of each well was measured with a microplate reader (Infinite 200 PRO; Tecan Group Ltd., Mannedorf, Switzerland) at 405 nm. The percentage of chondroitin sulfate degradation was calculated as (OD_{405} control - OD_{405} sample)/OD_{405} control × 100. Cultures were measured in triplicate. Activity was also examined using an agar plate assay. TYES agar containing a final concentration 400 µg/ml of chondroitin sulfate A and 4% bovine serum albumin (BSA) were used. *F. columnare* cultures were grown to mid-log phase (OD_{600} 0.5) and 3 µl was spotted on the plate, and incubated at 30°C for 24 h. To visualize chondroitin digestion, plates were flooded with acetic acid for five min. Digestion was identified as a clear zone around the bacterial growth.

Proteolytic activity. Proteolytic activity was quantified using azocasein as a substrate as previously described (13), with some modifications. *F. columnare* strains were grown in 25 ml TYES broth overnight at 28°C with shaking at 200 rpm. Overnight cultures were standardized to an OD_{600} of 0.5 and then 120 µl was used to inoculate 6 ml TYES broth. Triplicate cultures were incubated at 28°C with shaking at 200 rpm for 20 h. 5-ml volumes of cultures were centrifuged at 3,220 × g for 20 min and residual cells were removed from the supernatant by passage through 0.45 µm Pall Acrodisc filters. Protein levels were determined on the cell pellets using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA). Peptidase activities were determined for the cell-free spent culture fluids. A 2% azocasein (Sigma-Aldrich) solution was prepared in 0.05 M Tris-HCL pH 7.4. Cell-free spent culture fluid (50 µl) was mixed with 50 µl azocasein solution and incubated for 2 h at 30°C. The reaction was stopped by adding 130 µl of 10% TCA and incubating 10 min at room temperature. Samples were centrifuged for 20 min at 18,400 × g to remove precipitated azocasein. 100 µl of the soluble fraction of each sample was

added to a well of a flat-bottom 96-well plate. 200 µl of 1 M NaOH was added to each well and mixed. Triplicate assays were performed for each sample, and negative controls were included consisting of 50 µl of sterile TYES broth. The OD_{440} was determined using a CLARIOstar Microplate Reader. For each sample, the OD_{440} values from triplicate assays were averaged, and the mean negative-control OD_{440} was subtracted from these. The adjusted OD_{440} values were converted to units of proteolytic activity by dividing by 0.001 (37). Proteolytic activity was multiplied by 20 to obtain the total proteolytic activity per ml of culture and was divided by the cell protein concentration to obtain the extracellular proteolytic activity per milligram of total cell protein.

Hemolysis assay. Hemolytic activity was quantified essentially as described (70, 71) using defibrinated sheep blood (Hardy Diagnostics, Santa Maria, CA). *F. columnare* strains were grown in 50 ml TYES broth at 28°C with shaking at 200 rpm to an OD_{600} of 0.7. Five ml volumes of cultures were centrifuged at 3,220 × g for 20 min and the supernatants were passed through 0.45 µm Pall Acrodisc filters. A 2% suspension of sheep blood was prepared in phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). Cell-free spent media was diluted 1:10 with PBS. 300 µL of diluted cell-free spent media was mixed with 150 µL of the blood suspension and incubated for 30 min at 37°C. Positive and negative controls were included consisting of ddH_2O (which results in lysis) and PBS (which does not result in lysis), respectively. Following incubation, samples were centrifuged for 6 min at 2,500 × g to remove precipitated lysed blood cells. Hemolysis was determined by measuring OD_{541} of 75 µl of the soluble fraction of each sample in a flat-bottom 96-well plate. For each spent media sample, the raw OD_{541} values obtained from the triplicate assays were averaged, and

753 the mean negative-control OD_{541} was subtracted from these values. Data are presented as percent
754 of wild-type hemolysis.

755 **Zebrafish challenges.** *F. columnare* wild-type, mutant, and complemented mutant strains
756 were grown in TYES overnight at 28°C. 5 ml of overnight culture was diluted into 25 ml of fresh
757 TYES and grown until OD_{600} reached 0.5. To determine the number of live cells per ml, cultures
758 were serially diluted and plated on TYES agar. To test the virulence of each strain, naïve adult
759 Eckwill zebrafish were immersed in a solution of 0.5 ml *F. columnare* cells and 99.5 ml
760 dechlorinated Milwaukee municipal tap water for 60 min at 28°C. Control fish were exposed to
761 0.5 ml growth medium without *F. columnare* in 99.5 ml of water. After exposure fish were
762 moved to tanks with 2 l fresh water at 28°C and observed for up to ten days for signs of
763 infection. Each treatment was performed in triplicate tanks, with each tank containing five
764 zebrafish in 2 l water. Mortalities were recorded daily. A minimum of 20% of the fish that died
765 were examined for the presence of bacteria phenotypic of *F. columnare* (yellow, rhizoid,
766 tobramycin resistant colonies) by swabbing gills, fins, and skin, streaking on TYES agar
767 containing tobramycin, and incubating for 2 d at 30°C. No signs of disease were observed prior
768 to challenge and no indications of *F. columnare* or columnaris disease were observed in the
769 uninfected control tanks or in the maintenance tanks at any time.

770 In some cases, survivors of challenges with *F. columnare* mutants were examined for
771 resistance to later infection by wild-type *F. columnare*. For these experiments, fish were
772 maintained in 2 l of water for 28 d after the initial exposure to the mutant strain. Some of the fish
773 were then challenged with the wild type, as described above, and others were exposed to the
774 same volume of TYES medium without bacteria. Naïve fish were also challenged with the wild
775 type at the same time.

776 **Challenge of germ-free zebrafish larvae.** Germ-free zebrafish larvae were challenged
777 with wild-type, mutant, and complemented strains of *F. columnare* strain MS-FC-4 at 28°C
778 essentially as previously described (50). In brief, 10 to 12 larvae (6 days post fertilization) were
779 exposed to 10^4 CFU/ml *F. columnare* cells for 3 h in 25 cm^3 culture flasks with vented caps
780 containing 20 ml of sterile mineral water. They were then transferred to 24-well plates
781 containing 2 ml sterile water per well. Larvae were fed every 48 h with 50 µl of germ-free
782 *Tetrahymena thermophila* per well. Mortalities were counted daily and measured in days post
783 infection (dpi) with 0 dpi corresponding to the infection day. All zebrafish larval experiments
784 were stopped at nine dpi and zebrafish were euthanized with tricaine (MS-222) (Sigma-Aldrich
785 #E10521). Each experiment was repeated at least twice.
786 **Rainbow trout challenges.** Commercially available certified disease-free rainbow trout
787 (*Oncorhynchus mykiss*) eggs were acquired from Troutlodge Inc., Sumner, WA. Viable hatched
788 trout were hand fed daily to satiation using a commercially available trout feed (Ziegler Inc.,
789 PA). Trout were maintained at the USDA-ARS National Center for Cool and Cold Water
790 Aquaculture research facility in Kearneysville, WV in flow through water at a rate of 1 l/min, at
791 12.5°C, until the challenge weight of ~1.3 g was met. The fish in this facility are checked yearly
792 for multiple diseases including columnaris disease, and except for fish in the challenge room,
793 they are certified disease-free. No signs of disease were observed prior to challenge and no
794 indications of *F. columnare* or columnaris disease were observed in the uninfected control tanks
795 or in the maintenance tanks at any time. Fish were moved to challenge aquaria 1 week prior to
796 immersion challenge to acclimate to the elevated water temperature of 16°C.
797 Wild-type (strain MS-FC-4), mutant and complemented strains were each used for
798 immersion challenges. Frozen bacterial stocks were stored at -80°C in 60% TYES broth and 40%

799 glycerol. Bacterial cultures, for challenges, were grown as previously described (72). Briefly,
800 100 µl of each frozen stock was inoculated into 10 ml TYES broth and incubated overnight at
801 30°C with shaking at 200 rpm. These starter cultures were used to inoculate Fernbach flasks,
802 each containing 1 l TYES broth. These were each incubated at 30°C with shaking at 200 rpm
803 until an optical density of 0.7 to 0.75 at 540 nm was reached.
804 Challenges of fry were performed using triplicate 3 l tanks with restricted water flows
805 (~200 ml/min) at 16°C. Each tank contained 40 fish of approximately 1.35 g each. Water flows
806 were stopped for the immersion challenge and tanks were inoculated with bacterial cultures and
807 incubated for 0.5 h after which water flows were resumed. Control tanks were inoculated with
808 TYES broth. Serial dilutions of water samples from each tank after inoculation were plated on
809 TYES agar to determine CFU/ml. The final challenge concentrations for each experiment are
810 listed in the figures. Mortalities were removed and counted daily. The data for triplicate tanks of
811 each strain were pooled and survivor fractions for each strain were calculated. 20% of mortalities
812 were randomly tested by homogenizing gill tissue and streaking on TYES agar plates to
813 determine if *F. columnare* was present. Confirmation of *F. columnare* was determined by
814 morphological observation of yellow, rhizoid, adherent colonies and by amplifying and
815 sequencing 16S rRNA genes. *F. columnare* was detected in all mortalities tested. Genomovar
816 confirmation was determined by enzymatic digestion (HaeIII) of the 16S rRNA gene as
817 previously described (5, 11, 73) and all were genomovar I (and genetic group 1), as expected for
818 strain MS-FC-4. In some experiments, survivors of challenge with mutants were examined for
819 protection against later infection with the wild-type strain. These fish were maintained for
820 approximately 400-degree days (equivalent to 25 days at 16°C) after initial exposure to allow
821 development of possible adaptive immunity, and were then challenged with the wild-type strain

as described above. Challenges continued for 21 days or until 3 days without recorded mortalities post-exposure.

Challenges of rainbow trout alevin were performed as previously described (59). In brief, 100 alevin were challenged 3 days post-hatch in 3-liter tanks. Total CFUs are given in the figures. Mortalities were removed daily and whole alevin were homogenized and streaked on TYES agar plates to determine the presence of *F. columnare*.

Bioinformatic analyses. Genome sequences were analyzed for T9SS genes encoding proteins that belong to appropriate TIGRFAM multiple-sequence alignment families (74). This was accomplished using the Integrated Microbial Genomes (IMG version 4.0.1 [https://img.jgi.doe.gov/]) Function Profile Tool to examine the genome for sequences predicted to encode proteins related to GldK (TIGR03525), GldL (TIGR03513), GldM (TIGR03517), GldN (TIGR03523), SprA (TIGR04189) and PorP/SprF (TIGR03519). The genomes were also examined for genes encoding predicted secreted proteins that have type A CTDs (TIGR04183) and type B CTDs (TIGR04131 and pfam13585) (16). In each case, the trusted cutoffs assigned by The J. Craig Venter Institute (JCVI) that allow identification of the vast majority of family members with vanishingly few false positives (74) were used. The IMG Function Profile Tool was also used with appropriate pfam family identifiers to identify orthologs of SprE (pfam13181), SprT (pfam13568), PorE (pfam00691, pfam07676, and pfam13620), and PorU (pfam01364). Proteins with type C CTDs were identified by the presence of conserved domain corresponding to NCBI family accession number NF033708 and to conserved domain cl41395 (21, 31). Other predicted *F. columnare* T9SS proteins listed in Table S3 (PorF, PorG, PorQ, PorV, PorX, PorY, PorZ, Plug, and SprD) were identified by BLASTP analysis using the appropriate *F. johnsoniae* or *P. gingivalis* protein as query.

Statistical analyses. A value of $p < 0.05$ was considered significant. For characterization assays a one-way ANOVA with Tukey's post-test was used to analyze differences between treatment groups, unless otherwise noted. GraphPad Prism (version 9.1.2) was used to compute statistical tests. Error bars represent SEM (standard error of the mean).

Data Availability.

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

Ethics statements.

Experiments with adult zebrafish were performed at the University of Wisconsin-Milwaukee and followed protocols approved by the University of Wisconsin-Milwaukee Institutional Animal Care and Use Committee. Larval zebrafish experiments were conducted at the Institut Pasteur according to European Union guidelines for handling of laboratory animals (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm) and were approved by the relevant institutional Animal Health and Care Committees. Rainbow trout challenges were performed under the guidelines of NCCCWA Institutional Animal Care and Use Committee Protocol.

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Table 1. Genes deleted in this study that encode secreted proteins

Locus tag	Gene name	Predicted function of protein product	Conserved domains	Phenotypes of deletion mutants
C6N29_01705	*cslB*	chondroitin sulfate lyase	GAG_Lyase super family (cl21724) T9SS CTD (TIGR04183)	Δ*cslA* Δ*cslB* resulted in inability to digest chondroitin sulfate
C6N29_03400		endonuclease	NUC1 super family (cl30531)	
C6N29_04500	*cylA*	cytolysin	Thiol_cytolysin super family (cl03150)	Δ*cylA* resulted in partial reduction in virulence
C6N29_05590	*cslA*	chondroitin sulfate lyase	GAG_lyase (cd01083) T9SS CTD (cl41395)	Δ*cslA* resulted in a partial chondroitin sulfate digestion defect
C6N29_05800		peptidase	FTP (pfam07504) M4_TLP (cd09597) T9SS CTD (TIGR04183)	
C6N29_07385			Rhs_assc_core super family (cl37315) RHSA super family (cl34567)	
C6N29_08610			Sortilin-Vps10 super family (cl25791) PSII_BNR super family (cl29690) VPS10 super family (cl33391) PKD (cd00146) Laminin_G_3 (pfam13385) T9SS CTD (TIGR04183)	
C6N29_11340	*cylB*	cytolysin	Thiol_cytolysin super family (cl03150)	Δ*cylB* resulted in decreased hemolysis
C6N29_11545		peptidase	Zn_peptidase super family (cl19825)	Deletion of the region spanning C6N29_11545 and C6N29_11550 resulted in decreased proteolysis and decreased virulence in rainbow trout fry.
C6N29_11550		peptidase	Zn_peptidase super family (cl19825)	

Table 2. Strains and plasmids used in this study[a]

Strain or plasmid	Description[b]	Source or reference
***E. coli* strains**		
DH5αMCR	Strain used for general cloning	Life Technologies (Grand Island, NY)
S17-1 λ pir	Strain used for conjugation	(75)
***F. columnare* wild-type strains**		
IA-S-4	Wild type; genetic group 1	(13)
MS-FC-4	Wild type; genetic group 1	(30, 76)
CSF-298-10	Wild type; genetic group 1	(77, 78)
Mutants and chromosomally complemented strains derived from *F. columnare* MS-FC-4		
FCB8	Δ*porV*; deletion of T9SS gene C6N29_02900	This study
FCB14	Δ*gldN*; deletion of T9SS and motility gene C6N29_09600	This study
FCB20	ΔC6N29_05800 (Δpeptidase); deletion of gene encoding predicted peptidase	This study
FCB45	ΔC6N29_11545-C6N29_11550 (Δ2peptidases); deletion of two adjacent genes encoding predicted peptidases	This study
FCB48	Δ*gldN* complemented by *gldN* inserted ectopically in chromosome and expressed from *ompA* promoter ($\Delta gldN_{EC}$)	This study
FCB54	ΔC6N29_11545-C6N29_11550 ΔC6N29_05800 (Δ3peptidases); deletion of genes encoding three predicted peptidases	This study
FCB61	Δ*porV* complemented by *porV* inserted ectopically in chromosome and expressed from *ompA* promoter ($\Delta porV_{EC}$)	This study
FCB62	Δ*porV* complemented by *porV* inserted ectopically in chromosome and expressed from native *porV* promoter ($\Delta porV_{EC2}$)	This study
FCB86	Δ*cylA*; deletion of C6N29_04500 encoding predicted cytolysin	This study
FCB88	ΔC6N29_08610	This study
FCB93	ΔC6N29_07385	This study
FCB94	Δ*cylA* Δ*cylB*; deletion of genes encoding predicted cytolysins	This study
FCB118	ΔC6N29_03400; deletion of gene encoding predicted endonuclease	This study
FCB119	Δ*cylB*; deletion of C6N29_11340 encoding predicted cytolysin	This study
FCB122	Δ*cslB*; deletion of C6N29_01705 encoding chondroitin sulfate lyase	This study
FCB123	Δ*cslA* Δ*cslB*; deletion of genes encoding chondroitin sulfate lyases	This study
FCB127	Δ*cslA*; deletion of C6N29_05590 encoding chondroitin sulfate lyase	This study
Plasmids		
pAS43	Plasmid expressing GFP from pCP29; Ap^r (Em^r, Cf^r)	(14)
pBFC1	Used to construct deletion mutant FCB20. 2.7 kbp region	This study

	upstream of peptidase-encoding gene C6N29_05800 (amplified with primers 2038 and 2039) and 3.0 kbp region downstream of the same gene (amplified with primers 2040 and 2041) inserted in BamHI and SphI sites of pMS75; Ap^r (Tc^r)	
pBFC2	Used to insert genes expressed from *ompA* promoter at prophage region on *F. columnare* MS-FC-4 chromosome. Contains *ompA* promoter and regions upstream and downstream of insertion site (amplified using primers 2080-2085) in pMS75; Ap^r (Tc^r)	This study
pBFC5	Used to insert genes expressed from their native promoters at prophage region of *F. columnare* MS-FC-4 chromosome. Constructed by removing *ompA* promoter from pBFC2; Ap^r (Tc^r).	This study
pBR5	Plasmid used to construct pBFC2. Contains 2475 bp region spanning *F. columnare* MS-FC-4 genes C6N29_05040 and C6N29_05045 and the *F. johnsoniae ompA* promoter inserted into pMS75; Ap^r (Tc^r).	This study
pCP23	*E. coli-F. columnare* shuttle plasmid; Ap^r (Tc^r)	(29)
pJS1	2.9 kbp region upstream of peptidase-encoding genes C6N29_11545 and C6N29_11550 amplified with primers 2108 and 2109 and inserted into BamHI and SalI sites of pMS75; Ap^r (Tc^r)	This study
pJS2	Used to construct deletion mutant FCB45. 2.7 kbp region downstream of peptidase-encoding genes C6N29_11545 and C6N29_11550 amplified with primers 2110 and 2111 and inserted into SalI and SphI sites of pJS1; Ap^r (Tc^r)	This study
pJS3	Plasmid for ectopic complementation of Δ*gldN* resulting in strain FCB48; 1.2 kbp region spanning *gldN* amplified using primers 1648 and 2116 and inserted into BamHI and SalI sites of pBFC2; Ap^r (Tc^r)	This study
pJS4	Plasmid for ectopic complementation of Δ*porV* resulting in strain FCB61; 1.6 kbp fragment spanning *porV* amplified using primers 1967 and 2119 and inserted into BamHI and SalI sites of pBFC2; Ap^r (Tc^r)	This study
pJS5	Plasmid for ectopic complementation of Δ*porV* resulting in strain FCB62; 1.6 kbp region spanning *porV* amplified using primers 1967 and 2119 and inserted into BamHI and SalI sites of pBFC5. Contains native *porV* promoter.	This study
pLN5	Plasmid for complementation of Δ*gldN*; Ap^r (Tc^r)	(13)
pLN24	2 kbp region upstream of *porV* amplified with primers 1875 and 1876 and inserted into BamHI and SalI sites of pMS75; Ap^r (Tc^r)	This study
pLN25	Used to delete *porV*; 1.9 kbp region downstream of *porV* amplified with primers 1877 and 1878 and inserted into SalI and SphI sites of pLN24; Ap^r (Tc^r)	This study
pMS75	Suicide vector carrying *sacB* used to construct gene deletion mutants; Ap^r (Tc^r)	(12)
pNT5	2.1 kbp region downstream of C6N29_03400 amplified with	This study

	primers 2196 and 2197 and inserted into KpnI and XbaI sites of pMS75; Ap^r (Tc^r)	
pNT6	2.1 kbp region downstream of *cylA* amplified with primers 2200 and 2201 and inserted into KpnI and XbaI sites of pMS75; Ap^r (Tc^r)	This study
pNT7	2.1 kbp region upstream of C6N29_07385 amplified with primers 2204 and 2205 and inserted into BamHI and SalI sites of pMS75; Ap^r (Tc^r)	This study
pNT8	2.0 kbp region upstream of *cylB* amplified with primers 2208 and 2209 and inserted into KpnI and XbaI sites of pMS75; Ap^r (Tc^r)	This study
pNT9	2.1 kbp region downstream of C6N29_08610 amplified with primers 2212 and 2213 and inserted into KpnI and BamHI sites of pMS75; Ap^r (Tc^r)	This study
pNT11	Used to construct deletion mutant FCB118. 1.8 kbp region upstream of C6N29_03400 amplified with primers 2198 and 2199 and inserted into XbaI and PstI sites of pNT5; Ap^r (Tc^r)	This study
pNT12	Used to construct Δ*cylA*. 3.2 kbp region upstream of *cylA* amplified with primers 2202 and 2203 and inserted into XbaI and PstI sites of pNT6; Ap^r (Tc^r)	This study
pNT13	Used to construct deletion mutant FCB93. 0.9 kbp region downstream of C6N29_07385 amplified with primers 2206 and 2207 and inserted into SalI and SphI sites of pNT7; Ap^r (Tc^r)	This study
pNT14	Used to construct Δ*cylB*. 2.0 kbp region downstream of *cylB* amplified with primers 2210 and 2211 and inserted into XbaI and PstI sites of pNT8; Ap^r (Tc^r)	This study
pNT15	Used to construct deletion mutant FCB88. 2.0 kbp region upstream of C6N29_08610 amplified with primers 2214 and 2215 and inserted into BamHI and PstI sites of pNT9; Ap^r (Tc^r)	This study
pNT67	Expresses GFP in *F. columnare*. pOmpA and GFPMut1 from pAS43 amplified with primers 2578 and 2579A and inserted into BamHI and PstI sites of pCP23; Ap^r (Tc^r)	This study
pNT69	Plasmid for complementation of Δ*cylA*; region spanning *cylA* amplified using primers 2595 and 2596 and inserted into BamHI and PstI sites of pCP23; Ap^r (Tc^r)	This study
pRC04	3.0 kbp region upstream of *cslB* amplified with primers 2069 and 2070 and inserted into XbaI and SalI sites of pMS75; Ap^r (Tc^r)	This study
pRC05	2.9 kbp region upstream of *cslA* amplified with primers 2064 and 2066 and inserted into KpnI and BamHI sites of pMS75; Ap^r (Tc^r)	This study
pRC09	Used to delete *cslA*. 2.5 kbp region downstream of *cslA* amplified with primers 2067 and 2068 and inserted into BamHI and SphI sites of pRC05; Ap^r (Tc^r)	This study
pRC12	Plasmid for complementation of Δ*cslA*; 2.3 kbp region of *cslA* amplified using primers 2097 and 2098 and inserted into KpnI and XbaI sites of pCP23; Ap^r (Tc^r)	This study
pRC14	Plasmid for complementation of Δ*cslB*; 2.8 kbp fragment of *cslB*	This study

	amplified using primers 2099 and 2100 and inserted into XbaI and SphI sites of pCP23; Ap^r (Tc^r)	
pRC46	Used to delete *cslB*; 2.5 kbp region downstream of *cslB* amplified with primers 2484 and 2485 and inserted into SalI and SphI sites of pRC04; Ap^r (Tc^r)	This study
pYT365	2.9 kbp region upstream of *gldN* amplified with primers 1995 and 1996 and inserted into BamHI and SalI sites of pMS75; Ap^r (Tc^r)	This study
pYT371	Plasmid for complementation of Δ*porV*; *porV* amplified with primers 1967 and 1968 and inserted into BamHI and SphI sites of pCP23; Ap^r (Tc^r)	This study
pYT375	Used to delete *gldN*; 3.0 kbp region downstream of *gldN* amplified with primers 1997 and 1998 and inserted into SalI and SphI sites of pYT365; Ap^r (Tc^r)	This study

[a] Additional wild-type *F. columnare* strains examined for plasmid transfer are listed in Table S1.

[b] Antibiotic resistance phenotypes: ampicillin, Ap^r; cefoxitin, Cf^r; tetracycline, Tc^r. Unless indicated otherwise, the antibiotic resistance phenotypes are those expressed in *E. coli*. The antibiotic resistance phenotypes given in parentheses are those expressed in *F. columnare* but not in *E. coli*.

Table 3. Primers used to construct plasmids

Primer	Sequence (5' to 3')[a]	Plasmids constructed using this primer
1648	GCTAGGGATCCAGCATCTTTCTCAGGTATTG	pLN5, pJS3
1875	GCTAGGGATCCAAGGGCGTATGACAGGAACA	pLN24
1876	GCTAGGTCGACAAATGGCACTGCTGTGGTT	pLN24
1877	GCTAGGTCGACACTTGGGCAGCGGGATTA	pLN25
1878	GCTAGGCATGCTTGTTCTATGGTAGGGAATGGT	pLN25
1967	GCTAGGGATCCTACTCATAATAAGCCCTTTGAACCT	pJS4, pYT371
1968	GCTAGGCATGCTCTCTCATCATAAATGTTAAGGGGA	pYT371
1995	GCTAGGGATCCTTTGCCAACAGAAGCCGAATG	pYT365
1996	GCTAGGTCGACCAAATTTGACTGAGCGAAAGTTGAC	pYT365
1997	GCTAGGTCGACCGTGACTTTGAACAAGATATGTGG	pYT375
1998	GCTAGGCATGCTCCTCATTCATCATTTTCTCATTTC	pYT375
2038	GCTAGGGATCCAGATATACCTTCACAACCTTCAATA	pBFC1
2039	GCTAGGTCGACTTTTACGTACTTATTGCTTTCTTGG	pBFC1
2040	GCTAGGTCGACATAAGAACATTAGATGATAGAACGG	pBFC1
2041	GCTAGGCATGCGGTTCTCTCACATTGTTAGTAATAA	pBFC1
2064	GCTAGGGTACCGATACCCTGATAAGTGGAAAG	pRC05
2066	GCTAGGGATCCTTGTCTCATAATTTTTGTGAA	pRC05
2067	GCTAGGGATCCATTAACGAAAATAAAACAATAAAG	pRC09
2068	GCTAGGCATGCCCAAACAATCAAAAGAAA	pRC09
2069	GCTAGTCTAGAGAAGTAGAAATTCCGTTTGAC	pRC04
2070	GCTAGGTCGACACTTTGTTTCATTTGTTTTCTTAT	pRC04
2080	GCTAGGGTACCCATATTGGATAGTTCAGTTAGGAAA	pPBR5
2081	**GTTCTTTGGTATGCGCTGCC**GAGCAATTAGTAAAAGAAAAAGCTG	pPBR5
2082	CAGCTTTTTCTTTTACTAATTGCTC**GGCAGCGCATACCAAAGAAC**	pPBR5
2083	GCTAGGGATCCTTTTTTTAATTACAATTTAGTTAATTACAAGC	pPBR5
2084	GCTAGCTGCAGGAACGTTTTATTACTCCTATAAAACC	pBFC5
2085	GCTAGGCATGCGAGAATTTGCCTTGATGATTTTATC	pBFC5
2097	GCTAGGGTACCATAATAAATTAAAAACAAGCACTT	pRC12
2098	GCTAGTCTAGAGTTTGGTCTCTATAAAATTCA	pRC12
2099	GCTAGTCTAGAGAATTGCCATTAAGTCAAACT	pRC14
2100	GCTAGGCATGCTTGTATAGAGAATTAACAAACGC	pRC14
2108	GCTAGGGATCCGTTATTGTTATATTTGGATCGCTAAC	pJS1
2109	GCTAGGTCGACACAAGAAAATAATGCTACTGATGTAG	pJS1
2110	GCTAGGTCGACCGATTAATTATGAGTAAAATGCAGG	pJS2
2111	GCTAGGCATGCCCCTTTTAGATTCAAAGAATACTCT	pJS2
2116	GCTAGGTCGACAACGGGTAGGAGTTTTTTTA	pJS3
2119	GCTAGGTCGACTCTCTCATCATAAATGTTAAGGGGA	pJS4
2120	GCTAGGGATCCGAGCAATTAGTAAAAGAAAAAGCTG	pBFC5
2196	GCTAGGGTACCAACACCATAGCTCTTCCGCTTC	pNT5
2197	GCTAGTCTAGACTCAGAATTAAATGAAGGTATCGC	pNT5
2198	GCTAGTCTAGAACACTAATGTTTGACACGC	pNT11
2199	GCTAGCTGCAGGCTGAATTATTAGCTGAATACCC	pNT11
2200	GCTAGGGTACCACTATTAGATTGTAATATAGCCACC	pNT6
2201	GCTAGTCTAGATACAAGATAATATTCGAATTAGAGGAAC	pNT6
2202	GCTAGTCTAGACAACTTAGAACCGACAATTG	pNT12
2203	GCTAGCTGCAGATCCGTAGTGCATTATTGAATTAG	pNT12
2204	GCTAGGGATCCCAATGTTACAGGCTTAAGTGAAGG	pNT7
2205	GCTAGGTCGACCAATATATTGTGACCATCCCGC	pNT7

2206	GCTAGGTCGACGGTCTCAGATTAGAAGCGGAAC	pNT13
2207	GCTAGGCATGCTATCCGTTGCGACGGGATGTTATT	pNT13
2208	GCTAGGGTACCTCATGATTATGAACCAGCCG	pNT8
2209	GCTAGTCTAGAGATTCATTGGTCACAACGTC	pNT8
2210	GCTAGTCTAGAAGGTACTATTGCAACAGGAG	pNT14
2211	GCTAGCTGCAGGTAATTGCGTTGTTTGGAC	pNT14
2212	GCTAGGGTACCGATTCAACGAAGGTCTATGC	pNT9
2213	GCTAGGGATCCTGACAATAGAATTAACTTAGATAGTTC	pNT9
2214	GCTAGGGATCCTGGGAGTAGCTTAAATTAGC	pNT15
2215	GCTAGCTGCAGTGTGAATCGTATTCTTGACC	pNT15
2484	GCTAGGTCGACATAGGAAATCAGAGCATTACAGTC	pRC46
2485	GCTAGGCATGCTTGGGTTGATTGATATGGTTGA	pRC46
2578	GCTAGCTGCAGGGCAGCGCATACCAAAGAACA	pNT67
2579A	GCTAGGGATCCTTATTTGTAGAGCTCATCCATGCC	pNT67
2595	GCTAGGGATCCATGAATTTTTTAAAACTAAATGCCAAGTGG	pNT69
2596	GCTAGCTGCAGTTAGTTTATAACACTATTTCCTTTACCAGTAATCG	pNT69

[a] Underlined sequences indicate introduced restriction enzyme sites

[b] Bold sequences for primers 2081 and 2082 indicate reverse complementary sequences

Figure 1. Gliding of wild-type and mutant cells on glass. Cells were grown in TYES at 28°C to mid exponential phase (OD_{600} approximately 0.5). Ten microliters of cultures were introduced into a glass tunnel slide and observed for motility using an Olympus BH-2 phase-contrast microscope. Wild-type (WT) *F. columnare*, Δ*gldN* mutant, Δ*porV* mutant, Δ*gldN* complemented with wild-type *gldN* on pLN5 (Δ$gldN_C$), and Δ*porV* complemented with wild-type *porV* on pYT371 (Δ$porV_C$) are shown. This figure also includes Δ*porV* ectopically complemented with wild-type *porV* inserted at a neutral site on the chromosome and expressed from the *ompA* promoter (Δ$porV_{EC}$). In each case, a series of images were taken using a Photometrics Cool-$SNAP_{cf}^2$ camera. Individual frames were colored from red (time 0) to yellow, green, cyan, and finally blue (40 s) and integrated into one image, resulting in "rainbow traces" of gliding cells. The top six panels (in greyscale) show the first frame for each strain. The bottom six panels show the corresponding 40 sec rainbow traces. White cells in the bottom panel correspond to cells that exhibited little if any net movement. Bar at lower right (20 μm) applies to all panels. The rainbow traces correspond to the sequences in Movie S1 and Movie S2.

Figure 2. Colonies of wild-type, mutant and complemented strains of *F. columnare*. Colonies of wild type (WT), Δ*gldN* mutant, Δ*gldN* mutant complemented with wild-type *gldN* on plasmid pLN5 (Δ$gldN_C$), Δ*porV* mutant, and Δ*porV* mutant complemented with wild-type *porV* on plasmid pYT371 (Δ$porV_C$). This figure also includes colonies for Δ*gldN* ectopically complemented with wild-type *gldN* at a neutral site on the chromosome and expressed from the *ompA* promoter (Δ$gldN_{EC}$), Δ*porV* complemented with wild-type *porV* at a neutral site on the chromosome and expressed from the *ompA* promoter (Δ$porV_{EC}$), and Δ*porV* complemented with wild-type *porV* at a neutral site on the chromosome and expressed from the native *porV* promoter (Δ$porV_{EC2}$). Colonies arose from cells incubated for 45 h at 30°C on ¼ TYES agar. Photomicrographs were taken with a Photometrics Cool-$SNAP_{cf}^2$ camera mounted on an Olympus IMT-2 phase contrast microscope. Bar (1 mm) applies to all images.

Fig. 3. Chondroitin sulfate lyase, proteolysis, and hemolytic activities of material secreted by wild type (WT), Δ*gldN* mutant, Δ*porV* mutant, and complemented mutants. The *gldN* mutant was complemented by pLN5 (Δ$gldN_C$), or by insertion of *gldN* on the chromosome (Δ$gldN_{EC}$). The *porV* mutant was complemented by pYT371 (Δ$porV_C$), or by insertion of *porV* on the chromosome (Δ$porV_{EC}$). (A) Secreted chondroitin sulfate lyase activity. (B) Secreted proteolytic activity. (C) Secreted hemolytic activity normalized to wild type. Statistics correspond to one-way ANOVA with Tukey post-test comparing all conditions to wild type. ****: $p<0.0001$; ***: $p<0.001$; **: $p<0.01$; *: $p<0.05$; absence of star: non-significant.

Fig. 4. Adhesion, biofilm and sedimentation of wild-type, mutant, and complemented strains. Strains examined were wild-type *F. columnare* MS-FC-4 (WT), Δ*gldN* mutant, Δ*porV* mutant, Δ*gldN* mutant complemented with pLN5 (Δ$gldN_C$); Δ*gldN* complemented with wild-type *gldN* on the chromosome (Δ$gldN_{EC}$); Δ*porV* mutant complemented with pYT371 (Δ$porV_C$); Δ*porV* complemented with wild-type *porV* on the chromosome (Δ$porV_{EC}$), and ΔC6N29_07385. (A) Adhesion to polystyrene after 3 h of incubation at 30°C without shaking as determined by staining with crystal violet and measuring absorbance at 595 nm. Adhesion shown in relation to the wild-type strain, which was set as 100. (B) Biofilm formation on polystyrene of cells in half-

strength TYES broth incubated for 96 h at 30°C without shaking. (C) Cell sedimentation. Cells grown in half-strength TYES broth for 24 h at 28°C with shaking at 200 rpm were transferred to a centrifuge tube and allowed to stand at room temperature for 5 h without shaking before being photographed. Statistics correspond to one-way ANOVA with Tukey post-test comparing all conditions to wild type. ****: $p<0.0001$; **: $p<0.01$; absence of star: non-significant.

Fig. 5. Adhesion to zebrafish fins of wild type, Δ*porV* mutant, and Δ*porV* mutant complemented with *porV* inserted ectopically on the chromosome and expressed from the *ompA* promoter (Δ$porV_{EC}$). Cells of each strain carried pNT67, which expresses GFP. Live fish were exposed to the *F. columnare* strains for 60 min, rinsed twice in clean water, and pectoral fins were examined for attached cells by fluorescence microscopy. The bottom row shows part of a fin from a fish that was not exposed to *F. columnare* cells, and illustrates the background fluorescence not associated with *F. columnare* cells. Left column: phase contrast images. Middle column: fluorescence images. Right column: composite images. Six fins were examined for each strain. Representative images are shown. Bar indicating 100 μm applies to all images.

Fig. 6. T9SS is required for virulence in zebrafish. Zebrafish were exposed by immersion to *F. columnare* strains for one h at 28°C, transferred to fresh water, and percent survival was monitored for 10 d. (A) Strains examined were wild-type *F. columnare* MS-FC-4 (WT); Δ*gldN* mutant; Δ*porV* mutant; Δ*gldN* mutant complemented with plasmid pLN5 (Δ$gldN_C$); Δ*gldN* ectopically complemented with wild-type *gldN* at a neutral site on the chromosome and expressed from the *ompA* promoter (Δ$gldN_{EC}$); Δ*porV* mutant complemented with plasmid pYT371 (Δ$porV_C$); Δ*porV* complemented with wild-type *porV* at a neutral site on the chromosome and expressed from the *ompA* promoter (Δ$porV_{EC}$). The final challenge concentrations were 2.3×10^6 CFU/ml (WT), 2.8×10^6 CFU/ml (Δ*gldN*), 4.2×10^6 CFU/ml (Δ*porV*), 1.9×10^6 CFU/ml (Δ$gldN_C$), 2.5×10^6 CFU/ml (Δ$gldN_{EC}$), 1.9×10^5 CFU/ml (Δ$porV_C$), and 1.4×10^6 CFU/ml (Δ$porV_{EC}$). Fifteen fish were challenged with each strain as indicated in Materials and Methods. Five control fish that were exposed to an equal amount of growth medium without *F. columnare* cells were also included, and each of these control fish survived. (B). Fish that survived exposure to Δ*gldN* (Δ*gldN* survivors) and Δ*porV* (Δ*porV* survivors) were examined for resistance to wild-type cells. Naïve fish, Δ*gldN* survivors, and Δ*porV* survivors were maintained for 28 d and then exposed to wild-type cells at 2.9×10^6 CFU/ml for one h. Survival was monitored for 10 d. 'Control' indicates three additional fish that had survived exposure to Δ*gldN* and three fish that had survived exposure to Δ*porV* that were exposed to an equivalent volume of TYES medium instead of to wild-type cells.

Fig. 7. T9SS is required for virulence in rainbow trout. Rainbow trout were exposed by immersion to *F. columnare* strains and percent survival was monitored for 21 d. Strains examined were wild-type *F. columnare* MS-FC-4 (WT); Δ*gldN* mutant; Δ*porV* mutant; Δ*gldN* mutant complemented with plasmid pLN5 (Δ$gldN_C$); Δ*gldN* ectopically complemented with wild-type *gldN* at a neutral site on the chromosome and expressed from the *ompA* promoter (Δ$gldN_{EC}$); Δ*porV* mutant complemented with plasmid pYT371 (Δ$porV_C$); Δ*porV* complemented with wild-type *porV* at a neutral site on the chromosome and expressed from the *ompA* promoter (Δ$porV_{EC}$). The final challenge concentrations for each strain are given in the corresponding

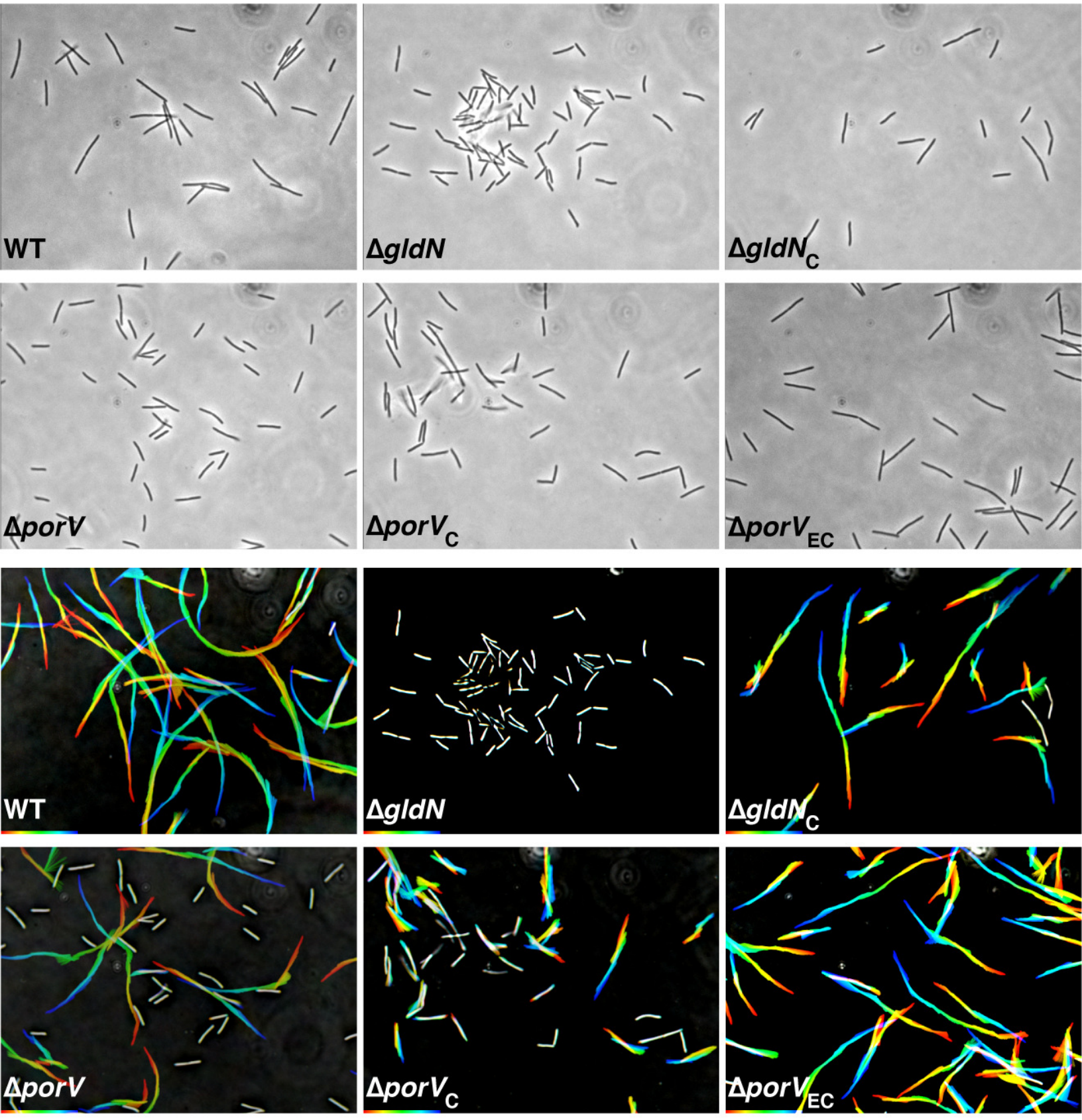
color and indicate CFU × 10^6/ml (example: 6.8 × 10^6 CFU/ml for wild-type challenges of alevin). Results for WT are in red, TYES control in black, Δ*porV* or Δ*gldN* in blue, plasmid complementation in green and ectopic integrated complemention in purple. Left column, rainbow trout alevin. Middle column, rainbow trout fry. Right column, Naive fry, and fry that survived exposure to Δ*gldN* (Δ*gldN* survivors) and Δ*porV* (Δ*porV* survivors) were examined for later resistance to challenge with wild-type cells.

Fig. 8. Chondroitin sulfate lyase, proteolysis, and hemolytic activities of material secreted by wild-type and mutant strains. A) Qualitative plate assay for chondroitin sulfate lyase activities of wild type, mutants (Δ*cslA*, Δ*cslB*, and Δ*cslA* Δ*cslB*) and mutants complemented with pRC12, which carries *cslA*, or with pRC14, which carries *cslB*. B) Quantitative assay for chondroitin sulfate lyase activities for the same strains as in (A) as determined by measuring the amount of undigested chondroitin sulfate A in each well of a 96-well plate after incubating the cells for 30 min at 30°C. Measurements were taken in triplicate. C) Secreted proteolytic activities of wild type, Δ*gldN*, ΔC6N29_05800 (Δpeptidase), ΔC6N29_11545-C6N29_11550 (Δ2peptidases), and ΔC6N29_05800 ΔC6N29_11545-C6N29_11550 (Δ3peptidases). D) Hemolysis activities of wild type and mutants (Δ*cylA*, Δ*cylB*, and Δ*cylA* Δ*cylB*). Statistics correspond to one-way ANOVA with Tukey post-test comparing all conditions to wild type. ****: $p<0.0001$; ***: $p<0.001$; **: $p<0.01$; *: $p<0.05$; absence of star: non-significant.

Fig. 9. Challenge of germ-free zebrafish larvae with *F. columnare* strains. Survival of germ-free zebrafish larvae exposed to wild-type, mutant, or complemented *F. columnare* strains. All strains were derived from wild-type (WT) *F. columnare* strain MS-FC-4. 'Δpeptidase' indicates deletion of C6N29_05800 and 'Δ3peptidase' indicates deletion of C6N29_05800, C6N29_11545, and C6N29_11550. Zebrafish larvae were infected at 6 days post fertilization by immersion for 3 h with 5 × 10^4 CFU/ml. Zero days post infection (dpi) corresponds to the day of infection. Mean survival is represented by a thick horizontal bar with standard deviation. For each condition, n = 10 to 12 zebrafish larvae. Larval mortality rate was monitored daily and surviving fish were euthanized at day 9 post infection. Statistics correspond to unpaired, non-parametric Mann-Whitney test comparing all conditions to non-infected larvae (GF). ****: $p<0.0001$; ***: $p<0.001$; absence of star: non-significant. Blue mean bar corresponds to larvae not exposed to the pathogen (GF) and red mean bars correspond to exposed larvae.

Fig. 10. Deletion of two genes encoding predicted peptidases resulted in partial defect in virulence in rainbow trout. Rainbow trout were exposed by immersion to *F. columnare* strains and percent survival was monitored. Strains examined were wild-type *F. columnare* MS-FC-4 (WT, red); Δ*gldN* (black); ΔC6N29_05800 (Δpeptidase, purple); ΔC6N29_11545-C6N29_11550 (Δ2peptidases, green); ΔC6N29_05800 ΔC6N29_11545-C6N29_11550 (Δ3peptidases, blue). The final challenge concentrations for each strain are given in the corresponding color. Panel A: challenge of rainbow trout alevin. Panel B: challenge of rainbow trout fry. 'Control' indicates fish exposed to equivalent amount of TYES growth medium instead of to *F. columnare*.

Fig. 11. Role of the predicted cytolysins CylA and CylB in virulence. Fish were exposed by immersion to *F. columnare* strains and percent survival was monitored. Strains examined were wild-type *F. columnare* MS-FC-4 (WT, red); Δ*gldN* (black); Δ*cylA* (green); Δ*cylB* (orange); Δ*cylA* Δ*cylB* double mutant (blue); Δ*cylA* Δ*cylB* complemented with pNT69, which carries *cylA* (Δ*cylA*$_C$ Δ*cylB*, purple). The final challenge concentrations for each strain are given in the corresponding color. Panel A: Challenge of zebrafish. Fifteen zebrafish were challenged with each strain. Panel B: Challenge of rainbow trout alevin (left) and fry (right). 'Control' indicates fish exposed to equivalent amount of TYES growth medium instead of to *F. columnare*.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ΔporV
ΔporVC
ΔporVEC
WT
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ΔgldNC
ΔporV
ΔporVC
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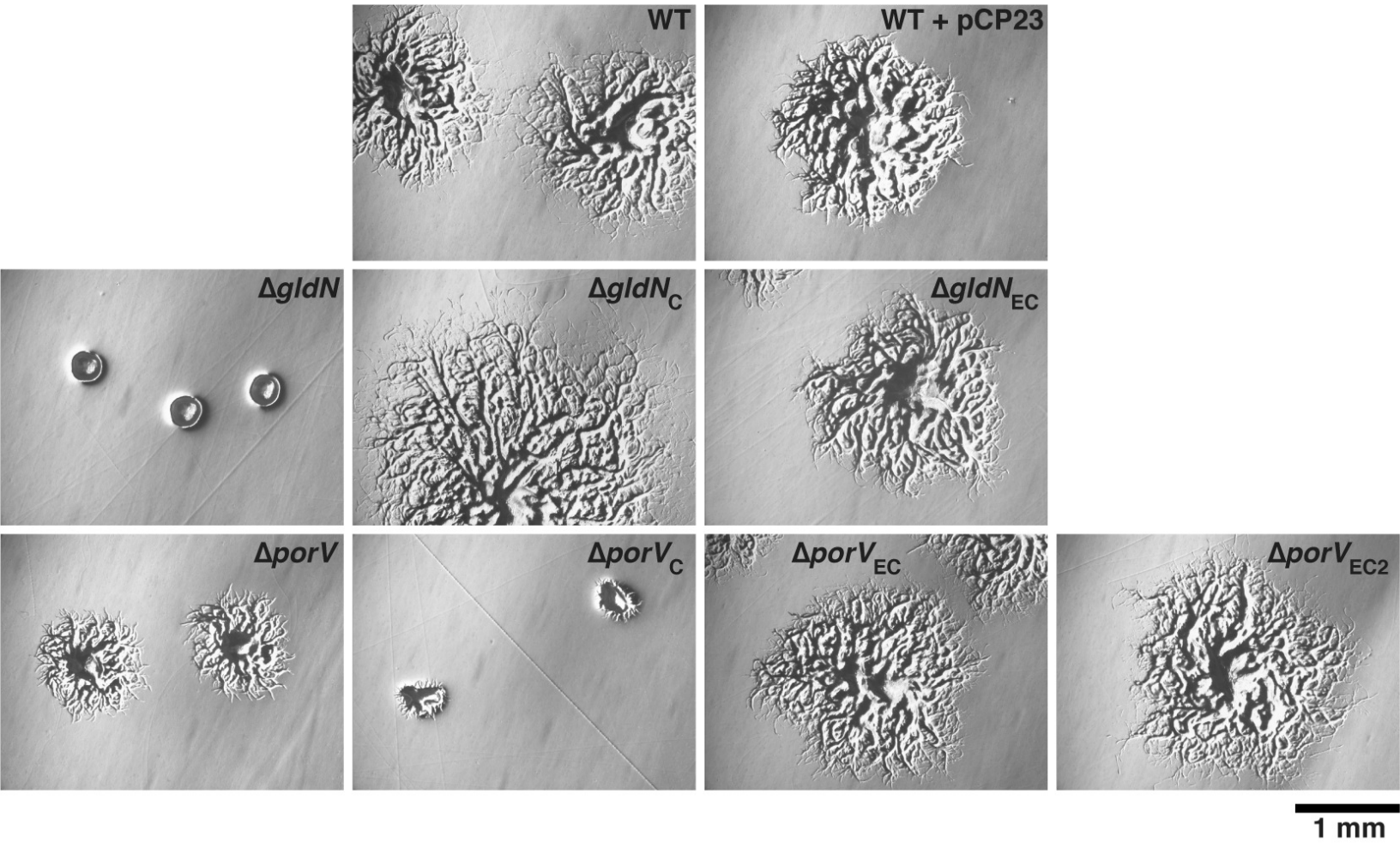
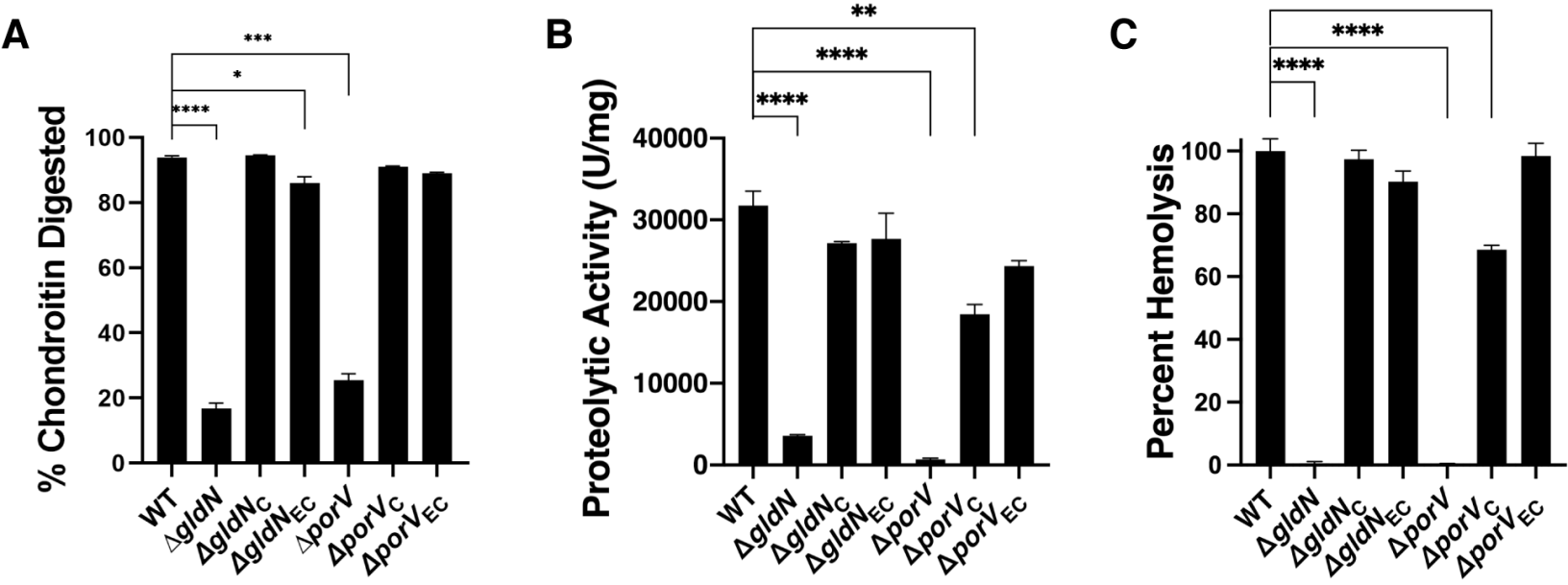
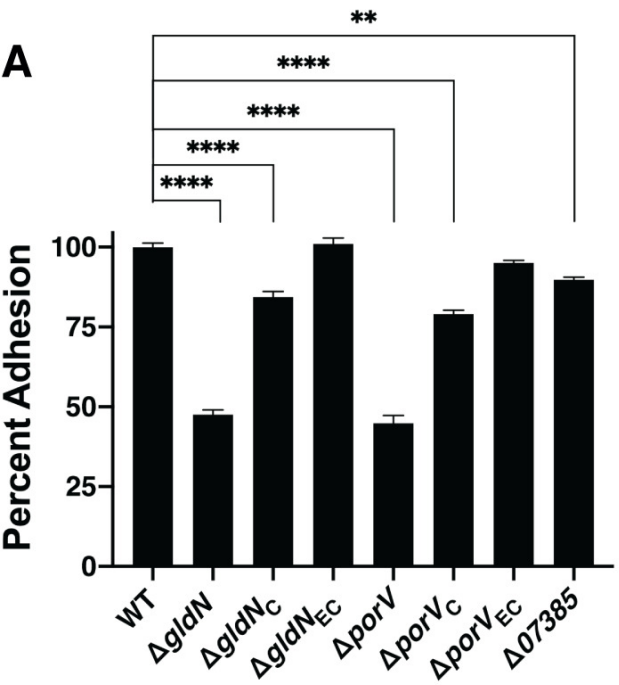
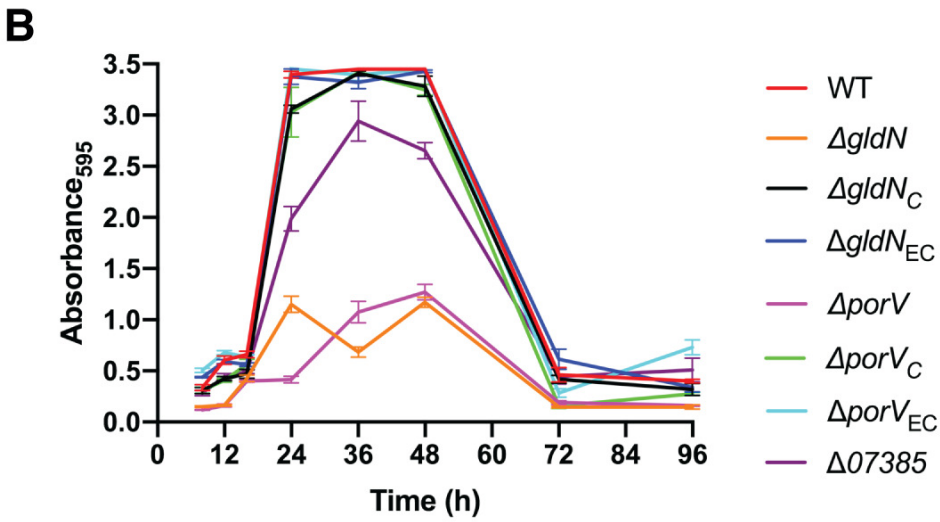
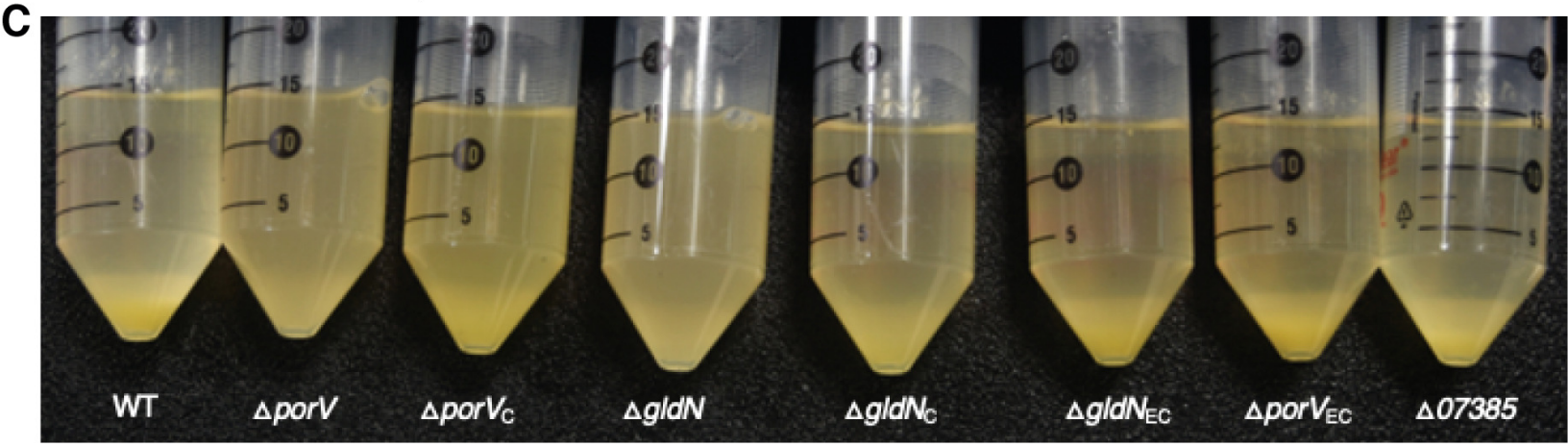
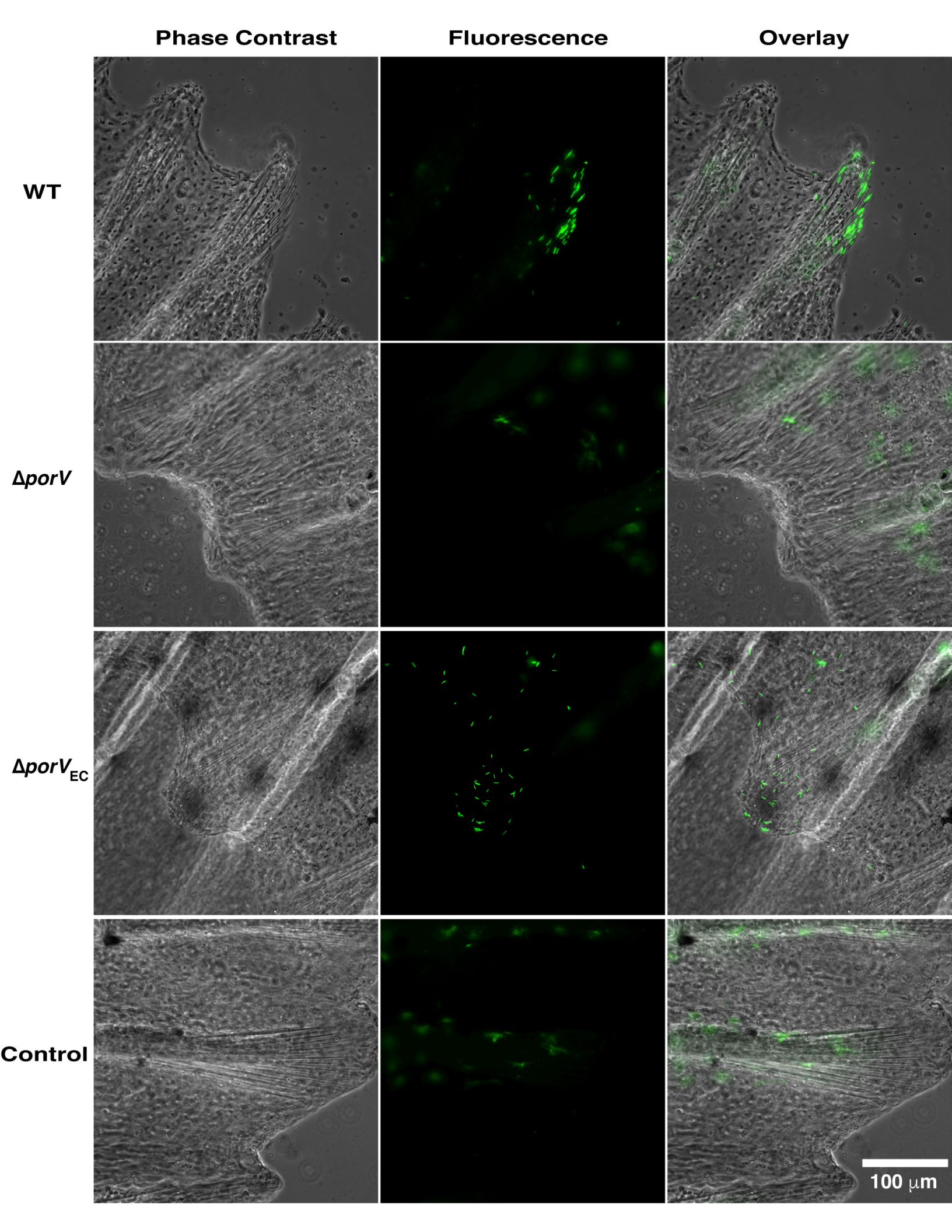

WT
WT + pCP23
ΔgldN
ΔgldN$_C$
ΔgldN$_{EC}$
ΔporV
ΔporV$_C$
ΔporV$_{EC}$
ΔporV$_{EC2}$
1 mm

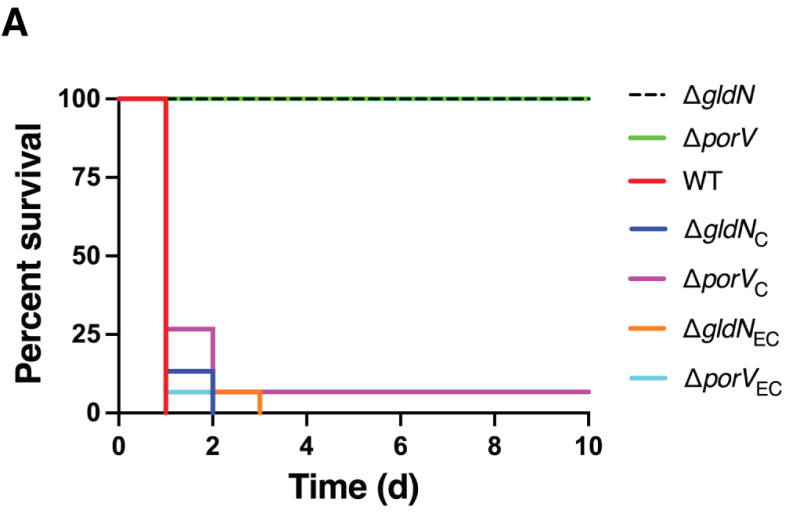

A
% Chondroitin Digested
100
80
60
40
20
0
****
*
***
WT
ΔgldN
ΔgldNC
ΔgldNEC
ΔporV
ΔporVC
ΔporVEC
B
Proteolytic Activity (U/mg)
40000
30000
20000
10000
0
****
****
**
WT
ΔgldN
ΔgldNC
ΔgldNEC
ΔporV
ΔporVC
ΔporVEC
C
Percent Hemolysis
100
80
60
40
20
0
****
****
****
WT
ΔgldN
ΔgldNC
ΔgldNEC
ΔporV
ΔporVC
ΔporVEC

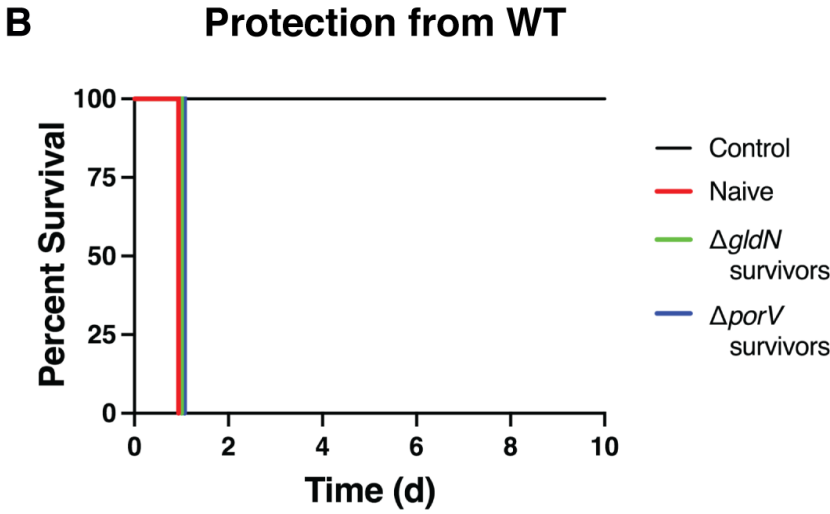

A
**
****
****
****
****
Percent Adhesion
100
75
50
25
0
WT
ΔgldN
ΔgldNC
ΔgldNEC
ΔporV
ΔporVC
ΔporVEC
Δ07385


B
Absorbance595
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
12
24
36
48
60
72
84
96
Time (h)
WT
ΔgldN
ΔgldNC
ΔgldNEC
ΔporV
ΔporVC
ΔporVEC
Δ07385

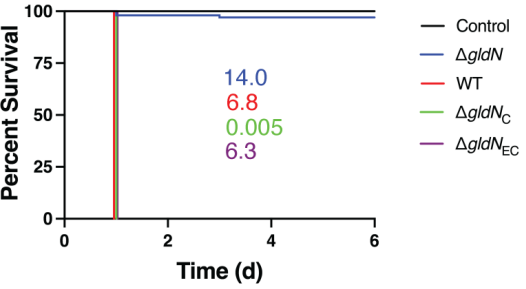

C
WT
ΔporV
ΔporVC
ΔgldN
ΔgldNC
ΔgldNEC
ΔporVEC
Δ07385

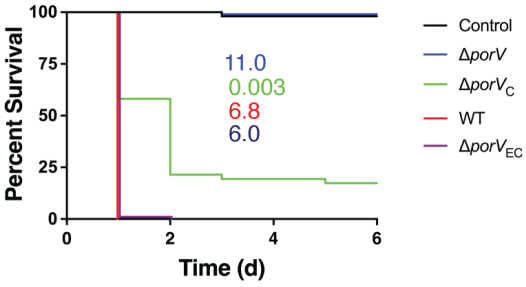

Phase Contrast
Fluorescence
Overlay
WT
ΔporV
ΔporV EC
Control
100 μm


A
Percent survival
100
75
50
25
0
0
2
4
6
8
10
Time (d)
ΔgldN
ΔporV
WT
ΔgldN$_C$
ΔporV$_C$
ΔgldN$_{EC}$
ΔporV$_{EC}$

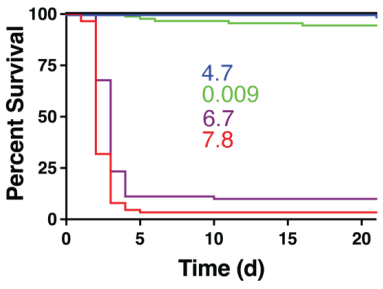

B
Protection from WT
Percent Survival
100
75
50
25
0
0
2
4
6
8
10
Time (d)
Control
Naive
ΔgldN survivors
ΔporV survivors

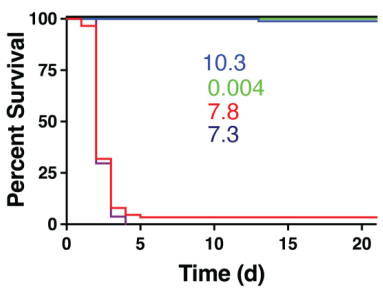
Alevin



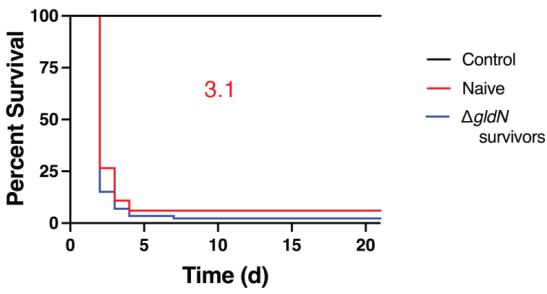


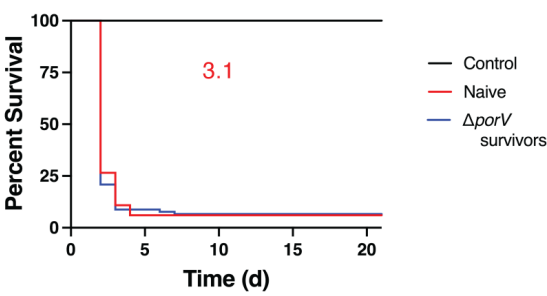
Fry ~2gr

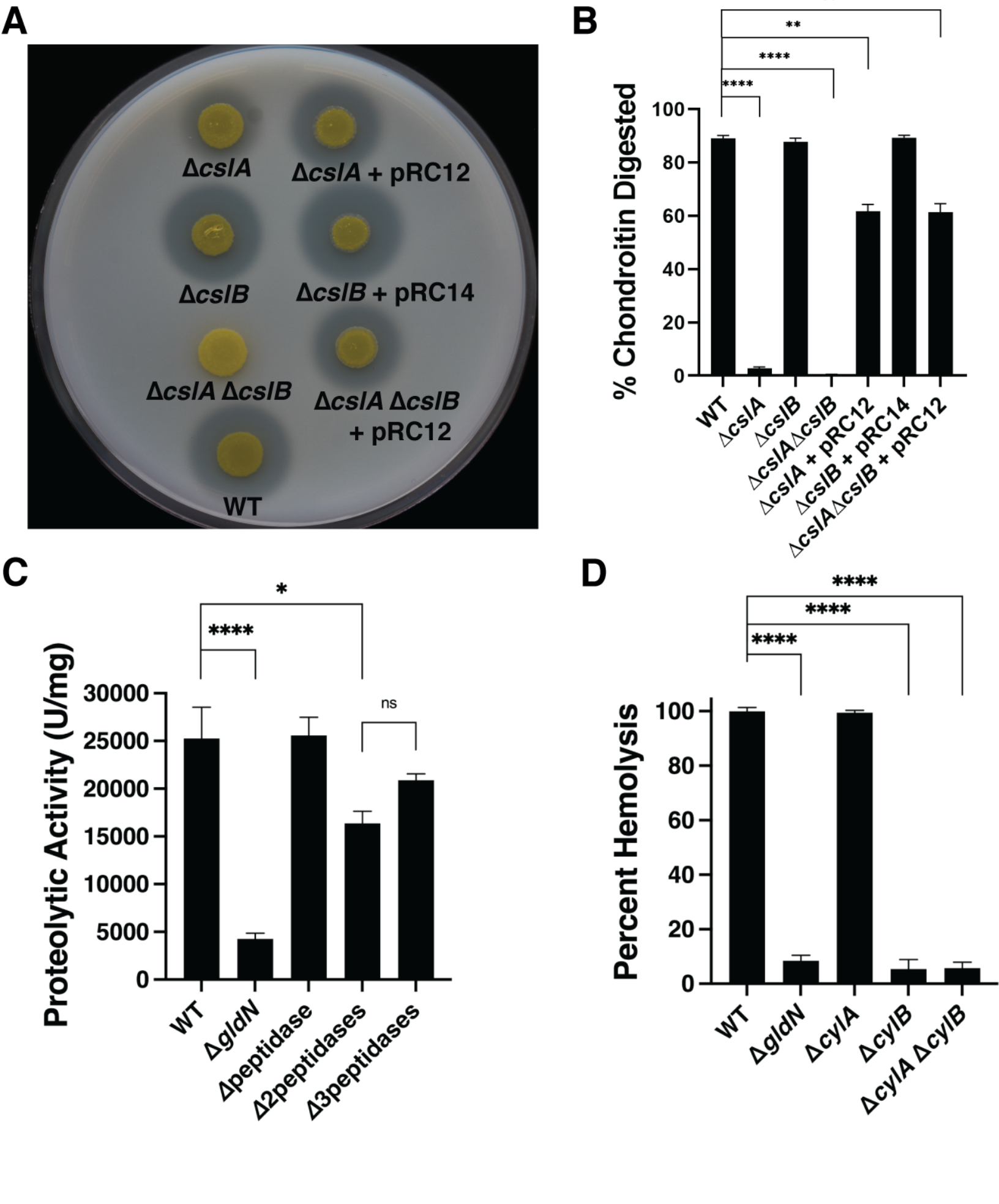


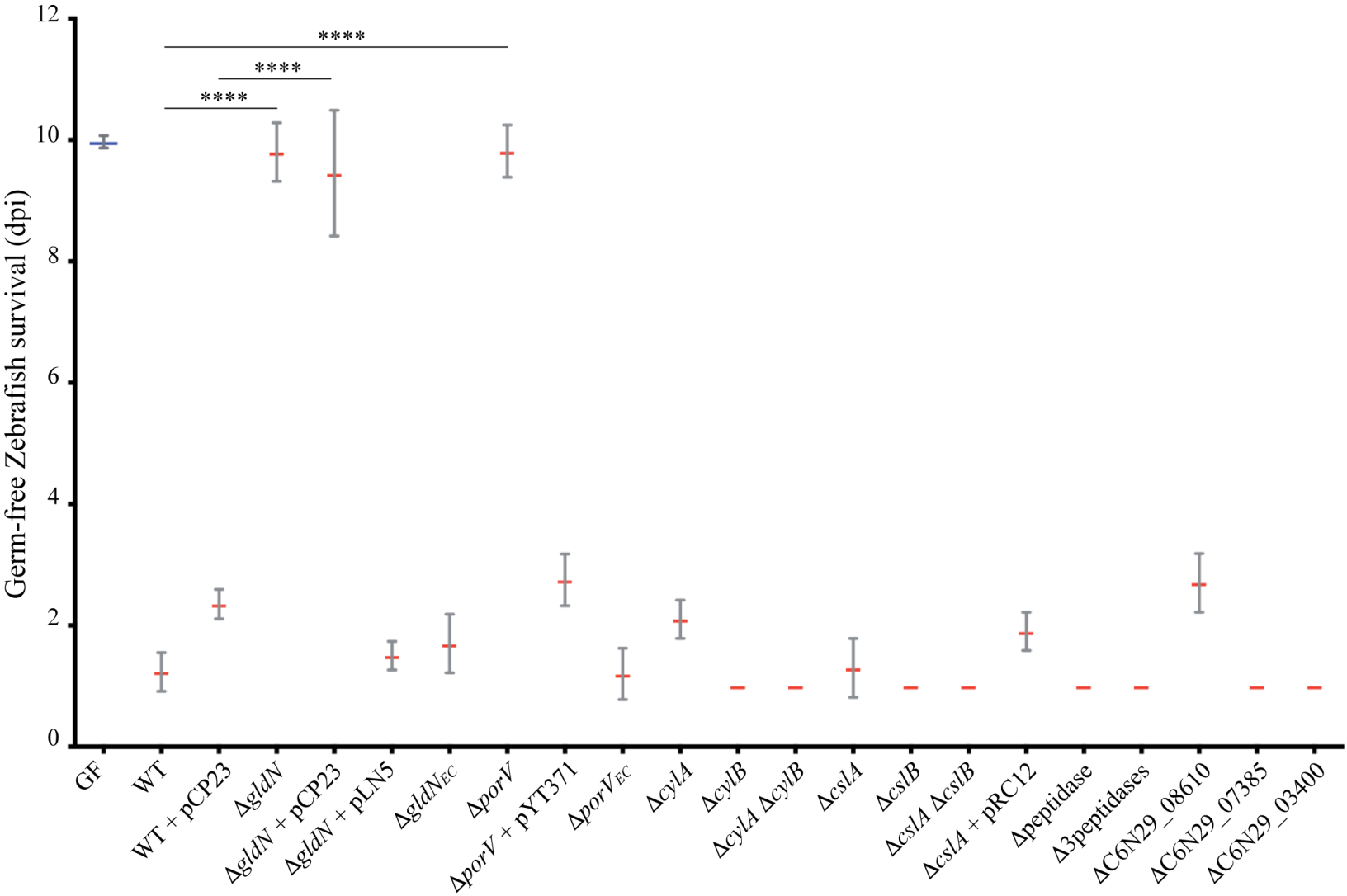


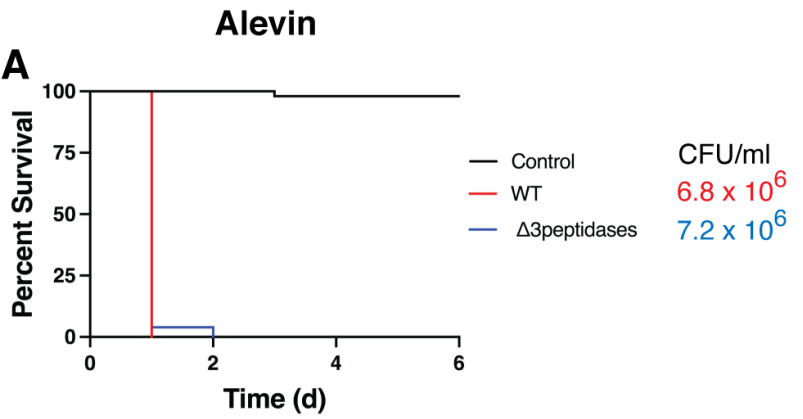
Protection from WT

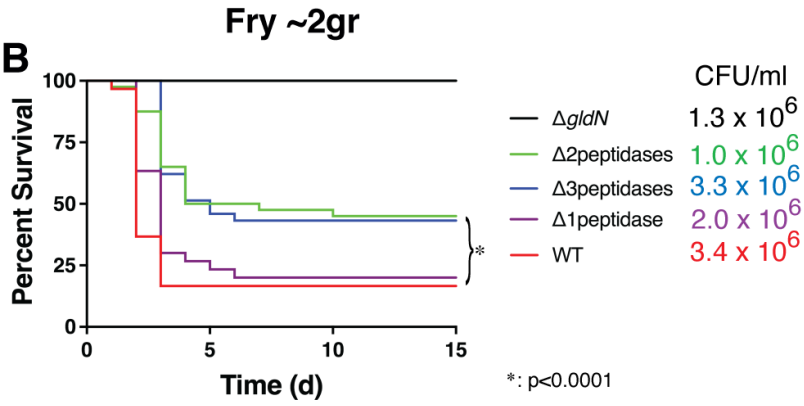





A
ΔcslA
ΔcslA + pRC12
ΔcslB
ΔcslB + pRC14
ΔcslA ΔcslB
ΔcslA ΔcslB + pRC12
WT
B
% Chondroitin Digested
100
80
60
40
20
0
**
**
****
****
WT
ΔcslA
ΔcslB
ΔcslAΔcslB
ΔcslA + pRC12
ΔcslB + pRC14
ΔcslAΔcslB + pRC12
C
Proteolytic Activity (U/mg)
30000
25000
20000
15000
10000
5000
0
*
****
ns
WT
ΔgldN
Δpeptidase
Δ2peptidases
Δ3peptidases
D
Percent Hemolysis
100
80
60
40
20
0
****
****
****
WT
ΔgldN
ΔcylA
ΔcylB
ΔcylA ΔcylB

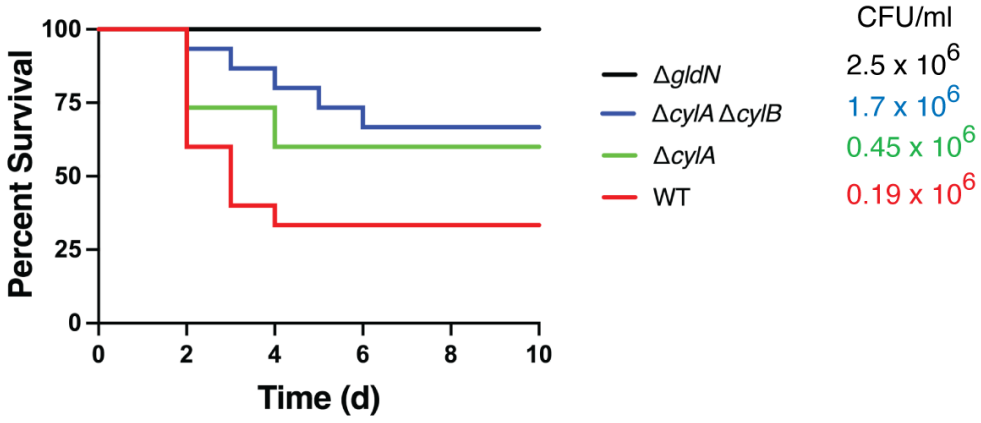

Germ-free Zebrafish survival (dpi)
12
10
8
6
4
2
0
****
****
****
GF
WT
WT + pCP23
ΔgldN
ΔgldN + pCP23
ΔgldN + pLN5
ΔgldNEC
ΔporV
ΔporV + pYT371
ΔporVEC
ΔcylA
ΔcylB
ΔcylA ΔcylB
ΔcslA
ΔcslB
ΔcslA ΔcslB
ΔcslA + pRC12
Δpeptidase
Δ3peptidases
ΔC6N29_08610
ΔC6N29_07385
ΔC6N29_03400


Alevin
A
Percent Survival
100
75
50
25
0
0
2
4
6
Time (d)
Control
WT
Δ3peptidases
CFU/ml
6.8 x 10^6
7.2 x 10^6


Fry ~2gr
B
Percent Survival
100
75
50
25
0
0
5
10
15
Time (d)
*
ΔgldN
Δ2peptidases
Δ3peptidases
Δ1peptidase
WT
CFU/ml
1.3 x 10^6
1.0 x 10^6
3.3 x 10^6
2.0 x 10^6
3.4 x 10^6
*: p<0.0001

A

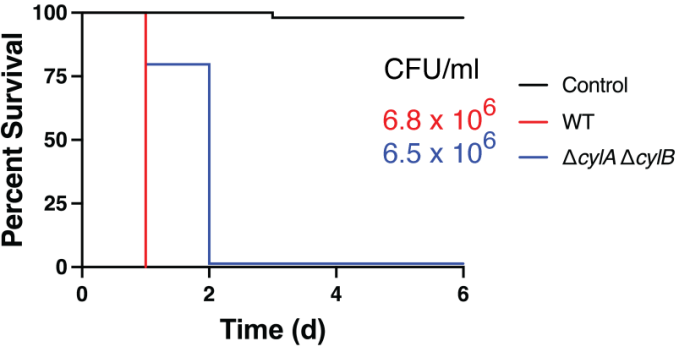
Zebrafish



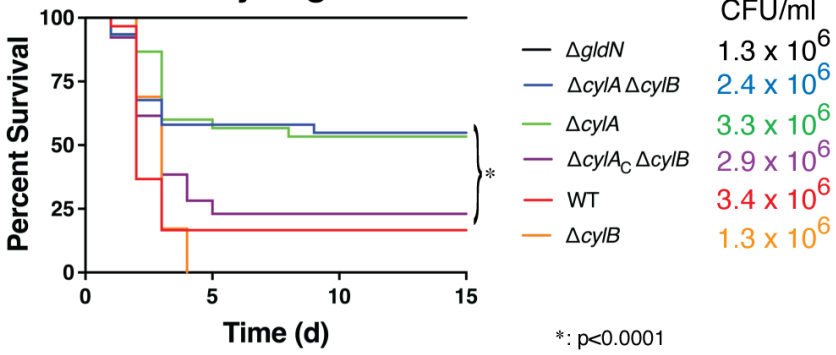
B

Rainbow trout

Alevin



Fry ~2gr



*: p<0.0001