

Review

# Supposed Virulence Factors of *Flavobacterium psychrophilum*: A Review

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**Abstract:** *Flavobacterium psychrophilum* is currently one of the most important pathogens in aquaculture worldwide, causing high losses to farmed salmonids particularly during early growth stages with significant economic impact. Despite previous attempts, no effective vaccine has been developed, and protection against introduction into farms is difficult due to the ubiquitous occurrence of the pathogen. A better understanding of the mechanism of disease development is essential for targeted therapeutic and preventive measures in farms. Unfortunately, the pathogenesis of diseases caused by *F. psychrophilum* has not been elucidated yet. Previously, several putative virulence factors have been identified. Some appear to be essential for disease development, while others are probably dispensable. The importance of some factors has not yet been explored. This review focuses on the supposed virulence factors of *F. psychrophilum* and the current knowledge about their importance in the pathogenesis of the disease.

**Keywords:** pathogenicity; fish disease; proteolytic enzymes; adhesins; iron uptake; motility

**Key Contribution:** This review summarizes the current information on the putative virulence factors of *F. psychrophilum*.



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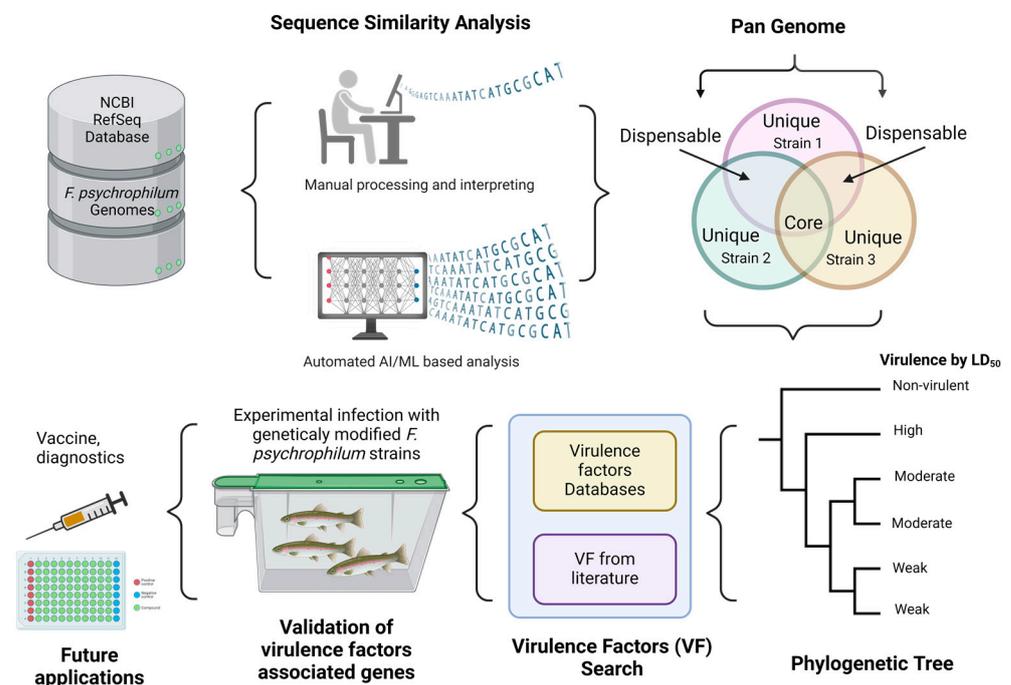
## 1. Introduction

*Flavobacterium psychrophilum* is a Gram-negative rod-shaped, yellow-pigmented bacterium. It is the etiological agent of fish diseases called Rainbow Trout Fry Syndrome (RTFS) and Bacterial Cold Water Disease (BCWD). The disease was first observed in 1946 by Davis in the United States and was named “peduncle disease” for its clinical symptoms [1]. The bacterium was first isolated two years later from diseased coho salmon (*Oncorhynchus kisutch*) [2]. Nowadays, this fish pathogen is found to cause infections among hatchery fish populations worldwide, including in European countries (e.g., United Kingdom, Spain, France, Denmark, Finland, Germany, Poland, Czech Republic), Asia (e.g., China, Japan, Turkey), or South America (e.g., Argentina, Chile) [3–13]. *Flavobacterium psychrophilum* is currently one of the most serious threats in fish aquaculture industry with a significant economic impact on the affected farms.

The most susceptible fish are salmonids [1,2], although the disease can occur in other fish species such as ayu (*Plecoglossus altivelis*) [6], eel (*Anguilla anguilla*), or cyprinids [14]. The entrance gate into the host organism is mainly injured skin or gills with reduced amounts of protective mucus [15,16]. Predisposing factors such as low water temperature, overcrowding, imbalance of chemical parameters of water, or inadequate nutrition increase the risk of development of the disease. RTFS manifests in rainbow trout 4–7 weeks after they start to feed [17] at water temperatures often below 10 °C [18]. Clinical signs of RTFS include superficial swimming, apathy to lethargy, inappetence, exophthalmia, dark body colouration, pale gills, and abdominal distension. In some cases, even skin ulcerations with subsequent ruptures of abdominal wall may appear [5,19]. By autopsy, anaemia, ascites,

inner organs petechiae and splenomegaly are often found [4,5,19,20]. The mortality rate is 50–60% [21] and in extreme cases can increase to 90% [22]. BCWD usually proceeds less dramatically compared to RTFS. The main findings are superficial changes: skin and fin necrosis, mostly in the caudal region, occasionally leading to vertebrae exposure [23,24]. Severity of the outbreak is always contingent on mutual interactions between pathogen, host, and the environment.

Although *F. psychrophilum* is an important pathogen of fish, mechanism of its pathogenicity remains unclear. Unlike other fish pathogens, it is difficult to determine the virulence of *F. psychrophilum* strains based on genomic analysis, because results do not necessarily correspond to the virulence of the strain. Determination of LD50 by experimental infections in fish is therefore needed to evaluate the virulence of a given strain (Figure 1) [25]. Identifying the factors required for virulence is important not only for understanding disease development, but also for discovering specific targets for the pathogen control.



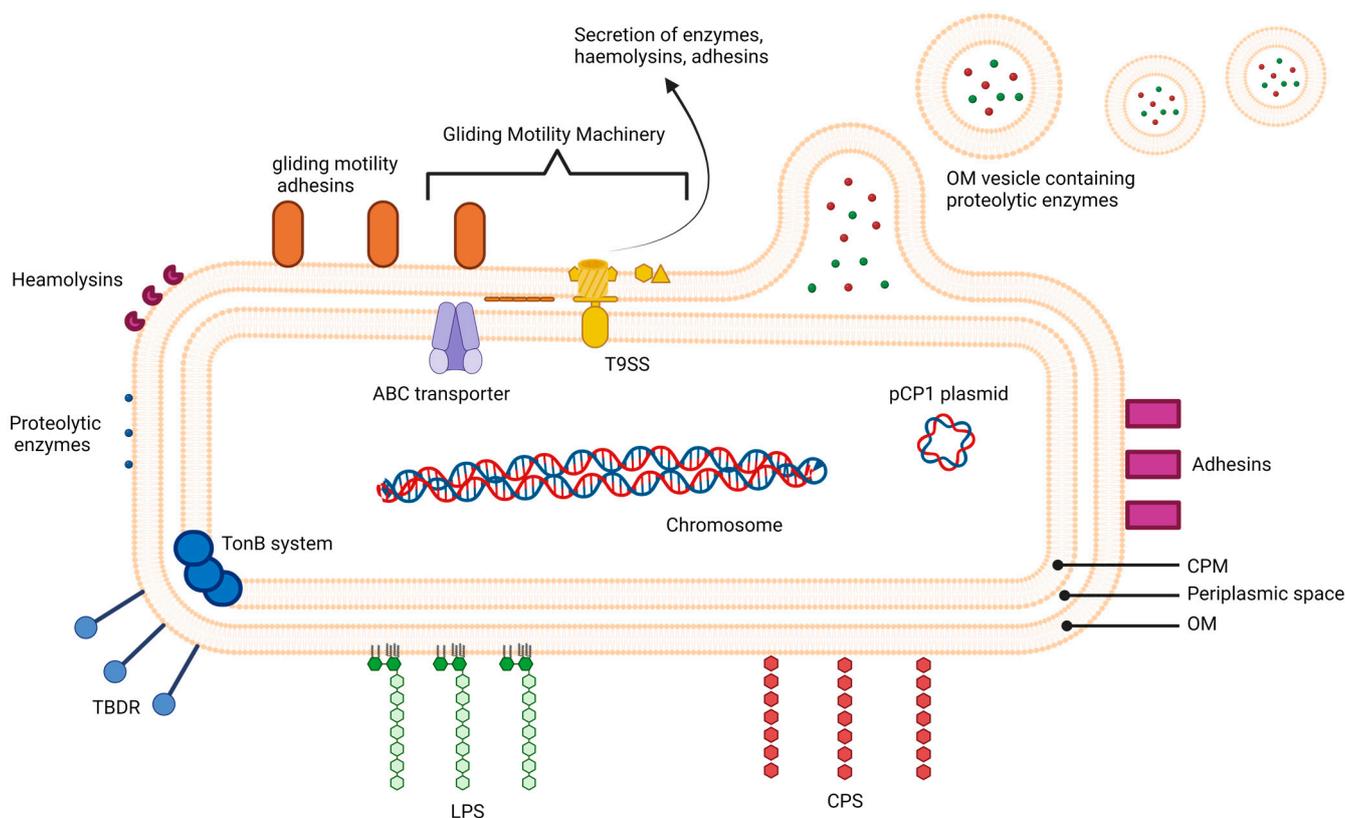
**Figure 1.** A simplified workflow for predicting virulence factors using bioinformatic approaches. Created with [BioRender.com](https://www.biorender.com) (accessed on 7 April 2024).

This review summarizes the current knowledge on supposed virulence factors of *F. psychrophilum* and their influences on disease development.

## 2. Adhesion and Biofilm Formation

Aquatic bacteria including *F. psychrophilum* show strong tendency to adhere to solid surfaces of inanimate materials as well as to animal tissues [13,26–28], while only small number of cells is found in planktonic phase [29]. Adhesion is realized through specific surface compounds (adhesins) that are secreted by the type IX secretion system (T9SS) [30,31] (Figure 2). Previously, 28 potential T9SS-secreted adhesins were reported [31–33]. Adhered cells form biofilms, a coherent layer with typical three-dimensional structure, in the environment that typically consist of mixed bacterial species [29]. Adhesion to the host body surface (gills or skin) is considered to be the initial step in the pathogenesis of infections caused by *F. psychrophilum* and is therefore an important virulence factor of the bacterium [26,34]. This assumption is supported by experiments in which highly virulent isolates appear to have strong adhesive activity, whereas the adhesion of low-virulent isolates is weaker [26], and three-dimensional structure may be absent [13]. However, some studies have noted that adherence ability is strongly related to colony morphological

type [35–37], regardless of virulence [35,37]. Thus, adhesion and biofilm formation are presumably not crucial determinants of strain virulence.



**Figure 2.** Schematic diagram showing the supposed virulence factors of *F. psychrophilum*. Abbreviations used: TBDR—TonB-dependent receptors; LPS—lipopolysaccharide; CPS—capsular polysaccharide; OM—outer membrane; CPM—cytoplasmic membrane; T9SS—type IX secretion system; ABC transporter—ATB binding cassette. Created with [BioRender.com](https://www.biorender.com) (accessed on 6 April 2024).

Adherence activity of *F. psychrophilum* depends on environmental conditions such as temperature or organic pollution [13,26,28]. At low temperatures, when BCWD/RTFS outbreaks usually occur, adhesion is strong, while higher temperatures inhibit adherence activity. Temperature can affect bacterial adhesion due to decline in viscosity, which can result in decrease of adhesive polymer efficiency [38]. Another possible explanation is temperature-dependent expression of genes regulating the adhesion, as was observed in *Vibrio alginolyticus* [39]. This phenomenon could partly explain why outbreaks are not common during higher temperatures. Organic pollution also seems to affect the adhesion of bacteria to fish surfaces. In more polluted water, adhesion is increased. This may be due to alterations in the expression of genes encoding adherence molecules in bacteria due to environmental conditions [26]. There may also be a direct effect of environmental conditions on the gills of fish. In polluted water, both circulatory and structural changes to the gills occur, which may facilitate bacterial adhesion [26,40]. Lack of  $\text{Ca}^{2+}$  or acid/alkaline pH inhibits adhesion [41], as has been described for other bacterial species [42–45]. Differences in adherence activity on various surfaces have also been reported in previous studies; *F. psychrophilum* adheres more readily to fin tissue, caudal peduncle, and lower jaw than to other fish body surfaces [36,46]. Regarding inanimate materials, *F. psychrophilum* shows a good ability to adhere to common materials used in aquaculture, such as glass, stainless steel, polyethylene plastic, or wood but lower ability to adhere to antibacterial plastic surfaces, even when tested under water [28].

In biofilms, gene expression of cells differs from the planktonic state. This is due to communication between cells via quorum sensing [29]. Some virulence-related genes are

up-regulated in biofilms compared to the planktonic state, and therefore biofilms may serve as reservoirs for virulence factors. These genes include probable TonB receptors, ABC transport components, putative adhesins, two-component system (TCS) proteins, or probable peptidases [13]. Compared to the planktonic phase of *F. psychrophilum*, biofilms with high cell densities show tolerance to several times higher concentrations of antimicrobial compounds commonly used in aquaculture (oxytetracycline, flumequine) and increased resistance after exposure to sub inhibitory concentrations. Cell survival in immature biofilms with low bacterial density is similar to that in the planktonic phase, suggesting that complex biofilm structure and sufficient extracellular polymeric substance are required for increased antibiotic resistance [47].

### 3. Enzymes

#### 3.1. Proteases (Tissue Destruction)

*Flavobacterium psychrophilum*, similarly to other fish pathogens, produces enzymes with proteolytic activity. These participate in the destruction of host tissue after colonisation of the fish surface and provide the bacterium necessary nutrients for its growth [48–52]. These enzymes are assumed to belong to the most important virulence factors because they lead to rapid and massive destruction of the host tissue [23,37,53].

Previously, 14 putative genes encoding extracellular proteases have been described in the *F. psychrophilum* genome, as shown in Table 1. These enzymes are mainly metalloproteases [32,33,54]. Secretion is mostly via T9SS [31], although some enzymes are independent of this system, e.g., recently described elastinase [54]. Some enzymes are secreted into the extracellular environment in membrane vesicles (e.g., gelatinase and Fpp1) while other enzymes are bound to the cell membrane and can only degrade their substrates in the presence of living cells [23,48,53–55] (Figure 2). Together, they have the ability to degrade a wide range of protein substrates, allowing damage to the connective and muscle tissue of affected fish. However, not all are functional or present in all strains [32,48,56]. Strains with impaired function of the gene for collagenase (*fpcol*) have been observed [32,56], but this does not appear to have a significant effect on pathogenesis in rainbow trout [32], suggesting that its activity may be substituted by other proteases. Interestingly, a positive correlation was noted between collagenase activity and pathogenesis in ayu (*Plecoglossus altivelis*) [56]. This finding suggests that different virulence factors may be involved in different fish species. Elastinase is also encoded only by certain strains [57], and these usually have enhanced virulence compared to their elastinase-free partners [48,54,58]. The best characterized proteases in *F. psychrophilum* are Fpp1 and Fpp2 (*F. psychrophilum* protease 1, *F. psychrophilum* protease 2). *Fpp1* and *fpp2* genes are transcribed together as they are part of the same operon (*fpp1-fpp2* operon) [59]. Fpp1 consists of four domains and belongs to the M12 family of metalloproteases [60]. It requires calcium ions for its activity, but strontium and barium also have the ability to activate the enzyme [49]. This could explain the affinity of the bacterium for fin tissue, which is a source of calcium ions [51,53]. Fpp1 is a psychrophilic and heat-sensitive protease with activity from 4 °C to 37 °C. This corresponds to the psychrophilic lifestyle of the bacterium. Calcium ions further stabilize the activity of the enzyme even at higher temperatures. Fpp1 degrades a wide spectrum of proteins, including gelatin; laminin; fibronectin; fibrinogen; collagen types I, II and IV; actin; and myosin [49]. Fpp2 also is composed of four domains and is most similar to M43 family enzymes [60]. Unlike Fpp1, Fpp2 is a psychrophilic metalloprotease with calcium ion-independent production, but calcium acts as an activator and a thermal stabilizer of the enzyme. Fpp2 is also a broad-spectrum protease, with a similar range to Fpp1 [50], but in addition appears to be the major caseinolytic enzyme of the bacterium [60]. The involvement of the Fpp1 and Fpp2 proteases in the pathogenesis of *F. psychrophilum* remains unclear, as deletion of either or both encoding genes does not seem to have any effect on the virulence of the bacterium [56,60].

**Table 1.** Proteolytic enzymes described in *F. psychrophilum*.

Locus Tag	Gene Name	Product Description	Family	Reference
FP0081		Putive zinc metalloprotease	M50	Duchaud et al., 2007 [32]
FP0082		Metalloprotease	M1	Duchaud et al., 2007 [32]
FP0086	<i>Pep0</i>	Metallopeptidase <i>Pep0</i>		Duchaud et al., 2007 [32]
FP0231	<i>Fpp1</i>	Psychrophilic metalloprotease <i>Fpp1</i>		Secades et al., 2001 [49]
FP0232	<i>Fpp2</i>	Psychrophilic metalloprotease <i>Fpp2</i>	M43	Secades et al., 2003 [50]
FP0280		Putative fungalysin metalloprotease	M36	Duchaud et al., 2007 [32]
FP0281		Putative fungalysin metalloprotease	M36	Duchaud et al., 2007 [32]
FP0506		Elastinase		Rochat et al., 2019 [54]
FP1024		Putative cytophagalysin metalloprotease	M43	Duchaud et al., 2007 [32]
FP1619		Putative cytophagalysin metalloprotease	M43	Duchaud et al., 2007 [32]
FP1763		Putative subtilisin family serine endopeptidase		Duchaud et al., 2007 [32]
FP1776		Collagenase	M43	Duchaud et al., 2007 [32]
+ FP1777				
FP2364		Putative membrane-associated zinc metalloprotease	M48	Duchaud et al., 2007 [32]
FP2396		Putative subtilisin family serine endopeptidase	S8	Duchaud et al., 2007 [32]

### 3.2. Glycosyltransferases

Although protein glycosylation was thought to be mainly a property of eukaryotic cells, it is now known that bacteria, including *F. psychrophilum*, are also capable of forming glycosylated proteins [61,62]. The effect of glycoproteins on the virulence of bacteria has been previously described [63]; they may participate in bacterial adhesion, contribute to stability of the attachment of the pathogen to the host cell, and alter immunogenicity [64]. Also, genes encoding specific glycosyltransferases may be directly linked to the production of virulence factors [63]. Several genes encoding putative glycosyltransferases have been detected in *F. psychrophilum* [32]. Among them, type-2 glycosyltransferase (*FpgA*) was found to be directly associated with virulence. Mutation of the *fpgA* gene leads to reduced motility, proteolytic activity, and virulence [65].

### 3.3. Cell Wall Hydrolases

Cell wall hydrolases (CWHs) are a group of enzymes that are involved in the degradation of peptidoglycan in the bacterial cell wall. This is necessary for the growth of the bacterial cell and its regulation. In addition, CWHs may also influence virulence of the strain, although their exact role in the pathogenesis of most species remains unclear. In *Staphylococcus aureus*, CWHs are involved in peptidoglycan structural alterations due to environmental conditions inside the host body, which is believed to be necessary for bacterial adaptation and survival [66]. Gram-negative bacteria were found to shed fragments of peptidoglycan, which leads to proinflammatory response in the host [67]. CWHs also contribute to cell adhesion by modulating bacterial surface charge and exposure of adhesins [66]. A recent study suggests that CWHs may affect the formation of outer membrane vesicles, which themselves carry a number of virulence factors [68]. In *F. psychrophilum*, CWH has been found to be significantly up-regulated in outer membrane vesicles during infection of a susceptible host, suggesting a possible influence of CWH on pathogenesis [69]. Further studies are needed to clarify the importance of CWH as a putative virulence factor.

## 4. Gliding Motility

*Flavobacterium psychrophilum* does not form any pili, fimbriae, or flagella [55,70]. Still, it is able to translocate on surfaces by means of so-called gliding motility. Gliding motility is a type of movement that does not depend on external appendages; instead it depends on protein motility apparatus that is unique to the phylum Bacteroidota and differs from the gliding motility mechanisms of other bacteria. Using this apparatus, *F. psychrophilum* moves in the direction of its long axis at an average speed of 2  $\mu\text{m/s}$  [71].

There are 12 *gld* genes required for gliding motility (*gldA*, *gldB*, *gldD*, *gldF*, *gldG*, *gldH*, *gldI*, *gldJ*, *gldK*, *gldL*, *gldM* and *gldN*); 7 *spr* genes essential for colony spreading, although

these are not completely necessary for single-cell motility (*sprA*, *sprB*, *sprC*, *sprD*, *sprE*, *sprF* and *sprT*); and several *rem* genes with redundant motility functions [32,33,72–86], as shown in Table 2. In addition, the *gldC* gene was described in the *F. psychrophilum* genome [32], but it is apparently not essential for gliding motility [73] and is not usually listed among gliding motility genes. All *gld* proteins are associated with the cell envelope, but none is localized to the cell surface. Their disruption leads to a failure of gliding motility [72–79]. Several of the gliding motility proteins (GldK, GldL, GldM, GldN, SprA, SprD, SprE, SprF, SprT) are simultaneously components of T9SS [87], and even other gliding motility proteins that are not considered T9SS components influence its function [30]; this reflects how intimately linked the function of T9SS and the gliding motility apparatus is. Motility adhesins, most notably SprB, are secreted onto the cell surface via T9SS. This motility adhesin forms long slender filaments that move rapidly over the cell surface, which generates cell movement [88,89]. A trans-periplasmic motor composed of GldL and GldM provides energy to this apparatus by proton motive force [89,90]. The gliding motility, along with proteases-mediated tissue damage, allows the bacteria to find entry into a host organism, which contributes enormously to the virulence of the bacterium [51]. Mutations in genes for gliding motility often result in less virulent or completely non-virulent strains [30,31,91–94].

**Table 2.** Proteins involved in the gliding motility apparatus of *F. psychrophilum*.

Protein Name	Description	Reference
GldA	ATP-binding component of ABC transporter	Agarwal et al., 1997 [72]
GldB	Membrane lipoprotein	Hunnicut & McBride, 2000 [73]
GldD	Membrane lipoprotein	Hunnicut & McBride, 2001 [74]
GldF	Channel-forming component of ABC transporter	Hunnicut et al., 2002 [75]
GldG	Putative accessory protein of ABC transporter	Hunnicut et al., 2002 [75]
GldH	Membrane lipoprotein	McBride et al., 2003 [76]
GldI	Membrane lipoprotein	McBride & Braun, 2004 [77]
GldJ	Membrane lipoprotein	Braun & McBride, 2005 [78]
GldK	T9SS component	Braun et al., 2005 [79]
GldL	T9SS component	Braun et al., 2005 [79]
GldM	T9SS component	Braun et al., 2005 [79]
GldN	T9SS component	Braun et al., 2005 [79]
SprA	T9SS component	Nelson et al., 2007 [80]
SprB	Filamentous surface motility adhesin	Nelson et al., 2008 [81]
SprC	Outer membrane protein	Rhodes et al., 2011 [85]
SprD	T9SS component	Rhodes et al., 2011 [85]
SprE	T9SS component	Rhodes et al., 2011 [84]
SprF	T9SS component	Rhodes et al., 2011 [85]
SprT	T9SS component	Sato et al., 2010 [82]
RemA	sprB-like surface motility adhesin	Shrivastava et al., 2012 [86]
RemF	Periplasmic protein	Rhodes et al., 2011 [83]
RemG	Outer membrane protein	Rhodes et al., 2011 [83]
RemH	Periplasmic protein	Rhodes et al., 2011 [83]
RemI	Outer membrane protein	Rhodes et al., 2011 [83]

## 5. Iron Acquisition Mechanisms

Iron is an essential growth factor for microorganisms; however, in vertebrates, the access of bacteria to free iron is limited as iron is mainly present in iron- or haem-binding proteins [95]. The ability to obtain iron in host tissue is one of the important virulence factors of pathogenic bacteria, including *F. psychrophilum* [95,96]. Therefore, bacteria have evolved multiple mechanisms to acquire iron for their metabolism, as listed below.

### 5.1. Haemolysis Activity

One of the ways to ensure iron supply within the host organism is through the production of haemolysins. This leads to damage to red blood cells and the release of

haem and haemoglobin, which can be further processed by the bacteria. *Flavobacterium psychrophilum* is able to lyse rainbow trout erythrocytes [70], which may explain the anaemic state of diseased fish. The intensity of haemolysis may depend in part on the growth phase of the bacteria; Högfors-Rönholm & Wiklund [97] observed that smooth cells show strong haemolytic activity, whereas haemolysis of rough cells is weak or absent. A few years later, Sundell et al. [37] did not confirm this finding, therefore the relation between haemolysis intensity and colony type remains unclear. Haemolysis is a two-step contact-dependent process wherein *F. psychrophilum* needs to be attached to an erythrocyte by sialic acid-binding lectins [97,98] and then lyses the erythrocyte using cell-bound protein haemolysins. *F. psychrophilum* produces two types of haemolysis on agar, indicating the presence of two distinct haemolysins [97]. So far, a putative haemolysin gene FP0063 has been identified in the genome of *F. psychrophilum* strains [32]. The secretion of haemolysin is probably mediated through T9SS, as this transport system seems to be essential for proper haemolysis [31,92]. Genes for putative haemolysin D transporter (*hlyD*) have been identified in some isolates [33,99]. HlyD is involved in transport of haemolysin A in *Escherichia coli* [100], but its role in *F. psychrophilum* has not been elucidated.

### 5.2. Siderophore Production

Siderophore production is one of the best described iron acquisition mechanisms in pathogenic fish bacteria [101]. The presence of siderophores/siderophore-like structures in *F. psychrophilum* was first described in 2005. They are predicted to be encoded on the small cryptic plasmid pCP1 and possibly belong to the catecholate group of siderophores [102]; but closer characterization is lacking. Detection of IucA/IucC family siderophore synthase production further indicates siderophore production [103]. In many other fish pathogens, siderophore production has been identified as a major virulence factor, for example in *Vibrio anguillarum*, *Photobacterium damsela* ssp. *piscicola*, *Aeromonas* spp., *Edwardsiella ictaluri* [101]. Also, in *F. psychrophilum*, a positive correlation between siderophore/siderophore-like structures and virulence is suggested [102].

### 5.3. Iron Transport Systems

Next to widespread ATB-binding cassette (ABC) transporters for active uptake of substances across the cell membrane [104], *F. psychrophilum* requires the functional TonB system for ferric iron ( $\text{Fe}^{3+}$ ) acquisition [105]. This system is present only in Gram-negative bacteria and is formed by a complex of inner membrane proteins—TonB-ExbB-ExbD—that works as a motor generating energy from the proton gradient power for active transport of substances through the outer membrane proteins—Ton-B dependent receptors (TBDRs) (Figure 2). TBDRs specifically bind various substrates, including iron-complexes [106]. Bacteria typically encode several types of TBDRs, allowing the use of different types of iron complexes [107]. In *F. psychrophilum*, four TBDRs participating in iron acquisition—FhuA, FecA, HfpR and BfpR—have been best described previously, as well as the haemophore HfpY [96,108]. HfpY belongs to the HmuY family of haem-binding proteins, and its function is to recruit haem from host haemoproteins and deliver it to HfpR, its cognate TBDR. The activity of HfpR and HfpY is enhanced in iron-deficient environments, which allows the bacteria to survive inside the host organism. Conversely, BfpR is upregulated when high levels of haemoglobin are present, which may help bacteria adapt to haemoglobin overload during haemorrhagic septicaemia in host fish [96]. FhuA and FecA are siderophore receptors identified in many Gram-negative bacteria, including the related *Flavobacterium columnare* [109]. In *E. coli*, FhuA binds catecholate and hydroxamate siderophores, while FecA also binds ferric citrate [110]. Other TBDRs without further characterization have been found in *F. psychrophilum* [32,103,108]. The TonB system and TBDRs are required for *F. psychrophilum* pathogenicity as both TonB system mutants and TBDR mutants show decreased virulence [96,105].

*Flavobacterium psychrophilum* cells furthermore produce components of the Feo system: the cytoplasmic protein FeoA and the iron permease FeoB [33,99]. The Feo system is a

widespread ferrous iron ( $\text{Fe}^{2+}$ ) transport in prokaryotes. Ferrous iron is present mainly in conditions of low pH or limited oxygen [111]. Under neutral pH and aerobic conditions, the Feo system can be useful when it cooperates with iron-reducing agents [112]. This system is considered to be important for bacterial pathogenicity primarily under anaerobic conditions [111]; the importance for pathogenicity of strictly aerobic fish pathogen is probably marginal.

## 6. Secretion System

The type IX secretion system (T9SS), also known as the Por secretion system (PorSS), ensures transport of various substances across the outer membrane in *F. psychrophilum*. This system has only recently been discovered and, so far, appears to be unique to bacterial species in the phylum Bacteroidota [113]. T9SS functionally cooperates with the universal Sec translocase, which transports substrates across the cytoplasmic membrane into the periplasm [114]. T9SS subsequently secretes these substrates from the periplasm into the extracellular space.

T9SS is composed of secretion complex proteins, attachment complex proteins, and regulatory proteins. Nineteen proteins have been described as involved in T9SS function, as shown in Table 3 [79,80,82,84,85,115–124], although recent studies suggest that even more components may be involved [125,126]. Five of the encoding genes are referred to as core genes—GldL and GldM, forming a trans-periplasmic motor utilizing the power of the proton gradient [89]; GldN and GldK, a periplasmic ring-shaped complex associated with the outer membrane [121]; and SprA, an outer membrane protein working as a channel through which substrates are transported to the cell surface [124] (Figure 2). The secreted substrates are either released into the supernatant or anchored in the cell membrane after being transported through it [31,53,55]. This is mediated by the cell-surface attachment complex proteins PorQ, PorU, PorV, and PorZ [127]. These proteins covalently link the substrates to an anionic lipopolysaccharide anchor in the cell membrane and enable surface exposure of the substrates [128]. However, the functions of the system and the roles of all its components are not yet fully understood [31,124,129].

**Table 3.** Components of the type IX secretion system (T9SS) in *F. psychrophilum*.

Classification	Name	Description	Reference
Secretion complex proteins	GldK <sup>1,2</sup>	Periplasmic lipoprotein anchored to outer membrane	Braun et al., 2005 [79]
	GldL <sup>1,2</sup>	Cytoplasmic membrane protein	Braun et al., 2005 [79]
	GldM <sup>1,2</sup>	Cytoplasmic membrane protein	Braun et al., 2005 [79]
	GldN <sup>1,2</sup>	Periplasmic protein	Braun et al., 2005 [79]
	SprA <sup>1,2</sup>	Outer membrane channel protein	Nelson et al., 2007 [80]; Saiki & Konishi, 2010 [117]
	(Sov)		
	SprD <sup>2</sup>	Outer membrane protein	Rhodes et al., 2011 [85]
	SprE <sup>2</sup>	Outer membrane lipoprotein	Sato et al., 2010 [82], Rhodes et al., 2011 [84]
	(PorW)		
	SprF <sup>2</sup>	Outer membrane protein	Sato et al., 2010 [82]; Rhodes et al., 2011 [85]
	(PorP)		
	SprT <sup>2</sup>	Outer membrane $\beta$ -barrel protein	Sato et al., 2005 [115]; Sato et al., 2010 [82]
	(PorT)		
	Plug	Outer membrane protein	Lauber et al., 2018 [124]
PorE	Outer membrane lipoprotein	Heath et al., 2016 [122]	
PorF	Outer membrane Ton-B dependent receptor	Saiki & Konishi, 2010 [118]	
PorG	Outer membrane $\beta$ -barrel protein	Gorasia et al., 2016 [121]	
Attachment complex protein	PorQ	Outer membrane $\beta$ -barrel protein	Sato et al., 2010 [82]
	PorU	Outer membrane protein, T9SS sortase	Sato et al., 2010 [82]
	PorV	Outer membrane protein	Ishiguro et al., 2009 [116]; Chen et al., 2011 [119]; Shoji et al., 2011 [120]
	PorZ	Outer membrane $\beta$ -barrel protein	Lasica et al., 2016 [123]
Regulatory proteins	PorX	Cytoplasmic putative response regulator	Sato et al., 2010 [82]
	PorY	Cytoplasmic membrane sensor histidine kinase	Sato et al., 2010 [82]

Notes: <sup>1</sup> core component of T9SS. <sup>2</sup> component of the gliding motility apparatus. If the protein was first described in *Porphyromonas gingivalis* under a different designation than that used for *Flavobacterium* spp., references are given for descriptions of the protein in *P. gingivalis* (protein name given in brackets) and later in *Flavobacterium* spp.

In previous studies, T9SS was described as an essential determinant of virulence in several pathogenic species, for example in related *Flavobacterium columnare* [52,130,131], or *Porphyromonas gingivalis* [132]. In *F. psychrophilum*, this system has been found to secrete adhesin proteins, proteases, nucleases, and other hydrolases, each of which is a potential virulence factor [31]. It also secretes motility adhesins, which is essential for gliding motility [89]. The importance of T9SS for virulence of *F. psychrophilum* is confirmed by virulence tests of T9SS core gene mutants. These mutations lead to secretion failure and decreased virulence of the mutants [30,31,94].

The mechanisms of T9SS regulation are not completely understood yet. Previously, a two-component system (TCS) regulating gene expression of T9SS components has been described [133]. However, other studies suggest that there are more systems that likely further regulate the secretory system, with TCS being only one of them [123,134].

## 7. Lipopolysaccharide (LPS) and Capsular Polysaccharide (CPS)

A major component of the outer membrane of most Gram-negative bacteria is the pro-inflammatory lipopolysaccharide (LPS), which is also referred to as an endotoxin due to its cell-associated toxicity [135,136]. LPS is formed by three components, all of which have been previously detected in *F. psychrophilum* isolates: lipid A; the core oligosaccharide; and the O-polysaccharide, also known as the O-antigen [137,138]. The O-antigen is an inter- and intra-species variable part of LPS and can sometimes be completely absent; such LPSs are referred to as “rough” LPS because the morphology of the colonies is typically rough, whereas LPSs containing all three components are referred to as “smooth” [136]. However, there are no differences between the LPS structure of rough and smooth *F. psychrophilum* colony types; therefore, the colony types of this species are probably conditioned by other surface structures [35], such as glycopeptidolipids described in *Mycobacterium abscessus* [139] or antigenic peptides found in *Escherichia coli* [140]. The structure of the *F. psychrophilum* O-antigen revealed a varying number of repeating trisaccharides containing the relatively rare sugar N-acetylated bacillosamine (2-acetamido-4-((3S,5S)-3,5-dihydroxyhexanamido)-2,4-dideoxy-d-quinovose [137,138]. This sugar has been previously described only in some bacterial species, such as *Litorimonas taeanensis* [141].

The loosely associated capsular layer formed by capsular polysaccharides (CPS), also referred to as the glycocalyx, on the cell surface may further contribute to *F. psychrophilum* virulence by hiding its surface structures, promoting cell adhesion and motility [135,142]. The thickness of this layer varies among isolates [55,143,144] but is generally thin compared to other capsula-forming bacterial species [55] such as the closely related *F. columnare* [145] or *Pseudomonas putida* [146]. Its immunogenicity makes it a potential target for vaccines [142].

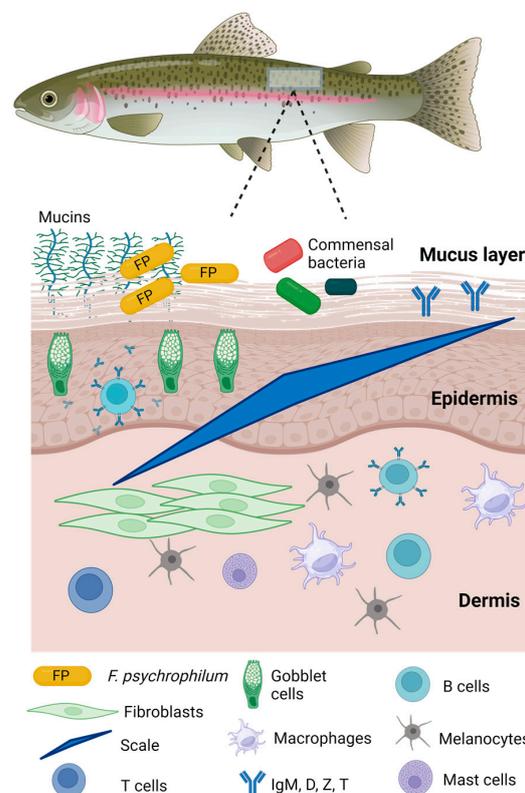
## 8. Influence of Serotype and Genotype on Virulence

*F. psychrophilum* isolates can be classified into three serotypes—Th, Fd, FpT—on the basis of their reaction to specific antisera [147]. In 2017, an alternative method of serotyping by mPCR was described; this method separates isolates into serotypes 0 (corresponding to FpT), 1 (corresponding to Fd), 2 (corresponding to Th), 3 (corresponding to FpT), and 4 (corresponding to FpT) [148]. Distinct correlation between serotype and virulence has been observed. The FpT serotype is considered less virulent for rainbow trout [37], as it is more frequently isolated from asymptomatic individuals [147,149,150]; also, previous studies suggest that this serotype occurs more frequently in other fish species [9,12,147,148,150,151]. The lower virulence of FpT serotype isolates may be related to the high frequency of elastinase absence [37,149,152]. However, the absence of elastinase cannot be considered a characteristic feature of the entire FpT serotype, as an elastinolytic-positive isolate of the FpT serotype has been described [9]. Serotypes Fd and Th are both commonly isolated from outbreaks in rainbow trout, and virulence for both serotypes has been confirmed by experimental infections [151,152]. However, serotype Th is thought to be associated with higher virulence, as it is the most frequently isolated serotype from outbreaks in rainbow trout [147,150,151].

Genetic typing, along with serotyping, is an important epidemiological technique. The standardized multilocus sequence typing (MLST) approach is commonly used. The selection of suitable regions for amplification was enabled by whole-genome sequencing data from strain JIP 02/86 [153], published in 2007 [32]. By identification of 7 loci (*trpB*, *gyrB*, *dnaK*, *fumC*, *murG*, *tuf*, *atpA*) [154], isolates are divided into sequence types and clonal complexes. A distinct correlation was found between sequence types/clonal complexes and host fish species [153]. In European countries, the clonal complex CC-ST10 with a dominant sequence type 2 (ST2) is significantly predominant. Its main host is rainbow trout [155–158]. Sundell et al. confirmed the high virulence of CC-ST10 isolates, and it is probably this high virulence that underlies the global spread of this complex [37]. Currently, the second largest clonal complex is CC-ST191, which has also been proven to be virulent in rainbow trout [158]. On the other hand, MLST analysis alone cannot reliably predict virulence given that isolates belonging to the same sequence type often show quite different virulence rate.

### 9. Interaction of *F. psychrophilum* with the Host Immune System

Once the pathogen enters the organism, the host fish activates immune mechanisms to defend itself against the infection (Figure 3). However, *F. psychrophilum* is able to partially evade these mechanisms. Highly virulent strains have been found to be able to survive inside macrophages [159,160], which provides the bacteria with a safe hiding place in the host body. A study by Wiklund et Dalsgaard (2002) shows that *F. psychrophilum* strains, regardless of their virulence, are relatively resistant to alternative complement activity and the effect of specific antibodies [161]. Like most fish pathogens, *F. psychrophilum* is sensitive to the action of lysozyme contained in skin mucus. However, a higher concentration is required to inactivate *F. psychrophilum* than for inactivation of many other fish pathogens, e.g., *Aeromonas salmonicida*. A lysozyme concentration of 2 mg/mL was found to reduce the amount of *F. psychrophilum* by only 44% in 90 min [162]. Meanwhile, the activity of lysozyme in salmonid skin mucus fluctuates significantly throughout the year, with the lowest concentrations being found in the first months of the year [163,164], when *F. psychrophilum* outbreaks occur most frequently.



**Figure 3.** Schematic depiction of the fish skin defense system. Adapted from Gomez et al. [165]. Created with BioRender.com (accessed on 7 April 2024).

## 10. Final Considerations

Previous studies have identified a number of properties that are believed to be associated with the pathogenicity of the bacterium. However, the exact mechanism of pathogenicity is still not fully elucidated. The source of infection for fish is either the environment where the bacterium survives for several months [166] or infected and dead fish that shed the bacterium in large numbers into the environment [15]. It is believed that the bacterium enters the body of the host organism through erosions on the body surface, even microscopic ones [16]. At this stage, the ability to adhere to the body surface and move to the site of damaged skin using gliding motility is probably involved. However, some studies have suggested that adherence is not crucial for disease development [35,37], and further studies are therefore needed to clarify its role. Gliding, on the other hand, appears to be essential for virulence, and its impairment results in avirulent strains [30,31,91–94]. This is probably due to the inability to translocate itself to the site where the bacterium can enter the organism. Closely linked to the ability to glide is the T9SS secretion system, the dysfunction of which also significantly affects the pathogenicity of the bacterium. After entering the organism, the bacterium rapidly attacks the host tissue by producing proteolytic enzymes. This leads to development of extensive lesions. Elastinase is probably the most important virulence factor among these enzymes [48,54,58]. In contrast, a number of proteolytic enzymes seem to be completely dispensable for pathogenesis, or their importance depends on the host fish species [32,56,60]. However, some proteolytic enzymes have not been characterized in detail and their importance in bacterial virulence is not yet known. During the life of a bacterium, it is essential to acquire iron for its metabolism, but iron is not freely available inside a host fish. The ability to obtain iron therefore determines the ability of the bacterium to survive in the host. The iron transport system TonB has been proven to be essential for *F. psychrophilum* virulence [105]. The role of haemolysin and siderophore production in pathogenesis remains to be investigated. As can be seen, many unknowns remain about the pathogenesis of this bacterial agent. Further research into the mechanisms of action is therefore needed. Currently, with new technologies (WGS), bacterial genomes can be sequenced and identified in ever shorter times and at lower costs. This influx of information is driving the need for bioinformatic tools and databases to analyse and make available vast amounts of data to better understand virulence and genome evolution affecting the ability of pathogens to cause disease [167]. In the case of *F. psychrophilum*, several works have been published in the last few years that dealt with the comparative genome analysis focusing on pathogenicity [33], relationships between genomic diversity and virulence traits [25], genomic diversity, and evolution of the analysed *F. psychrophilum* isolates [168]. The data obtained from these studies, which are available in public databases, indicated the genomic diversity of *F. psychrophilum* on a global scale. It can be assumed that the obtained results will become the basis for genetic manipulation of *F. psychrophilum*, which could shed more light on the importance of putative virulence factors in the pathogenesis of infection in the future. A better understanding of the infection processes will help us to better target preventive and therapeutic measures and reduce the losses caused by this infection.

## 11. Conclusions and Recommendations

The mechanism of pathogenesis and the exact involvement and role of the virulence factors described so far are not yet fully understood. Unfortunately, genomic or phenotypic analysis alone is not sufficient to predict the virulence of individual strains, and experimental infection with LD50 determination is essential. Comparison of LD50 and concurrent analysis of virulence factors is essential to better understand the infection process.

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