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# Development of Genetic Manipulation Techniques for the Fish Pathogen Flavobacterium psychrophilum

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# Development of Genetic Manipulation Techniques for the Fish Pathogen *Flavobacterium psychrophilum*

By

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SEADA SLOBODA

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
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## ABSTRACT

*Flavobacterium psychrophilum* is a known fish pathogen, causing bacterial cold-water disease (BCWD) and rainbow trout fry syndrome (RTFS), predominantly in salmonids. The disease causes tissue damage and tail rot in young and adult fish. The issue is prevalent in fisheries in the Pacific Northwest and the treatments often entail the use of antibiotics. Genetic manipulations in *F. psychrophilum* are scarce, due to most of the strains' ability to destroy foreign DNA via restriction enzymes. The virulence mechanisms of *F. psychrophilum* are not well understood. Lack of genetic manipulation tools in *F. psychrophilum* has also hampered the development of live attenuated vaccines to prevent BCWD and RTFS in aquaculture. In this study, we identified two methyltransferases, HpaIIM and ScrFIM, in *F. psychrophilum* strain CSF259-93, the most problematic strain in rainbow trout fisheries in the United States. A helper plasmid pSS05 carrying both HpaIIM and ScrFIM encoding genes was constructed and used to pre-methylate the target DNA to improve the efficiency of DNA transfer by conjugation. By using pSS05 in combination with a previously developed markerless deletion system, we constructed a mutant lacking *gldN*, a core component of the type IX secretion system. The *gldN* deletion mutant was deficient in secreted proteolytic activity, colony spreading, single cell motility, and virulence on rainbow trout. The pre-methylation system developed in this study also functions in other *F. psychrophilum* strains. It may assist identification of virulence factors and development of live attenuated vaccines in multiple *F. psychrophilum* strains.

# Chapter 1: Introduction

*Flavobacterium psychrophilum* is a fish pathogen and the causative agent of bacterial cold-water disease (BCWD). Over the past seventy years, numerous isolates of *F. psychrophilum* have been retrieved from salmonids in different geographic locations. Some isolates caused severe tissue degradation in the fish, and affected internal organs, while other isolates caused relatively mild symptoms. The greatest obstacle in the development of preventative measures and treatments of BCWD caused by *F. psychrophilum* is the lack of commercially available vaccines that can be easily administered to a large number of fish. In addition, *F. psychrophilum* isolates have been known to develop antibiotic resistance over time, and some of the strains possess genetic components that make genetic manipulation an arduous task. The goal of this research project was to develop an efficient genetic manipulation system in *F. psychrophilum* strain CSF259-93, which is most problematic in rainbow trout aquaculture in the United States. Successful development of genetic manipulation techniques such as gene deletions for *F. psychrophilum* is crucial to understanding how this bacterium causes disease, and propelling the science of vaccine advancements in salmonid aquaculture.

Treatments of BCWD are insufficient due to development of antibiotic resistant strains of the pathogen. This resistance is widespread and is found in many strains of *F. psychrophilum.* Specifically, chlortetracycline, bacitracin, chloramphenicol, erythromycin, neomycin, and penicillin were ineffective in the past in some strains of the pathogen.

General sanitation of the hatcheries and fisheries may remedy some of the issues related to abundant use of antibiotics. Currently, several methods need to be employed to achieve partial protection of the fish, especially for juvenile populations, which are more susceptible to disease.

Researchers are actively exploring vaccines as preventative measures to combat BCWD. Despite all the efforts there is only one commercially available vaccine for preventative treatment of *F. psychrophilum* infections in Atlantic salmon (ALPHA JECT® IPNV-Flavo). Several attempts at vaccine development have been made, but none have yielded a commercially available product in the United States. Similarly, biological controls via bacteriophages have not yielded tremendous success.

The issues that many researchers faced during vaccine development and during the use of preventative treatments were multifold. First, the genetic diversity revealed by genotyping in *F. psychrophilum* isolates is high. Novel genome sequence types are being discovered (1). Second, there are unknown genetic factors that potentially contribute to increased pathogenicity of the pathogens and susceptibility of the host to the disease. Additional problems were faced concerning applicability of different vaccines to juvenile and adult fish. However, the greatest challenge is the lack of genetic manipulation tools for *F. psychrophilum* isolates.

To address the lack of genetic manipulation tools for *F. psychrophilum*, this research project addresses the role of Restriction-Modification (R-M) systems in preventing gene transfer. The development of efficient genetic techniques required overcoming the restriction enzyme barriers to DNA transfer. Specifically, this research

project aimed to identify critical *F. psychrophilum* modification enzymes (often referred to as methyltransferases or MTases) and use them to pre-methylate the DNA of interest, and thus protect it from digestion by the restriction enzymes present in the *F. psychrophilum* host during DNA transfer. Successful and efficient DNA transfer will allow genetic techniques such as gene deletion systems to function in *F. psychrophilum* so that mutants lacking essential virulence factors can be generated. These mutants can be developed as potential live attenuated vaccines.

## Chapter 2: Literature Review

### Bacterial Cold-Water Disease

Bacterial cold-water disease (BCWD), occurring typically in waters below 15$^{o}$C, (2–4) is prevalent in salmonids raised in hatcheries in the Pacific Northwest and in Northern Europe (3, 5). BCWD was first identified in salmonids in the 1950s (6) and it is characterized by open lesions and tail rot in proximity to caudal peduncle (Figure 1) (7), necrotic myositis, and cephalic osteochondritis (3, 8, 9). Species that have been affected by BCWD include sockeye salmon (*Oncorhynchus nerka*), chinook salmon (*O. tshawytscha*), coho salmon (*O. kisutch*), rainbow trout (*O. mykiss),* lake trout (*Salvelinus namaycush*), and cutthroat trout (*O. clarki*) (10). The disease was also found in non-salmonids such as eel (*Anguilla anguilla*), and cyprinids such as crucian carp (C*arassius carassius*), and common carp (*Cyprinus carpio*) (11).

As the disease progresses the tissue degradation may lead to exposure of skeletal processes and fins (3, 12). In Europe, the predominant term used to describe the BCWD is rainbow trout fry syndrome (RTFS) because of the tendency of recently hatched young fry

exhibiting the symptoms of the disease in form of tissue degradation (4, 13). Juvenile rainbow trout at 10 weeks of age (1 g weight) or younger were shown to be more susceptible to the disease than were adults (14). The disease can be transferred horizontally by transmission among the fish of the same generation living in close proximity, but it is also possible that the disease is transferred vertically from the mother to the offspring (14).

***Flavobacterium psychrophilum***

*F. psychrophilum*, the causative agent of BCWD, was first isolated from kidney and from external lesions of juvenile silver (coho) salmon (*O. kisutch*) at Washington, USA in 1960 (6). *F. psychrophilum* belongs to the family *Flavobacteriaceae* in the phylum Bacteroidetes (2), which was recently re-named as *Bacteroidota* (16). *F. psychrophilum* is a Gram-negative, rod shaped bacterium with gliding motility (2). Historically, the organism was assigned to genus *Cytophaga* when it was first isolated (6), but after DNA-rRNA hybridization analyses in the early 1990s the organism was reassigned to the genus *Flavobacterium* (17).

Numerous isolates of *F. psychrophilum* have been isolated from different fish species and geographic locations. Specifically, THCO9/20 isolates from salmon (*O. kisutch*), CSF259-93 from rainbow trout (*O. mykiss*), and G3724 isolated from ayu (*P. altivelis*) (18). *F. psychrophilum* strains are generally unable to grow at temperatures above 25 ºC and they prefer temperatures between 5 ºC and 20 ºC (3, 12, 19). The pathogenicity declines as the temperature of the water increases over 13 ºC (3). In their natural habitat the strains can survive for several months even after the completion of cell division (growth

arrest) (20). Salinity tolerance varies by strain but generally *F. psychrophilum* isolates have shown tolerance to NaCl at concentrations lower than 0.8% (3, 5).

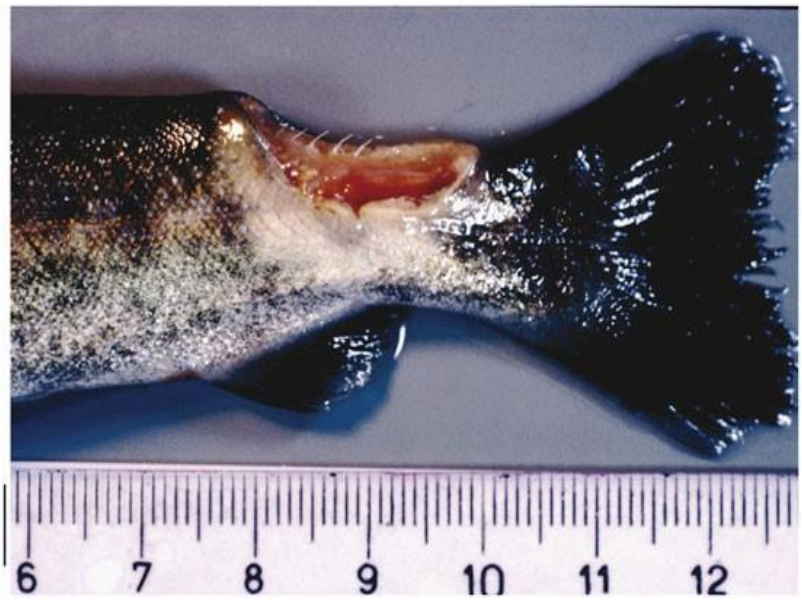



**Figure 1.** Lesions caused by *Flavobacterium psychrophilum* and bacterial cold-water disease in rainbow trout *(O. mykiss)* (7).

**Treatments and Prevention**

Infected salmonids are often treated with a variety of antibiotics because *F. psychrophilum* is known to develop resistance to antibiotics. Careful treatment options are crucial to treating the disease. In the past, chlortetracycline, bacitracin, chloramphenicol, erythromycin, neomycin, penicillin, and tetracycline had inhibitory effects on the pathogen but new solutions are continuously being explored (3, 21). Oxytetracycline, amoxicillin and oxalinic acid were also used for treatments in Europe in the 1990s, but the use of some of these antibiotics was discontinued due to antibiotic resistance (19, 22). In one recent study, florfenicol, oxytetracycline and erythromycin were successfully used to treat the

invasive virulence of *F. psychrophilum* (19). However, some *F. psychrophilum* strains are resistant to tetracycline (23). The genome of strain CSF259-93 contains two tetracycline resistance genes (FPSM_00635 and FPSM_00640) (24).

In addition, general sanitation of hatcheries is crucial and compounds such as quaternary ammonium, hydrogen peroxide, and removal of nitrogen and iron from the water, in combination with antibiotic use, have shown promise in preventing fish diseases, including BCWD (25). Despite the fact that there is a high availability of antibiotics that could be used as treatment options, *F. psychrophilum* strains continue to develop antibiotic resistance. Thus, it is difficult for fisheries to find appropriate treatment options.

**Vaccines**

Although chemical disinfectants, practices like removal of nitrogen and iron, in combination with antibiotic use, have shown promise at preventing fish diseases, these solutions are not comprehensive. Another avenue for treatment is vaccination. Specifically, immersion vaccines are an excellent preventative option due to their economical nature and relatively easy application to fish populations.

Several different types of vaccines are offered as preventative measures against fish pathogens. Vaccines commonly used in aquaculture are: killed whole-cell, toxoid, subunit vaccines, and live-attenuated vaccines (26). Killed-whole cell vaccines function by inactivation using heat or chemical, subunit vaccines are composed of an antigenic component that illicits an immune response, and live-attenuated vaccines are strains (mutants) of the disease causing organism that exhibit reduced virulence (27).

Immersion or oral vaccines are preferred for use in salmonid aquiculture due to ease of delivery to young disease susceptible fry to prevent RTFS (28). Several vaccines have been developed for the treatment of fish diseases including a live attenuated vaccine developed for the treatment of infections (Columnaris) caused by *Flavobacterium columna*re, a fish pathogen from the same genus as *F. psychrophilum* (29). However, there are no commercially available vaccines to prevent BCWD in the United States. Currently, the only commercially available vaccine is an inactivated vaccine used to prevention necrotic lesions caused by *F. psychrophilum* in Atlantic salmon (ALPHA JECT® IPNV-Flavo) and approved for use in Chile (30). A major limitation of this vaccine is that it is administered by injection, which limits its use for juvenile fry.

A few pilot studies exploring potential candidate vaccines were conducted. One vaccine, formalin-inactivated polyvalent vaccine (containing *F. psychrophilum* whole cells), was tested in a preliminary study (28). The vaccine was delivered to trout by immersion in 1 L of vaccine for 30 seconds and 84% relative percent survival (RPS) was conferred following the vaccination (28). Another attempt at an immersion vaccine was made, using live attenuated rifampicin resistant isolates of *F. psychrophilum*, 259-93A.16 and 259-93B.17, and the results yielded production of antibodies (31). Rainbow trout immunized (via intraperitoneal injection) using the live-attenuated 259-93B.17 resulted in a significant production of antibodies (31). However, the issues with many of the vaccines are that they may or may not confer protection against diverse strains, they are not always suitable for use in juvenile fish, and the method of administering the vaccine may be too tedious or cost-ineffective. This is why it is crucial to find a cost-effective solution that can

be applied to juvenile and adult fish immunization, such as development of live attenuated vaccines.

**Biological Control: Bacteriophages**

Bacteriophages are viruses that serve as biological agents against bacteria by infecting the host resulting in cell death without downstream adverse effects on humans or animals (32). A study conducted on Danish *F. psychrophilum* isolates, obtained from fisheries, as well as a strain obtained from a freshwater stream, showed promising results regarding the use of bacteriophage therapy to treat and prevent BCWD and RTFS (33). Bacteriophages FpV-9 and FpV-10 caused lysis in over 20 out of the 28 *F. psychrophilum* isolates (33). However, the authors stated that due to the fact that the experiments were conducted under controlled conditions it is difficult to conclude that phages can ensure a major control of *F. psychrophilum* in their natural habitat (33). While bacteriophages are a good alternative to antibiotics, they often have a very narrow host range (34) and tend to be an expensive treatment option.

**Target Strain Molecular Components: Genomic Islands and Virulence Elements**

*F. psychrophilum* strain CSF259-93, isolated from the spleen of diseased rainbow trout (*O. mykiss)* in 1993 in Southern Idaho (35), was chosen for this research project due to the fact that it is among the most virulent strains and due to a general lack of understanding of pathogenesis of *F. psychrophilum* (36). This strain and close relatives are the most problematic in rainbow trout aquaculture in the United States.

Genomic Islands (GIs) are clusters of genes that are prominent among prokaryotes and are involved in virulence, antibiotic resistance (37), bacterial fitness, and metabolic capabilities (38). GIs were identified in *F. psychrophilum* strains CSF259-93 and 950106-1/1 and they were thought to be associated with toxins and possible antibiotic resistance in these strains (37). CSF259-93 has a large GI (46.8 kb) containing toxin (Fic) encoding genes, virulence associated protein E (*VapE*), and type II (37) and type III (24) restriction modification systems.

Fic proteins produced by pathogens such as CSF259-93 strain (37), were previously identified in other bacterial species and were known for their interference in cell signaling cascades of the host, ultimately resulting in a destruction of actin cytoskeleton of the host cell and cell death (37, 39). Presence of Fic proteins in CSF259-93 and strain 950106-1/1 indicates that in reference to their mode of pathogenicity these isolates possibly differ from other *F. psychrophi*lum isolates (37).

Another major protein identified in CSF 259-93 strain is virulence associated protein E (VapE), first identified in *Dichelobacter nodosus* (40), and as the name of the protein suggests, it is involved in virulence, but the mechanism of action has yet to be determined (37).

Some of the pathogenesis of this strain is tied to molecular components on the outer membrane of the bacterium (41). Specifically, lipopolysaccharides (LPS) present on the cell surface, exotoxins and secreted proteins (35, 41). In one of the past studies, O-antigen polysaccharide (O-PS) was evaluated as a potential candidate for genetic manipulations

that would yield resulting mutants that can be used as vaccine candidates (41). This lipopolysaccharide was later identified as a CSF259-93 antigen of 15-16 kDa (35).

Strain CSF259-93 was compared to a non-virulent *F. psychrophilum* strain ATCC49418 and several different proteins were identified as potential virulence factors (35). Fifteen immunogenic proteins were identified in the virulent CSF259-93 strain and none of those proteins were found in the non-virulent strain (35). The proteins were identified using two-dimensional gel electrophoresis (2-DE) and western blotting techniques. Western blot analysis, using the immune sera collected from CSF259-93 immunized juvenile rainbow trout, indicated that several of the proteins that may be involved in virulence were >50 kDa in size (35). While CSF259-93 strain seems to have unique components in its genome a lot of the components of CSF259-93 are presumed to also be shared with other members of Bacteroidetes and one of these components is the type IX secretion system.

**The Type IX Secretion System**

Protein secretion systems and their substrates are important for cell propagation, pathogenesis, and survival of numerous bacteria. The newly discovered type IX secretion system (T9SS), is no exception. The T9SS is regulated by a two-component system, and is important for cell function and proliferation (42, 43). In *F. psychrophilum* and other Bacteroidetes fish pathogens, the T9SS is linked to virulence (43–45).

The T9SS, originally called the Por secretion system (PorSS), is a set of proteins involved in secretion of proteolytic enzymes, adhesins, endonucleases, and many other proteins (42, 46). The T9SS is confined to the phylum Bacteroidetes 11/11/2022 4:08:00

AM. It was firstly discovered in the non-motile human oral pathogen *Porphyromonas gingivalis* and the motile environmental bacterium *Flavobacterium johnsoniae* (47). Majority of the structural biology identifiers for T9SS have been performed in *P. gingivalis,* but these identifiers could potentially be applicable to other members of the phylum (42, 46, 48).

The location of the T9SS translocon is in the outer membrane (OM) of the bacteria and proteins that undergo secretion steps typically have a highly conserved C-terminal domain (CTD) that will lead sets of proteins across the OM translocon (Figure 2) (42, 49).

Studies of *P. gingivalis* have shown that the T9SS transports different proteins including virulence factors across the OM (42, 48). A recent study on the *F. psychrophilum* THCO2-90 strain showed that T9SS also contributes to the infectiveness of the pathogen (43). Gliding motility, proteolytic and hemolytic activity were reduced due to the deletion of T9SS components (43). However, questions that need to be explored are what proteins secreted by the T9SS are the virulence factors and would deletion of these virulence genes produce non-virulent mutants of *F. psychrophilum*. THCO2-90 is the only strain in which genetic manipulation has been feasible among the identified *F. psychrophilum* strains. Failure in genetic manipulation of other strains, including CSF259-93, may be attributed to the presence of restriction-modification systems (2).

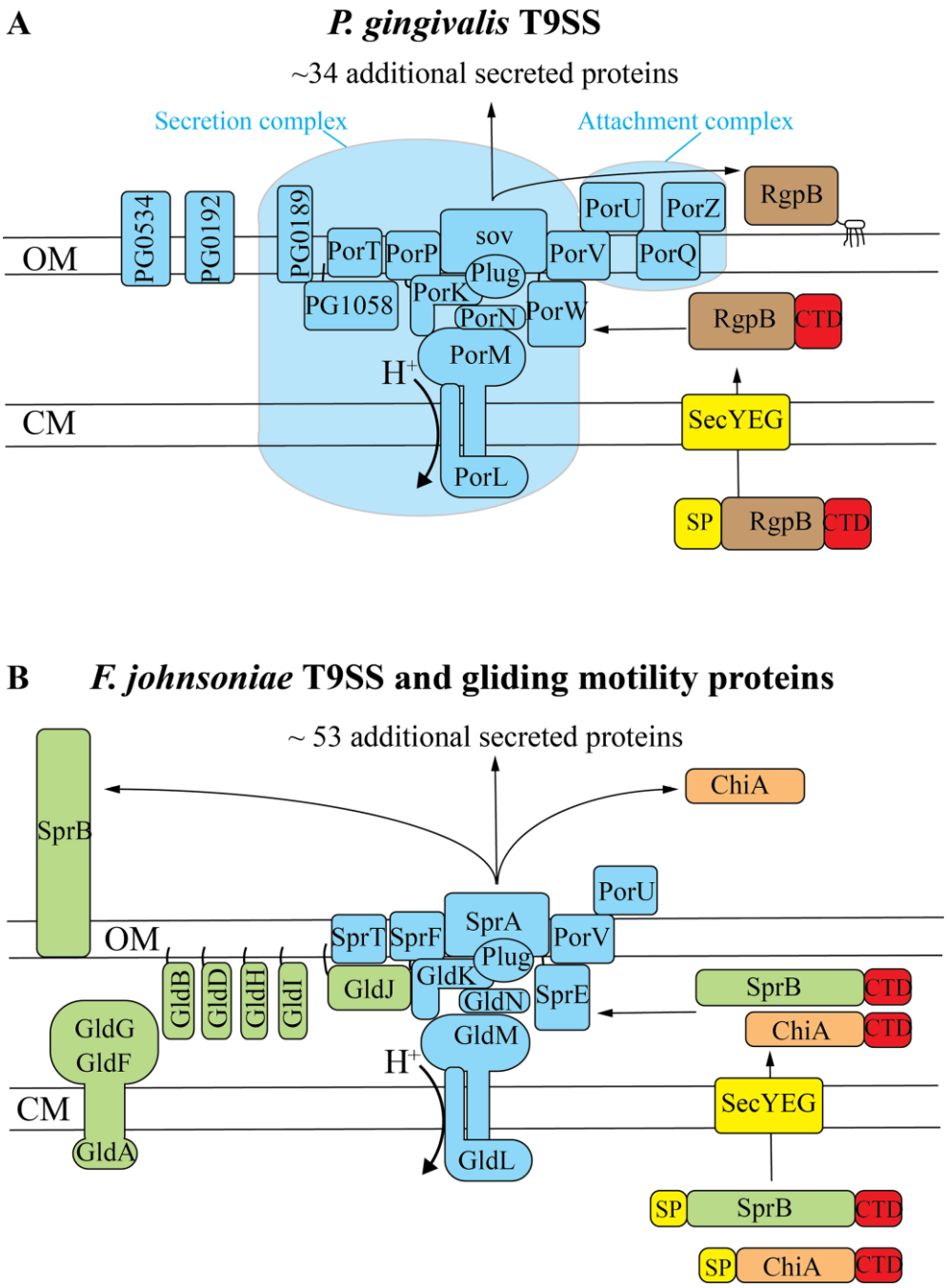



**Figure 2.** Schematic representation of different components of the T9SS and Sec System (50).

**Restriction-Modification Systems**

The restriction-modification (R-M) systems work by preventing transfer of genetic information from donor or environment to the recipient host (Figure 3). Specifically, the R-M systems may control the access of foreign DNA to the recipient cells and catalyze cleavage (51) of foreign DNA via the use of restriction endonucleases (REases) (52). On the other hand, use of modification enzymes, methyltransferases (MTases), may confer protection to particular sites on the target DNA and protect those sites from DNA cleavage (53). The REases involved in cleavage are assigned names based on the name of the organism they originate from, an example would be *Haemophilus influenzae* (bacteria species name) and HindII (REase name) (54). The R-M systems are considered primitive immune systems in prokaryotes.

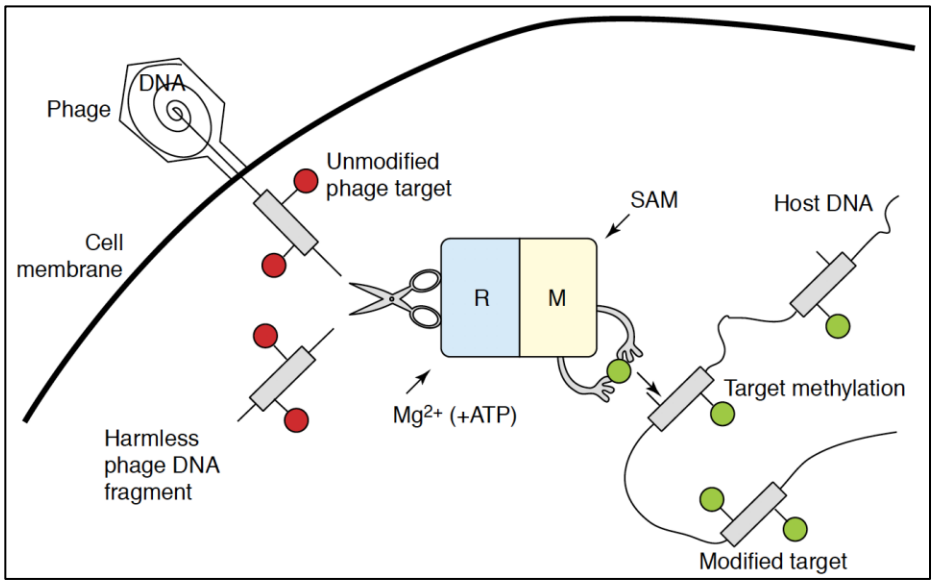



**Figure 3.** The function of R-M systems, as illustrated by the Type I R-M enzymes and phage infection. DNA with green circles is methylated and DNA with red circles is un-methylated (55).

The authors of a study conducted on *F. psychrophilum,* strains THCO2-90 and THCO4-90, hypothesized that restriction endonucleases (present as a part of R-M system) were possible culprits for impairing the acceptance of *E. coli* DNA into *F. psychrophilum* (21). Similarly, it is possible that these restriction enzymes are responsible for the lack of success in DNA transfer from *E. coli* to *F. psychrophilum* CSF 259-93. Successful foreign DNA transfer is imperative for a gene deletion strategy and for the development of a live attenuated vaccine.

There are four different types of R-M systems present in prokaryotes: type I, II, III, and IV. Type I R-M systems are found in numerous prokaryotes and these systems are considered relatively complex compared to type II (56). There are three major components that comprise type I R-M systems and they control both restriction and modification (54). The three components within type I R-M systems are: one Specificity (encoded by the *hsdS* gene) unit, two MTase (encoded by *hsdM* gene) units, as well as two REase (encoded by the *hsdR* gene) subunits (57). Hsd is an abbreviation for host-specificity for DNA. MTases utilize S-adenosylmethionine (SAM) as a methyl group donor and they methylate $N^6$ location on adenine (56) although recent studies have found protection by $N^4$-methyl cytosine (58). For the restriction reaction to occur the requirements are the presence of SAM, ATP, and $Mg^{2+}$ (53, 59). The cleavage process involves adenosine triphosphate (ATP) hydrolysis and subsequent translocation of DNA (57). There are several families of type I R-M systems IA, IB, IC, ID, IE that have been briefly reviewed in the literature (56).

Type II R-M systems could possibly be one of the largest groups of R-M systems consisting of over 3000 different members (55). They are also continuously being

discovered. The MTases in these R-M systems often have fairly similar amino acid motifs, while REases do not have similar amino acid sequences and they are classified in 11 classes (55). Similar to type I R-M system restriction requires presence of $Mg^{2+}$ (60) and modification requires SAM (55). Type II R-M systems enzymes (MTases and REases) recognize 4-8 bp sequences and modify or cleave according to their role (55). The REases may often be homodimers that consist of the duplicate of the same enzyme with each of the enzymes cutting a single strand of the DNA at the same time (60). Other REases may bind multiple copies of their recognition sites before the DNA cleavage occurs (60). The MTases within type II R-M systems usually methylate $N^4$ or $C^5$ on cytosine, and $N^6$ on adenine of their recognition sites on each strand (61).

Type III R-M systems function similar to type I R-M systems. Structurally they are hetero-oligomers that are composed of a "mod" subunit, necessary for modification and recognition of the substrate, and a "res" restriction endonuclease subunit (62). R-M enzymes in this group interact with a pair of inversely oriented sequences of 5-6 bp that are usually asymmetric (53). The "mod" subunits methylate $N^6$ on adenine on one strand of the DNA duplex to confer protection.

Type IV R-M systems are different from other types of R-M systems in that the REases selectively cleave DNA that has been methylated (53). Type IV R-M systems are not of interest in this study because their REases cleave methylated DNA.

In a recent study in the cyanobacterium *Synechocystis* sp., it was shown that pre-methylation of DNA before introduction to the host bacteria increased the chance of successful transformation (52). *Synechocystis sp.* strain 6803 methyltransferase genes were

introduced to *E. coli* on a compatible plasmid pACYC184 to pre-methylate the co-existing plasmid used in transformation (52). Successful pre-methylation of the tested plasmids yielded higher efficiency in transformation (52).

Although, *Synechocystis* is not closely related to *F. psychrophilum*, we used a similar strategy of DNA pre-methylation to improve the DNA transfer efficiency in *F. psychrophilum* CSF259-93.

**Research Strategies Used in This Study**

For this project, the plan was to clone different methyltransferase genes from *F. psychrophilum* CSF259-93 individually or in combination onto pACYC184, generating helper plasmids for pre-methylation of target DNA in *E. coli*. pACYC184 is compatible in *E. coli* with the commonly used plasmids in manipulation of Bacteroidetes such as pCP11 (63) because they have different origins of replication (Figure 4). A pACYC184 derived helper plasmid and the shuttle vector pCP11 (reporter plasmid) were co-transformed into the conjugation donor *E. coli* strain S17-1 λ pir. We expected that pCP11 would be methylated in this *E. coli* strain by the expression of the methyltransferase gene(s) from the helper plasmid. The co-transformed *E. coli* S17-1 λ pir strains were then tested by conjugation (introduction of methylated single-stranded DNA into *F. psychrophilum* CSF259-93) to assess the efficiency of pCP11 transfer.

A B

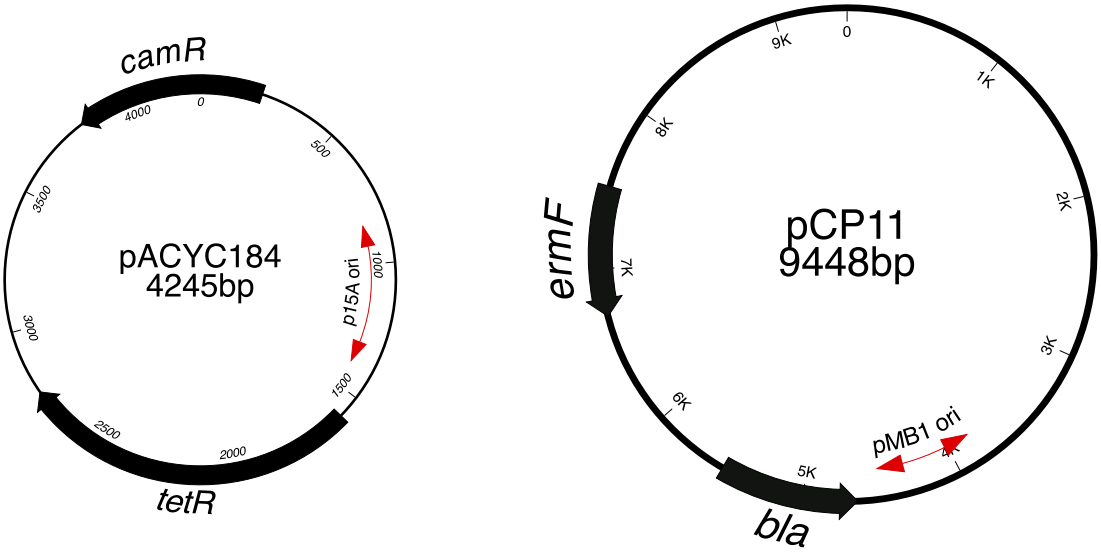



**Figure 4.** Maps of the pACYC184 (A) and pCP11 (B) plasmids. The red double arrow on each plasmid indicates the origin of replication (ori) used in *E. coli*.

Once an efficient DNA transfer system overcoming the restriction barrier in *F. psychrophilum* CSF259-93 is developed, we proposed to delete a potential virulence gene using this system in combination with the published *sacB*-mediated deletion system (64). There are several possible mutants that could be created by deletion of the core components of the T9SS: *gldK*, *gldL*, *gldM*, *gldN*, *sprA*, *sprE*, and *sprT* (42). *gldN* deficient cells were avirulent in recent studies in strain THCO2-90 isolated from *O. kisutch* (Coho salmon) kidney (43). The virulence in *ΔgldN* mutant was decreased to an extent that proteolytic activity, hemolytic activity, and colony spreading were reduced (when compared to the

wild type) (43). This implies that *gldN* and the T9SS have an essential role in secretion of proteolytic, hemolytic and adhesive factors, which could be potential virulence factors. Due to success in deletion of *gldN* in THCO2-90 we aimed to delete *gldN* in strain CSF259-93. We predicted that the deletion of *gldN* in *F. psychrophilum* CSF259-93 would induce a similar change in virulence.

# Chapter 3: Methods

## Strains and Growth/Storage Conditions

*E. coli* strain DH5αMCR was used for cloning of target methyltransferase genes onto pACYC184 to generate the helper plasmids. *E. coli* strain S17-1 λ pir was used in DNA pre-methylation and in conjugation as a donor due to the presence of pili required for conjugation. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) medium. LB was prepared by adding 10 g Bacto Tryptone, 5 g yeast extract, combined with 10 g NaCl and added up to 1 l by double distilled $H_2O$ (dd $H_2O$) and adjusted to pH 7.3 with 1 M NaOH (15 g agar can be added prior to autoclaving for agar plates).

Wild type *F. psychrophilum* strains CSF259-93, CSF201-91, CSF408-92, CSF060-99, and THCO2-90 (Table 1) were used for genetic manipulations and inoculated on tryptone yeast extract salts (TYES) agar or liquid media. The following components were combined for 1 l TYES: 1 l dd$H_2O$, 4.0 g tryptone, 0.4 g yeast extract salts, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g $CaCl_2$ and the pH was adjusted to 7.2 (using NaOH). For TYES 15 g agar was added if needed and for TYES+ 2.5% sucrose 975 ml dd$H_2O$ was used in combination with 25 ml of 50 % sucrose (added post autoclaving).

The cells inoculated on TYES were incubated over 48-72 hours at 20°C or 18°C. For liquid cultures shaking incubator set at 200 rpm was used. Antibiotics were used at the following concentrations when needed: ampicillin, 100 µg/ml; chloramphenicol, 10 µg/ml; and erythromycin 20 µg/ml.

One milliliter of cultured cells was added to a cryotube containing 250 µl of 75% glycerol solution and stored at -80°C. Two copies of each strain were stored separately in different freezers.

**Table 1.** The wild type *F. psychrophilum* strains used in this study.

| Strain | Description | Reference |
|---|---|---|
| CSF259-93 | Wild type; isolated in spleen of *O. mykiss* in Idaho | (2, 3) |
| CSF201-91 | Wild type; isolated in spleen of *O. mykiss* in Idaho | (4) |
| CSF408-92 | Wild type; isolated in spleen of *O. mykiss* in Idaho | (4) |
| CSF060-99 | Wild type; isolated in spleen of *O. mykiss* in Idaho | (4) |
| THCO2-90 | Wild type; isolated in kidney of *O. kisutch* in Oregon | (65) |

**Phusion PCR and DNA Purification**

Polymerase Chain Reaction (PCR) was used for amplification of DNA fragments using Phusion DNA polymerase (Fisher Scientific) or Taq DNA polymerase (New England BioLabs). For Phusion PCR 50 µL reaction was set up. 25 µL of 2 X Phusion Master Mix was combined with 1.5 µL of 10 mM forward primer, 1.5 µL of 10 mM reverse primer, 1 µL of *F. psychrophilum* (CSF259-93) genomic DNA, and 21 µL of sterilized dd$H_2O$. This was the first step of gene cloning process (Figure 5).

Following the Phusion PCR 2 µL of the DNA was combined with 1 µL of the DNA loading dye and loaded onto 0.7% agarose gel with SYBR safe DNA stain (Fisher Scientific) ran at 100V for 35-45 min for a visual confirmation of a successful amplification of the DNA, as well as the concentration of the DNA under blue or UV light. The DNA

was then purified following IBI Scientific DNA Purification/Gel Extraction protocol and eluted to 35 µL and stored at -20 °C.

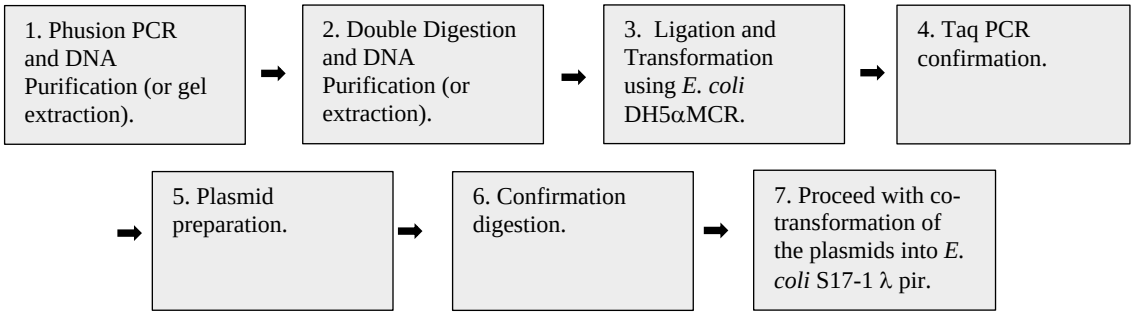



**Figure 5.** Representation of the gene cloning process for constructing the plasmids.

**Digestion and Ligation**

Thirty-five µL PCR product or 5 µl plasmid (~200 ng/µl) was digested in a 50 µL reaction. 5 µl of buffer CutSmart or 3.1 (dependent on the enzyme compatibility with the buffer verified on NEB tools app) was added and 1 µl of each of the two necessary restriction enzymes (refer to Table 2 for more information) required for double digestion. The digestion mixtures were incubated for 1.5 hours at 37°C. The digested PCR product and plasmid DNA were purified using following IBI Scientific DNA Purification/Gel Extraction protocol and eluted to 25 µL. The concentration of the DNA components was determined via gel electrophoresis similarly to Phusion PCR agarose gel confirmation. Based on band brightness the proportion of plasmid DNA and PCR product were determined for ligation.

Ligation reaction setup typically included 9 µL PCR product, 8 µL plasmid DNA, 2 µL ligase buffer and 1 µL T4 DNA ligase (Fisher Scientific) to initiate the joining of the PCR product and plasmid. Ligation occurred at room temperature over 24-72 hours

depending on the size of the PCR product and it was deactivated by 10 min heat inactivation at 65°C.

**Transformation**

Transformation was conducted by adding 10 µL of ligation product into $CaCl_2$ treated competent cells (DH5αMCR). The cells mixed with DNA were incubated on ice for 30 minutes and subsequently heat shocked at 42°C for 90 seconds allowing the cell membrane to accept the DNA. $CaCl_2$ and the heat shock of the cells have an important role in allowing for a change in cell membrane properties which allows for an easier acceptance of the DNA (65). Following this step the cells were kept on ice for 5 min and 1 ml LB was added (66). After LB was added the cells were incubated at 37 °C for 1 hour and subsequently centrifuged at 4,665 x g for 3 minutes. Supernatant was discarded to remove un-transformed DNA and 200-400 µl of LB was added. The cells (100 µl) were spread on the appropriate agar plate (LB and chloramphenicol for *E. coli* containing pACYC184 based plasmids) and incubated at 37°C for up to 18 hrs.

Co-transformation of pairs of compatible plasmids into *E. coli* strain S17-1 λ pir was performed similarly. One of the plasmids contains the methyltransferase genes (ex. pSS01), and the other one is either a reporter plasmid (ex. pCP11) or a deletion construct (pYT313 based plasmid). However, 2 µl of each plasmid was added instead. The cells were plated on LB containing both chloramphenicol and ampicillin.

**Taq Colony PCR**

Taq Colony PCR was used to confirm successful transformation of the plasmid containing desired DNA insert by screening individual colonies for the presence of a particular sequence. For the Taq PCR, the master mix was prepared following the instructions for 25 µL reaction set up. 2.5 µL standard *Taq* buffer (10 X), 0.5 µL dNTPs (10 mM), 0.5 µL of each primer (forward and reverse, 10 µM), 3 µL lysis product from boiled colonies (or 1 µL of genomic DNA and 2 µL sterilized $ddH_2O$ as the control), 0.125 µL *Taq* DNA polymerase, and $ddH_2O$ was needed to add up to 25 µL for a reaction set up.

**Plasmid Preparation**

The plasmids used in this study are listed in Tables 2 and 3. The first step during the plasmid preparation protocol was to inoculate the desired *F. psychrophilum* or *E. coli* strains on their respective media, TYES and LB agar, respectively, and allow for overnight growth (up to 72 hours for *F. psychrophilum*). The following day one colony from the streaked plates was selected and inoculated into the appropriate media with the appropriate antibiotics (refer to Table 2) and incubated at the appropriate temperature.

The HI-speed mini plasmid kit (IBI Scientific) was used for plasmid preparation following the manufacturer's instructions. Target cells were obtained, lysed and purified (3 ml total volume for *E. coli* and 30 ml for *F. psychrophilum*). Plasmids in the supernatant were then extracted following a series of wash steps and purification steps provided in the plasmid kit guide. The samples were labeled and placed in the freezer.

**Table 2.** Plasmids used in this study.

| Plasmid | Characteristics[a] | Purpose or role for this study | Growth Condition (*E. coli*) | Reference |
|---|---|---|---|---|
| pACYC184 | Low copy, p15A ori, $Tc^r$, $Cm^r$ | Compatible vector for cloning of pre-methylation helper plasmids. | LB and Cm | (67) |
| pCP11 | Shuttle vector, pMB1 ori, $Ap^r$ ($Em^r$) | Reporter plasmid for conjugation. | LB and Ap | (63) |
| pYT313 | *sacB* suicide vector, pMB1 ori, $Ap^r$ ($Em^r$) | Suicide vector used for gene deletion. | LB and Ap | (64) |

[a]Antibiotic resistance phenotypes: ampicillin, $Ap^r$; chloramphenicol, $Cm^r$; erythromycin, $Em^r$; 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. psychrophilum* but not in *E. coli*.

**Construction of Pre-methylation Helper Plasmids**

A 1.8 kbp fragment, spanning CSF259-93 *hpaIIM* (FPSM_02394) and its 450 bp upstream region and 80 bp downstream region, was amplified by PCR using Phusion DNA polymerase and primers YZ0090 (introducing a BamHI site) and YZ0091(introducing a SalI site). The fragment was digested with BamHI and SalI and ligated into pACYC184, which had been digested with the same enzymes, to generate pSS01. The *tetR* gene was partially removed from pACYC184 by the above process. pSS02 carrying *scrFIM* (FPSM_00612) and pSS03 carrying *mboIM* (FPSM_01581) were constructed using the same procedure and primers listed in Table 3.

To construct pSS05 carrying both *scrFIM* and *hpaIIM*, *scrFIM* was amplified with primers YZ0133 and YZ0134, digested by SalI and NarI, and ligated into SalI and NarI digested pSS01. To construct pSS07 carrying *M.FpsJIIM* (FPSM_00552), *M.FpsJIIM* was amplified with primers YZ0138 and YZ0139, digested by BamHI and NarI, and ligated into BamHI and NarI digested pSS01. The same procedure was used to construct pSS08 carrying *tauIM* (FPSM_00649) and pSS11 carrying *bstUIM* (FPSM_01519) using the primers listed in Table 3. All the constructed helper plasmids in this study are listed in Table 3.

**Table 3.** Primers to amplify the DNA-methyltransferase encoding genes in *F. psychrophilum* CSF259-93 and the constructed helper plasmids.

| Primer Name | Sequence | Gene Name | REase Underlined | Helper Plasmid |
|---|---|---|---|---|
| YZ0090 F | 5' – GCTAGGGATCCTATTATTGGGGTTAGAGGAATC – 3' | *hpaIIM* | BamH1 | pSS01 |
| YZ0091 R | 5' – GCTAGGTCGACTATTCAAATTGGCATCTCCA – 3' | | Sal1 | |
| YZ0092 F | 5' – GCTAGGGATCCAGCAGGGAAAAGTTTTGAAG – 3' | *scrFIM* | BamH1 | pSS02 |
| YZ0093 R | 5' – GCTAGGTCGACGAAGTGGCGGATTTAGAA – 3' | | Sal1 | |
| YZ0094 F | 5' – GCTAGGGATCCACCGTATCAAAGACGATTTT – 3' | *mboIM* | BamH1 | pSS03 |
| YZ0095 R | 5' – GCTAGGTCGACACCTTAGAAGATTTTCAATATGC – 3' | | Sal1 | |
| YZ0133 F | 5' – GCTAGGTCGACAGCAGGGAAAAGTTTTGAAG – 3 | *scrFIM* and *hpaIIM* | SalI | pSS05 |
| YZ0134 R | 5' – GCTAGGGCGCCGAAGTGGCGGATTTAGAA – 3 | | NarI | |
| YZ0138 F | 5' – GCTAGGGATCCCCAATACTTCCCTAATGCAA – 3 | *M.FpsJIIM* | BamH1 | pSS07 |
| YZ0139 R | 5' – GCTAGGGCGCCTGCTGCGGTGTATAATATAA – 3 | | NarI | |
| YZ0140 F | 5' – GCTAGGGATCCAGCGCACTTAGAAACTTAAA – 3 | *tauIM* | BamH1 | pSS08 |
| YZ0141 R | 5' – GCTAGGGCGCCTACAATCCAAGCCAATCATT – 3 | | NarI | |
| YZ0145 F | 5' – GCTAGGGATCCATTTAGCCAAAAATTGAGCG – 3 | *bstUIM* | BamH1 | pSS11 |
| YZ0146 R | 5' – GCTAGGGCGCCATTGTTTCAAGTCCGTTTTT – 3 | | NarI | |

**Conjugation**

*E. coli* S17-1 λ pir cell cultures containing co-transformed plasmids (pSS01 + pCP11 for example) were inoculated into LB (with ampicillin and chloramphenicol). *E. coli* S17-1 λ pir containing only pCP11 (un-methylated) was used as a control. *E. coli* cultures were grown at 37$^{o}$C on a rotator for ~16 hours. *F. psychrophilum* strains were streaked on TYES agar from the -80$^{o}$C freezer and incubated at 20$^{o}$C for 3 days. The cells were re-streaked (lawn inoculated) on TYES plates and incubated at 20$^{o}$C for the duration of ~36 hours.

The *E. coli* cells (5 ml per test tube) were aseptically transferred into sterile 15 ml conical centrifugation tubes labeled with their respective names, centrifuged for 5 min at 4, 000 x g at 20°C, and washed once with 10 ml TYES. *F. psychrophilum* cells were scraped off the plates using sterilized cell lifters and added into appropriately labeled centrifugation tubes, containing 10 ml of TYES, and centrifuged for 5 min at 4, 000 x g at 20°C. The resulting *F. psychrophilum* cell pellets were washed once with 10 ml of TYES.

Supernatants were removed and cells were resuspended in 1 ml of TYES. Optical density (OD) at 600 nm were measured following a preset protocol using a microplate reader. To measure OD 600 cells were diluted tenfold (20 µl cells into 180 µl TYES) and pipetted (in duplicates) onto 96 well microplates. TYES only was used as a blank.

After the measurement of OD600, *E. coli* and *F. psychrophilum* cell culture concentrations were adjusted to OD600~4.0 and ~7.0, respectively. Following this step 500 µl of *E. coli* was mixed with 250 µl of *F. psychrophilum.* The tubes were centrifuged at 4,000 x g for 5 min. The supernatant was removed. The cells were resuspended into 50 µl

TYES and spotted on appropriately labeled TYES plates. The spots were allowed to dry and the plates were incubated for 48-72 hours at 20°C.

After incubation, the conjugants were scraped using the cell lifters and resuspended into 1 ml of TYES. If abundant CFU growth was expected the cells were diluted tenfold. Using a flame-sterilized plate spreader (hockey stick) 100 µl of cells was evenly distributed onto each TYES (with erythromycin) plates (in replicates of 6-8 per conjugation). The number of colonies post-conjugation on each plate was counted and recorded after incubation for 5 – 10 days. Randomly selected erythromycin resistant colonies were re-streaked on TYES erythromycin plates for purification and stock preparation. PCR was used to confirm the presence of plasmids in *F. psychrophilum* by amplifying the erythromycin resistance gene *ermF*. Figure 6 represents a brief summary of the cloning steps leading up to conjugation.

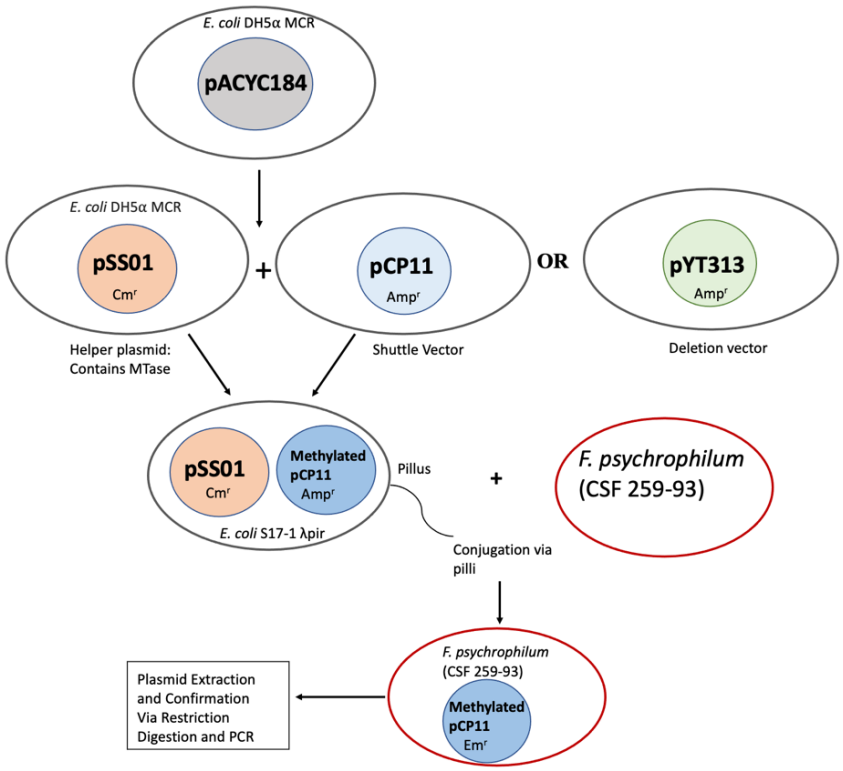



**Figure 6.** Development of DNA pre-methylation technique for manipulating *F. psychrophilum* strain CSF259-93.

### Construction of *gldN* Deletion Mutant

The *sacB* gene-mediated deletion strategy is an in-frame markerless deletion strategy utilized in the phylum *Bacteroidetes* (64). The process of gene deletion starts with DNA cloning of homology arms into a suicide vector carrying *sacB* and ends with two rounds of DNA recombination in *F. psychrophilum* resulting in relatively equal chance of cells containing the wildtype or deletion mutants (Figure 7).

The first step for *sacB*-mediated deletion is cloning of the upstream and downstream regions of the target gene (ex. the T9SS core gene *gldN*) into the suicide

plasmid pYT313 (Figure 7A) (64) that is unable to replicate independently in *F. psychrophilum.* Un-replicable plasmid introduced into *F. psychrophilum* will presumably integrate into the chromosome of the host via recombination. Upstream region of *gldN* was cloned into BamH1and SalI sites on pYT313 using the forward primer, YZ0117 (5' – GCTAGGGATCCGATGCAATGTTAAAAGAGGC – 3' engineered with BamHI site) and reverse primer, YZ0118 (5'–GCTAGGTCGACAGCATTCAACAAGTTAGATTG – 3' engineered with SalI site), generating pSS04. The downstream region of *gldN* was cloned into SalI and SphI sites on pSS04 using the forward primer, YZ0119 (5' – GCTAGGTCGACGTTCGTAATTTCGAGCAAGA – 3' engineered with SalI site) and reverse primer, YZ0120 (5' – GCTAGGCATGCCGGTTCTTCCTTCTTCAAAA – 3' engineered with SphI site), generating the final deletion construct, pSS12, containing both upstream and downstream regions of *gldN*. pSS12 and the helper plasmid pSS05 were co-transformed into *E. coli* S17-1 λ pir as the donor strain for conjugation.

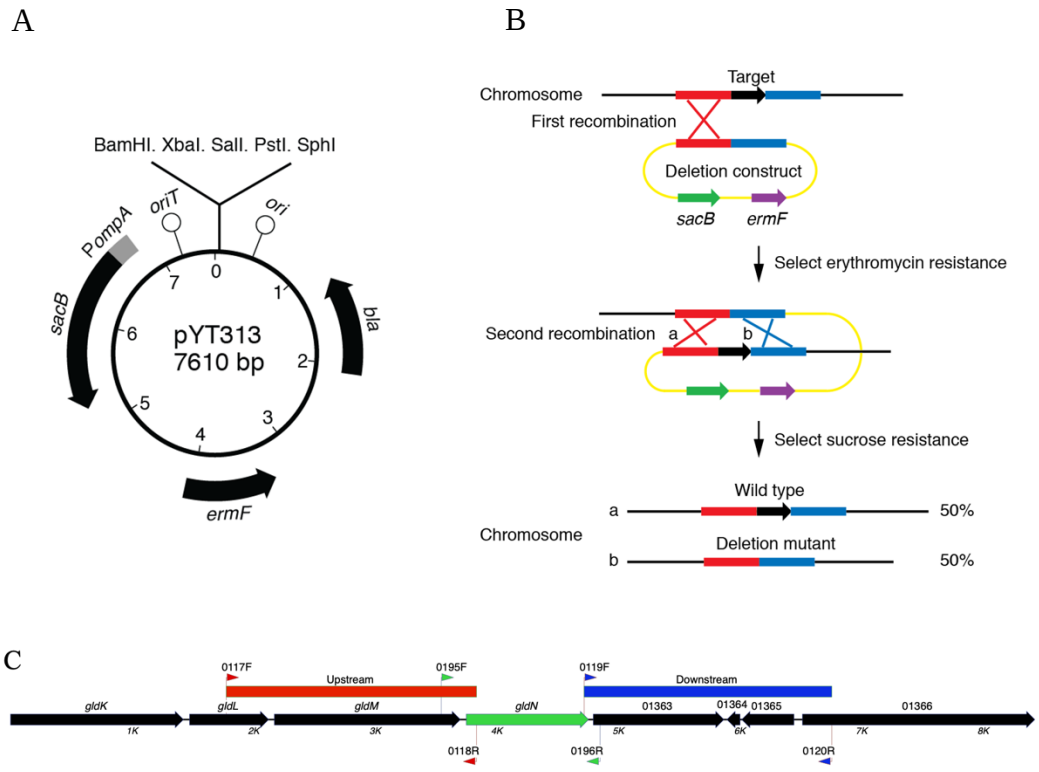



**Figure 7.** Strategy used to delete *gldN* in *F. psychrophilum* CSF259-93. (A) Map of the suicide vector used for constructing chromosomal gene deletions (64). Numbers immediately inside of the ring (pYT313) refer to kilobase pairs of sequence. *ori* refers to the origin of replication that functions in *E. coli*, but not in *F. psychrophilum*. *oriT* refers to the conjugative origin of transfer. *bla* confers ampicillin resistance on *E. coli*, but not on *F. psychrophilum*. *ermF* confers erythromycin resistance on *F. psychrophilum*, but not on *E. coli*. *sacB* confers sucrose sensitivity on *F. psychrophilum*. (B) Illustration of the *sacB*-mediated deletion system. Regions shown in red and blue in panel B are those that lie upstream and downstream of the target gene. Yellow, purple and green denote regions of the plasmid pYT313. (C) Map of the regions containing *gldN* on the *F. psychrophilum*

CSF259-93 genome. Numbers below the map refer to kilobase pairs of sequence. Binding sites for primers used in PCRs to generate deletion construct or to complement the final deletion mutants are shown above and below the maps, with the blunt ends indicating the actual binding sites. The region between the upstream and downstream fragments is deleted in the mutant.

The first recombination allowed conjugated *F. psychrophilum* cells containing pSS12 plasmid with *ermF* gene to grow on TYES erythromycin plates. The resulting colonies were streaked on fresh TYES erythromycin plates for pure culture isolation. Cells containing the plasmid that was integrated into the chromosome in the upstream or downstream region was confirmed via Taq PCR screening for *ermF* presence and gel electrophoresis. Following this step cells were grown in TYES broth without erythromycin for 24 hours, allowing the second recombination to occur.

The second recombination occurs either in the upstream or the downstream region on pSS12, and the homologous regions on the chromosome. Suppose the first recombination occurs in the upstream region (red box in Figure 7B), if the second recombination also occurs in the upstream region the gene will not be deleted (the cells remain wildtype), but if the recombination occurs in the downstream region (blue box in Figure 7B) the cells will lose the target gene. The second recombination may not occur in some cells, resulting in a portion of cells that still have the plasmid and *sacB* on the chromosome. These cells will be killed by plating on TYES agar with 2.5 % sucrose. The *sacB* gene, originally found in *Bacillus subtilis*, encodes a periplasmic levansucrase that

catalyzes the hydrolysis of sucrose and synthesis of high molecular weight levans (68). Growth of the *sacB*-containing cells on sucrose will activate the synthesis of levans and it will be lethal (69) to these cells. Only cells that have gone through the second recombination and lost the plasmid will be able to grow on sucrose. Taq colony PCR was used to confirm the absence of the target gene resulting in smaller PCR product compared to the wildtype. Primers used for the confirmation were: YZ0195 (5'-GCTAGGGTACCTAGTTGTTTCTGGAAGCAAA-3' engineered with KpnI site) and YZ0196 (5'- GCTAGGCATGCCGGAGCCAATAATTAGGTAG-3' engineered with SphI site) (Figure 7C). The same primers were used to complement the *gldN* mutant described below.

**Complementation**

After each deletion mutant was constructed, a good copy of the gene that had been deleted was inserted into a shuttle plasmid and introduced into the mutants to verify if the complemented strains will return to pre-deletion properties. This process is called complementation. The shuttle vector pCP11 was used for the complementation of the *gldN* deletion mutant. Primers YZ1095 and YZ1096 were utilized to clone *gldN* and a putative promoter region into KpnI and SphI sites on pCP11, generating pSS13, which was then utilized for co-transformation with pSS05 and conjugation into the *F. psychrophilum* CSF259-93 *gldN* deletion mutant, generating the complemented strain.

**Colony Spreading**

*F. psychrophilum* CSF259-93 wildtype, *ΔgldN*, and complement strains were streaked on 5% TYES agar. 5% TYES is 100 % TYES that has been diluted 20-fold and was then solidified with 10 g/l agar (43). The plates were subsequently incubated at 18°C for up to 8 days. Colony spreading was observed and documented using a Moment CMOS camera (Teledyne Photometrics, Tuscon, AZ) mounted on a Nikon Eclipse TS100 inverted microscope.

**Individual Cell Motility Analysis**

Motility of individual cells was analyzed by spotting 5 µl of fresh cells (grown for 24 hours at 18°C with shaking) on 1% TYES agar pad on a glass slide and covering the slide with $O_2$ permeable Teflon membrane (Yellow Springs Instrument Co., Yellow Springs, OH) (43). Utilizing an Olympus CX41 phase-contrast microscope, the Moment CMOS camera, and Ocular, an image acquisition software, numbers of images of cell movements were captured in a form of a movie. The software captured 175 frames in 30 seconds. The cell movements were processed with color-coded schematic representation. The images captured were transferred to Fiji (Image J) to be processed as "rainbow traces" and converted to one image with color-coded cell movements, described above (43) (Figure 13). White cell color indicated little to no movement, red represented initial movement of the cell and yellow, green, cyan, and blue indicated subsequent cell movements until time elapsed.

**Analysis of Proteolytic Activity - Spot Plate Assay**

Cell cultures of *F. psychrophilum* CSF259-93 wildtype, *ΔgldN*, and complement strains were grown at 18 ºC for 24 hours and adjusted to OD600 0.5. Three µl of the supernatant was spotted on TYES supplemented with 1.5% of casein (C-3400, Sigma) or skim milk (Thermo Fisher). To prepare the media, the 2X TYES and 3% casein or skim milk stock solutions were autoclaved separately and then combined. Proteolytic activity was observed on the plates with agar clearing surrounding the area of growth. The plates were incubated at 18 ºC for 5-10 days and pictures were obtained using a Bio-Rad imaging system under visible light.

**Rainbow Trout Genetic Lines, Rearing Conditions, and Water Quality Parameters**

Rainbow trout were obtained as eyed-eggs (Troutlodge Inc, WA) and reared under standard feed conditions with 13ºC flow-through spring water with dissolved oxygen at or above 12 mg/L. The genetic stock of fish in Exp 1, were from the Troutlodge February spawning line, and fish had a mean body weight (bwt) of 2.7 g at the time of challenge (age 72 days post-hatch). In Exp 2, Troutlodge May spawning line was used and fish were 14.2 g bwt at the time of challenge (age 127 days post hatch). Animal husbandry procedures and challenge experiments were approved by the NCCCWA institutional animal care and use committee (NCCCWA IACUC protocol 192).

**Standardized Laboratory Challenge**

*F. psychrophilum* strains were grown and prepared as described (70). Briefly, a dilution of a -80 °C frozen stock was plated on TYES plates at 15 °C for five days, and the harvested bacteria re-suspended in Dulbecco's PBS (Sigma). Cell concentration was measured by optical density of the suspension at 525 nm and challenge dose and volume were adjusted for body weight. Bacterial cell number was verified by direct plate counting of triplicate cultures. Fish were challenged by intramuscular injection in the mid-point between the start of the dorsal fish and lateral line using a 26 g needle attached to an Eppendorf repeating syringe. Triplicate tanks were used for all treatments. In Exp 1, each fish was injected with 20 µL bacterial suspension while in Exp 2, each fish was injected with 50 µL bacterial suspension. Control fish were injected in the same location with the corresponding volume of sterile PBS per experiment. Moribund or dead fish were removed daily between the times of 7:30 and 9:30 am. Fish were fed twice daily prior to challenge and not fed the day of challenge. Fish were fed to satiation once per day during the challenge.

**Bioinformatic Analyses**

REBASE (http://rebase.neb.com/rebase/rebase.html) was used to analyze the R-M systems present in *F. psychrophilum* CSF259-93 (71). Genome and gene sequences of *F. psychrophilum* CSF259-93 were obtained from IMG (https://img.jgi.doe.gov/). All primers, plasmid maps, and gene maps were generated using MacVector (Version 18.2.5).

# Chapter 4: Results

## Multiple R-M systems exist in the genome of *F. psychrophilum* CSF259-93

A couple of sources on *F. psychrophilum* have discussed the possibility of restriction barriers being some of the main culprits in successful genetic manipulations (2, 21, 24). *F. psychrophilum* strains are thought to destroy foreign DNA that is unmodified (commonly unmethylated) at sites that are a target for restriction enzymes produced by *F. psychrophilum.*

However, none of these studies have directly explored the function of the *F. psychrophilum* R-M systems. A recent study on R-M systems was conducted in the cyanobacterium, *Synechocystis* sp. strain 6803 (72). The researchers identified two type II methyltransferase-encoding genes. Introduction of one of these, *slr0214,* into a vector resulted in 11- to 161-fold increase in transformation efficiency (72). A similar strategy was used in this study where we identified several R-M systems in *F. psychrophilum* strain CSF259-93.

The genome of *F. psychrophilum* strain CSF259-93 was analyzed on REBASE (http://rebase.neb.com/rebase/rebase.html) and both type II and type III R-M systems were identified (Table 4). Among these R-M systems, HpaII methyltransferase and ScrFI methyltransferase were predicted to be important for protection of pCP11 because there are 23 HpaII sites and 22 ScrFI sites on the plasmid.

**Table 4.** R-M systems in *F. psychrophilum* CSF259-93 identified on REBASE.

| **Modification Enzyme** | **Type of R-M** | **Recognition Sequence** | **Restriction Enzyme** | **Number of Sites on pCP11** | **Helper Plasmid Constructed** |
|---|---|---|---|---|---|
| Fps93ORF2394P (HpaIIM) | II | CCGG | HpaII | 23 | pSS01 |
| Fps93ORF612P (ScrFIM) | II | CCNGG | ScrF1 | 22 | pSS02 |
| M.Fps93ORF1581P (MboIM) | II | GATC | MboI | 17 | pSS03 |
| Fps93IP (M.FpsJIIM) | III | CGCAG | M.FpsJII | 8 | pSS07 |
| Fps93ORF649P (TauIM) | II | GCSGC S = G/C | TauI | 19 | pSS08 |
| Fps93ORF1246P (AcuIM) | II | CTGAAG | Eco57I/AcuI | 3 | |
| Fps93ORF1519P (BstUIM) | II | CGCG | FnuDII /BstUI | 14 | pSS11 |
| Fps93ORF572P (Jma19592IM) | IIG | GTATNAC | Jma19592I | 0 | |
| Fps93ORF273P | IIG or Type I | Unknown | Unknown | Unknown | |
| Fps93ORF28P | II | Unknown | Unknown | Unknown | |
| M.Fps93ORF646P | II | Unknown | Unknown | Unknown | |

**Construction of pre-methylation helper plasmids**

Six helper plasmids carrying individual methyltransferases (pSS01/HpaIIM, pSS02/ScrFIM, pSS03/MboIM, pSS07/ M.FpsJIIM, pSS08/TauIM, and pSS11/BstUIM) and one plasmid carrying two methyltransferases (pSS05/both HpaIIM and ScrFIM) (Table 3) were constructed. All the regions with inserted MTase encoding genes were confirmed by sequencing. The maps of pSS01 and pSS05 are shown below as examples (Figure 8).

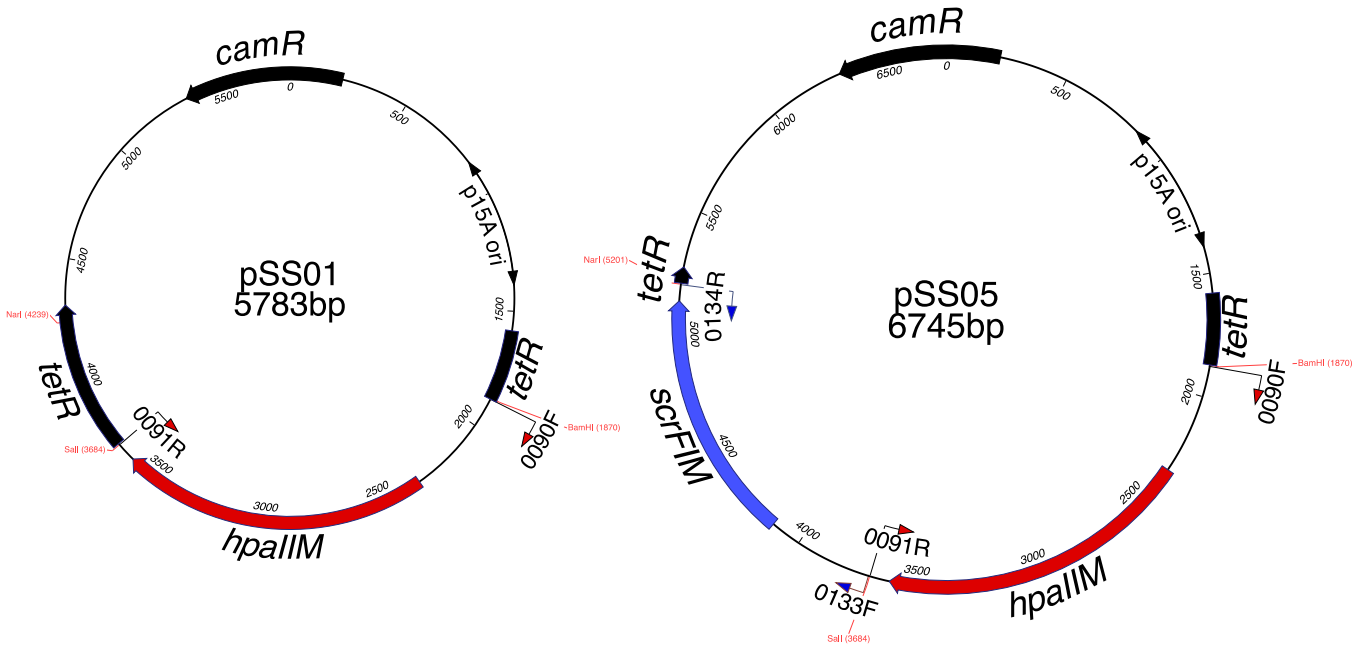



**Figure 8.** Maps of pSS01 and pSS05. Numbers immediately inside of the ring refer to base pairs of sequence. *ori* refers to the origin of replication that functions in *E. coli*, but not in *F. psychrophilum*. *camR* confers chloramphenicol resistance on *E. coli*, but not on *F. psychrophilum*. *tetR* confers tetracycline resistance on *E. coli* but it is disrupted by the inserted MTase genes and is non-functional. Binding sites for primers used in PCRs to

clone the MTase genes are shown by the arrows inside and outside of the rings, with the blunt ends indicating the actual binding sites.

**Confirmation of the presence of plasmids in *E. coli* S17-1 λ pir**

After the molecular cloning of target methyltransferase genes and co-transformation, the presence of plasmids in their respective *E. coli* strains was confirmed (Figure 9). For example, in Figure 9 the DNA gel electrophoresis imaging information indicates a presence of a plasmid at ~5,000 base pairs in size in lane 2. This indicates the presence of pSS01. Co-transformed pSS01 and pCP11 plasmids were also evident in lane 3. Similar information is visible in the imagining of other helper plasmids (Figure 9).

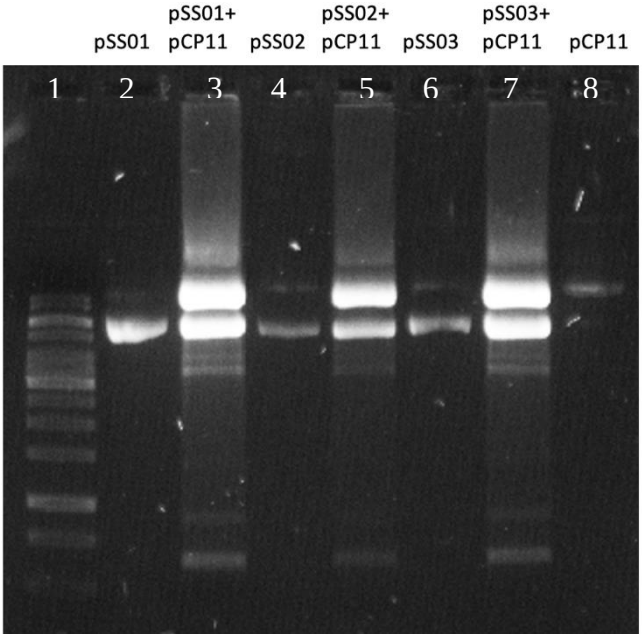



**Figure 9.** Confirmation of co-presence of pSS01, pSS02, and pSS03 with pCP11 in *E. coli* S17-1 λ pir by DNA agarose gel electrophoresis.

**Digestion of unmethylated and pre-methylated pCP11**

Once the presence of plasmids was confirmed additional experiments were conducted. For example, we examined if pCP11 present in a cell that also had pSS01, pSS02, and pSS03, was protected from restriction enzymes (Figure 10). Lane 1 was the gene ruler and lane 2, 4 and 6 were all isolated from *E. coli* with a presumably unmethylated plasmids. Lanes 3, 5 and 7 were isolated from *E. coli* with presumably methylated HpaII, ScrFI and MboI sites, respectively. Plasmids were isolated by gel extraction to separate them from the co-existing pCP11. Lanes 2 and 4 have visible fragments of DNA when exposed to HpaII and ScrFI, respectively. In lane 6 there is only one DNA fragment (indicated by one DNA band). We later noticed that the MboI site is blocked by dam methylation in our *E. coli* strain no matter if the helper plasmid is present or not and that is why there is no fragmentation of DNA in both lane 6 and lane 7.

However, it is interesting to note that in lanes 3 and 5 that were thought to be methylated by HpaII MTase and ScrFI MTase there are still multiple bands visible. If pCP11 was efficiently methylated, we would not expect to see fragmentation of the plasmid after restriction digestion. This indicates that methylation and protection of pCP11 in *E. coli* by the action of helper plasmids may not be efficient enough to be noticed by the *in vitro* experiments. Duration of growth of *E. coli* strains, stage of growth, expression level of the enzymes, and "actions" of MTases *in vivo* may be some of the reasons for the presence of multiple bands on presumably methylated plasmids. More efficient methylation may occur during the conjugation process. Indeed, successful DNA transfer results were observed in the conjugation experiments described below.

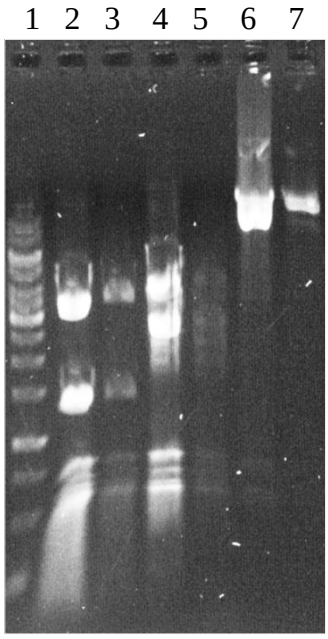



**Figure 10.** Digestion of pCP11 using restriction endonucleases that cut unmethylated DNA sites. 1: DNA ladder. 2, 3: pCP11 digested by HpaII. 4, 5: pCP11 digested by ScrF1. 6, 7: pCP11 digested by MboI. pCP11 used in 2, 4, and 6 was isolated from *E. coli* without pSS plasmids. pCP11 used in 3, 5, and 7 was isolated from *E. coli* carrying pSS01, pSS02, and pSS03, respectively.

**Conjugation Results**

Table 5 represents average CFU counts after plating the cells on erythromycin plates post conjugation. The data indicates that pCP11 co-transformed with helper plasmids containing the methyltransferase genes *hpaIIM* or/and *scrFIM* results in successful DNA transfer via conjugation.

**Table 5.** Conjugative transfer of pre-methylated pCP11 and pSS12 into *F. psychrophilum* CSF259-93.

| **Conjugation Plasmids** | **Site of Pre-Methylation** | **CFU Count per Erythromycin Plate (Trial 1)** | **CFU Count per Erythromycin Plate (Trial 2)** |
|---|---|---|---|
| pCP11 | N/A | 0 | 0 |
| pCP11+pSS01 | HpaII | 6 | 184 |
| pCP11+pSS02 | ScrFI | 2 | 1 |
| pCP11+pSS03 | MboI | 0 | 0 |
| pCP11+pSS05 | HpaII/ ScrFI | 666 | 353 |
| pCP11+pSS07 | M.FpsJII | 0 | 0 |
| pCP11+pSS08 | TauI | 0 | 0 |
| pCP11+pSS11 | BstUI | 0 | 0 |
| pSS12+pSS01 | HpaII | 0 | ND |
| pSS12+pSS02 | ScrFI | 0 | ND |
| pSS12+pSS05 | HpaII/ ScrFI | 6 | 10 |

As indicated in Table 5, the conjugation was unsuccessful when pCP11 without a helper plasmid was used (as expected). Among the six helper plasmids carrying individual MTases, only pSS01 carrying HpaII MTase and pSS02 carrying ScrFI MTase protected pCP11, resulting erythromycin resistant colonies after conjugation. This indicates pre-methylation of HpaII or ScrFI sites individually prevented digestion of pCP11 to a certain

extent, and thus the DNA transfer was successful although the numbers of erythromycin resistant colonies were low. When pSS05 containing both HpaII and ScrFI MTases was utilized during conjugation with pCP11, there was a substantial increase in the number of CFUs/ per TYES + erythromycin plate, indicating it is essential to methylate both HpaII and ScrFI sites on pCP11 for high efficiencies of DNA transfer. pSS05 also protected the transfer of pSS12 into *F. psychrophilum* CSF259-93, making deletion of *gldN* possible. Pre-methylation of other MTases individually did not result in successful DNA transfer. It is possible that the highest protection can be achieved by cloning all the MTases onto one helper plasmid, which can be done in the future.

**Construction of the *gldN* mutant in *F. psychrophilum* CSF259-93**

Due to such a great success utilizing pSS05 in conjugation, the plasmid was tested in methylation of the *gldN* deletion construct pSS12 and the deletion was successful. A colony PCR was done to screen for the *gldN* deletion mutants from 11 colonies grown on sucrose plates after the second recombination (Figure 11). The PCR results showed five of the 11 colonies were deletion mutants and six of them were wild type.

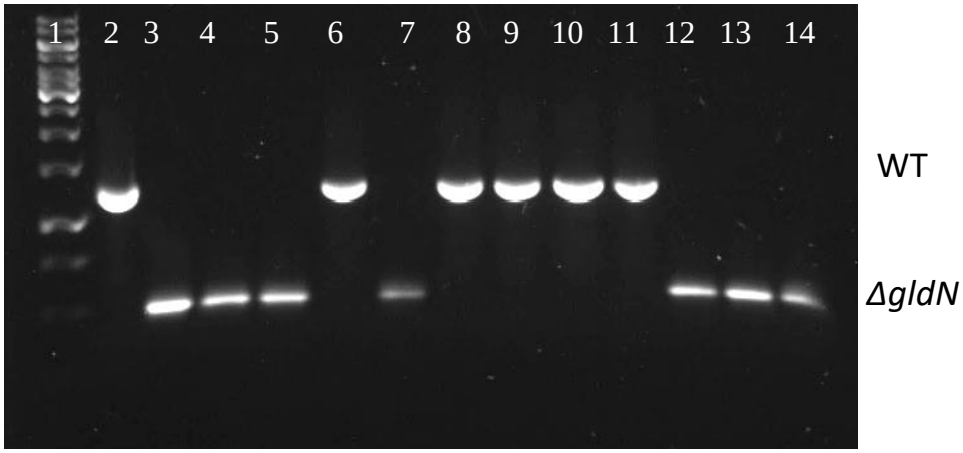



**Figure 11.** PCR confirmation of *gldN* deletion in CSF259-93 using primers YZ0195 and YZ0196. 1, DNA ladder; 2, WT control; 3, pSS12 control; 4-14, colonies growing on sucrose plates. Colonies in lanes 6, 8, 9, 10, and 11 are wild type and colonies in lanes 4, 5, 7, 12, 13, and 14 are deletion mutants.

Based on the results the use of methyltransferase containing pSS helper plasmids confers protection against the restriction enzymes present in *F. psychrophilum* CSF259-93, and pre-methylation of foreign DNA prior to conjugation improves conjugation efficiency. pSS05 was also used to assist creating a few other gene deletion mutants in our laboratory, indicating it can be generally used in manipulation of *F. psychrophilum* CSF259-93. The development of a genetic manipulation system involving pre-methylation of foreign DNA is successful.

**pSS05 protects DNA transfer in other *F. psychrophilum* strains**

Genetic manipulation in most of the *F. psychrophilum* strains has not been

successful. The restriction barrier can be also the issue in other strains. To demonstrate that, we also tested the protection of pCP11 by pSS05 during conjugation in *F. psychrophilum* strains CSF201-91, CSF408-92, CSF060-99, and CSF016-90.

The results showed that pCP11 transfer was successful in all the tested strains only when it was pre-methylated by pSS05 (Table 6). It is likely pSS05 can be used in more strains that have similar R-M systems.

**Table 6.** Conjugative transfer of pre-methylated pCP11 into different *F. psychrophilum* strains.

| ***Flavobacterium psychrophilum* Strains** | **Plasmids** | **Plasmid Transfer Successful Yes or No** |
|---|---|---|
| All | pCP11 | No |
| CSF 259-93 | pSS05 + pCP11 | Yes |
| CSF 201-91 | pSS05 + pCP11 | Yes |
| CSF 408-92 | pSS05 + pCP11 | Yes |
| CSF 060-99 | pSS05 + pCP11 | Yes |
| CSF 016-00 | pSS05 + pCP11 | Yes |

### *gldN* mutant is deficient in colony spreading and gliding motility

Similar to the findings in a 2020 study on *F. psychrophilum* strain THCO2-90 (43), we found that the *gldN* deletion mutant lacks spreading on TYES agar surface (Figure 12). The spreading is restored upon complementation by re-introducing the deleted component

(*gldN*) back to the cells lacking this gene. Similarly, it was shown that the cells of *gldN* mutant lacks gliding motility (Figure 13). The WT and complemented strain have an identical phenotype (indicating motility).

This type of motility is an identifying marker of many bacteria in family *Flavobacteriacea*e of the Bacteroidetes (2) and in case of *F. psychrophilum* it is thought to be important in pathogenicity (37) and this may lead to spread of the pathogen and invasion of the host.

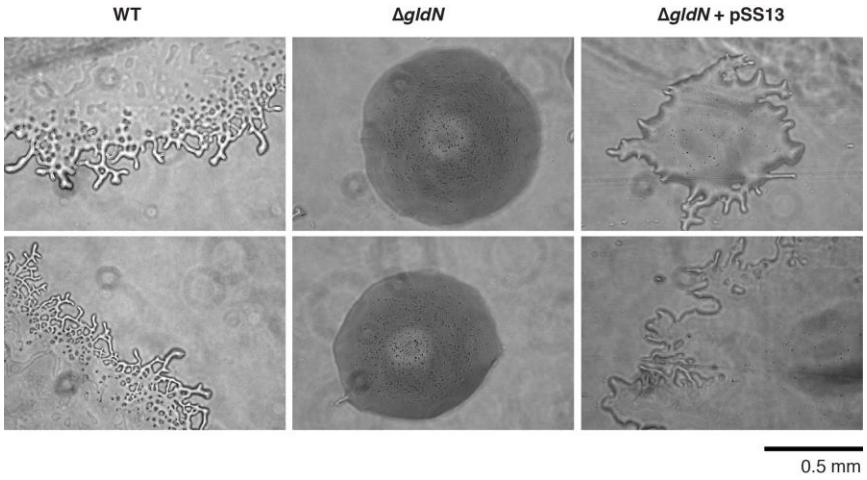



**Figure 12.** Colony spreading of *F. psychrophilum* CSF 259-93 on 5% TYES agar. WT: wild type, *⊿gldN*: *gldN* deletion mutant, *⊿gldN* + pSS13: complemented strain with restored phenotype.

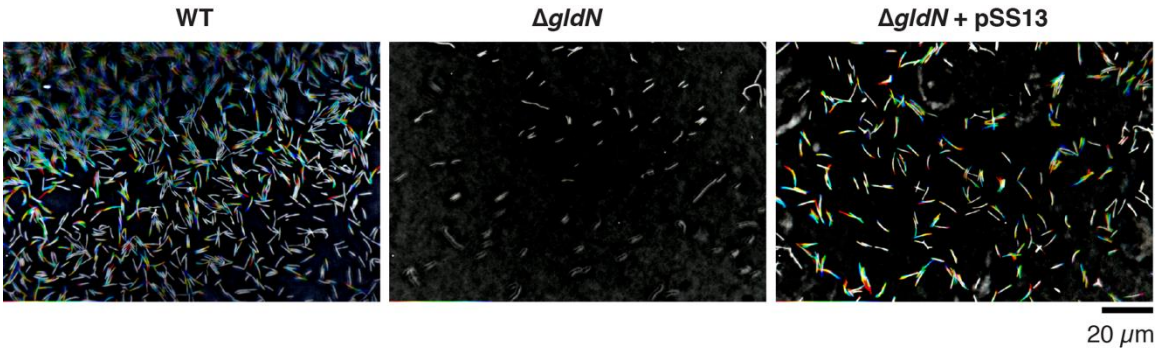



**Figure 13.** The images above represent individual cell motility on agar surface. A series of images were taken for 30 s. Individual frames were colored from red (time 0) to yellow, green, cyan, and finally blue (30 s) and integrated into one image, resulting in "rainbow traces" of gliding cells. The white color indicated no movement.

### *gldN* mutant is deficient in proteolytic activities

Proteolytic activity of WT, *gldN* mutant**,** and the complemented strain is shown in Figure 14. The *gldN* mutant was deficient in the ability to digest casein and skim milk. This indicates that *gldN* may play an important role in secretion of proteases in *F. psychrophilum* strain CSF 259-93. Similar results were observed in another strain of the pathogen, THCO2-90 (43).

Proteolytic activity of *gldN* can often be considered as one of the markers of virulence. The proteolysis of host tissue is often a mode of invasion of the whole organism utilized by many pathogens and *F. psychrophilum* is likely utilizing this system. Therefore, if *gldN* is deleted the proteolytic activity and virulence may decrease.

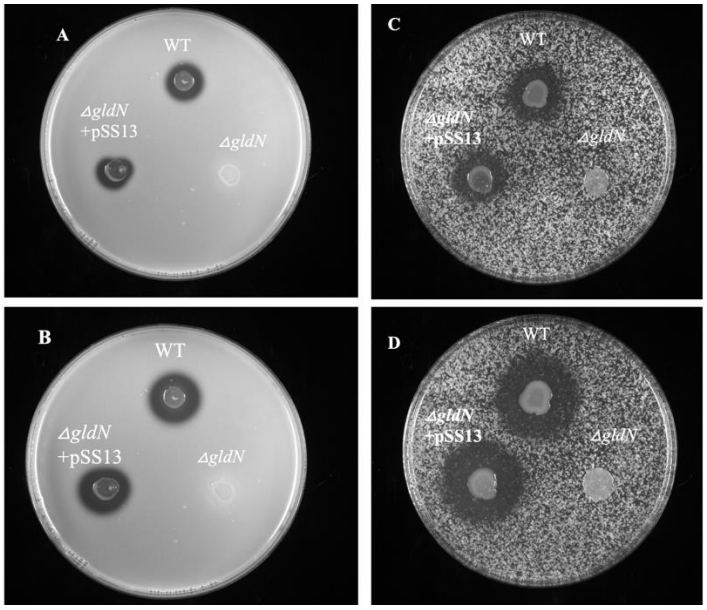
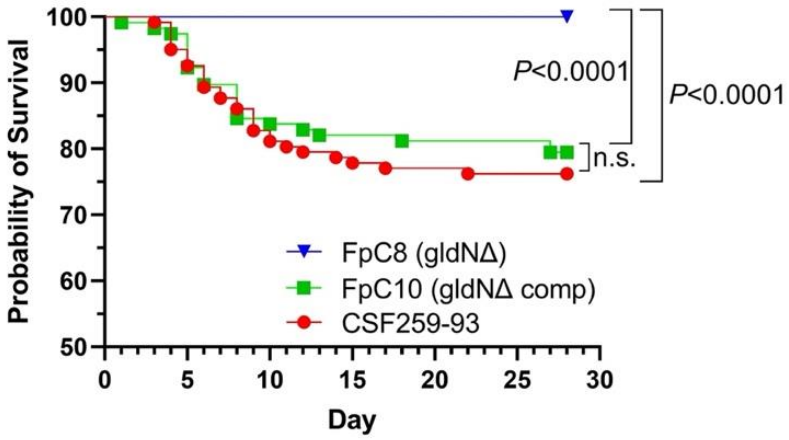



**Figure 14.** Proteolytic activity of WT, *gldN* mutant, and the complemented strain on TYES + 1.5% skim milk after 5 and 7 days, A and B respectively. Proteolytic activity of WT and complemented strain on TYES + 1.5% casein after 7 and 12 days respectively, C and D.

**Rainbow Trout (*O. mykiss*) Challenge Study**

Figure 15 represents data supplied by the collaborator on this project, Gregory D. Wiens at National Center for Cool and Cold Water Aquaculture USDA-Agricultural Research Service. The preliminary data indicates that the final percent mortality of rainbow trout exposed to the *gldN* deletion mutant is 0% (Exp 1) or 3.3% (Exp 2) when two different genetic stocks of rainbow trout were tested. The mortality rates for WT and complemented strain are 23.7% and 20.5% for Exp 1, 78.3% and 86.7% for Exp 2, respectively. Consistent with the previous report, the *gldN* mutant has nearly lost the ability to cause disease in fish (43).

Exp 1




Exp 2

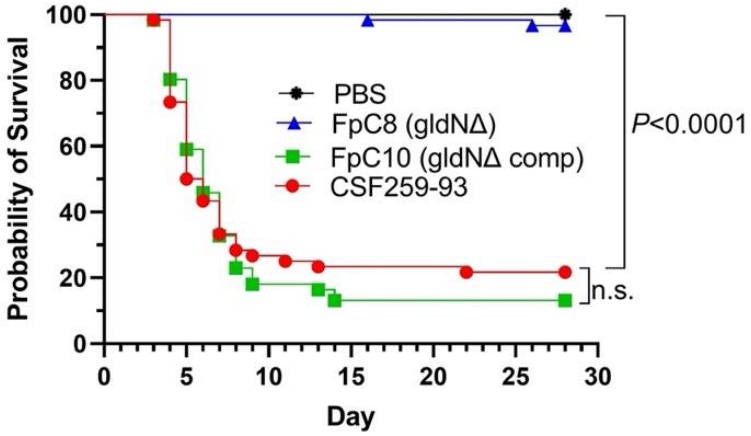



**Figure 15.** The percent survival of rainbow trout after 30 days. The fish were injected (per protocol) with PBS, *gldN* mutant (FpC8)**,** the complemented strain (FpC10), and WT (CSF259-93) and percent survival was observed and measured.

# Chapter 5: Discussion and Conclusions

*F. psychrophilum* is a known fish pathogen and a causative agent of BCWD. This pathogen has a daily impact on fisheries across the globe. Currently, there are studies on *F. psychrophilum* being conducted in the United States, France, Finland, as well as in China and Japan. The magnitude of the issue is enormous not only because of the global impact on the fisheries, but also because of overuse of antibiotics and lack of available vaccines.

Due to an earlier development of pCP11 (shuttle vector) and pYT313 (deletion vector) plasmids that can be utilized in family *Flavobacteriaceae*, genetic manipulation in *F. psychrophilum* THCO2-90 strain and other members was successful in recent years (43). However, many of the strains of *F. psychrophilum* have restriction enzymes that destroy foreign DNA and plasmids required for genetic manipulation such as gene deletions. In order to develop a viable genetic manipulation system, we used the strategy to pre-methylate the foreign DNA for protection during DNA transfer.

## Significance of R-M Systems in the Development of the Technique

We accessed REBASE (http://rebase.neb.com/rebase/rebase.html) where we found information regarding the presence and types of R-M systems in *F. psychrophilum* CSF259-93. We chose several methyltransferase (MTase) genes that we used to develop helper plasmids (Table 3) based on the number of their corresponding restriction sites on pCP11. All of the MTase genes inserted into the helper plasmids (based on pACYC184) encode either type II or III methyltransferases. There are still many unknowns when it comes to the expression level of these *F. psychrophilum* MTases and their activities in *E.*

*coli*. However, the conjugation experiments were successful when certain helper plasmids were used.

When restriction enzymes were utilized *in vitro* to cleave the presumably methylated pCP11 by helper plasmids we first needed to gel extract the plasmids and separate them from the helper plasmids (Figures 9 and 10). Following this we used respective REases for each of the presumably protected pCP11 and found that cleavage still occurred on some of the sites as multiple bands were seen on the gel electrophoresis (Figure 10) for pSS01 and pSS02. This led to a conclusion that perhaps *in vivo* mechanisms of action are different than *in vitro*. Specifically, we think that it is possible that even partial pre-methylation can protect and allow for acceptance of foreign DNA during conjugative gene transfer.

It is worth to mention that *F. psychrophilum* favors lower temperatures for their growth and their enzymes including the MTases probably are more active at lower temperatures as well. *E. coli* was grown at 37$^{o}$C in the above experiment. The high growth temperature may inhibit the activity of the MTases, making them inefficient in protecting pCP11 from restriction digestion *in vitro*. During the conjugation process, the donor *E. coli* cells are incubated at lower temperatures (18-20$^{o}$C) mixed with *F. psychrophilum* for 2 days. More efficient methylation of pCP11 may occur during this period of time.

We are not sure why the other tested MTases did not protect pCP11 individually during conjugation. Cloning of a combination of different MTases on the helper plasmids may be required for more efficient protection. It is also possible that these MTase genes are never expressed or are not expressed enough in *E. coli*. qPCR and other biochemical

analyses can be used to determine the expression levels of MTases in *E. coli*. It is also possible that some of the F. psych restriction enzymes are not expressed or are expressed at low levels. It may not be necessary to modify sites for which the restriction enzymes are not expressed or are less active.

This is the first study to explore the R-M systems to aid in a development of a genetic manipulation technique in *F. psychrophilum*. The developed system was successfully applied to delete the T9SS core gene *gldN* and a few other genes.

**The Role of Colony Spreading and Single Cell Motility in Pathogenesis**

A group of researchers in a recent study on *F. psychrophilum* strains analyzed genetic predictors for pathogenicity and virulence 5 (73). They analyzed dozens of isolates for characteristics such as colony spreading, elastinase activity, caseinase activity, and cell motility. They measured these characteristics for 26 isolates, 25 virulent and one non-virulent. They found that the virulent isolates have a much better colony spreading capacity $10.3 \pm 0.7$ mm to $45 \pm 2.1$mm, while the non-virulent isolate exhibited colony spreading at $7.7 \pm 0.3$ mm (73). Similar outcomes were explored in a recent study conducted on THCO2-90 isolate and virulent wildtype strain was motile while the non-motile mutant was not (43).

This study also discovered that motility is only present in the wildtype strain CSF259-93 when compared to the *gldN* deletion mutant. The deletion mutant did not exhibit colony spreading as presented on Figure 12 and the motility of single cells was also absent (Figure 13). This indicates that *gldN* has a crucial role in motility of the pathogen. As previously confirmed *gldN* is a core gene in the type IX secretion system and the

absence of this gene may impact secretion of motility adhesins as well as other potential virulence factors (74).

**Proteolytic Activity and Virulence**

Proteolytic activity of bacteria found in *Flavobacteriaceae* family has often been mentioned in the literature (2, 43, 47). Ability of pathogenic bacteria to metabolize a wide variety of proteins is an evolutionarily advantageous trait. A study on Fpp1 in *F. psychrophilum* uncovered that this particular protein exhibits characteristics of a metalloprotease found in eukaryotic organisms. Fpp1 is involved in hydrolysis of actin and myosin in fish and according to the article this enzyme may be involved in tissue invasion and pathogenicity (75).

This extensive breakdown of tissue can be exemplified in other experiments where specific proteins can be used. In another study a team of researchers found that only one *F. psychrophilum* isolate did not breakdown casein while other isolates had caseinase activity by forming clearing zones in ratios ranging from 2.5 to 4.2 (73). Similarly, in our study casein and skim milk were used to demonstrate that deletion mutant *⊿gldN* lacks the ability to break down proteins (Figure 14). This indicates that *⊿gldN* may not have the necessary characteristics to invade host tissue.

Similar results were obtained in recently published research on THCO2-90 strain of *F. psychrophilum* where the researchers demonstrated the importance of *gldN* in proteolytic as well as hemolytic activity (43). Hemolytic activity of *F. psychrophilum* is not often mentioned in the literature, but it is essential in understanding the comprehensiveness of virulence mechanisms present in this pathogen.

**Rainbow Trout Challenge**

Rainbow trout challenge study data indicates that *ΔgldN* causes very little to no mortality to the fish when applied via injection route. This was expected due to the fact that *gldN* is one of the essential components in type IX secretion system, which is required in secretion of potential virulence factors. On the other hand, the use of WT and complemented strain causes much lower survival, which was also expected (Figure 15).

**Conclusions**

The development of genetic manipulation techniques for the fish pathogen *F. psychrophilum* has been a tremendous challenge for many years. The pre-methylation method developed in this project can circumvent the R-M systems in several different strains of the pathogen and improve the DNA transfer efficiency significantly. The method can be used in combination with the *sacB* mediated deletion system to generate mutants in *F. psychrophilum* CSF259-93 and likely in other strains as well. This study lays a foundation for the development of live attenuated vaccines for prevention of BCWD and RTFS. This study also shed light on development of genetic manipulation techniques in other organisms where restriction barriers need to be overcome.

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