Flavobacterium 2007

May 2-4th, 2007
National Conservation Training Center
Shepherdstown, West Virginia

Program and Abstracts

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Flavobacterium 2007 Workshop,
National Conservation Training Center,
May 2-4th Shepherdstown, WV

Welcome to the first workshop dedicated to flavobacterial biology with specific emphasis on how these bacteria impact aquaculture. The genus *Flavobacterium* includes over 30 species of which *F. psychrophilum*, *F. columnare*, and *F. branchiophilum* are important disease agents for salmonids, catfish, and other cultured species. Flavobacteria are significant as they are ubiquitous in the soil, freshwater and marine environments and are noted for their novel gliding motility and ability to degrade polymeric organic matter. The motivation for this workshop was the desire to promote interactions between diverse groups of scientists and resource managers as well as to disseminate information arising from completed and ongoing genome sequencing projects. This meeting has sessions devoted to the following topics: an overview of the impact on aquaculture, genomics, metabolism and proteomics, pathogenesis, transmission and treatment, vaccines and immunity, systematics and diagnostics, graduate student presentations, nonpathogenic Flavobacteria, and future research directions. In addition, there will be a poster session and a tour of the National Center for Cool and Cold Water Aquaculture. We are pleased at this large gathering of scientists, fish health practitioners, and resource managers from academic, state and federal governments, aquaculture, biotech, and pharmaceutical fields. Approximately twenty-five percent of the participants are international and we especially welcome their important contribution to this meeting.

We look forward to a valuable exchange of scientific ideas. We hope that this meeting will be the springboard for future collaborations and coordinated progress toward reducing the impact of these diseases.

The Flavobacterium 2007 Steering Committee

Doug Call
Dave Hunnicutt
Scott LaPatra
Tim Welch
Greg Wiens

We specifically thank the following people for their help putting together this conference: Annette Martin, Kathy Root, Tim Ashton, Clayton Birkett, Nate Johnson, Jason Prochazka, Jen Harper, Katie Hovatter, Scott Gahr, Mark Hostuttler, Yniv Palti, Greg Weber, Jeff Silverstein, Caird Rexroad and Bill Hershberger.

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WEDNESDAY, May 2, 2007 8:20am – 5:10pm

7:30-8:20 **Registration**

8:20-8:30 **Introduction/Welcome** Bill Hershberger, Director NCCCWA

Session I – **Overview of the Impact and Challenges to Aquaculture**. Moderator: *Greg Wiens*

8:30-8:55 *Inger Dalsgaard* – The Impact of CWD/RTFS on Rainbow Trout Farming in Denmark.

8:55-9:20 *Rich Holt – Flavobacterium psychrophilum* Infections in Oregon Fish.

9:20-9:45 *Scott LaPatra – Flavobacterium psychrophilum* and Rainbow Trout: An Industry Perspective.

9:45-10:10 *Mark Lawrence – Flavobacterium columnaris* and its Impact on the Catfish Industry.

10:10-10:30 **Break**

Session II – **Genome Sequencing and Analysis. –** Moderator: *Kenneth Cain*

10:30-11:00 *Eric Duchaud* – Complete Genome Sequence of *Flavobacterium psychrophilum.*

11:00-11:30 *Greg Wiens* – Genome Sequence of *Flavobacterium psychrophilum* strain CSF 259-93 and Characterization of a Large Cluster of Genes Containing Short Repeats.

11:30-12:00 *Mark Lawrence* – The *Flavobacterium columnare* Genome Sequencing Project.

Lunch –NCTC Commons Room

Session III – **Metabolism and Proteomics.** Moderator: *Tom Wiklund*

1:30-1:55 *Anamitra Bhattacharyya – Flavobacterium psychrophilum*: from genome sequencing and metabolic profiling to comparative genomics.

1:55-2:20 *Devendra Shah* – Effects of codon usage bias on recombinant expression of *Flavobacterium psychrophilum* proteins in *E. coli.*

2:20-2:45 *Alison Morgan* – Western blot analysis reveals four distinct antigenic profiles for *Flavobacterium psychrophilum*.

2:45-3:10 *Kenneth Cain* – Electrophoretic and Western Blot Analysis of the Lipopolysaccharide and Glycocalyx of *Flavobacterium psychrophilum*.

3:10-3:30 **Break**

Session IV **Pathogenesis.** Moderator: *Tim Welch*

3:30-3:55 *José A. Guijarro* – A Mutant in the ExbD Locus of a TonB System in *Flavobacterium psychrophilum* Shows Attenuated Virulence and Confers A High Level of Protection Against Cold Water Disease**.**

3:55-4:20 *Patricia Noguera* – Investigations into "Red Mark Syndrome" (RMS) in Scotland.

4:20-4:45 *Andrew Staroscik* – The Influence of Culture Conditions on Biofilm Formation in *Flavobacterium columnare*.

4:45-5:10 *Cova Arias* – Intraspecies characterization of *Flavobacterium columnare*.

6:00-9:00 **Poster Session and Reception**

POSTERS

DEVELOPMENT OF A DISEASE MODEL TO PRODUCE EXTERNAL COLUMNARIS (*FLAVOBACTERIUM COLUMNARE*) INFECTIONS IN CHANNEL CATFISH Maren T. Tuttle, Mark P. Gaikowski, William H. Gingerich, Becky A. Lasee and Susan M. Schleis

SUSCEPTIBILITY OF ZEBRA FISH (*DANIO RERIO*) TO INFECTION BY *FLAVOBACTERIUM COLUMNARE* AND *FLAVOBACTERIUM JOHNSONIAE*. Thomas R. Moyer, Todd Eckroat, Erin Mathes, and David W. Hunnicutt

PROGRESS TOWARD AN IMMUNOGENETIC MAP FOR RAINBOW TROUT
M.R. Fincham, G.D. Wiens, C.E. Rexroad III, R.L. Vallejo, Y. Palti

GENETIC VARIATION OF *FLAVOBACTERIUM PSYCHROPHILUM* EXAMINED BY PULSE-FIELD GEL ELECTROPHORESIS John Cheng, Stacey A. LaFrentz, Margaret A. Davis, Scott E. LaPatra, Ken Cain, and Douglas R. Call

***FLAVOBACTERIUM PSYCHROPHILUM* IN NORWAY: PHENOTYPIC IDENTIFICATION AND GENETIC VARIATION**
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ASSOCIATIONS OF MARKERS LINKED TO MAJOR HISTOCOMPATIBILITY (MH) REGIONS WITH RESISTANCE TO *FLAVOBACTERIUM PSYCHROPHILUM* IN RAINBOW TROUT
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IDENTIFICATION OF CELL-SURFACE PROTEINS INVOLVED IN *FLAVOBACTERIUM JOHNSONIAE* GLIDING MOTILITY
Shawn S. Nelson, Soumya Pochiraju, Jun Liu, Sriram Subramaniam, Sreelekha Bollampolli and Mark J. McBride[1]

THURSDAY, May 3, 2007 8:30am – 4:30pm

Session V Transmission and Treatment. Moderator – *Jean-François Bernardet*

8:30-8:50 *Christopher Good* – A Prospective Case-Control Study of Bacterial Gill Disease Outbreaks in Ontario, Canada Government Salmonid Hatcheries.

8:50-9:10 *Norihisa Oseko* – Distribution of *Flavobacterium psychrophilum* in Wild Adult Salmonids Returning to Rivers in Hokkaido, Northern Japan.

9:10-9:30 *Kenneth Cain* – A Quantitative Enzyme-Linked Immunosorbent Assay (ELISA) and Filtration-Based Fluorescent Antibody Test as Potential Tools for Screening *Flavobacterium psychrophilum* in Broodstock.

9:30-9:50 *Ahmed Darwish* – Minimum inhibitory concentration testing of *Flavobacterium columnare.*

9:50-10:10 *Tom Wiklund* – Effect of Biofilm Formation on Antimicrobial Resistance of *Flavobacterium psychrophilum*.

10:10-10:30 Break

Session VI Vaccines and Immunity. Moderator: *Scott LaPatra*

10:30-10:55 *Sima Hadidi* – Rainbow trout Innate Immunity against *Flavobacterium psychrophilum.*

10:55-11:10 *Elizabeth Crump* – Diagnostics and Vaccine Design for *Flavobacterium psychrophilum*.

11:10-11:35 *Craig Shoemaker* – A Modified Live Vaccine Against *Flavobacterium columnaris*.

11:35-12:00 *Oriol Sunyer* – Recombinant FspA, a Surface Antigen from *Flavobacterium psychrophilum*, Induces Protective Antibody Responses in Rainbow Trout.

12:00-1:30 **Lunch at NCTC**

Session VII - Systematics and Diagnostics. Moderator: *Doug Call*

1:30-1:55 *Jean-François Bernardet* – Flavobacterium: History of a Genus.

1:55-2:20 *Douglas Call – Flavobacterium psychrophilum* is composed of two distinct genetic lineages.

2:20-2:45 *Pierre Nicolas* – Clonal Complexes and Recombination in *Flavobacterium psychrophilum* as revealed by Multi-Locus Sequence Analysis.

2:45-3:10 *Victor Panangala* – Rapid Detection of *Flavobacterium columnare*, the Bacterium Causing Columnaris Disease in Fish.

3:10-3:30 **Break**

Session VIII Graduate Student Presentations. Moderator: *Devendra Shah*

3:30-3:45 *Eva Högfors* – Immunization of Rainbow Trout with a Low Molecular Fraction Protein Extracted from *Flavobacterium psychrophilum*.

3:45-4:00 *Shawn Nelson* – Cell-Surface Proteins Involved in *Flavobacterium johnsoniae* Gliding Motility.

4:30-5:15 **Tour of the National Center for Cool and Cold Water Aquaculture**

5:30-7:00 **Picnic Dinner at the NCCCWA**

FRIDAY, May 4, 2007

Session IX - Environmental Flavobacterium (non-pathogenic). Moderator: *Dave Hunnicutt*

8:30-9:10 *Mark McBride – Flavobacterium johnsoniae* Gliding Motility: Mechanisms and Mysteries.

9:10-9:35 *Shicheng Chen* – Unique transcriptional signals in ubiquitous Bacteroidetes

9:35-10:00 *Dave Hunnicutt* – Biofilm Formation by *Flavobacterium johnsoniae.*

10:00-10:30 **Break**

Session X - Future Research Directions. Moderator: *Greg Wiens*

a. Aquaculture Industry Perspective (*S. LaPatra*, Clear Springs Foods, Inc.)
b. Academic/Government Perspective (*K. Cain*, University of Idaho)
c. Pharmaceutical/Biotech Perspective (*J. Zinn*, Novartis; *E. Crump* Microtech; *P. Jordan*, Schering Plough)
d. International Perspective (*I. Dalsgaard*, Danish Institute for Fisheries Research)

12:00-1:00 **Lunch at NCTC or on own**

1:00 **End of Workshop!**

Presentation Abstracts

THE IMPACT OF CWD/RTFS ON RAINBOW TROUT FARMING IN DENMARK

Inger Dalsgaard *, Morten S. Bruun and Lone Madsen

**Danish Institute for Fisheries Research, Fish Disease Laboratory, Stigbøjlen 4, DK-1870 Frederiksberg C, Denmark. id@difres.dk*

Rainbow trout fry syndrome (RTFS) has since the mid 1980s caused serious losses in the production of rainbow trout (*Oncorhynchus mykiss*) fry in Denmark as well as other European countries. In Denmark it was estimated that RTFS in 1998 caused the death of 88 million fry yielding a financial loss of 18 million Dkr (approximately 2.5 mill Euro). Outbreaks usually occur at water temperature of 8-14°C with mortalities of 50-60%. Infected fry exhibit lethargy, loss of appetite, ascites and anaemia. In fingerlings and larger fish the disease is referred to as coldwater disease (CWD) and causes mortalities of 2-10%, mostly at water temperatures of 2-10°C. The affected fish show ulcers and may suffer from blindness. Both CDW and RTFS are caused by *Flavobacterium psychrophilum.*

No registered, commercial vaccines against infections caused by *F. psychrophilum* are available. Other possibilities for preventing RFTS have been demonstrated by results of several Danish projects. *F. psychrophilum* was found in ovarian fluid and milt indicating that broodstock may serve as a reservoir of the pathogen. Results showed that *F. psychrophilum* was present on the surface of eggs. Disinfection of fertilized eggs with an aqueous iodophore solution is commonly practised by fish farmers. Transmission of *F. psychrophilum* from wild fish has been studied and the pathogen was not found in the wild fish upstream a farm. The same study showed that the fish farm might have an impact on the wild fish downstream.

At present, RTFS can be controlled by antimicrobial agents. Florfenicol is the drug of choice. Varying resistance patterns in *F. psychrophilum* to the licensed drugs oxolinic acid and trimethoprim/sulfadiazine are seen and the use of these drugs cannot be recommended. Due to increasing problems with development of antibiotic resistance the need for research in alternative treatments is important. However, prevention of the disease by proper stock management and husbandry is important. Hatcheries based entirely on groundwater systems have been suggested as a possibility for eliminating *F. psychrophilum* or at least keeping the amount of this bacterium low. Investigations on the occurrence of the bacterium in groundwater recirculation systems contra surface water flow-through systems were done on a hatchery where outbreaks of RTFS often occurred. *F. psychrophilum* was isolated from broodstock in both water systems, and in the groundwater recirculation system the bacterium was isolated from fish that had been kept in the system for minimum four months. Fry also harboured *F. psychrophilum*, but in the groundwater recirculation system the bacterium was first isolated from the fry after they had been graded. *F. psychrophilum* was found in other sections of the hatchery at the time of grading. This suggests that ground water recirculation systems and good management procedures might be a possible method for hatcheries to avoid disease outbreaks with *F. psychrophilum*.

A similar trend was expected from an ongoing project combining increased production on rainbow trout farms and reduced environmental impact. Eight traditional flow-through farms have been redesigned to "model farms" based on recirculation technology. *F. psychrophilum* was isolated from fish on all eight farms mainly from gills and skin mucus, but also from ulcers and internal organs. The significance of the occurrence of *F. psychrophilum* not in combination with disease outbreak is unclear. There were, however, indications that outbreaks of CWD had been a problem during winter time. The results obtained in the monitoring period yielded an important basis for studies to further improve our knowledge of infections caused by *F. psychrophilum*.

FLAVOBACTERIUM PSYCHROPHILUM INFECTIONS IN OREGON FISH

R.A. Holt[1], C.R. Banner[2], R.J. Kaufman[2] and T. Amandi[2]

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2 Oregon Department of Fish and Wildlife, 220 Nash Hall, Oregon State University, Corvallis, OR 97331

Bacterial cold-water disease (BCWD) caused by *Flavobacterium psychrophilum* is considered one of the most prevalent and troublesome infections of hatchery salmonids in the Pacific Northwest, U.S.A. In Oregon, BCWD is the most frequent cause of elevated loss requiring treatment in juvenile and yearling salmonids in Oregon Department of Fish and Wildlife hatcheries. Annually, about one third of all fish health examinations conducted to investigate elevated losses found *F. psychrophilum* infections. This high prevalence can be explained by this bacterium's ability to infect all salmonids and some non-salmonids, cause disease in most life stages from fry to adults and is associated with many different types of lesions and disease signs. In the 1960s, BCWD caused serious losses mostly in hatchery juvenile coho salmon (*Oncorhynchus kisutch*) and occasionally in rainbow trout (*O. mykiss*). Since then, more BCWD epizootics occur annually in juvenile rainbow trout than any other species. Some of the disease signs attributed to *F. psychrophilum* include: the classic eroded skin and muscle lesion of the peduncle, ventral body, snout or lower jaw, vertebra deformities, anemia and dark pigmentation. Late in BCWD epizootics the fish often display no external lesions or signs, but are infected with the bacterium. Juvenile rainbow trout display enlarged spleens, often with lesions through the body wall over the spleen, clear fluid in the stomach or yellow fluid in the lower gut. In recent years unusual disease signs similar to the lesions of furunculosis, i.e. blood filled skin blisters and bleeding gills have been observed in rainbow and steelhead trout. One outbreak in hatchery reared juvenile white sturgeon (*Acipenser transmontanus*) occurred with the fish having typical BCWD lesions in the peduncle. Adult Chinook and coho salmon often suffer external skin lesions from *F. psychrophilum* especially in areas where mechanical abrasions have occurred. This bacterium is often found in the internal tissues of the spawning salmon and trout. Infections of *F. psychrophilum* are found in association with fish viruses such as infectious hematopoietic necrosis virus (IHNV) and erythrocytic inclusion body syndrome virus. During the 1990s, several large die-offs of wild kokanee salmon (*O. nerka*) occurred in Lake Billy Chinook in the Central Oregon Deschutes River Basin. The fish were found to have mixed infections of IHNV, BCWD and external fungi. During the winter of 2004, a fish kill involving thousands of feral mature goldfish (*Carassius auratus*) occurred in Fern Ridge Reservoir near Eugene, Oregon. The dying fish, nearly all females with large gray descaled areas on skin posterior to the head or in the peduncle region, were infected with *F. psychrophilum* and external parasites such as Ichthyobodo. In Oregon, *F. psychrophilum* continues to be a significant cause of fish losses in hatchery and naturally reared fish stocks.

FLAVOBACTERIUM PSYCHROPHILUM AND RAINBOW TROUT: AN INDUSTRY PERSPECTIVE

Scott E. LaPatra
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Flavobacterium psychrophilum is an economically important pathogen of the rainbow trout (*Oncorhynchus mykiss*) industry in the Hagerman Valley of southern Idaho. The disease caused by this pathogen, bacterial coldwater disease (CWD), is primarily a disease of young life stages of fish. Major outbreaks are often associated with co-infections of both *F. psychrophilum* and infectious hematopoietic necrosis virus (IHNV). Although the disease is associated with low temperatures, rainbow trout in this region are produced at a constant temperature of 15^{o}C. Clinical signs vary between outbreaks and age of the fish affected. In most cases, the younger the fish, the greater the severity of disease. Of particular concern is that fish surviving an epizootic of CWD may develop spinal deformities. The most common deformities in survivors are spinal compression in the anterior, mid or posterior portions of the fish. This is a significant economic impact when the fish go to market because the product is often downgraded and the efficiency of the automated processing equipment is impacted. To presumptively diagnose CWD imprints of the spleen and anterior kidney are made on glass slides that are stained with Diff Quik. *F. psychrophilum* cells are very long, thin rods which makes their identification relatively easy. To isolate the organism spleen and anterior kidney are cultured on tryptone-yeast extract (TYE) and incubated for 2-4 days at 15^{o}C. Although evidence is mounting for egg - associated and vertical transmission, we have not been able to detect the bacteria in the ovarian fluid of sexually mature rainbow trout or on or within the egg. Standard 100 ppm iodophor disinfection is done at the egg water-hardening step and also when the eggs are shipped and subsequently received. Additionally, all adult rainbow trout are injection vaccinated with a non-adjuvanted whole cell killed bacterin prior to entering the spawning population. Our working hypotheses are that egg-associated and vertical transmission are negligible risks with our current management program. We feel the primary mode of transmission is through the water which can be enhanced during below freezing temperatures that commonly occur during the winter in our area. Because the water is a constant 15^{o}C heavy fogging due to below freezing temperatures will occur resulting in aerobiological spread of the pathogen. It is interesting to note that while we consider waterborne occurrence of *F. psychrophilum* to be the primary risk no one has been able to reproducibly infect rainbow trout and cause disease in the laboratory using a bath or immersion challenge method. Prevention of CWD is difficult due to the ubiquitous nature of the organism. Prudent fish culture management practices that focus on pond hygiene, proper fish loadings and feed rates are the core of any fish health management program. However, if infection and/or disease are detected rapid treatment with U.S. Food and Drug Administration approved compounds should be implemented although these are short-term strategies. Long-term strategies include vaccine development and selective breeding. There are effective vaccines that are available, however, a direct inoculation is required. The development of mass immunization strategies is as important if not more important than the development of the vaccines themselves for this industry. Selective breeding for disease resistance is extremely powerful but labor and facility intensive but holds tremendous potential for the future.

COMPLETE GENOME SEQUENCE OF *FLAVOBACTERIUM PSYCHROPHILUM*

Eric Duchaud, Pierre Nicolas, Jean-François Bernardet & Abdenour Benmansour

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Infections caused by the bacterium *Flavobacterium psychrophilum* in salmonid fish are responsible for heavy mortalities and considerable economic losses in fish farms worldwide. Many publications have contributed to the description of the bacterium but virulence mechanisms remain poorly known so far. In order to characterize the molecular bases of virulence in *F. psychrophilum* and to improve our understanding of host-pathogen relationships, the sequence of the whole genome of strain JIP 02/86 (ATCC 49511) has been determined. The genome consists of a 2,861,988–base-pair (bp) circular chromosome with 2,432 predicted protein-coding genes. The in silico analysis of the *F. psychrophilum* genome has identified candidate genes likely involved in pathogenicity. Among them, genes encoding putative "true (exo)-toxins", e.g. putative hemolysines and proteases, some of which were identified from their similarity with proteins involved in the virulence in other pathogenic species ; endotoxins, i.e lipopolysaccharide encoding genes ; and fitness factors that probably play important roles in virulence have been detected.

GENOME SEQUENCE OF *FLAVOBACTERIUM PSYCHROPHILUM* STRAIN CSF 259-93 AND CHARACTERIZATION OF A LARGE CLUSTER OF GENES CONTAINING SHORT REPEATS.

Gregory D. Wiens[1], Timothy W. Welch[1], Caird Rexroad III[1], Scott LaPatra[2], Douglas R. Call[3], David Hunnicutt[4], Anamitra Bhattacharyya[5], John Campbell[5], and Theresa Walunas[5]

[1]National Center for Cool and Cold Water Aquaculture, Leetown, WV 22602; [2] Clear Springs Foods Inc., Research Division, PO Box 712, Buhl, ID 83316; [3] Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA 99164; [4]Penn State Erie, The Behrend College, School of Science, 5091 Station Road, Erie PA 16563; [5]Integrated Genomics, Chicago, IL;

At the National Center for Cool and Cold Water Aquaculture, we have initiated an integrated approach to understand the pathogenesis and host response of rainbow trout to *F. psychrophilum* infection. As part of this program, and in cooperation with Clear Springs Foods Inc, we initiated a project to completely sequence the genome of *F. psychrophilum* strain CSF 259-93 in order to identify virulence factors and protective antigens. This strain was chosen as it is representative of lineage II strains causing significant disease in U.S. trout aquaculture, it is freely available, and a challenge protocol had been established. At the outset of this project, a single colony was isolated, virulence verified, and a large stock of DNA and viable bacteria were archived for future reference and challenge work. Genome sequencing and assembly was carried out by Integrated Genomics and a total of 40,527 shotgun sequence reads and 2966 fosmid sequences were assembled (8-fold sequence coverage). The completed genome consists of a single circular chromosome of 2,900,735 bp with a G+C content of 32.49%. A total of 87.96% of the genome is composed of open reading frames (ORFs) and 2634 ORFs were identified by Integrated Genomics ORF-calling software. The genome contains many transposase ORFs and they are associated with four regions of genome variability. A total of 1674 ORFs (63%) were assigned function and 1,086 ORFs are in asserted pathways. A particularly interesting set of 19 highly similar ORFs were identified that contain variable numbers (3 to 11 copies) of a 23 aa repeated motif. These ORFs were all in the same orientation and formed a large (21.5 kb) contiguous gene cluster. These repeats have homology to leucine-rich motifs while ends of these proteins are highly conserved with each other. A PCR assay was developed to distinguish between genes containing various numbers of repeats and this locus is highly polymorphic between strains. One of these ORFs, designated *F. psychrophilum* repeat protein N (*frp*N) was cloned and recombinant protein purified. This protein was weakly immunogenic in rainbow trout and did not induce a protective immune response against challenge. In summary, the genome of *F. psychrophilum* CSF 259-93 is relatively small compared to other sequenced bacteria from the Cytophaga-Flavobacterium-Bacteroides group and it contains abundant insertion elements that are associated with several regions of genome variability. The complete genome sequence is the first step toward a better understanding of metabolic and pathogenic processes, and the identification of potential vaccine candidates.

THE *FLAVOBACTERIUM COLUMNARE* GENOME SEQUENCING PROJECT

Mark L. Lawrence[1], David W. Dyer[2], Allison F. Gillaspy[2], Attila Karsi[1], and Nicole Benton[2]

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Flavobacterium columnare is a bacterial pathogen that affects commercial aquaculture, the ornamental fish industry, and wild fish populations in both the U.S. and worldwide. In particular, it is the second most prevalent disease of farm-raised channel catfish, which is the largest aquaculture industry in the U.S. In spite of the magnitude of this disease problem, very little is known about how *F. columnare* causes disease, and an effective preventive method is still not available. The *F. columnare* genome sequence will enable new research methods to identify disease-causing genes and will accelerate vaccine development research. The *F. columnare* genome also has biological significance because it is a member of the Bacteroidetes, which is a group of bacteria that has few sequenced genomes but contains some of the most abundant bacterial species in mammalian intestines and in aquatic environments.

Genome sequencing was accomplished by constructing small and medium insert libraries from *F. columnare* strain ATCC 49512 in pJAZZ-KA (Lucigen, Middleton, WI) (3-6 kb, 6-10 kb, and 10-12 kb average insert size), end sequencing, and assembling using Phred/Phrap. Three-fold coverage of the genome was reached on July 17, 2006, at which time the sequence data was released and is being provided as assembled contigs (http://micro-gen.ouhsc.edu/f_columnare/f_columnare_home.htm). As of October 21, 2006, 8X coverage of the genome was achieved; 52,512 sequencing reads have been generated with a 78.4% success rate, yielding 41,157 high quality reads. Average read lengths have been 807 bp. The assembly currently contains 36 contigs >2 Kb in length. This project is funded through the USDA-CSREES (award number 2006-35600-16571).

FLAVOBACTERIUM PSYCHROPHILUM: FROM GENOME SEQUENCING AND METABOLIC PROFILING TO COMPARATIVE GENOMICS

Anamitra Bhattacharyya, Ph.D.

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We report the completion of the genome sequencing of the bacterial fish pathogen, *Flavobacterium psychrophilum*. The 2.9 Mb *F. psychrophilum* genome comprises a single main chromosome encoding 2,634 open-reading frames (ORFs) of which over 63% have functional gene assignments. The complete *F. psychrophilum* genome assembly has been analyzed using the ERGO™ Genome Analysis Suite by connecting functions of ORFs to our extensive, curated pathway database collection, comprising more than 6,000 metabolic and non-metabolic pathways. Numerous genes relevant to pathogenesis, secretion and various functional sub-systems have been identified in the genome to date. Results of a nutritional profiling analysis for this pathogen will be presented that will aid growth media development. Progress on *F. psychrophilum* genome comparison with other available *Flavobacterium* spp. will also be discussed together with the availability of technologies to produce new and affordable expression microarray chips for the academic and industrial research communities.

EFFECTS OF CODON USAGE BIAS ON RECOMBINANT EXPRESSION OF *FLAVOBACTERIUM PSYCHROPHILUM* PROTEINS IN *E. COLI*

Devendra H Shah[1], Kenneth D. Kein[2], and Douglas R. Call[1]

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Using data from a draft genome sequence of *Flavobacterium psychrophilum* (strain FS-CSF259-93), we cloned and expressed several putative virulence associated genes using an E. coli expression host. Initial expression experiments using conventional protocols produced either absent or very low levels of full length protein products and these were often associated with one or more minor molecular weight species. We hypothesized that difficulties arose due to codon bias between *F. psychrophilum* and the expression host *E. coli* BL21. Subsequently, co-expression of seven rarely occurring tRNA species in the host along with a fusion of trxA gene (encoding 11.7 kDa thioredoxine protein) to the N-terminal of the target proteins dramatically improved the expression of FS proteins and reduced the numbers of minor molecular weight species of expressed products; the latter were not eliminated entirely. We subsequently analyzed complete ORFs from 97 putative virulence genes of *F. psychrophilum* relative to frequency of usage and the relative adaptiveness of each of 61 codons compared with E. coli. This analysis led to the following observations: (i) *F. psychrophilum* genes have a strong bias in codon usage at the 3rd position "wobble base" of codons with the position-specific mol% G+C at 1st, 2nd and 3rd position being 40.7%, 34%, and 24%, respectively; (ii) *F. psychrophilum* uses 19 codons considered rare in *E. coli* of which 11 codons appear to be used with relatively high frequency in *F. psychrophilum*; (iii) for the estimation of relative adaptiveness, the codon with highest frequency value for each amino acid was set to 100% relative adaptiveness and all other codons for the same amino acid were scaled accordingly. This resulted in identification of 3 additional codons that are being used optimally (100% relative adaptiveness) in *F. psychrophilum* but, rarely in the *E. coli* (<55% relative adaptiveness); (iv) although all the 61 codons are found within the coding sequences of *F. psychrophilum*, it uses nearly half the numbers (n=49) of tRNAs compared to *E. coli* (n=86), indicating that use of base wobble in the 3rd position might allow a given tRNA gene to preferentially utilize certain codons. These observations confirm that inefficient expression of *F. psychrophilum* genes in E. coli is likely due to condon bias that can be partially remedied by inclusion of supplemental tRNA's, but it is likely that conditions for expression of each gene will require individual optimization.

WESTERN BLOT ANALYSIS REVEALS FOUR DISTINCT ANTIGENIC PROFILES FOR *FLAVOBACTERIUM PSYCHROPHILUM.*

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Flavobacterium psychrophilum is the causative agent of Rainbow Trout Fry Syndrome (RTFS) and Bacterial Cold-Water Disease (BCWD). Both presentations cause severe economic losses worldwide. Commercially licensed vaccines against *F. psychrophilum* are not yet available, and fish are currently treated using antibiotics. An effective vaccine against *F. psychrophilum* will need to incorporate as many serovars as possible, however, there is no international agreement on a serotyping method for *F. psychrophilum* and a variety of serotyping methods using different serological schemes are used. There is also a further problem of isolates changing serotype following challenge and altering serotypes when cultured on different media. This has far greater implications due to the requirement for TSE-Free vaccines and the fact those previous serotyping systems all utilised bacteria grown on non-TSE free media.

Twenty-six isolates from different sources were cultured on TSE-Free agar. The profiles of these bacteria were compared using Western Blot using a cocktail of two *F. psychrophilum* anti-rabbit polyclonals against a virulent (B97026) and a non-virulent (32/97) isolate. It was observed that there were only four different profiles for the 26 isolates, allowing the bacteria to be divided into four serotypes. Importantly, these serotypes do not appear to change if the bacteria are passaged through fish and then recultured.

To provide conclusive evidence of the existence of 4 serotypes, more isolates need to be analysed. The determination of distinct serotypes is important in the development of an effective vaccine.

ELECTROPHORETIC AND WESTERN BLOT ANALYSES OF THE LIPOPOLYSACCHARIDE AND GLYCOCALYX OF *FLAVOBACTERIUM PSYCHROPHILUM*

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Flavobacterium psychrophilum is the aetiological agent of bacterial coldwater disease (CWD) and rainbow trout fry syndrome (RTFS) and it has emerged as one of the most significant bacterial pathogens in salmonid aquaculture worldwide. Previous studies have suggested that the O-polysaccharide (O-PS) component of the lipopolysaccharide (LPS) of *F. psychrophilum* is highly immunogenic and may be involved in eliciting a protective immune response in rainbow trout (*Oncorhynchus mykiss* Walbaum). In the present study, SDS-PAGE and Western blotting techniques were used to analyse the carbohydrate antigens of *F. psychrophilum*. Our analysis identified two distinct carbohydrate-banding patterns. One banding pattern corresponds with LPS, and we hypothesize that the other carbohydrate-banding pattern is that of the loosely associated glycocalyx of *F. psychrophilum*. Electron *F. psychrophilum* microscopy of cells immunogold labeled with a monoclonal antibody specific for this banding pattern supports this hypothesis as the outermost layer of the bacterium was heavily labeled. This is a significant finding because the immunogenic antigens that have been referred to as the O-PS of LPS, and implicated as potential vaccine candidate antigens, appear to be components of the glycocalyx of *F. psychrophilum*. This research suggests that the glycocalyx of *F. psychrophilum* may be an important antigen to consider for the development of a vaccine to control CWD and RTFS.

A MUTANT IN THE EXBD LOCUS OF A TONB SYSTEM IN *FLAVOBACTERIUM PSYCHROPHILUM* SHOWS ATTENUATED VIRULENCE AND CONFERS A HIGH LEVEL OF PROTECTION AGAINST COLD WATER DISEASE.

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Flavobacterium psychrophilum is a psychrotrophic, fish pathogenic bacterium that causes the "cold water disease" (CWD) in salmonids. In an attempt to go inside the pathogenesis mechanisms of this bacterium, a set of mutants were isolated using a Tn4351-mutagenesis system. One of these named FP1033, deficient in growth on iron depleted medium as well as in extracellular proteolytic activity recovered the parental phenotype in the presence of iron, hemin and hemoglobin. The gene disrupted by the transposon in this mutant encoded for a protein with similarity to ExbD proteins which are members of the TonB complex system involved in iron uptake mediated by siderophores. In pathogenic bacteria, these iron acquisition systems allow the bacteria to scavenge iron and grow inside the host. Therefore, the TonB system is associated with mechanisms involved in the progress of the infection in several important pathogens including fish infecting bacteria such as *Yersinia ruckeri* and *Vibrio anguillarum*. Analysis of the DNA surrounding the transposon insertion into the *F. psychrophilum* chromosome showed the presence of a tonB cluster of genes composed by exbB, two exbD (exbD1 and exbD2) and tonB loci. RT-PCR analysis and complementation studies indicated that these genes are transcribed as an operon and that the exbD2::Tn4351 phenotype was caused by the lack of ExbD2. FP1033 presented decreased virulence and conferred high level of protection in rainbow trout fry after vaccination. This is the first report of a *F. psychrophilum* attenuated strain that successfully induced a protective immune response in rainbow trout fry against CWD. These results suggest that the exbD2 locus from this particular tonB system is a suitable target to generate a live attenuated vaccine.

INVESTIGATIONS INTO "RED MARK SYNDROME" (RMS) IN SCOTLAND

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During the autumn of 2003 haemorrhagic lesions on the body surface of freshwater reared rainbow trout (*Oncorhynchus mykiss*) were described and reported as a new condition termed 'red mark syndrome RMS'. Affected fish are generally over 100 g, show single to multiple areas of swelling on the skin, particular over the flanks, with a light coloured to well demarcated petechial haemorrhagic reaction (red coloured marks). Scale loss may be observed at the centre of the lesion, which can eventually become ulcerated. Sporadic exophthalmia and skeletal deformity have also been recorded. Histologically, a chronic dermatitis is observed with infiltration involving primarily the basement membrane and scale pockets. Ellipsoid dilatation, renal haemorrhage and degenerative lesions and liver focal necrosis, are occasionally observed. Acute necrotizing myocarditis has been also recorded in smaller fish.

The condition has been reported from approximately 25 freshwater aquaculture sites and fisheries in Scotland and several cases have also been reported in England. Although it is considered to produce no direct mortality, RMS does have an economic impact as affected fish may become susceptible to further infection with associated increased labour costs and antibiotic treatment. Additionally, fish become unmarketable as they are downgraded at harvest. The apparent spread of the condition and response to antibiotic treatment suggests an infectious aetiology.

Despite efforts by several laboratories, no virus, parasite or fungal agent has been isolated, and bacterial isolates showed no dominant organism hence the aetiology of the condition remains unexplained. FRS has been involved in the diagnosis of RMS and has gathered significant clinical, pathological and bacteriological information, indicating RMS might be a multifactorial condition. Certain environmental factors and farming practices might be rendering fish stocks immune compromised, triggering a hypersensitivity reaction to the presence of chronic, septic, sub-lethal condition against *Flavobacterium psychrophilum* and related species.

THE INFLUENCE OF CULTURE CONDITIONS ON BIOFILM FORMATION IN *FLAVOBACTERIUM COLUMNARE*

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Flavobacterium columnare is the causative agent of columnaris disease. This disease is responsible for significant economic losses to the US channel catfish (*Ictalurus punctatus*) industry. Specific mechanisms of invasion by this pathogen are not well understood. Since the bacteria can grow to high density in external lesions on the fish surface, we sought to define conditions that stimulate *Fla. columnare* to grow as a biofilm. Growth and biofilm formation of 10 strains of *Fla. columnare* was assessed under a variety of culture conditions including the addition of different salts, glucose and mucus scraped from the surface of Atlantic salmon (*Salmo salar*). The addition of a complex salt mixture to the base medium (Ordals medium) was found to dramatically increase cell density in shaking cultures. The addition of individual salts revealed that the divalent cat ions Mg^{2+} and Ca^{2+} both cause dense biofilms to form. The addition of glucose or salmon skin mucus also resulted in increased biofilm formation relative to the base medium. The degree to which different strains produced biofilms under each condition varied, but only two strains failed to produce a film under any conditions tested. Confocal microscopy revealed that the biofilms were complex multi-layered structures and showed that biofilms induced by different substrates had differing architectures. The mucus- and glucose-induced films were relatively uniform mats of cells while the films induced by the addition of Ca^{2+} and Mg^{2+} were uneven clumps of cells, some of which were large enough to be seen by the unaided eye on the bottom of the culture vessels. These results demonstrate that the degree to which *Fla. columnare* grows as a biofilm is dependent on culture conditions. Most strains tested formed biofilms under conditions designed to mimic the environment cells experience when they first encounter the fish host. Further characterization of these biofilms and the conditions in which they form will increase our understanding of the early stages of the infection process of this economically important fish pathogen.

INTRASPECIES CHARACTERIZATION OF *FLAVOBACTERIUM COLUMNARE*

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In recent years, several studies have been devoted to clarify the intraspecies diversity of *Flavobacterium columnare* in an attempt to better understand the epidemiology of columnaris disease. *F. columnare* is a phenotypically homogeneous species and, therefore, the use of standard biochemical tests or chemotaxonomic markers are not suitable for strain characterization. Nevertheless, this bacterial species harbors an intrinsic genetic diversity that was first revealed by 16S rDNA-RFLP analysis. This molecular marker split the species into three different genomovars that can be further subdivided using more powerful fingerprinting methods.

For the past four years our group has carried out an extensive intraspecies characterization of *F. columnare* isolates recovered from farm raised fish as well as from natural populations in the Southeast of the USA. We have ascribed these isolates to the genomovar level by 16S rDNA-RFLP. To date, only genomovars I and II are represented in our collection of more than 140 isolates. In addition, we have shown the high degree of polymorphisms present in both the 16S rDNA and the ISR sequence by SSCP analysis. Besides ribosomal markers, we have used AFLP to snap shot the genomic diversity of the species. As expected, the highest diversity index for the species was defined by AFLP. The deep division between genomovars I and II was observed not only when ribosomal markers were used but also when a whole genome fingerprinting method such as AFLP was employed. Interestingly, genomovar II displayed a higher diversity index than genomovar I regardless of typing method.

Recently, we have identified and sequenced four *F. columnare* putative virulence genes and found differences in the nucleotide and the derived aminoacid sequences between both genomovars. We also found significant differences between the two genomovars in terms of host specificity and virulence.

DEVELOPMENT OF A DISEASE MODEL TO PRODUCE EXTERNAL COLUMNARIS (*FLAVOBACTERIUM COLUMNARE*) INFECTIONS IN CHANNEL CATFISH

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Columnaris disease, caused by the gram-negative bacterium *Flavobacterium columnare*, is characterized by "saddleback" lesions on the dorsal fin, extensive necrosis of the gill filaments, and fin and tail lesions. The ability of columnaris to rapidly infect and cause large-scale mortality in a diverse array of cultured fish is of major economic concern to the aquaculture industry. Therapeutic agents needed to control the disease are limited, mainly because of the difficulty in conducting and collecting field effectiveness data. Disease models offer researchers year round access to diseased fish that reduce trial costs, and minimize the confounding variables that are usually present during field effectiveness trials. In the present study, columnaris disease was induced using four strains of columnaris isolated from clinically infected fish representing diverse fish species (hybrid striped bass, channel catfish, rainbow trout and carp) and geographic locations (Iowa and California). Columnaris disease was induced by abrading fish then immersing fish in a columnaris bath at 10^3 and 10^4 colony forming units (CFU)/mL. Mortality patterns were initially determined with the channel catfish strain in August of 2005 and reassessed in August of 2006 to determine storage effects on pathogenicity and assess repeatability of the disease model. Frozen storage of the catfish strain did not alter pathogenicity or our ability to induce columnaris disease. All four strains were used in disease challenges to evaluate mortality patterns and disease progression differences between strains. Characterization of mortality patterns and disease progression will aid researchers in the ability to test efficacy of waterborne therapeutants for columnaris disease treatment.

SUSCEPTIBILITY OF ZEBRA FISH (*DANIO RERIO*) TO INFECTION BY *FLAVOBACTERIUM COLUMNARE* AND *FLAVOBACTERIUM JOHNSONIAE*.

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Flavobacterium columnare is a serious pathogen in a wide range of fish species. *Flavobacterium johnsoniae* is an opportunistic pathogen of certain fish. Both are gliding bacteria. These species were tested for their ability to infect the zebra fish, *Danio rerio*. Both injection and bath infection methods were tested. The results indicate that *F. johnsoniae* is not an effective pathogen in *D. rerio*, but that *F. columnare* is an effective pathogen. *F. johnsoniae* did not cause increased death rates following bath infection, but did cause increased death rates following injection, with an LD50 of approximately 3×10^{10} cfu. Non-motile mutants of *F. johnsoniae* produced a similar LD50. *F. columnare* caused increase death rates following both injection and bath infections. There was considerable strain variation in LD50, with the most lethal strain tested producing an LD50 of 3.2×10^6 cfu injected and 1.1×10^6 cfu ml^{-1} in bath experiments including skin damage. The LD50 of *F. columnare* in zebra fish without skin damage was $>1 \times 10^8$, indicating and important effect of skin damage. Additional experiments using *D. rerio* eggs and fry will be discussed.

GENETIC VARIATION OF *FLAVOBACTERIUM PSYCHROPHILUM* EXAMINED BY PULSE-FIELD GEL ELECTROPHORESIS

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The long term goal of this project is to examine the molecular epidemiology of *Flavobacterium psychrophilum* in salmon and trout hatcheries to better understand transmission dynamics within and between host species. The immediate objective of this project is to develop a suitable pulsed-field gel electrophoresis (PFGE) assay for this work. We initially screened two isolates (CSF 259-93 and ATCC 49418) with a panel of 26 restriction enzymes. Ten enzymes were suitable for restricting genomic DNA from both strains whereas ten other enzymes failed to restrict either source of DNA. Six enzymes selectively digested one source of DNA but not the other. We retrieved *F. psychrophilum* isolates from a bank of national and international diagnostic isolates and using a single enzyme we tested 17 strains that originated from salmon and 8 strains that originated from trout. PFGE analysis showed that overall genetic similarity as assessed by a Dice band sharing coefficient was 60.2% with similarity between salmon isolates lower (64.0%) than similarity between trout isolates (71.9%). A cluster analysis (UPGMA) showed a division isolates consistent with a published hypothesis that trout and salmon isolates represent distinct genetic lineages of *F. psychrophilum*. We are applying this assay to assess clonality of *F. psychrophilum* recovered from six epizootics at three proximal aquaculture facilities. Preliminary analysis indicates that individual epizootics are dominated by single clones, but that these clones are distributed amongst production locations.

PROGRESS TOWARD AN IMMUNOGENETIC MAP FOR RAINBOW TROUT

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QTL (quantitative trait loci) mapping utilizes a series of methods for finding pieces of DNA that are closely linked to genes that confer a desired phenotype. One of our research goals is to map QTL in rainbow trout that associate with innate resistance to *Flavobacterium psychrophilum*. A high density genetic map of over 1200 microsatellite markers has been recently constructed in our lab to enable whole genome association studies. We are now adding candidate immune response genes to the genetic map. The process begins by using known mouse and human immune genes to identify putative homologous genes in the trout and salmon expressed-sequence-transcript (EST) datasets. PCR primers are then developed from the partial or complete coding sequences to screen a rainbow trout BAC (bacterial artificial chromosome) library in order to isolate clones that harbor the genes of interest. The selected BACs are mapped by HindIII restriction enzyme digestion to construct locus specific physical maps (contigs). These physical maps are used to link a single gene to a single locus of the trout genome. A representative BAC from each locus is fragmented, sub-cloned, and partially sequenced to isolate microsatellites that are then added onto the genetic map. To date we have mapped 14 immune response genes using this process and we are currently in the process of isolating microsatellites for another set of 14 genes. Our immuno-focused genetic map will be used as a cost effective tool in future studies that seek to associate rainbow trout genetic variations with disease resistance. Additionally, it will provide useful information for comparative genome mapping with other model fish species and for integration of the physical and genetic maps of rainbow trout.

FLAVOBACTERIUM PSYCHROPHILUM IN NORWAY: PHENOTYPIC IDENTIFICATION AND GENETIC VARIATION

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Isolation of Flavobacterium spp. from diseased fish is relatively uncommon in Norway. Most *F. psychrophilum* isolations occur during the winter months in salmon (Salmo salar) and trout (*Salmo trutta*) reared for mitigation purposes. The most significant mortalities (40-50%) in recent years have, however, been associated with systemic infections in relatively large farmed rainbow trout during the summer months. As *F. psychrophilum* is relatively refractive to many of the commonly utilised phentotypic test methods, APIzym (Biomerieux) tests have been widely used in our laboratory for identification of this bacterium and differentiation of this species from other related bacteria. To both test our phenotype based diagnostic testing system and investigate the genetic variation in *F. psychrophilum* in Norway, we sequenced the nearly complete 16S rRNA gene in 14 isolates classified by phenotypic testing as *F. psychrophilum* and 12 isolates identified as non-psychrophilum *Flavobacterium* spp. The results indicate that our limited range of phenotypic tests are adequate for identification of *F. psychrophilum*, as all Norwegian and the single Swedish isolate identified by these methods have identical 16S sequences with one of the two North American genotypes described by Soule et al. 2005. This genotype, which is associated with elastase production (at least in the American strains) does not, however, contain any *F. psychrophilum* reference strains. The existence of two conserved *F. psychrophilum* 16S genotypes and the absence of intermediate genotypes within the cluster indicates the need for a more in depth investigation e.g. multi-locus sequence analysis, of the relationship between these two groups.

Reference

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SELECTING FOR RESISTANCE AGAINST BACTERIAL COLD-WATER DISEASE

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At the National Center for Cool and Cold Water Aquaculture (NCCCWA) reducing the negative impact of diseases on rainbow trout culture is a primary objective. Improving innate resistance is particularly important for fish that are <4 g because they typically respond poorly to vaccines. Our selective breeding program to improve resistance to bacterial cold-water disease, a chronic disease of rainbow trout caused by *Flavobacterium psychrophilum*, began in 2005 with 75 families of rainbow trout, each with hundreds of offspring. We conducted a challenge study in which family groups with 40 fish each, the average fish weight at injection was 2.4grams (~200 fish/lb), were injected with a strain of *Flavobacterium psychrophilum* bacteria kindly provided by Clear Springs Foods, Inc. The number of days from injection until mortality was recorded for each fish in each family until day 21 of the challenge. This was the trait (days to death) evaluated for genetic analysis. Over the course of the 21 day challenge, approximately 70% of all fish died. Survival rates among family groups ranged from near 0 to greater than 70%. Thus there was large variation between families in resistance. The heritability of resistance was between 0.3 and 0.4. This means that of all the variation seen between fish and families, approximately 30 to 40% is attributable to genetic, not environmental differences and suggests that selective breeding could be an effective method for improving resistance to bacterial coldwater disease. Selective breeding of resistant families took place in January 2007. Results from challenge of the 2007 families (May to June 2007) will help to verify the magnitude of genetic variation for resistance to bacterial cold water disease.

ASSOCIATIONS OF MARKERS LINKED TO MAJOR HISTOCOMPATIBILITY (MH) REGIONS WITH RESISTANCE TO *FLAVOBACTERIUM PSYCHROPHILUM* IN RAINBOW TROUT

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The 2005 NCCCWA brood year (75 full-sib families) were challenged with *Flavobacterium psychrophilum*, the causative agent of bacterial coldwater disease and rainbow trout fry syndrome. Overall mortality rate was 70% with large variation among families. Resistance to the disease was assessed by monitoring post challenge days to death. Phenotypic variation and additive genetic variation were estimated using mixed models of survival analysis. Microsatellite markers were previously isolated from BAC clones that harbor genes of interest and mapped on to the rainbow trout genetic linkage map. The parents and grandparents of the 2005 brood year families were genotyped with markers linked to the four major histocompatibility (MH) genomic regions to assess linkage disequilibrium (LD) between those genomic regions and resistance to the bacterial disease. The impact of MH sequence variation on selective breeding for disease resistance in aquaculture is discussed.

IDENTIFICATION OF POTENTIAL VACCINE TARGET ANTIGENS BY IMMUNOPROTEOMIC ANALYSIS OF A VIRULENT AND A NON-VIRULENT STRAIN OF THE FISH PATHOGEN *FLAVOBACTERIUM PSYCHROPHILUM*

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Flavobacterium psychrophilum is the etiological agent of bacterial coldwater disease (CWD) and rainbow trout fry syndrome (RTFS). To identify antigens associated with virulence or host immunity, we compared total and immunogenic proteins of cellular and extracellular products (ECP) between a virulent (CSF-259-93) and non-virulent (ATCC 49418) strain of *F. psychrophilum*. One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis of total cellular proteins revealed only minor differences between the strains; however, separation of ECP showed that proteins were differentially expressed. Western blot analysis using rainbow trout (*Oncorhynchus mykiss*) anti-CSF-259-93 sera showed greater reactivity to proteins of the virulent strain, including many >50 kDa. Further analysis by 2-dimensional electrophoresis (2DE) identified numerous differences between the strains. Western blot analysis combined with 2DE identified several immunogenic proteins that reacted with the antisera and were shared between the 2 strains. However, at least 15 immunogenic proteins appeared to be unique to the virulent strain, while 4 such proteins were identified in the non-virulent strain; 8 proteins unique to the virulent strain and 6 shared proteins were further analyzed for identification by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis. Of these, 3 immunogenic proteins (HSP 60, HSP 70, and ATP synthase) and 2 other proteins (Arginine tRNA ligase and thermolysin) were conclusively identified. The 2 highly immunogenic heat shock proteins were shown to share extensive homology with heat shock proteins of related bacteria. This approach for antigen identification may provide a basis for targeted vaccine development against CWD and RTFS.

IDENTIFICATION OF CELL-SURFACE PROTEINS INVOLVED IN *FLAVOBACTERIUM JOHNSONIAE* GLIDING MOTILITY

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Cells of the gliding bacterium *Flavobacterium johnsoniae* move rapidly over surfaces by an unknown mechanism. Previous genetic analyses identified 12 Gld proteins that are required for motility. These proteins are thought to form a complex in the cell envelope but surprisingly, none of them appear to be exposed on the cell-surface. We hypothesize that the cell-surface components of the machinery are redundant and that the genes encoding these proteins were not previously identified because only completely nonmotile mutants were analyzed. To identify redundant cell-surface components of the machinery *HimarEm1* induced mutants with partial motility defects were isolated. Three contiguous genes (*sprC*, *sprD*, and *sprB*) that encode abundant cell-surface proteins were identified. Mutations in any of these genes or a deletion spanning the entire region resulted in cells that failed to form spreading colonies on agar but that exhibited rapid gliding on glass in wet mounts. Antibodies raised against SprB inhibited motility of wild-type cells, confirming the importance of SprB in gliding. Cryo-EM analysis revealed fibrils on the surface of wild-type cells that were absent from cells of the *sprCDB* deletion strain CJ1584. *HimarEm1* mutagenesis was used to identify mutations that further compromised the motility of CJ1584. Four mutants with dramatically reduced motility had insertions in a homolog of *sprB* which we named *sprF*. SprB and SprF are similar to cell-surface adhesins, and SprF has a lectin-like domain that may bind carbohydrates. Three additional *HimarEm1* induced motility mutants of CJ1584 had insertions in genes predicted to be involved in exopolysaccharide synthesis or export. Fibrils composed of Spr proteins might be the outermost components of the motility machinery. Exopolysaccharides might enhance motility by coating the substratum and interacting with these fibrils.

A PROSPECTIVE CASE-CONTROL STUDY OF BACTERIAL GILL DISEASE OUTBREAKS IN ONTARIO, CANADA GOVERNMENT SALMONID HATCHERIES

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Bacterial gill disease (BGD) is an important concern in freshwater aquaculture, and affects many species of farmed fish worldwide. This disease has been a persistent problem at Ontario Ministry of Natural Resources (OMNR) salmonid hatcheries and the Alma Aquaculture Research Station (AARS); BGD outbreaks at these locations have, on occasion, been associated with rapid and very high morbidity and mortality levels. The causative agent of BGD, *Flavobacterium branchiophilum*, is considered ubiquitous in fresh water, and therefore outbreaks of BGD are thought to be precipitated by environmental conditions and host factors favoring opportunistic infections. Despite the importance of BGD, very little epidemiological research has been conducted to examine factors associated with episodes of this disease. This paper presents a 14-month (July, 2002 – September, 2003) rearing unit-level prospective nested matched case-control investigation at five OMNR hatcheries and the AARS, with the objective of identifying, and quantifying the effects of, important risk factors for BGD outbreaks. Daily data were collected on putative BGD risk factors for all early-rearing (<9 months of age) fish tanks at participating hatcheries, and all outbreaks of BGD were confirmed by light microscopy at the Fish Health Laboratory (University of Guelph) during the study period. Control tanks were selected at the end of the study and matched to individual case tanks based on time, hatchery, and species. The case-control data were then analyzed using multivariable logistic regression modeling, controlling for fish age. The results of the final model indicated that tanks with confirmed BGD outbreaks were significantly more likely to have lower fish numbers, lower individual fish weights, higher mortality levels and higher feeding rates during the week preceding observed BGD outbreaks than were asymptomatic control tanks. Refinements in the observation and manipulation of these factors will therefore aid in the prevention of fish losses associated with BGD outbreak mortality spikes. The predictive (as opposed to causal) nature of the identified risk factors indicates the need for further research to understand the relationships between these factors and BGD.

DISTRIBUTION OF *FLAVOBACTERIUM PSYCHROPHILUM* IN WILD ADULT SALMONIDS RETURNING TO RIVERS IN HOKKAIDO, NORTHERN JAPAN

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Beginning in 1990, bacterial coldwater disease (BCWD) has been associated with mass mortality among coho salmon (*Oncorhynchus kisutch*) reared at hatcheries in Miyagi and Iwate prefectures of Japan. Since that time, the occurrence of BCWD has increased among cultured coho salmon and rainbow trout (*O. mykiss*), but there has been no information about this disease among the important populations of wild salmonids that inhabit the rivers in the island of Hokkaido in northern Japan until 2005. In 2005, Misaka et. al. detected *Flavobacterium psychrophilum*, the causative agent of BCWD, in wild chum salmon (O. keta) brood stock collected from eight rivers in Hokkaido. To further investigate the distribution of *F. psychrophilum* among other adult salmonid populations in the Hokkaido region, kidney tissues and ovarian fluid samples were obtained from adult brood stocks of wild chum salmon, masu salmon (*O. masou*), sockeye salmon (*O. nerka*), and pink salmon (*O. gorbuscha*) in the autumn of 2006. *Flavobacterium psychrophilum* was detected in chum salmon from ten rivers (Tokushibetsu, Abashiri, Shari, Iwaobetsu, Shibetsu, Nishibetsu, Tokachi, Shizunai, Chitose, and Yurappu) at the rate of 53 - 98% (average 85%), while masu salmon from three rivers (Tokushibetsu, Shari, and Shiribetsu), sockeye salmon from Abira river, and pink salmon from Shibetsu river produced bacterial isolates at the rate of 32 - 93%, 67%, and 50%, respectively. The present study indicates that *F. psychrophilum* is widely distributed in Hokkaido among wild brood stocks of many salmonid species.

A QUANTITATIVE ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) AND FILTRATION-BASED FLUORESCENT ANTIBODY TEST AS POTENTIAL TOOLS FOR SCREENING *FLAVOBACTERIUM PSYCHROPHILUM* IN BROODSTOCK

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Flavobacterium psychrophilum, the etiologic agent of coldwater disease (CWD), is a significant bacterial pathogen in salmonid aquaculture. Vertical transmission of this pathogen has been demonstrated, and it is hypothesized that disease management may be improved through broodstock screening. This study describes the development of two assays to screen broodstock kidney tissue homogenates and ovarian fluid for the presence of *F. psychrophilum*. Four mouse hybridoma clones producing monoclonal antibodies against *F. psychrophilum* (CSF 259-93) outer membrane proteins were generated. Western blot, dot blot, and ELISA assays demonstrated the specificity of these antibodies. One antibody in particular, monoclonal antibody FL-43, was reactive with 67 different *F. psychrophilum* isolates, but did not react with *Flavobacterium columnare* and a spectrum of other *Flavobacterium* species. Native FL-43 was used as a capture antibody and FL-43 conjugated to horseradish peroxidase was the secondary detection antibody in a sandwich ELISA. This assay appears to be suitable for sensitive detection and quantification of *F. psychrophilum* in infected tissue homogenate, and was capable of detecting *F. psychrophilum* at a lower boundary of 1.28×10^2 cfu/ml. Naturally infected Coho salmon broodstock (n=50 samples) had an estimated infectious load of 1.6×10^3 - 5.0×10^6 cfu/ml. The ELISA was not suitable for ovarian fluid, therefore, FL-43 was conjugated to Alexa Fluor®-488 and a filtration based fluorescent antibody test (FAT) was developed to detect *F. psychrophilum* in spiked ovarian fluid samples and from naturally infected fish. This FAT demonstrated a high incidence of *F. psychrophilum* in ovarian fluid samples collected from Coho salmon and rainbow trout. All results were confirmed by culture and nested PCR.

MINIMUM INHIBITORY CONCENTRATION TESTING OF *FLAVOBACTERIUM COLUMNARE*

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A simple, accurate and reliable microdilution method has been developed to test the susceptibility of *Flavobacterium columnare* to antibiotics. The method has been used to determine the minimum inhibitory concentration (MIC) of 23 *F. columnare* isolates. The developed method conducted at 28 °C for 48 h used standardized inoculum, Mueller Hinton broth at 1/5 the full strength (4g/l), reference isolate, a positive and a negative control wells and a standardized custom made microtitre plates, Sensititre® Susceptibility Plates for Aquaculture (Trek Diagnostic Systems, Inc.). *Escherichia col*i, ATCC25922 was used as the reference isolate and produced MIC values within the range published by the Clinical and Laboratory Standards Institute (CLSI), formally the National Committee for Clinical Laboratory Standards (NCCLS). Mueller Hinton broth at 1/5 the full strength (4 g/l) was found to support significantly better growth for *Escherichia coli*, ATCC25922 and *F. columnare* type strain, ATCC23463, than Mueller Hinton broth at 1/7 the full strengt**h.**

EFFECT OF BIOFILM FORMATION ON ANTIMICROBIAL RESISTANCE OF *FLAVOBACTERIUM PSYCHROPHILUM*

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The use of antimicrobial agents in finfish aquaculture has resulted in the emergence of antibiotic resistant aquatic bacteria, in an increase of antibiotic resistance in fish pathogenic bacteria and in alterations of the bacterial flora. In aquaculture, the treatment of bacterial fish diseases can be complicated by the development of resistant bacterial biofilms. Pathogenic bacteria found in these biofilms cause recurrent exposure of fish to infections and the presence of asymptomatic carriers. Biofilms constitute a protected mode of growth that allows bacteria to survive in hostile environments and provide an ideal niche for rapid exchange of bacterial DNA containing genes for antibiotic resistance. In the present study, the effect of biofilm formation on antimicrobial resistance of *Flavobacterium psychrophilum* and environmental bacterial isolates was examined. Planktonic and biofilm cells were exposed to oxytetracycline and flumequine in 96-well microtiter plates after which growth and viability was measured by plating cells onto agar plates. One *F. psychrophilum* strain was further used to study the development of antimicrobial resistance in biofilm cells. The results suggest that in high bacterial densities, cells of *F. psychrophilum* forming biofilms are less susceptible to the two antimicrobials. Furthermore, biofilm cells of *F. psychrophilum* may rapidly develop resistance to both oxytetracycline and flumequine if exposed to subinhibitory concentrations of these antimicrobials.

RAINBOW TROUT INNATE IMMUNITY AGAINST *FLAVOBACTERIUM PSYCHROPHILUM*.

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Flavobacterium psychrophilum infection is associated with significant loss of rainbow trout production in the U.S. and other parts of the world. In 2005, a selective breeding program was initiated at the National Center for Cool and Cold Water Aquaculture to improve rainbow trout innate resistance against *F. psychrophilum* challenge (See Silverstein et al. Abstract). After an initial evaluation at ~2 g size, several resistant and susceptible families were chosen for further study. Cohorts were raised until their average weight was10 g size and 800 g and then naive fish were challenged with strain CSF 259-93. Six of the eight families maintained their original relative resistant or susceptible phenotypes indicating that these are stable family traits as fish increase over 300-fold in size. Inflammatory cytokine, IL-1β and TNF-α1, mRNA levels were significantly lower in spleen tissue of resistant trout compared to susceptible trout. Interestingly, naive and challenged resistant fish had a significantly larger spleen-somatic index (spleen weight normalized to total weight) as compared to treatment-matched susceptible fish at days 1, 3 and 5 post-challenge. This observation led us to test whether spleen size would predict innate resistance in an unrelated cohort of fish from another brood year (2006). Average spleen indices were surveyed in 103 families, and fish from twelve families were pooled into groups having large (SI=1.46), medium (SI= 1.10), and small (SI= 0.73) spleen-size. Each of the three groups was challenged with *F. psychrophilum* or *Yersinia ruckeri*. Consistent with our previous observations, trout with larger spleens were significantly more resistant to *F. psychrophilum* challenge. However, this result was pathogen specific as there was no correlation between spleen size and survival following *Y. ruckeri* challenge. Histological analyses identified that there were higher hemosiderin levels, and a higher frequency of melanomacrophages in the spleens of resistant fish. To our knowledge, this is the first report of a positive, and pathogen-specific, association between innate immunity, spleen phenotype and cytokine response in a teleost fish. These results also suggest that selective breeding may be a promising and complementary approach to reduce loss from bacterial coldwater disease.

DIAGNOSTICS AND VACCINE DESIGN FOR *FLAVOBACTERIUM PSYCHROPHILUM*

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Several yellow-pigmented species within the family Flavobacteriaceae are commonly associated with diseases in fish. Of these, *Flavobacterium psychrophilum* has emerged as a major pathogen, primarily in salmonid aquaculture, however many non-salmonid species are also affected. Paramount to controlling disease is the ability to easily discriminate, and reliably identify the causative agent, especially in the field, but also in vaccine trials. We have developed several methods for the identification of *F. psychrophilum*, based on RAPD PCR, latex bead agglutination and discriminating growth media. We are developing immersion and injectable vaccines against *F. psychrophilum* for fry and broodstock. Our approach to recombinant vaccine design involves the selection of a cocktail of protective protein antigens, immune modulators and optimal adjuvants for each vaccine. Much effort has been devoted to the optimization of large-scale protein expression and purification, vaccine formulation and delivery.

A MODIFIED LIVE VACCINE AGAINST *FLAVOBACTERIUM COLUMNARE*

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Flavobacterium columnare is an aquatic bacterium that is highly infectious in both warm and cold water species of fish. In the channel catfish (*Ictalurus punctatus*) industry it causes columnaris disease and has a significant impact on production (~$30 million annually). Development of a successful vaccine against columnaris required the vaccine to be safe and easily administered to young fish. The vaccine also needed to stimulate protective immunity of long duration. We developed a modified *F. columnare* isolate by passage on an antibiotic (rifampicin). Changes in the lipopolysaccharide (LPS) profile were shown by immunoblots that revealed the higher molecular weight bands of LPS were absent in the rifampicin modified isolate. The modified *F. columnare* was attenuated and did not cause disease. In vivo reversion to virulence studies (back-passage studies) demonstrated the vaccine to be safe at ten times the normal immunization dose in 10 day post hatch channel catfish fry. Efficacy of the modified live *F. columnare* vaccine was demonstrated in 10 and 48 day post hatch (RPS from 71-94%) and 3 month old channel catfish. Recently, we demonstrated vaccine efficacy in eyed channel catfish eggs immunized 24 to 48 hours prior to hatching. Relative percent survivals ranged from 50-77 % following challenge of the resulting immunized fry at greater than 100 days post vaccination. Intervet, Inc., licensed the vaccine from USDA-ARS (CRADA), obtained USDA-APHIS-CVB approval and is marketing the vaccine under the trade name AQUAVAC-COL™[1].

[1] Mention of trade names or commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the United States Department of Agriculture.

RECOMBINANT FspA, A SURFACE ANTIGEN FROM *FLAVOBACTERIUM PSYCHROPHILUM,* INDUCES PROTECTIVE ANTIBODY RESPONSES IN RAINBOW TROUT

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Flavobacterium psychrophilum is the causative agent of bacterial cold water disease (CWD) and rainbow trout fry syndrome (RTFS). Coldwater disease is a rapidly growing concern in salmonid aquaculture worldwide. In the United States, CWD and RTFS are prevalent in the Pacific Northwest where coho salmon, rainbow trout and steelhead trout from commercial and conservation hatcheries suffer the greatest losses. Control and prevention of the disease is currently being addressed with the use of antibiotics. At present, no vaccines exist against *F. psychrophilum* and up until very recently nothing was known about *F. psychrophilum* antigen candidates that could be used as subunit vaccines. Here we have produced recombinant FspA, a surface *F. psychrophilum* antigen, and tested its ability to induce protective antibody responses in passive immunization studies. A fragment of FspA encoding for a 195 amino acid protein was expressed in *E. coli*. The recombinant protein was refolded and injected intraperitoneally into fish (10 ug/fish) with Freunds Complete Adjuvant (FCA). Antiserum from immunized fish contained high anti - FspA antibody titers (3200-25600) and recognized both the recombinant protein as well as the surface of *F. psychrophilum* bacterium. Passive immunization studies (n = 125/per group) showed that antiserum from immunized fish protected animals challenged with *F. psychrophilum (*strain, CSF 259-93). Thus, antiserum containing the highest anti - FspA titers (25,600) induced the highest protection (~67% relative percentage survival [RPS]). In contrast, animals passively immunized with non-immune sera suffered 66-82% mortality. These results strongly suggest that recombinant FspA induces a good antibody response that is responsible for conferring immunity to infected animals. This is the first time that a recombinantly produced *F. psychrophilum* antigen is shown to induce protective antibodies. Our results suggest that FspA is a good antigen candidate that may be used in the future as subunit vaccine.

FLAVOBACTERIUM: HISTORY OF A GENUS

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This presentation aims at explaining the history and evolution of the genus *Flavobacterium* since its original description in 1923. Flavobacterium is a striking example of the disruption of conventional bacterial taxonomy that resulted from the introduction of molecular techniques. The scant original description had let widely different organisms be grouped in the genus. Most of them had been reclassified in other or new genera following successive emendations, and the genus seemed to have reached a reasonable level of phenotypic homogeneity in the early nineties, when techniques for analyzing the 16S rRNA gene were introduced. They have demonstrated (i) that most species grouped in the genus *Flavobacterium* were actually quite distant from the type strain of the genus, *F. aquatile*, and that their reclassification was necessary; and (ii) conversely, that a number of organisms previously assigned to the genera *Cytophaga* and *Flexibacter* were actually closely related to *F. aquatile*. As this species had to remain the type species of the genus, these organisms had to be reclassified as Flavobacterium species. These results, supported by phenotypic and chemotaxonomic (fatty acid and whole-cell protein profiles) data, allowed for a thorough emendation of the genus description and for the description of the family Flavobacteriaceae in 1996.

As a result of this emendation, the genus *Flavobacterium* comprised 10 species. Since then, it has progressively grown to encompass more than 40 validly described species representing an interesting variety of ecological niches and ways of life, including the fish pathogens *F. branchiophilum*, *F. columnare*, and *F. psychrophilum*. Without any doubt, the genus *Flavobacterium* will continue to evolve. As culture-dependent and -independent investigations of bacterial communities have revealed Flavobacterium strains in various environments, new species will keep being described. However, as some of the species proved to share only moderate 16S rRNA gene sequence similarity values with other members of the genus, they may be removed from the genus and reclassified in the future. It is for instance the case of *F. columnare.*

FLAVOBACTERIUM PSYCHROPHILUM IS COMPOSED OF TWO DISTINCT GENETIC LINEAGES

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Flavobacterium psychrophilum is the etiologic agent of bacterial coldwater disease, which is a significant disease for both commercial and conservation aquaculture. We used suppression subtraction hybridization to examine genetic differences between virulent (CSF 259-93) and non-virulent (ATCC 49418) strains to identify virulence factors and potential vaccine targets for this organism. Virulence in this study was defined by the outcome of an in vivo challenge model. During the course of this work we also employed comparative microarray hybridizations to examine the distribution of gene fragments amongst a battery of isolates representing a worldwide distribution. This analysis found two distinct genetic lineages for this pathogen. One lineage was most closely associated with disease in Pacific salmon while the other lineage was most closely associated with disease in rainbow trout. This finding has since been independently corroborated by polymorphisms in 16S rRNA loci, mixed-genome microarray hybridizations, differential restriction enzyme methylation systems, pulsed-field gel electrophoresis, and using a novel microsatellite assay. Phenotypic characteristics including elastin hydrolysis and tetracycline resistance also correlate with the lineage subdivisions. These data lead to two hypotheses whereupon lineage subdivision is associated with either host-specificity or ecological association. A recent challenge study provided data to reject the host-specificity hypothesis. The unique life-history strategies of salmon and rainbow trout are consistent with lineage differences being associated with an ecological origin.

CLONAL COMPLEXES AND RECOMBINATION IN *FLAVOBACTERIUM PSYCHROPHILUM* AS REVEALED BY MULTI-LOCUS SEQUENCE ANALYSIS.

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We will report the first results on the investigation of the population structure of the salmonid pathogen *F. psychrophilum* using multi-locus sequence typing (MLST). This method is becoming very popular with the availability of complete genome sequences and consists of sequencing a limited number of loci representative of the conserved core genome to characterize the isolates. Compared to traditional molecular typing methods such as RAPD or RFLP, one of its advantages is to produce highly reproducible data allowing rapid and global comparisons between results from different laboratories. MLST data are also directly amenable to population genetics and phylogenetic analyzes as well as to epidemiological studies. Our preliminary results, obtained on a limited number of isolates, already revealed the existence of a few clonal complexes responsible for most outbreaks in fish-farms. These clonal complexes tend to have a worldwide distribution and to be associated with different fish species. Beside, a great genetic diversity within the species was also revealed when isolates collected from various environments were considered. Intra-species recombination played a great role in generating this diversity. We will finally discuss the advantages and limits of the MLST approach in the context of Flavobacterium studies and emphasize the expected benefits of adopting a standardized typing scheme.

RAPID DETECTION OF *FLAVOBACTERIUM COLUMNARE*, THE BACTERIUM CAUSING COLUMNARIS DISEASE IN FISH

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Flavobacterium columnare is a ubiquitous bacterium that causes columnaris disease in a wide variety of fish resulting in devastating losses particularly in the commercial aquaculture industry worldwide. Timely diagnosis of disease is imperative for prevention of spread and to reduce the economic loss to fish farmers. Two diagnostic tests for the rapid detection of *F. columnare* in infected fish were developed. An indirect immunofluorescenece (IFA) test with dual spectral properties was compared with bacteriological culture (accepted standard) for simultaneous detection of *Edwardsiella ictaluri* (the cause of enteric septicemia of channel catfish) and *F. columnare*. A total of 303 samples (derived from kidney, brain and nares) from 101 experimentally infected fish examined concurrently by IFA and culture, revealed that the IFA test compared favorably in sensitivity (EI = 80.7%; FC=87.2%) and specificity (EI = 83.9%; FC=88.9%) with the standard culture.

In a separate experiment, the sensitivity and specificity of a TaqMan real-time polymerase chain reaction (RT-PCR) targeting a 113 bp nucleotide region of the chondroitin AC lyase gene of *F. columnare* was evaluated. Specificity of the assay evaluated with 20 isolates of *F. columnare* and 15 other taxonomically or ecologically related bacteria revealed that the primers and probe were 100% specific for detection of *F. columnare*. In tissues (blood, gills and kidney) of *F. columnare* experimentally infected fish, the bacterial numbers assessed by RT-PCR ranged from 3.4×10^0 to 9.5×10^5 CFU/ml. The diagnostic assays developed are sensitive and specific for detection of *F. columnare* in infected fish.

IMMUNIZATION OF RAINBOW TROUT WITH A LOW MOLECULAR FRACTION PROTEIN EXTRACTED FROM *FLAVOBACTERIUM PSYCHROPHILUM*

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Flavobacterium psychrophilum was isolated from rainbow trout (*Oncorhynchus mykiss*) in Finland for the first time in 1993. Since then, it has become a widespread fish pathogen in Finnish aquaculture, especially in rainbow trout farms. We investigated the immunogenic efficacy of a crude low molecular fraction (LMF) protein, extracted from the surface of *F. psychrophilum*, in rainbow trout under laboratory conditions. The LMF protein, with an approximate molecular weight of 25-33 kDa, was found in several different serotypes of *F. psychrophilum*, both virulent and avirulent, but not in other well known fish pathogenic bacteria. Two independent intraperitoneal immunization trials were carried out using LMF with and without Freund´s Complete Adjuvant (FCA). The first trial included LMF (two different concentrations) with FCA, a FCA control and a saline control. The second trial included LMF (one concentration) without FCA and two treatments with formalin-inactivated and sonicated *F. psychrophilum* cell preparations without FCA and a saline control. In this trial a booster immunization with corresponding preparations was given to the fish in all groups six weeks after the initial immunization. Specific plasma antibody titers against *F. psychrophilum* were analyzed with an indirect enzyme-linked immunosorbent assay (ELISA) and the efficacy of the immunizations was determined by an intramuscular challenge with *F. psychrophilum*. Rainbow trout immunized with LMF and FCA in the first trial showed elevated antibody titers three weeks after immunization and the titers increased furthermore during the following three weeks. The immunized fish also showed a significant protection against the challenge with *F. psychrophilum*. The FCA by itself did not enhance the antibody response in the injected fish nor induce a protection against the artificial infection. Rainbow trout immunized twice with LMF without adjuvant in the second trial did not, however, show an increased antibody response and the fish were not protected from the challenge. In contrast to the LMF treatment, rainbow trout immunized with formalin-inactivated and sonicated *F. psychrophilum* cells did show significantly increased antibody titers three weeks after the initial immunization. The booster immunization did not increase the antibody response, but the fish were highly protected from the challenge with *F. psychrophilum*. These results suggest that isolated surface proteins from *F. psychrophilum* only enhance a protective immune response in rainbow trout when combined with oil-based adjuvants, e.g. FCA, and not when administered in water based solutions. Formalin-inactivated and sonicated *F. psychrophilum* cell preparations triggered the antibody production and protected the rainbow trout from an artificial infection more efficiently than the LMF without the adjuvant, suggesting that whole and sonicated cell preparations would be better candidates for an immersion vaccine against RTFS than extracted proteins.

CELL-SURFACE PROTEINS INVOLVED IN *FLAVOBACTERIUM JOHNSONIAE* GLIDING MOTILITY

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Cells of the gliding bacterium *Flavobacterium johnsoniae* move rapidly over surfaces by an unknown mechanism. Previous genetic analyses identified 12 Gld proteins that are required for motility. These proteins are thought to form a complex in the cell envelope but surprisingly, none of them appear to be exposed on the cell-surface. We hypothesize that the cell-surface components of the machinery are redundant and that the genes encoding these proteins were not previously identified because only completely nonmotile mutants were analyzed. To identify redundant cell-surface components of the machinery HimarEm1 induced mutants with partial motility defects were isolated. Three contiguous genes (sprC, sprD, and sprB) that encode abundant cell-surface proteins were identified. Mutations in any of these genes or a deletion spanning the entire region resulted in cells that failed to form spreading colonies on agar but that exhibited rapid gliding on glass in wet mounts. Antibodies raised against SprB inhibited motility of wild-type cells, confirming the importance of SprB in gliding. Latex spheres carrying anti-SprB were rapidly propelled along wild-type cells, whereas spheres lacking antibody did not interact with cells under the conditions used. Cryo-EM analysis revealed fibrils on the surface of wild-type cells that were absent from cells of the sprCDB deletion strain CJ1584. SprB may be part of adhesive fibrils that contact the substratum and are propelled by the Gld protein motility machinery during cell movement. Cells with mutations in sprB and in sprF (a homolog of sprB) were much more severely impaired in motility than cells with single mutations in either gene, confirming the redundant nature of the outermost components of the motility apparatus.

FLAVOBACTERIUM JOHNSONIAE GLIDING MOTILITY: MECHANISMS AND MYSTERIES

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Cells of the gliding bacterium *Flavobacterium johnsoniae* move rapidly over surfaces by an unknown mechanism. Genetic analysis identified 24 genes involved in gliding. Twelve Gld proteins are thought to be essential components of the 'motor' in the cell envelope. Among these, GldA, GldF, and GldG comprise an ATP-binding cassette transporter that may have a role in assembly or functioning of the motility apparatus. Many of the other Gld proteins required for motility are not similar in sequence to proteins of known function, and their exact roles in cell movement remain unknown. All of the Gld proteins appear to localize to the cell envelope and six of them are lipoproteins, but none are exposed on the cell surface. Immuno-electron microscopic analysis demonstrated that GldJ is arrayed helically in the periplasm. Genetic and biochemical experiments indicated that many of the Gld proteins interact. Six of the remaining motility genes encode redundant cell-surface proteins. SprB and SprF appear to be interchangeable cell-surface adhesins that may function as the outermost components of the machinery. SprF has a lectin-like domain that may bind carbohydrates. Antibody against SprB inhibited motility, and spheres coated with anti-SprB attached to cells and moved rapidly along them suggesting that SprB is propelled by the Gld motor. Mutations in genes predicted to be involved in exopolysaccharide synthesis or export also compromised motility. Cryo-electron microscopy revealed fibrils on the surface of wild-type cells that were absent from cells of a nonmotile gldFG mutant, and of an sprCDB deletion mutant. A model of *F. johnsoniae* gliding is beginning to emerge. In this model the Gld proteins constitute the 'motor' in the cell envelope that converts chemical energy into movement. The motor is connected to cell surface adhesive fibrils composed of proteins such as SprB and SprF. These fibrils are propelled along the cell surface. Cells may express different adhesins to allow movement on different types of surfaces. Exopolysaccharides might enhance motility on some surfaces by coating the substratum and interacting with the adhesive fibrils.

UNIQUE TRANSCRIPTIONAL SIGNALS IN UBIQUITOUS BACTEROIDETES

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Studies on molecular regulatory mechanisms in bacteria of the phylum Bacteroidetes are underdeveloped due to lack of genetic tools and poorly characterized genetic elements. Genes expressed in Bacteroidetes are usually not expressed when transferred into proteobacteria, whilst proteobacterial genes (antibiotic resistance genes, genes required for plasmid replication, and others) are not expressed in Bacteroidetes. To address these deficits, we developed a promoter-trap technique using a green fluorescent protein reporter system. A library of 9,000 clones containing chromosomal fragments of *F. hibernum* strain W22 in pSCH03 was screened for their ability to drive expression of the promoterless *gfpmut3* gene. Twenty strong promoters were identified for further study. The transcription start points were determined from seven promoter clones by the 5' rapid amplification of cDNA ends technique. Promoter consensus sequences from *Flavobacterium* were identified as TAnnTTTG and TTG, where *n* is any nucleotide, centered approximately 7 and 33 bp upstream of the transcription start site, respectively. A putative novel ribosome binding site consensus sequence is proposed to be TAAAA, identified by alignment of the 20-bp regions upstream of the translational start site in 25 genes. The majority of identified *Flavobacterium* promoter elements showed homology to those of other Bacteroidetes, but not of proteobacteria, and they functioned poorly in *E. coli*. In order to analyze systematically promoter structure, we measuring the effects of site-directed mutations on the –33 consensus element, –7 consensus element, and spacer length on activity of the *Flavobacterium ompA* promoter. Most of the base substitutions in these regions caused large decreases in promoter activity, allowing us to define the context of the *ompA* promoter and preferred bases at specific positions. The optimal –33/-7 motifs (TTTG/TAnnTTTG) found here are identical to *Bacteroides fragilis* σ^{ABfr} consensus –33/-7 promoter elements, but lack similarity to the *E. coli* σ^{70} promoter elements. The length of the spacer separating the –33 and –7 motifs of *ompA* promoter also had a pronounced effect on the promoter activity, with 19 bp being optimal. The non-conserved sequences in the spacer region and in regions close to the consensus motifs were randomized in order to determine their importance for promoter activity. We found that in addition to the consensus promoter elements and spacer length, GC content of the core promoter sequences had a pronounced effect on *Flavobacterium* promoter activity.

BIOFILM FORMATION BY *FLAVOBACTERIUM JOHNSONIAE*

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Many bacteria have the ability to grow attached to a surface in a biofilm, an assemblage of bacterial cells enclosed in a polysaccharide. Biofilm formation has several benefits to the bacteria including close association with each other, maintenance of a favorable ecological niche, and protection from physical forces, toxins, and/or other cells. Flavobacteria have been shown to exhibit gliding motility on surfaces, and, as surface associated organisms, may be expected to form biofilms. The ability of several species of *Flavobacterium* to form biofilms was tested under various conditions such as media type, media concentration, media content, incubation temperature, and incubation length. *Flavobacterium johnsoniae* wild type strains (UW 101 and FJ 1) that are good chitin digesters produced robust biofilms; whereas those that do not digest chitin well (MM 101 and CJ 101) did not produce biofilms. The speed of formation and density of the biofilm increased with dilution of the media. Biofilms began forming after about 8 hours of incubation and were only about twice as dense after 5 days of incubation. Non-motile mutants (also chitin-degestion deficient) did not produce biofilms, while motile non-spreading mutants did form biofilms.

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DIRECTIONS TO THE
NATIONAL CENTER FOR COOL AND COLD WATER AQUACULTURE
11861 LEETOWN ROAD
KEARNEYSVILLE, WV 25430

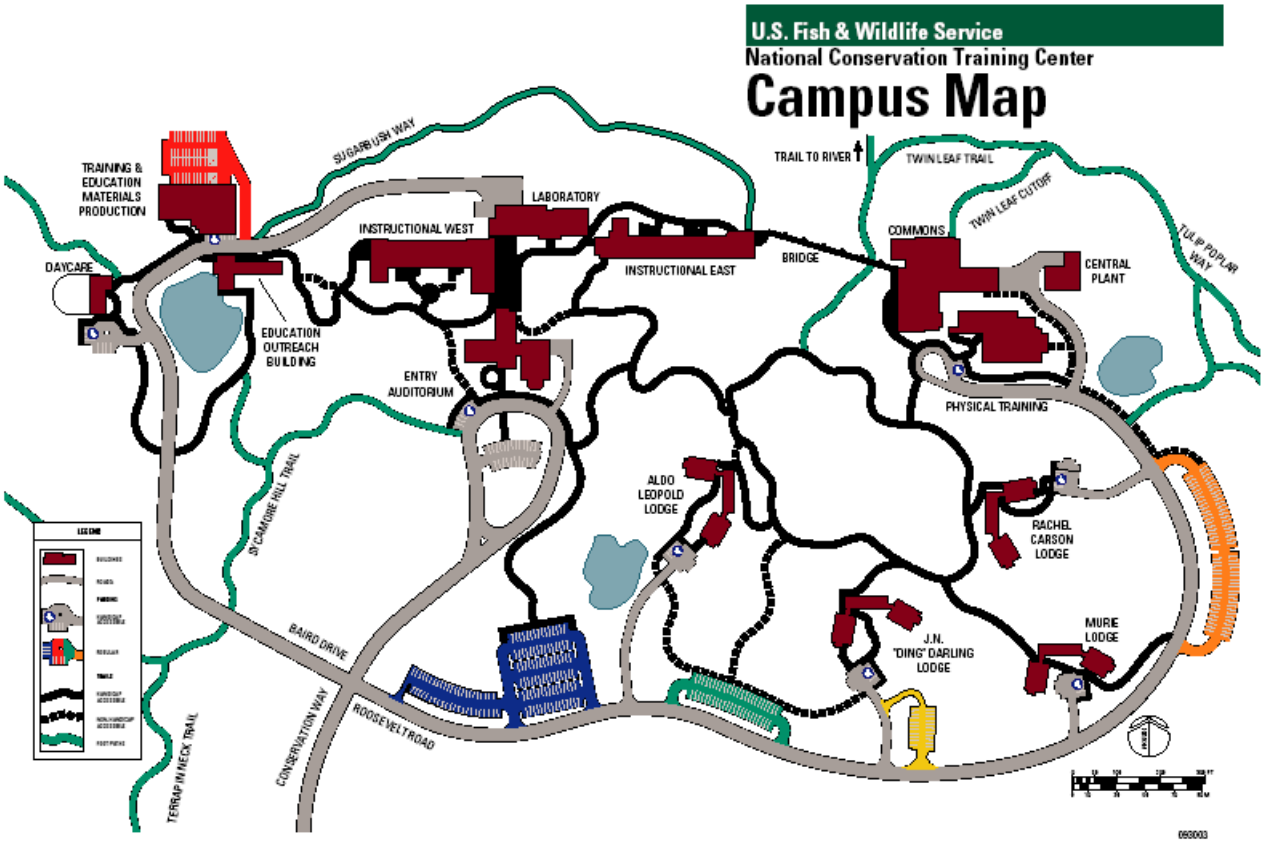
Leaving NCTC (U.S. Fish & Wildlife National Conservation Training Center) at end of driveway turn left onto Shepherd Grade Road.

Go approximately 3 – 3 ½ miles until you come to a stop sign. At the stop sign turn/bear to the right onto Route 480

Follow Route 480 to the next stop sign. Go straight (approximately 5 miles) until you come to a 4 way stop light. (Sheetz will be on your right and BC&T bank will be across the street on the left.

Continue to go straight through the light (480 changes to Leetown Road) go approximately 4 miles. The National Center for Cool and Cold Water Aquaculture will be on your right hand side. It's a big building with a green roof. There is a sign in front of the building.

If you have any problems please phone: 304-724-8340.


U.S. Fish & Wildlife Service
National Conservation Training Center
Campus Map
TRAINING & EDUCATION MATERIALS PRODUCTION
SUGARBUSH WAY
TRAIL TO RIVER
TWIN LEAF TRAIL
TWIN LEAF CUTOFF
TULIP POPLAR WAY
LABORATORY
INSTRUCTIONAL WEST
INSTRUCTIONAL EAST
BRIDGE
COMMONS
CENTRAL PLANT
DAYCARE
EDUCATION OUTREACH BUILDING
ENTRY AUDITORIUM
PHYSICAL TRAINING
ALDO LEOPOLD LODGE
RACHEL CARSON LODGE
SYCAMORE HILL TRAIL
BAIRD DRIVE
J.N. "DING" DARLING LODGE
MURIE LODGE
CONSERVATION WAY
ROOSEVELT ROAD
TERRAPIN NECK TRAIL
LEGEND