The Genomic, Phenotypic, and Immunologic Characterization of Flavobacterium fallonii sp. nov.

Presented to the faculty of Lycoming College in partial fulfillment of the requirements for Departmental Honors in

Biology

by
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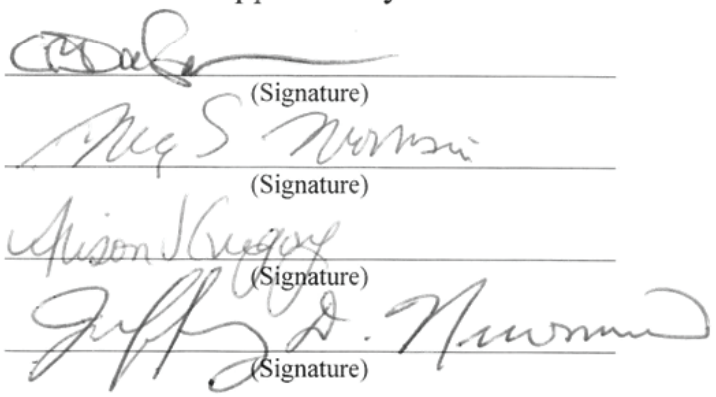
Approved by:



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I. Abstract:

Three yellow-orange pigmented, Gram negative, rod-shaped, motile bacterial strains, designated strains JRM, KMS, and AJR, were isolated from a creek in north-central Pennsylvania during December of 2013. Comparative 16srRNA sequences identified the closest matches as *Flavobacterium hibernum* and *Flavobacterium hydatis*. Full genome sequencing of the three isolates and the reference type strains was completed for comparative genomic analysis. Based on the genotypic and phenotypic results of this study, strains JRM, KMS, and AJR represent a novel species within the *Flavobacteriaceae* that will be tentatively named *Flavobacterium fallonii*. To further compare the organisms, polyclonal antiserum against *Flavobacterium fallonii* JRM was made and demonstrated cross reactivity within the species *Flavobacterium fallonii* and with the reference organisms.

II. Introduction:

DNA-DNA Hybridization

From the invention of the microscope and Leeuwenhoek's first look at the world of microbes until the middle of the 20th century, bacteria were classified by phenotypic characteristics utilizing physical attributes of the organism and biochemical test results. This system stayed relatively intact until the 1950s, when the introduction of nucleic acid analysis and the increase in computational technology made a significant impact in microbe taxonomy. A technique discovered in the late 1950s called DNA-DNA hybridization improved over the next few decades and became a crucial experiment for comparing genomes between microbes.

The first use of DNA-DNA hybridization in bacterial taxonomy was published in 1961 by C.L. Schildkraut (Schildkraut et al., 1961). DNA-DNA hybridization is still the gold standard for species delineation in bacterial taxonomy (Goris et al., 2007). The basis of DNA-DNA hybridization stems from the relatively weak hydrogen bonds connecting double stranded DNA molecules and the idea that more closely related organisms will have more complementary base pairs in their genomes. Once isolated, the DNA from two different organisms is heated to in order to break the hydrogen bonds between the complementary base pairs of the two strands. Once in single strand form, the mixture of DNA between the two organisms is able to slowly cool. Complementary strands will begin to re-anneal as the hydrogen bonds reform (Brenner et al., 1969). The resulting DNA hybrid is then re-heated. Since the DNA hybrid is not a perfect match, the temperature that the DNA separates should be lower than the original melting temperature as less energy needs to enter the system to break the hydrogen bonds. A matrix using the thermal stability or the hybrid re-association can be used to make determinations about the relatedness of the two species (Mora, Urdiain, and Lopez, 2011). A value of 70% was proposed as the basis for species differentiation (Wayne et al., 1987). However, DNA-DNA hybridization does have its pitfalls.

The experiment itself is tedious and time consuming, yields results that are not easily reproduced, and are often inaccurate. The cost of doing DNA-DNA hybridization requires either specialized instruments or contracting out to labs that still perform this test which can be expensive (Goris et al., 2007). According to quotes taken from the DSMZ website, DNA preparation for DNA-DNA hybridization costs €250 and then the cost of actually performing the hybridization is €125 (https://www.dsmz.de/) In addition, one of the major criticisms behind DNA-DNA hybridization is its inability to produce a cumulative database to be shared with the

rest of the scientific community (Rosellò-Mora, Urdiain, and Lopez, 2011). Although DNA-DNA hybridization is the current standard for bacterial taxonomy, there is need to replace this practice with a more accurate and reproducible measure accounting for the advances in genomic technologies (Stackebrandt et al., 2002).

rRNA Analysis

In the late 1970s, another important breakthrough came in cataloging and comparing ribosomal ribonucleic acids (RNA). In particular, the 16S rRNA gene sequence quickly became an extremely useful molecular sequence in determining phylogenetic relationships (Mora and Amman, 2001). The 16S rRNA gene is a universal genetic marker as it is an essential gene derived from a common ancestor, highly conserved, and very genetically stable (Henz et al., 2005). All organisms have a 16S rRNA gene sequence, so it can be used to determine relationships between organisms. The small subunit rRNA sequence was originally proposed as a phylogenetic marker in 1987 (Woese, 1987). The gene itself consists of about 1,500 bases, which makes it a practical size for sequencing easily. In addition, it also provides enough variability in sequence and is large enough in size for bioinformatics (Janda and Abbott, 2007). In 2006, the proposed threshold for delineating species increased from 97% to 98.5% as this value correlated to a 70% DNA-DNA hybridization value (Stakenbrandt and Ebers, 2006). Although widely accepted, the threshold of 98.5% similarity is still a soft value. In 2014, a cutoff of 98.7% pairwise similarity was proposed as the new species classification for 16S rRNA sequences in correlation with average nucleotide identity (Kim et al., 2014).

Despite the ease and universal nature of 16S rRNA sequencing it still is not the ideal method for taxonomically classifying bacteria. For example, type strains *Bacillus globisporus*

and *Bacillus psychrophilus* have 16S rRNA sequences are over 99.5% identical. This would make them the same species using the proposed 98.7% species threshold. However, when DNA-DNA hybridization is performed, the two species only have a 23% to 50% relatedness value, which is far below the 70% gold standard. Also, when comparing organisms based on16S rRNA sequence, there is no hard value for distinguishing organisms at the genus level (Janda and Abbott, 2007). However, further improvements in genome sequencing has provided innovative ways to compare bacteria.

Whole Genome Analysis

The ability to classify organisms using their entire genome has increased dramatically as genome sequencing technology has advanced. The reduced cost of sequencing full genomes and the access to computer programs to help to organize and analyze this data has allowed for more accurate classification of new organisms, particularly in prokaryotic fields. In order to capitalize on full genome sequences and computing technology, a database called the GBPD program was invented in 2005 as a way to calculate intergenomic distances (Henz et al., 2005). Building on this idea, a useful free online tool was released in 2010 on the DSMZ website that calculated DDH (DNA-DNA hybridization) *in silico* (performed on a computer) (Auch et al., 2010). Comparing organisms on a computer was much more cost effective than performing physical DNA-DNA hybridization. For the amount of money spent on contracting a physical DNA-DNA hybridization, two organisms could have their whole genome sequenced and compared via online tools such as the Genome-Genome Distance Calculator (Meier-Kolthoff et al., 2013). The proposed DDH value corresponding to the species level is still 70% using this method.

A second *in silico* method for genomic comparison is called Average-Nucleotide-Identity (ANI). This test has been called the "*possible next generation gold standard for species delineation*" due to its widely accepted use in the prokaryote taxonomist community (Kim et al., 2014). ANI is an algorithm first released in 2005 that compares shared genes between two organisms (Konstantinidis and Tiedje, 2005). Unlike eDDH, ANI examines the similarity of the sequences themselves, and not their ability to hybridize. This fact can be seen when comparing the two algorithms. An ANI value of 95% correlates to a 70% DDH value (Konstantinidis and Tiedje, 2005). While it is a prominent and heavily used parameter for species comparison, ANI does not have the capability to compare organisms that are distantly related. Distantly related organisms would not be expected to have high levels of similarity in their nucleotide sequences which would result in low ANI values. ANI values that appear lower than 60% have been declared insignificant (Kim et al., 2014).

A third *in silico* method for genetic comparison called Average Amino Acid Identity (AAI) is similar to ANI in that it compares similarities in the amino acid sequences themselves and not probability of hybridization. AAI is calculated by examining protein-coding genes between whole genomes in pairwise comparisons. The calculated value represents the similarity of the amino acids found in conserved genes (Thompson et al., 2013). Similarly to ANI, AAI values of <95% correlate to a 70% DDH value. Although not as widely accepted as ANI, AAI does have the strategic advantage of being able to compare organisms that are more distantly related (Thompson et al., 2013). However, although technology has advanced by way of whole genome sequencing and computational comparisons, many wet-lab type experiments are still required in this field.

The Bacteriological Code

Along with genomic technology for classifying novel species, other methods of classification are required. To classify new species of bacteria, the procedure uses comparative testing of the novel species to its closest genetic relatives both phenotypically and genetically. This procedure has been outlined and revised in what is called the Bacteriological Code (Tindall et al., 2010). The Code was first released in 1958, with major revisions coming in 1975 and then again in 1990, as a guideline for the publication, classification, and nomenclature of a new prokaryotic species (Lapage et al., 1990). The Bacteriological Code requires specific phenotypic tests that must be submitted: the 16S rRNA sequence, deposition in two public culture collections on two different continents, and for the 16S rRNA similarity to be >97% to a reference organism, another form of genomic comparison (Tindall et al., 2010). However, despite being outdated in phylogenomic guidelines, the Code is still followed extremely closely by many prokaryotic taxonomists.

Introduction to Flavobacterium

For the purpose of this study, the physical tests required by the Code were performed along with genomic comparison. Reference organisms were chosen by coupling with the comparison of 16S rRNA sequences and their proximity on a neighbor joining phylogenetic tree. All of the aforementioned parameters for categorizing prokaryotes by *in silico* methods were also performed between *Flavobacterium fallonii JRM* ^{T}, strains *Flavobacterium fallonii KMS* and *Flavobacterium fallonii AJR,* and reference species. Results showed that the closest species to *Flavobacterium fallonii JRM*^{T} are *Flavobacterium hibernum* and *Flavobacterium hydatis*.

Flavobacterium are Gram negative, non-spore forming bacteria that are aerobic and have an optimal growing temperature between 25-35 degrees Celsius. The genus *Flavobacterium* has cells pigmented by carotenoid and/or flexirubins, giving a yellow/orange coloration. Bacteria in this family have been shown to produce both pigments; usually, carotenoid pigments are produced by marine *Flavobacterium* while flexirubins are more common in bacteria found in soil or freshwater environments (Bernardet et al., 2002). The family *Flavobacteriaceae* was first proposed by Jooste in 1985, and was included in the First Edition of Bergey's Manual of Systematic Bacteriology in 1923. However, the description of the family was emended by Bernardet in 1996 to include that the family *Flavobacteriaceae* contains rod shaped cells that are short to moderately long with rounded or slightly rounded ends (Bernardet et al., 2002).

As previously stated, *Flavobacterium* are classified into a category known as Gram negative. Gram staining is a classification procedure developed over 130 years ago by Danish scientist Hans Christian Gram to differentiate the two main classes of microbes (Gram, 1884). Most bacteria stain either Gram positive or Gram negative based on the physical structure of their cell wall. All bacterial cell envelopes utilize a multilayered structure to provide protection from the surrounding environment as well as to maintain homeostasis. Gram positive bacteria have a thick layer of peptidoglycan that retains the crystal violet used in the first stain. Gram negative bacteria do not retain the crystal violet stain since they have a much thinner wall of peptidoglycan and are instead counter-stained with a chemical called safranin. This causes the Gram positive bacteria to appear purple while Gram negative bacteria stain pink.

Gram negative bacteria have three main layers of the cell envelope: an outer membrane, a thin peptidoglycan cell wall, and a cytoplasmic membrane (Silhavy et al, 2010). Similar to other biological membranes, the outer membrane is a lipid bilayer that lightly utilizes phospholipids,

but unlike eukaryotic membranes, the outer leaflets of the outer membrane of Gram negative bacteria contain glycolipids. The most prominent glycolipid is lipopolysaccharide (LPS), which is made of a combination of fat and sugar molecules. (Silhavy et al, 2010). LPS makes up around 75% of the surface and 5–10% of the total dry weight of Gram negative bacteria (Erridge et al, 2002). This molecule has not been extensively studied in non-pathogenic species of bacteria. However, there has been an abundance of valuable information describing the potential for variation in both the sugars of the LPS, as well as the fatty acid makeup of the molecule (Tindall et al., 2010). Upon closer examination, the LPS complex is made up of three parts: an outer glycan polymer called O antigen, a middle non-repeating oligosaccharide R core, and a hydrophobic lipid A inner domain. Lipid A is a glucosamine-based phospholipid that anchors the complex to the outer membrane (Parija, 2009). Lipid A also serves as an endotoxin, which elicits a strong innate immune response when in contact with most animals.

Immune Response to Bacterial Antigens

At a basic level, the immune system can be classified into two components: the innate and the adaptive. Innate immune cells such as macrophages can detect endotoxins on TLR4 receptors. Toll-Like Receptors (TLR4) are ancient immune mechanisms that trigger the release of inflammatory proteins such as TNF-α and IL1-β (Raetz, 2002). These inflammatory compounds attract immune cells to infected areas of the body and to fight the pathogen. However, the adaptive immune system can also be invoked in response to specific antigens such as endotoxins, exotoxins, other bacterial proteins, or LPS. Since LPS are on the outer surfaces of the bacteria, these molecules make great targets for antibodies. Antibodies or immunoglobulins are glycoproteins made by immune system B cells that bind to very specific molecules called antigens. These proteins are secreted in large quantities from specialized B cells, called plasma

cells, and form one of the key components of adaptive immunity. The molecules are Y shaped and consist of two heavy polypeptide chains and two light polypeptide chains all linked by disulfide and non-covalent bonds. The heavy chains are approximately ~55kD and the light chains are ~25kD (Janeway, 2001). The key to the practicality of antibodies is their two hyper-specific antigen binding sites on the top branches of the Y. The fact that each antibody has two antigen binding sites allows for the cross linking of antigens.

There are three main functions of antibodies in the immune system: neutralization, opsonization, and stimulation of the complement system. In the process of neutralization, antibodies interfere with toxins simply by binding to the pathogens and effectively blocking the binding of the toxin to host cells (Parija, 2009). Opsonization is a mechanism where antibodies bind directly to a pathogen. The Fc region on the stem of the Y shaped protein can stimulate the uptake and destruction of the antibody-antigen complex by phagocytic cells. The third function of antibodies is to stimulate the complement system, which ultimately ends in the lysis of the bacteria through the complement cascade and the attraction of additional inflammatory cells by its soluble byproducts (Parija, 2009). In mammals, there are five different classes of immunoglobulins based on structural variations: IgG, IgM, IgA, IgD, and IgE (Lipman et al, 2005). Due to the hyper-specificity of antibodies, these proteins are extremely useful in both research and clinical laboratory settings. This degree of high affinity has led to antibodies making major contributions as reagents in the 20th and 21st centuries. The use of antibodies in diagnostic assays has made a dramatic impact on the medical field for overall health improvement.

There are two kinds of antibodies used in lab: polyclonal and monoclonal. Antibodies can be examined using measures of affinity, specificity, and avidity. The measured strength of the

antibody-to-epitope binding is called affinity. The avidity of an antibody refers to its overall binding strength to antigens presenting many epitopes. Specificity examines an antibody's ability to bind to a specific epitope in an environment containing multiple epitopes (Lipman et al, 2005).

Polyclonal antibodies (PAbs) are made by the activation of multiple B cells that all respond to specific epitopes on the pathogen. This ends up in a large quantity of antibodies all with varying degrees of binding affinity for different parts of the antigen. PAbs are most often made by injecting the antigen into rabbits, sheep, or goats. The animal responds to the pathogen, and generates antibodies against that antigen. The animal is then given booster shots with the pathogen at designated time intervals. The serum from the animal will then contain PAbs against the pathogen. The larger the animal, the more serum is produced and the easier it is to access their vascular system. Rabbits are most often used for the production of PAbs because of their low cost to house. However, it has been reported that rabbits have inconsistencies in their antigen response, so multiple rabbits must be inoculated to ensure a good result (Harlow and Lane, 1999).

On the contrary, monoclonal antibodies are the result of a single B lymphocyte clone producing antibodies (Lipman et al, 2005). Monoclonal antibodies (MAbs) were discovered as a result of a myeloma patient's sera. Seeing the potential scientific gain from monoclonal antibodies, Kohler and Milstein created a Nobel Prize winning procedure in the 1970s for producing monoclonal antibodies using fused splenic B cells and cancer cells (Kohler and Milstein, 1975). This fused cell is called a hybridoma, and once made, it can generate MAbs continuously.

In research, both polyclonal and monoclonal antibodies have their pros and cons. PAbs are far cheaper and easier to produce than monoclonals and can be made much quickly. PAbs

can be generated in several months, whereas on average, MAbs require more time. They are also usually more stable over a wider range of conditions such as pH or saline content, making them easier to work with (Lipman et al, 2005). PAbs also have the ability to target multiple epitopes on an antigen, which can increase ease of signal detection (Lipman et al, 2005). MAbs guarantee precise affinities to their epitope as a result of their homogenous makeup. This principle is valuable when conducting experiments that utilize immunochemical techniques, as more antigens can be bound in a shorter amount of time. MAbs are very important in research focusing on molecular structure and protein-to-protein interactions, but minor changes in epitope structures as a result of polymorphism, glycosylation, or slight denaturation can render MAbs ineffective because of their high specificity (Lipman et al, 2005). For this study, PAbs from two rabbits were used. For the purpose of this study, it was hypothesized that based on the genetic similarities between *Flavobacterium fallonii* isolates, polyclonal antibodies specific to whole *Flavobacterium fallonii JRM* cells will cross link strains *KMS* and *AJR* strongly, while having little cross linking effect on other genetically similar species of Flavobacteria.

Two major sets of lab experiments performed with antibodies are Western blots and Enzyme Linked Immunosorbent Assays (ELISA). Western blotting evolved from a discovery that detected DNA sequences in separated DNA fragments in gel electrophoresis called Southern blotting. In 1979, this idea was applied to the use of proteins. The Towbin lab successfully transferred proteins from the gels to nitrocellulose membranes by using an electric field (Towbin and Staehelin, 1979). A couple of years later, another improvement on the procedure created sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Burnette, 1981). Gel electrophoresis uses electric current to separate molecules based on their molecular weight, electric charge, or isoelectric point (Jensen, 2012).

The most common technique for western blotting still uses Sodium-Dodecyl-Sulfide Poly Acrylamide Gel Electrophoresis (SDS-PAGE). The SDS acts as a denaturing agent to unwind and coat proteins, giving them a uniform charge-to-mass ratio. Comparing the distance, the protein travelled in the gel to standards provides an estimate of the size of the proteins used. Using electricity, the proteins from the gels are transferred to a membrane made of nitrocellulose, polyvinylidene difluoride, activated paper, or activated nylon to create a more stable and workable medium (Towbin and Staehelin, 1979). This transfer is achieved by making a sandwich with the gel and the membrane and using electric current to pull the proteins through the porous gel onto the membrane. Since they are extremely specific, antibodies are used to check for the target proteins on the membrane. To ensure there is no background nonspecific binding of the antibodies on the membrane, it is applied in a dilute solution of protein to block all nonspecific binding sites on the membrane. A primary antibody against the target protein is then washed over the membrane with the idea that the antibody will bind only to that specific protein and nowhere else.

Both PAbs and MAbs can be used when doing a western blot. However, PAbs are more popular because they are less specific and have a greater chance of binding more of the antigen, yielding greater signals than MAbs. Also, since MAbs are so sensitive, if there was any conformational change to the antigen during the denaturation, electrophoresis, or membrane transfers, the antibody might not bind. This makes PAbs a more popular choice for western blots (MacPhee, 2009).

One of the key technical aspects to this step is determing the optimal antibody concentration for binding (Burnette, 1981). After an incubation period and a series of washes, the secondary antibody is applied to the membrane. This antibody targets the primary antibody. The

secondary antibody is linked to a radioactive, fluorescent, or chemiluminescent compound so it can be visualized. Horseradish peroxidase is a commonly used enzyme linked to secondary antibodies as it can use color changing substrate to show a visual color change (Jensen, 2012).

This concept of using antibodies linked to compounds designed to visualize the antigen is also the key principle behind ELISAs. The concept of an ELISA was developed from radioimmunoassays, which were first used in the 1940s. The modern ELISA was pioneered in 1971 by multiple research labs by modifying the procedures from radioimmunoassays (Aydin, 2015). Since then, with modifications along the way, this has become a practical laboratory technique in both research and clinical settings. ELISAs can be categorized as either a homogenous enzymatic or a heterogeneous enzymatic immunoassay. There are four main types of heterogeneous enzymatic immunoassays: Direct, Indirect, Sandwich, and Competitive ELISAs. Each of the two main groups and four types have their own pros and cons for use (Aydin, 2015).

For this study, Indirect ELISAs were performed. Indirect ELISAs were developed in 1978 as a modification of the procedure for Direct ELISAs (Lindstrom and Wager, 1978). An Indirect ELISA works by binding the target antigen to a surface such as a plastic microplate. The plates are then incubated with a primary antibody to target the desired antigen. After a few washes, a secondary antibody conjugated to an enzyme against the primary antibody is washed over the plate and incubated. After an additional series of washes, the substrate to the enzyme is added to the plates. Where the enzyme is present, the substrate will react, and a visual signal will be produced. This technique is called an Indirect assay as the signal is not coming directly from the primary antibody, but the secondary antibody.

III. Methods:

Flavobacterium fallonii sp. JRM, *KMS,* and *AJR* were isolated at a depth of 1cm from the freshwater Loyalsock Creek in Montoursville, Pennsylvania USA in December 2013. The water sample was spread onto tryptic soy agar and incubated in the micro lab. The bacteria were isolated into colonies and grown at 22˚C for two days. Phylogenetic tests were completed as the organisms were grown at varying temperatures, pH, salt concentrations, aerobic and anaerobic conditions.

Genome Sequencing and Comparisons

16S rRNA- To identify the organism, the 16S rRNA was originally sequenced using the 27f primer in Polymerase Chain Reaction. The bacteria were first frozen and thawed before 12.5uL of 2x Taq PCR and 12.5uL of 2x rRNA primer mix was added along with the 27f primer before the bacteria underwent thermal cycling to replicate the 16S rRNA sequence. The sequencing was done by the Sanger dideoxy chain termination method by the University of Arizona. This primary sequence provided information that *Flavobacterium fallonii JRM* was a potentially novel species after submission to EZTaxon (http://www.ezbiocloud.net). According to the percent similarity to the closest relative was *Flavobacterium hibernum* at 97.26%. The full sequence was performed using the following primers, the 27f, rRNA1, 785f, 1492r, and the 810r primer for full coverage.

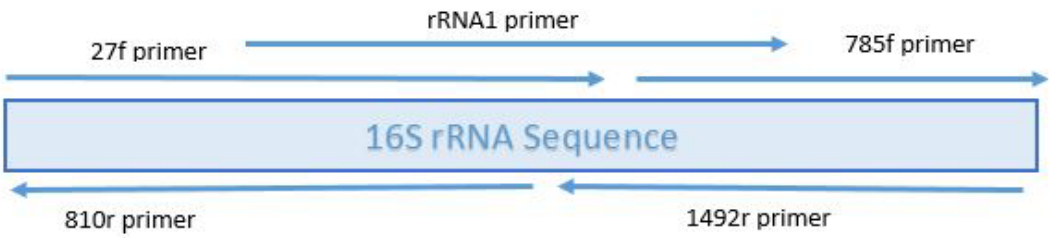


Figure 1. Schematic of how multiple 16S rRNA primers work together for full coverage of the 16SrRNA gene

This sequence was assembled using the program CAP3 (Huang and Madan, 1999 http://doua.prabi.fr/software/cap3). Then it was re-submitted to EZ-Taxon where the highest percent similarity was 98.26%, still lower than the 98.5% threshold for identifying different species. (Kim et al 2012). The most related organisms based on 16S rRNA sequence to Flavobacterium JRM were still *Flavobacterium hibernum* and *Flavobacterium hydatis* respectively. The 16S rRNA sequences were aligned in a program called MEGA6 (http://www.megasoftware.net/) where the probable phylogenetic relationship was examined. A phylogenetic tree was created using the 16srRNA sequences for members of the genera Flavobacterium downloaded from EZTaxon to examine neighbor joining maximum likeness. This provided information that led to using Flavobacterium hibernum and Flavobacterium hydatis as reference organisms.

Whole Genome Sequencing- The DNA of *Flavobacterium sp. JRM, KMS*, and *AJR* were prepared for sequencing using the Qiagen DNeasy DNA kit according to the manufacturer's instructions for Gram-positive bacteria. They were then sent to be sequenced on an Illumina

MiSeq (V3 26300 base) by the Indiana University Center for Genome Studies as part of a Genome Consortium for Active Teaching NextGen Sequencing Group run (GCAT-SEEK). The reads were assembled using the paired-end de novo assembly option in SoftGenetics NextGENe V2.3.4.2 (http://www.softgenetics.com/NextGENe.php). The assembled genomes were uploaded to the Rapid Annotation with Subsystem Technology web service (http://rast.nmpdr.org/) for analysis and annotation. (Ross Overbeek et al., 2014) The genome was edited for contamination and bad reads by *BLASTing* the sequences in RAST and in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). If the average coverage of the read was under 7X, the sequence was deleted. RAST was used to check for repeats and bad contigs, while NCBI was used to check for contamination. *Flavobacterium sp. JRM* was contaminated with *Cornyebacterium* DNA. NCBI matched uploaded reads to similar sequences and if the sequences were positively identified, the sequences were removed. A similar approach was taken with the other genomes. The assembled genome for *Flavobacterium fallonii ii so. JRM* was 169 contigs at 5,386,118 base pairs. The average coverage was 27x coverage. The assembled genome of *Flavobacterium fallonii sp. KMS* was 58 contigs with a size of 5,620,217 base pairs. The average coverage was 28X. The assembled genome for *Flavobacterium fallonii sp. AJR* was 204 contigs with a size of 5,401,825 base with an average coverage of 33X. The genomes were then uploaded and published to GenBank. When publishing an organism's genome in GenBank, the full sequence and sequencing stats are available in NCBI for public use.

Phylogenomics- Digital DNA-DNA Hybridization between *Flavobacterium fallonii JRM, Flavobacterium fallonii KMS, Flavobacterium fallonii AJR* and the reference organisms was calculated using the Genome-to-Genome Distance Calculator (http://ggdc.dsmz.de/)developed at the Leibniz Institute DSMZ German Collection of Microorganisms and Cell Cultures (Meier-

Kolthoff et al., 2013). To get the Average Nucleotide Identity (ANI) the genomes were uploaded to EZ BioCloud (http://www.ezbiocloud.net/) and calculated using the Average Nucleotide Identify tool (Yoon et al., 2017). The Kostas Lab ANI Calculator (http://enve-omics.ce.gatech.edu/ani/) and OAT (Orthologous Average Nucleotide Identity Tool) was also used to calculate ANI. The difference between OrthoANI and ANI is that OrthoANI produces identical reciprocal similarities. The values generated from original ANI and OrthoANI are comparable and both have a proposed cut-off for species demarcation at 95-96% (Lee et al., 2016). The assembled genomes were also uploaded to an online genome annotating program called RAST (Aziz et al., 2008). In the Newman lab, a method for calculating Average Amino Acid Identity (AAI) was developed that uses RAST exported files (http://lycofs01.lycoming.edu/~newman/AAI/) . These. tsv files were then downloaded and used for the AAI.

Phenotypic Tests

Biolog- Biolog was completed to quantitatively compare phenotypic data between *Flavobacterium fallonii JRM, KMS, AJR* and the reference organisms. Over the course of 36 hours, Biolog takes pictures of the 96-well plates containing different energy sources or growth inhibitors. By redox reaction, if growth occurs, the wells will turn purple. This occurs as the Biolog dye uses tetrazolium redox chemistry. These dyes measure the flux of NADH production from the bacteria grown in each well. This NADH then causes the color change in the dye outside the cells. The computer assigns numbers to how well the organisms grow from 0-100 based on the intensity of the color change. (Biolog, Inc.). Bug+ Blood agar was the medium used

as suggested by Biolog. The data provided by multiple Biolog runs was combined and averaged for more accurate and standardized results for use in this project.

Fatty Acid Methyl Ester Analysis- Overnight samples of the organisms were grown on either TSA or R2A. Using a plastic loop, 3-5 mg of log phase cells were smeared lower part of the labeled GC vial 0.25 mL of reagent 1 was added to each vial to saponify the phospholipids. Reagent 1 contained dH20, HPLC grade methanol, and NaOH. The vials were vortexed for 10 seconds and placed in a 100ºC heating block for 5 minutes. Then the vials were cooled for 2 minutes, vortexed for 10 seconds, and placed back in heating block for another 25 minutes. Then the vials were removed from the heating block and cooled for 1 minute. Then, 0.5 mL of reagent 2 was added to the vials in order to methylate the fatty acids. Reagent 2 contains a mixture of 325 ml 6.00M HCl in 275 mL of methanol. They were then vortexed for 10 seconds and placed in an 80ºC heating block for 10 minutes. After 1 minute of cooling, 0.5 mL of reagent 3 was added to each vial to extract the fatty acid methyl esters from the aqueous solution. Reagent 3 contained 200 mL Methyl tert-butyl ether in 200 mL of hexane. Then the vials were tightly sealed and placed vertically on the orbital shaker at 75 rpm for 10 minutes. The vials were then centrifuged for 30 seconds at 10,000rpm. Using a Pasteur pipette, the lower, aqueous phase was discarded. Next, 0.75 mL of reagent 4 was added to the organic layer to remove non-esterified fatty acids. Reagent 4 was 10.8g of NaOH in 900mL of dH20. Then the vials were placed back on the orbital shaker at 75 rpm for 5 minutes. The vials were then centrifuged for 30 seconds at 10,000rpm. 200 µL of upper organic phase was transferred to a glass insert within an appropriately labeled second vial. These vials were then analyzed on the Gas Chromatograph. The samples were run according to the standard method.

Antibiotic Sensitivity- Cultures of liquid TSB medium for each organism were incubated overnight in the shaker. The following day 100 µL of the each culture was pipetted onto a respective plate and spread with a disposable plastic spreader. In each plate, forceps were used to arrange four sterile filter paper disks around the periphery and one in the center of the plate in the patterns seen below.

amp carb clav amox clavamox	clin kan ery ctc cm	nal str pen rif tet

5 µL of the appropriate antibiotics were then pipetted onto the disks. These plates were incubated at room temperature for 48 hours. Using a ruler, the diameter of the zone of inhibition in millimeters around each antimicrobial agent was measured and reordered.

Immunological Comparisons

Antibody Production- Polyclonal antibodies were generated by the Custom Antibodies and Proteins group at Thermo Fisher Pierce Scientific at their facility in Rockford Illinois. To prepare the sample, an overnight culture of *Flavobacterium fallonii* sp. *JRM* grown in TSB was used. To estimate cell density, the culture was observed on a hemacytometer. A hemacytometer is a microscope slide that has extremely fine lines arranged in a grid-like pattern. By counting bacterial cells in the grids and knowing the volume of the culture, an estimation can be made as to the total cell density of that culture. By using this method, cell density for the culture was estimated at $1.9X10^7$ cells/mL. For the whole cell immunization protocol for Thermo Fisher, each sample needed to be 8-9 $X10^6$ or $1.6\text{-}1.8X10^7$ for two samples. To prep the samples for shipment to Thermo Fisher Scientific, ~2mL of JRM culture was centrifuged at 10,000 rpm for 3 minutes. The supernatant was discarded and the cells were re-suspended in a 0.85% saline buffer

in 2mL micro centrifuge tubes. Two identical samples were then put in a plastic box and placed in a Styrofoam cooler with an ice pack and shipped to Thermo Fisher Scientific. At the facility the Standard 70 Day Protocol for rabbit polyclonal antibodies was followed. On Day 0, two Specific Pathogen Free New Zealand White rabbits (rabbits 226 and 227) were pre bled for 5mL of serum. For the Primary Injection on Day 1, using the supplied JRM whole cells the test rabbits were injected subcutaneously in 10 sites with 0.5mg of antigen in Complete Freud's Adjuvant (CFA) . After 14 days, the rabbits were given a booster shot of 0.25mg of JRM in CFA. On Day 28, another booster shot of 0.25mg of JRM was injected subcutaneously into each rabbit. On Day 35, each rabbit was bled and ~25mL of serum was collected from each rabbit. A 3rd booster shot was injected into each rabbit on Day 42 with 0.25mg of JRM. A final bleed was performed on Day 56,58 and ~50mL of serum per each rabbit was collected. Serum was processed, packaged and prepared for shipment on Day 60. Upon arrival at Lycoming College on December 21, 2016, the serum samples were stored in the -80℃ freezer until use. Thermo Fisher Scientific provided 12 vials of crude antibody sera. For rabbit 226, 6 samples were provided: two Day 0 samples of 5mL and 0.5mL, two Day 35 samples of 1mL and 20mL, and two Day 56,58 samples of 1mL and 47mL. For rabbit 227, 6 samples were provided: two Day 0 samples of 0.5mL and 6mL, two Day 35 samples of 1mL and 23mL, and two Day 56,58 samples of 1mL and 37mL.

Agglutination Assay- A simple agglutination assay was performed to test for antibody concentrations and the reactivity of the serum. 1 mL of overnight cultures of strain JRM, KMS, AJR, or the reference organisms were pipetted into microcentrifuge tubes and spun at 10,000rpm for 1 minute. The supernatant was then decanted and the pellet was re-suspended in 1 mL of PBS. The samples were then diluted 10 fold five times. Antibody dilutions were also performed

diluting out the Day 56,58 rabbit 227 sera to working concentrations of 1:250, 1:500, 1:1000, and 1:2000. 5 uL of the respective antibody dilutions and 5 uL of cell dilutions were added to a microscope slide and mixed lightly before being cover slipped. The samples were then observed under a microscope using the 10X and 40X objectives. Pictures were taken with the lab camera.

Protein Extraction- 1. For the protein purification and extraction, 1.75 mL of culture was spun in the microcentrifuge tube at 14,000 rpm for 3 min. The supernatant was decanted and another 1.75 mL of culture was added and spun again. 0.5 mL of lysis buffer was added and mixed by vortexing. Each sample was placed in a heating block for 5 minutes at 95℃. Then the samples were centrifuged at 14,000 rpm for 10 minutes in the cold room at 4℃. The lysates were transferred to a new tube and placed on ice. To standardize the protein concentrations, using BioRad Protein Assay Reagent, Bovine Serum Albumin dilutions, and the Spec-20 at an absorbance of 595nm. Using the protein curve, protein concentrations of each sample were then diluted accordingly to reach a concentration of 1mg/1ml. This was important to both standardize the concentrations, as well as ensure a workable concentration of protein for Western Blotting. The tubes were then labeled and placed in the freezer.

Lipid Extraction- LPS extraction was performed using an Alpha Diagnostic Bacterial Lipopolysaccharides Extraction Kit based on the instruction manual. For each bacteria sample, 3 mL of culture in TSB were spun down in a centrifuge at 13,000 for 5 minutes. The supernatant was discarded and 1mL of the lysis buffer was added and vortexed. Then, 200 uL of chloroform was added to the microcentrifuge tube, vortexed vigorously, and left to incubate for 5 minutes at room temperature. The chloroform was added to separate the RNA and genomic DNA from the

other cell components. The samples were then centrifuged for 10 minutes in the cold room. 400 uL of the supernatant was then added to a new microcentrifuge tube. To this new tube, 800 uL of the Purification Buffer was added, vortexed, and incubated for 10 minutes. Then the samples were spun at 13,000 rpm for 15 minutes in the cold room. The supernatant is discarded leaving the pelleted LPS in the tube. 1mL of 70% ethanol was used to wash the LPS pellet, and then it was centrifuged for 3 minutes at 13,000 rpm in the cold room. The LPS pellet was then dried at room temperature for 2 hours, after the supernatant was discarded. Once dry, 40mL of 10mM Tris-HCL buffer at pH 8 was added to the tubes and vortexed. The tubes were then placed in the heating block at 100℃ for 2 minutes. 3 uL of proteinase K was added to each tube to improve each sample by breaking down any of the extra proteins of each sample and then incubated for 30 minutes at 50℃. Samples were stored at 4℃.

Western Blot- Procedure adapted from pages 31-42 of the Immunology 347 Lab Manual (Morrison, 2017). For the Western Blot, samples were used from the previous LPS and protein extractions. For the protein samples, 40 µL of the protein extracts at were pipetted into labelled 0.5 mL microcentrifuge tubes. Then 20 µL of 3x SDS sample buffer was added to each tube. The sample buffer was made of a 62.5 mM Tris-HCl, pH 6.8 with 2% SDS, 25% glycerol, 0.01% bromophenol blue, and 5% 2-mercaptoethanol. The samples were then placed in the heating block at 95°C for 5 minutes. The samples were then placed on ice until further use. The LPS samples were treated with the same protocol as the protein preps. Four 15% BioRad poly acrylamide Ready Gels were used for this experiment. The gels were taken out of their respective packages and the tape at the bottom of the gel was removed along the black line. The green plastic combs were pushed out of the gels. On gel 3, this did not happen smoothly, and the gel

wells of rows 1-4 were unusable. The gel cassettes were placed in the electrode assembly with the shorter plate facing the middle of the assembly, and the longer plate facing outwards. The gels were pushed into place to form tight, leakproof seal on the buffer dam. The electrode assembly clamped in place to ensure the gels would not move. The upper buffer chamber was filled with ~125 ml of running buffer. The running buffer was a solution of 25 mM Tris, 192 mM glycine, 0.1% SDS, at pH 8.3. Using the same buffer, the lower buffer tank was filled with ~200 ml of running buffer to the marked line. The samples were then loaded into the gel wells according to the following-

Gel 1- Protein	**Gel 2- LPS**
Lane 1 – empty	Lane 1 – empty
Lane 2 – 25 µL Escherichia coli	Lane 2 – 25 µL Escherichia coli LPS
Lane 3 – 25 µL Flavobacterium hibernum	Lane 3 – 25 µL Flavobacterium hibernum LPS
Lane 4 – 25 µL Flavobacterium hydatis	Lane 4 – 25 µL Flavobacterium hydatis LPS
Lane 5 – 5 µL Kaleidoscope marker	Lane 5 – 25 µL Flavobacterium sp. JRM LPS
Lane 6 – 25 µL Flavobacterium sp. JRM	Lane 6 – 25 µL Flavobacterium sp. KMS LPS
Lane 7 – 25 µL Flavobacterium sp. KMS	Lane 7 – 25 µL Flavobacterium sp. AJR LPS
Lane 8 – 25 µL Flavobacterium sp. AJR	Lane 8 – 5 µL Kaleidoscope marker
Lane 9 – 25 µL Escherichia coli **LPS**	Lane 9 – 25 µL Flavobacterium sp. JRM protein
Lane 10 – empty	Lane 10 –empty

Gel 3- Protein	**Gel 4- LPS**
Lane 1 – empty Lane 2 – empty Lane 3 – empty Lane 4 – 25 µL Escherichia coli Lane 5 – 25 µL Flavobacterium hibernum Lane 6 – 25 µL Flavobacterium hydatis Lane 7 – 25 µL Flavobacterium sp. JRM Lane 8 – 25 µL Flavobacterium sp. KMS Lane 9 – 5 µL Kaleidoscope marker Lane 10 –25 µL Flavobacterium sp. AJR	Lane 1 – empty Lane 2 – 25 µL Escherichia coli LPS Lane 3 – 25 µL Flavobacterium hibernum LPS Lane 4 – 25 µL Flavobacterium hydatis LPS Lane 5 –25 µL Flavobacterium sp. JRM LPS Lane 6 –25 µL Flavobacterium sp. KMS LPS Lane 7 – 25 µL Flavobacterium sp. AJR LPS Lane 8 – 5 µL Kaleidoscope marker Lane 9 – 25 µL Flavobacterium sp. JRM protein Lane 10 – empty

The kaleidoscope marker was a Precision Plus Dual Color marker. The lids were then placed on the tank and ran for 30 minutes at 200 Volts. Upon completion the running buffer was poured out and the gel cassettes were removed from the assembled electrophoresis contraption. The gels were then taken out of the gel cassettes by cracking the plastic around the gels. Gels 1 and 2 were then placed in staining trays filled with dH_20 for 5 minutes. After 5 minutes, the water was changed. This was to wash the gels free of the SDS. After the washing was complete, Gel 1 was placed in a Bio-Safe Coomassie staining solution on the shaker. Gel 2 was placed in a solution of dH_20, methanol, and acetic acid in a 50/40/10 volume ratio. These gels were then placed on the shaker and left overnight. After rehydrating the gels with dH_20, pictures were taken with the lab camera. Gels 3 and 4 were used for Western Blotting. The proteins and LPS were transferred to a nitrocellulose membrane. This was accomplished by taking the gels out of the plastic gel cassettes and washing the gels in transfer buffer for 15 minutes on the shaker. The transfer buffer

was a solution of 2.5 mM Tris, 19.0mM glycine, 200mL of 20% methanol, and distilled water. The transfer apparatus fiber pads were also equilibrated in transfer buffer in a staining tray. A large staining tray filled with transfer buffer was used to assemble the blotting sandwich apparatus. On the bottom of the sandwich, was the black plastic plate. Then a presoaked fiber pad was added and then a piece of whatman paper. The gel was then placed on top of that with the nitrocellulose membrane placed on top of that. Another fiber pad and the white plastic blotting apparatus piece made up the remainder of the sandwich. The sandwich was inserted into the transfer apparatus with the transfer membrane closest to the positive electrode. The tank was then filled with transfer buffer, a stir bar, and a freezer pack. This whole piece was then placed on top of a stir bar in the cold room and left overnight to run. It was ran at 20-25 volts/93 mA in the cold room. Upon completion, the blots were washed with 1X PBS and stored in plastic wrap. PBS is 1mM sodium phosphate, 15mM NaCl and pH 7.4. The following day, the blots were rehydrated in 1X PBS for 30 minutes. Next, the blots were blocked in a staining tray with 30 mL of blocking solution. The blocking solution was 1X PBS, 0.025% Tween20, and 5% nonfat dried milk by weight. The gels were left in the blocking solution on the shaker for 2 hours. After that, the blocking solution was removed and the primary antibody blocking was added. The blocking solution was 1X PBS and BSA with day 56,58 primary antibody from rabbit 227 diluted to 1:2000. This incubated on the shaker overnight. The next day the blots were washed in wash buffer of 1X PBS with 0.025% Tween20 3 times for 5 minutes each. 25mL was used per wash. The secondary antibody solution was then added to each of the blots. The secondary antibody was Goat Anti-Rabbit IgG HRP and was diluted into the 1X PBS with 0.025% Tween20 at a concentration of 1: 10,000. 30mL of the secondary antibody solution was then added to the blots and left to incubate on the shaker for 45 minutes. After the 45 minutes, the blots were washed 2

times for 5 minutes with 25 ml PBST. Afterwards the gels were washed again with 40 mL of 1X PBS for 5 minutes each time. The blots were then placed in a substrate solution of Substrate B, ethylene-glycol, Solution A, and hydrogen peroxide. After 5 minutes, the blots began disintegrating in the substrate solution. The blots were then washed in PBS and photographed.

Quantitative ELISA- Procedure adopted from pages 1-4 of the Immunology/Microbiology experiment (Morrison and Newman, 2017). For the ELISA, overnight cultures of *Flavobacterium fallonii JRM, Flavobacterium fallonii KMS, Flavobacterium fallonii AJR*, *Flavobacterium hibernum, Flavobacterium hydatis, Flavobacterium aquatile, Flavobacterium johnsoniea, Chryseobacterium balustinum*, and *Escherichia coli* were grown in either TSB or R2A liquid medium. 1 mL of culture was placed in microcentrifuge tubes and centrifuged at 7000 rpm for 2 min. The supernatant was poured off and the cells were re-suspended in 1 mL PBS. For each re-suspended culture 2.5 mL of PBS was added to a 13 x 100 mM glass tube with 100 µL of each cell suspension and then vortexed. To standardize the bacteria cell densities, each sample was diluted to an OD600 of 0.10 in spectrophotometer at 600 nm. The ELISA was performed using a flat bottom 96 well Microtiter plate. In each well, 50 µL of standardized cell suspensions was pipetted according to the following pattern.

All yellow shaded squares received 50uL of the respective culture samples

H	G	F	E	D	C	B	A		
1:1k	1:1k	1:2k	1:4k	1:8k	1:16k	1:32k	PBS	←Ab	
PBS								E.coli	1
								F. hib	2
								F.hyda	3
									4
PBS								F. JRM	5
								F. KMS	6
								F.AJR	7
									8
PBS								F. aqau	9
								F. john	10
								C. balus	11
									12

Each 96-well plate was then incubated in the Biosafety Cabinet until all liquid evaporated. Several hours later once the wells were dried, 100 µL of methanol was added to each well to fix the bacteria to the well. The plates were incubated for 1 minute at room temperature and then shaken off over the sink. The plates were then set to air dry. Once dried, 100 µL of a blocking solution of 10mg/mL BSA in PBS was added to each well and left to incubate at 37℃ for 30 min. Afterwards, the plates were then shaken off and 100 µL of PBS was added to each well and incubated at 37℃ for 10 min. This wash was then completed a second time. The plate was then left overnight in the Biosafety Cabinet to air dry. The following day, 100 µL of PBS was added to each well used and incubated at room temperature to rehydrate the antigens. The following dilutions were used for the primary antibodies-1:1000, 1: 2,000, 1: 4,000, 1: 8,000, 1: 16,000, and 1: 32,000. 1.0 mL PBS was added to a 2.0 mL microcentrifuge tube for each primary antibody dilution. 1.5 mL of 1:1000 antiserum was added to the 1k tube and 1.0 mL of the same 1:1000 antiserum stock was added to your 2k tube. For one plate, Day 0 sera from rabbit 227 was

used and for the other plate Day 56,58 sera from rabbit 227 was used. Serial dilutions were performed to make the desires dilutions. After shaking out the PBS from the wells, 100 µL of the diluted antiserum was added to respective wells. The plates were then incubated at 37℃ for 60 min. After the incubation, the plates were inverted and shaken off to remove the primary antibody. To wash the wells, 100 µL of PBS was added to each well and let sit at room temperature for 20 minutes. This wash was then completed two more times. For the secondary antibody, 100 µL of Goat anti-rabbit antibody conjugated to Horseradish peroxidase (HRP) at a dilution of 1: 50,000 was added to each well. The plates were then incubated at 37℃ for 60 min. After the incubation time, the plates were inverted to shake off the secondary antibody. To rinse each plate, 100 µL of PBS was added to each well and then shaken off. This rinse was then repeated two more times. To visualize the wells, 100 µL of tetramethylbenzidine (TMB) substrate was pipetted into each well. Once added, the plates were incubated at 37℃ for 25 minutes. This made each well a blue color. Then 100 µL 1 M HCl was added to each well to stop the reaction and turn the products yellow. The plates were then placed in the plate reader and viewed at 450nm.

Immunofluorescence Staining – Procedure adapted from Laboratory Excersise#9 in the 347 Immunology Class (Morrison, 2017). Using the OD600 dilutions from the ELISA, the cultures of *Flavobacterium fallonii JRM, Flavobacterium fallonii KMS, Flavobacterium fallonii AJR*, *Flavobacterium hibernum, Flavobacterium hydatis, Flavobacterium aquatile, Chryseobacterium balustinum*, and *Escherichia coli.* 1 µL was pipetted onto microscope slides in a predetermined pattern. The slides were left to air dry in the BSL hood. Once dried, methanol was pipetted over the areas where the bacteria samples were spotted to fix the cells to the

microscope slide. These were left to dry at room temperature for 2 hours. Once dry, the slides were blocked with 250uL of a 10% NGS (natural goat serum) solution in PBS. The slides were then incubated in a humidified box for 30 minutes. The slides were then washed with PBS and the primary antibody was added. The primary antibody solution was 1% NGS and anti-JRM antibodies of day 56,58 rabbit 227 at a dilution of 1:1,000 in PBS. 250uL of this solution was washed over the microscope slides and incubated for 30 minutes at room temperature in a humidified box. For this step, the negative control just received a 1% NGS in PBS solution. The slides were washed in PBS twice before the secondary antibody solution was added. The secondary antibody solution was 1% NGS, Hoechst 33258 at a concentration of 1:50,000, and Goat Anti-Rabbit IgG H&L Alexa Fluor® 488 at a dilution of 1:2,000. 250uL of the secondary antibody solution was then washed over the slides and left to incubate at room temperature for 30 minutes in a humidified box. Upon competition, the slides were washed off with PBS utilizing multiple washes. Then, 5 drops of Aquamount was used as a mounting medium for cover slipping the slides. The slides were left over night to gel and then sealed with nail polish. The microscope slides were examined under a student-grade Nikon E200 fluorescent microscope. The Hoechst 33258 stains DNA and should fluoresce blue and the Alexa 488 should only stain where the primary anti-JRM bound and should fluoresce green. For Alexa488, the excitation light is 488nm and the emission light is 520nm. The Hoechst 33258 stain has an excitation of 352nm and an emission light of 461nm.

LPS Stain- After electrophoresis, LPS gels were oxidized in 100 mL of a 1% periodic acid (HIO4), 40% MeOH and 5% acetic acid (HAc) solution for 20 minutes. Then the gels washed with a 1% vitamin C solution for 5 min to remove the excess HIO4 in the gel. Next, the

gels were immersed in 100 mL staining solution of 0.001% UGF202, 40% EtOH, 5% DMF, and 0.25% MgCl2 for 20 minutes. For the synthesis of UGF202, a suspension of 1-Pyrenecarboxaldehyde (0.20 g, 0.87 mM) in 20 mL methanol (MeOH) was refluxed at 60℃ for 20 min. A solution of carbohydrazide (548 mg, 608 mM) in 5 mL deionized water was then added to solution. After stirring for additional 2 hours, the product was cooled and stored in a screw capped vial. This staining protocol was adapted from the Wang lab (Wang et al., 2015). Images of the stain were taken with the lab camera in the gel viewer. Since the UGF202 stain fluoresces at a 532nm a green laser pointer was also used to try to visualize the stain with no success.

Genome Analysis

Genome Analysis- The genomes of each organism were evaluated by a combination of methods. The Newman lab Venn Diagram Tool developed by Tom Sontag ’14 and Andrew Gale ’15 was used to sort and compare .tsv file exports from RAST (https://rast.nmpdr.org/) . The RAST sequence based function was used to compare a reference organism and five other organisms. For this study the three isolates of *Flavobacterium fallonii*, *Flavobacterium hydatis,* and *Flavobacterium hibernum* along with *Flavobacterium fallonii, Flavobacterium KMS, Flavobacterium hydatis, Flavobacterium hibernum,* and *Flavobacterium aquatile.* The first comparison was used to analyze unique genes to the species *Flavobacterium fallonii* and the second was used for comparing genes between *Flavobacterium fallonii,* the reference organisms, and then *Flavobacterium aquatile* as the type strain for the genus *Flavobacterium.* RAST sorts the sequences according to the bidirectional and unidirectional hits. *Figure 23* shows the format as seen in the RAST Seed Viewer. These tables were then exported as .tsv files and pasted into

the Newman lab Venn Diagram Tool and sorted by shared and unique genes between organisms. These unique gene lists were saved for further examination. The Diagram output in Microsoft Excel was used to sort the genes to be examined. Lists of genes were then compared with the Biolog results. Differences between organisms under a single growth condition were examined by looking in the genome for specific genes related to the particular metabolic pathway used in that growth condition. Predictions based on the Biolog results were made and then specific genes were looked for in the organisms. A second method for genome analysis was comparing the RAST sub-systems in the Seed Viewer. RAST annotates the genomes and sorts the full genome into a visual and interactive graph where number of genes pertaining to sorted metabolic functions. These graphs as well as the Biolog and other test results were then compared between the organisms.

IV. Results:

Genome Sequencing and Comparisons

16S rRNA- To identify the organism, the 16S rRNA was originally sequenced in the Microbiology course using just the 27f primer in Polymerase Chain Reaction (PCR). These sequences were then uploaded to EZ Taxon through EZ BioCloud All three organism's sequences yielded values under 98.5% with the next closest sequence in the EZ Taxon database. This suggested all three were potentially novel species. The full sequence was obtained using the following primers, the 27f, 330f, 785f, 1492r, and the 810r primer in PCR. Sanger Sequencing was used to determine the full 16S rRNA sequence. These sequences were then uploaded to EZ Taxon through EZ BioCloud. For *Flavobacterium fallonii JRM,* the closest match was *Flavobacterium hibernum* at 98.26%, with the next two closest organisms being *Flavobacterium*

aquidurense at 98.26% and *Flavobacterium tiangeerense* at 98.10% (*Figure 2).* *Flavobacterium hydatis* had a percent match of 98.02%. The full 16S rRNA sequence of *Flavobacterium fallonii KMS* was most closely related to *Flavobacterium hibernum* at 98.82% (*Figure 3).* The next two reference organisms based off of the EZ Taxon matches were *Flavobacterium tiangeerense* at 98.53% and *Flavobacterium collinsii* at 98.31%. *Flavobacterium hydatis* had a 16S rRNA similarity of 98.30% For *Flavobacterium fallonii AJR*, the highest EZ Taxon match was *Flavobacterium hibernum* with a value of 98.33% (*Figure 4).* The next highest match was *Flavobacterium aruacananum* also at 98.33%. The third highest reference organism match for *Flavobacterium fallonii AJR* was *Flavobacterium aquidurense* at 98.12%. *Flavobacterium hydatis* had a similarity of 97.98%. In comparison to each other, *Flavobacterium KMS* had a value of 98.89% similar to *Flavobacterium JRM*. *Flavobacterium AJR* was 99.93% similar to *Flavobacterium JRM.*

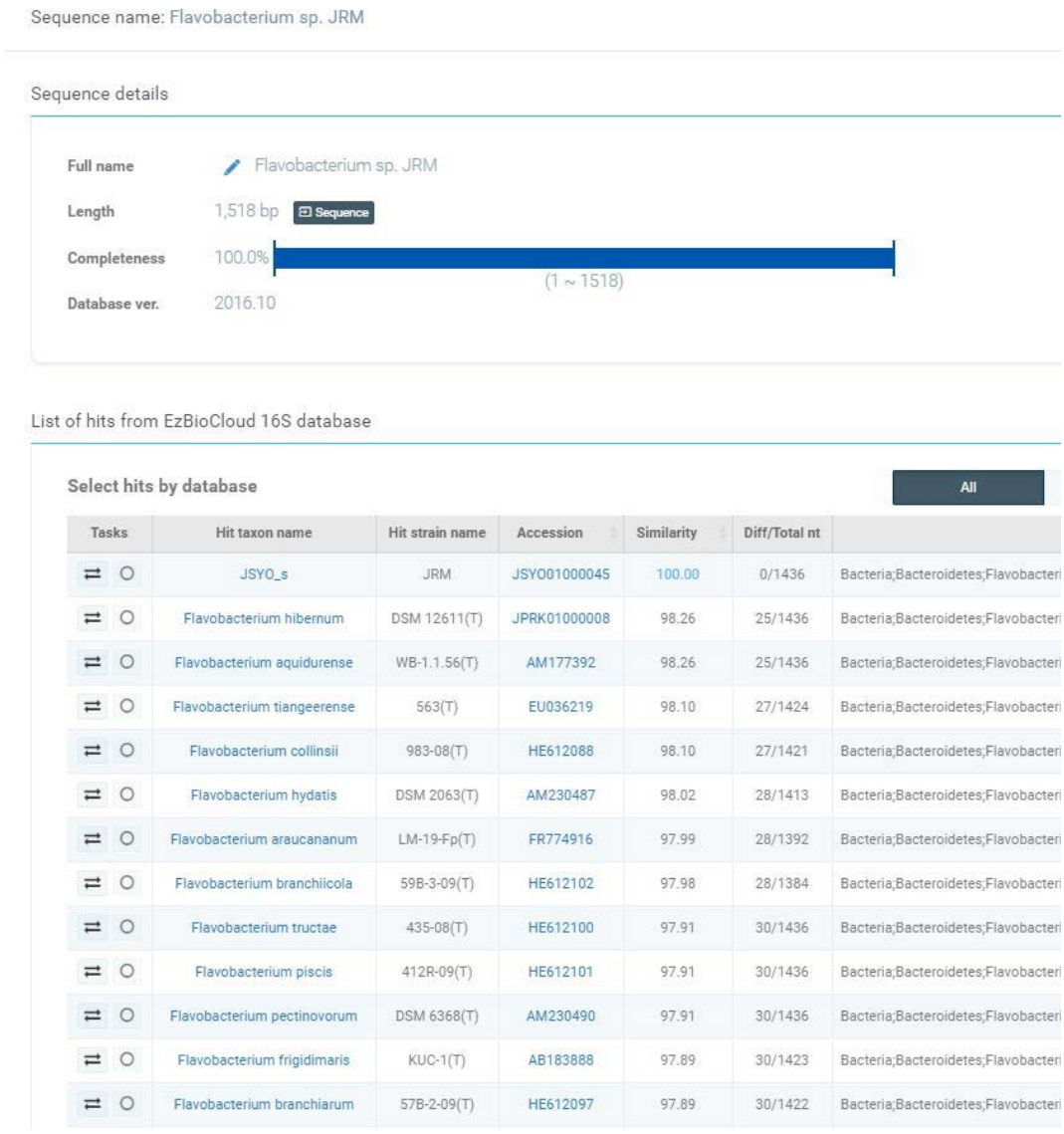


Figure 2. 16S rRNA Matches for *Flavobacterium fallonii JRM* in EZTaxon through EZBioCloud

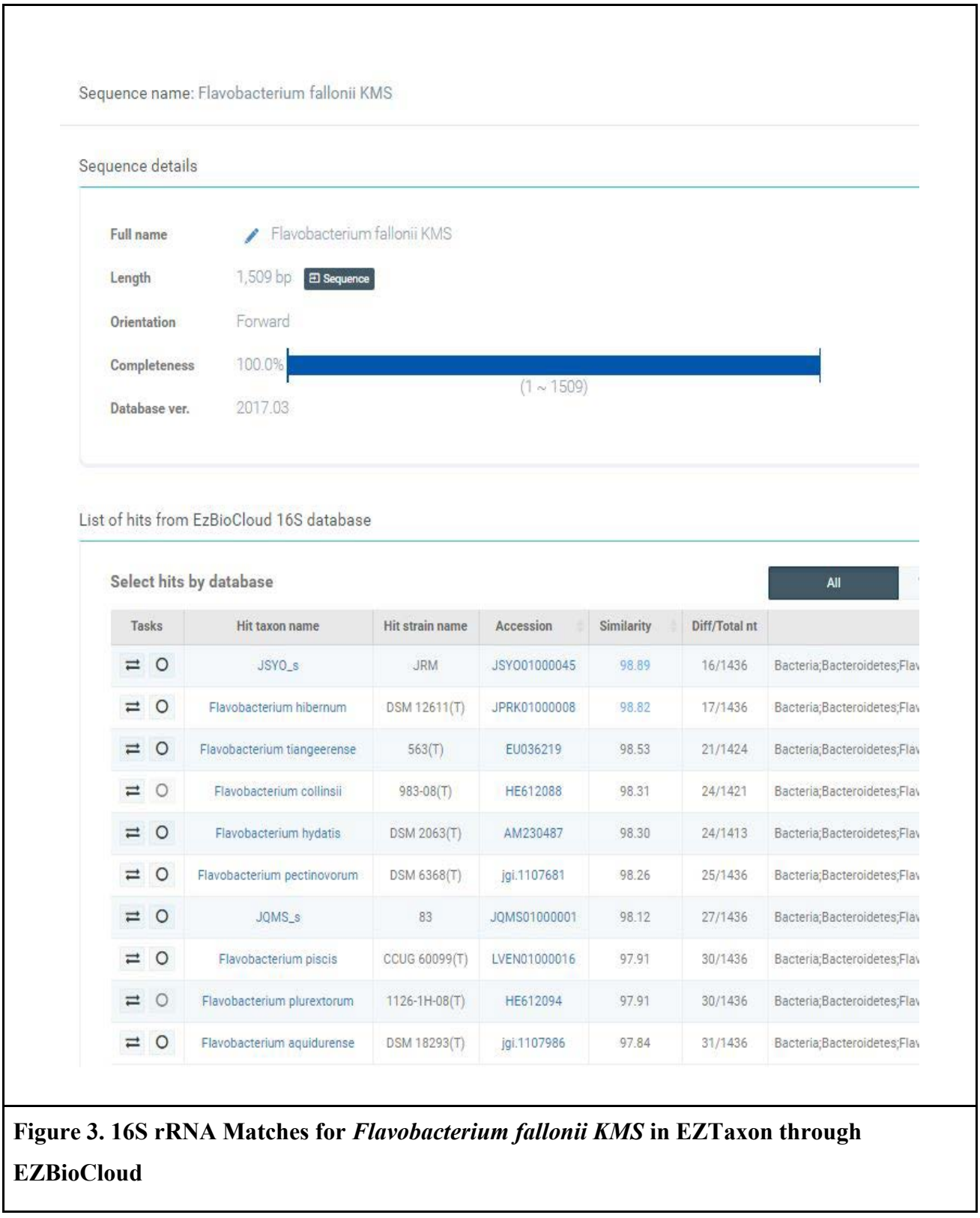


Figure 3. 16S rRNA Matches for *Flavobacterium fallonii KMS* in EZTaxon through EZBioCloud

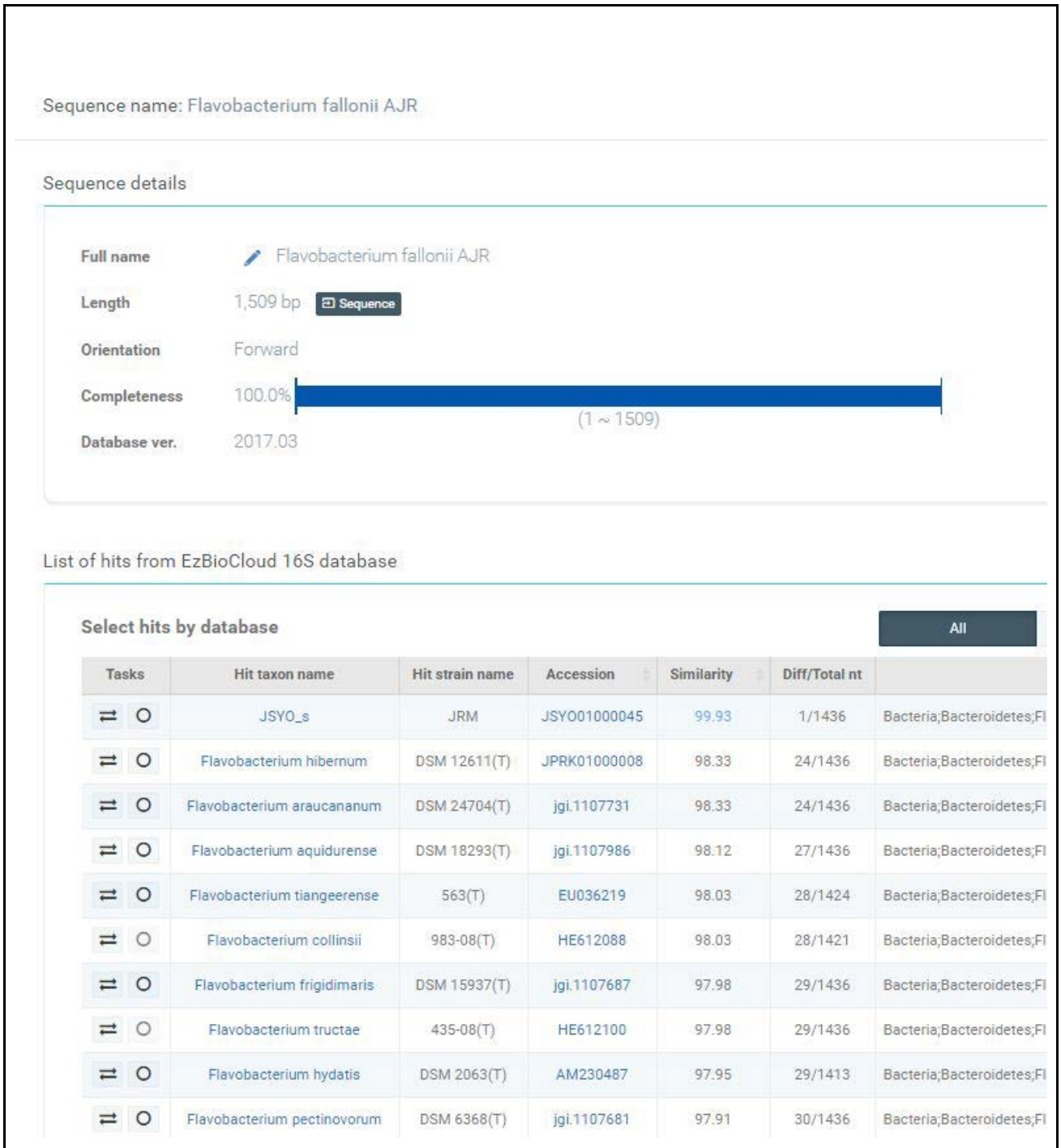


Figure 4. 16S rRNA Matches for *Flavobacterium fallonii AJR* in EZTaxon through EZBioCloud

16S rRNA EZ BioCloud Best Match

Flavobacterium fallonii sp. JRM		Flavobacterium fallonii sp. KMS		Flavobacterium fallonii sp. AJR	
Name	Pairwise Similarity (%)	Name	Pairwise Similarity (%)	Name	Pairwise Similarity (%)
Flavobacterium fallonii sp. JRM	100.00	Flavobacterium fallonii sp. JRM	98.89	Flavobacterium fallonii sp. JRM	99.93
Flavobacterium hibernum	98.26	Flavobacterium hibernum	98.82	Flavobacterium hibernum	98.33
Flavobacterium aquidurense	98.26	Flavobacterium tiangeerense	98.53	Flavobacterium araucananum	98.33
Flavobacterium tiangeerense	98.10	Flavobacterium collinsii	98.31	Flavobacterium aquidurense	98.12
Flavobacterium collinsii	98.10	Flavobacterium hydatis	98.30	Flavobacterium tiangeerense	98.03
Flavobacterium hydatis	98.02	Flavobacterium pectinovorum	98.26	Flavobacterium collinsii	98.03
Flavobacterium araucananum	97.99	Flavobacterium piscis	97.91	Flavobacterium frigidimaris	97.98
Flavobacterium branchiicola	97.98	Flavobacterium plurextorum	97.91	Flavobacterium tructae	97.98
Flavobacterium pectinovorum	97.91	Flavobacterium aquidurense	97.84	Flavobacterium hydatis	97.95
Flavobacterium tructae	97.91	Flavobacterium tructae	97.84	Flavobacterium pectinovorum	97.91
Flavobacterium piscis	97.91	Flavobacterium limicola	97.84	Flavobacterium branchiicola	97.90
Flavobacterium frigidimaris	97.89	Flavobacterium branchiarum	97.82	Flavobacterium piscis	97.84
Flavobacterium branchiarum	97.89	Flavobacterium reichenbachii	97.77	Flavobacterium branchiarum	97.82
Flavobacterium limicola	97.77	Flavobacterium xueshanense	97.77	Flavobacterium saccharophilum	97.70
Flavobacterium chilense	97.70	Flavobacterium araucananum	97.70	Flavobacterium limicola	97.70
Flavobacterium spartansii	97.61	Flavobacterium frigidimaris	97.63	Flavobacterium spartansii	97.69
Flavobacterium reichenbachii	97.56	Flavobacterium saccharophilum	97.63	Flavobacterium chilense	97.63
Flavobacterium oncorhynchi	97.52	Flavobacterium omnivorum	97.63	Flavobacterium oncorhynchi	97.59
Flavobacterium panaciterrae	97.48	Flavobacterium branchiicola	97.62	Flavobacterium xueshanense	97.55
Flavobacterium chungangense	97.42	Flavobacterium chilense	97.56	Flavobacterium reichenbachii	97.49
Flavobacterium plurextorum	97.42	Flavobacterium spartansii	97.54	Flavobacterium panaciterrae	97.41
Flavobacterium saccharophilum	97.34	Flavobacterium oncorhynchi	97.38	Flavobacterium chungangense	97.35
Flavobacterium glaciei	97.31	Flavobacterium chungangense	97.35	Flavobacterium plurextorum	97.35
Flavobacterium psychrolimnae	97.14	Flavobacterium psychrolimnae	97.35	Flavobacterium granuli	97.28
Flavobacterium hercynium	97.14	Flavobacterium panaciterrae	97.34	Flavobacterium glaciei	97.24
Flavobacterium granuli	97.13	Flavobacterium glaciei	97.31	Flavobacterium psychrolimnae	97.21
Flavobacterium succinicans	97.08	Flavobacterium granuli	97.28	Flavobacterium sinopsychrotolera	97.17
Flavobacterium resistens	97.08	Flavobacterium succinicans	97.21	Flavobacterium hercynium	97.07
Flavobacterium fluvii	96.94	Flavobacterium sinopsychrotolera	97.17	Flavobacterium resistens	97.01
Flavobacterium omnivorum	96.73	Flavobacterium hercynium	97.14	Flavobacterium aquicola	97.01
Flavobacterium chungbukense	96.65	Flavobacterium aquicola	96.94	Flavobacterium succinicans	97.01
Flavobacterium micromati	96.52	Flavobacterium chungbukense	96.86	Flavobacterium fluvii	96.87

Figure 4a. 16S rRNA Matches for *Flavobacterium fallonii* in EZTaxon through EZBioCloud.

The evolutionary relatedness of these strains was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 1.93656837 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree (*Figure 5)*. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site (Kimura, 1980). The analysis involved 122 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1188 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). Using this tree and the 16S rRNA sequence matches through EZ Taxon, reference organisms were chosen. On the tree, *Flavobacterium fallonii* most closely branched with *Flavobacterium hibernum*. While *Flavobacterium hydatis* did not necessarily branch with *Flavobacterium fallonii* it was chosen as a reference organism as well based on the preliminary 16S rRNA sequences and phenotypic similarities. *Flavobacterium aquatile* was used as a reference organism as it is the type strain for the genus *Flavobacterium.*

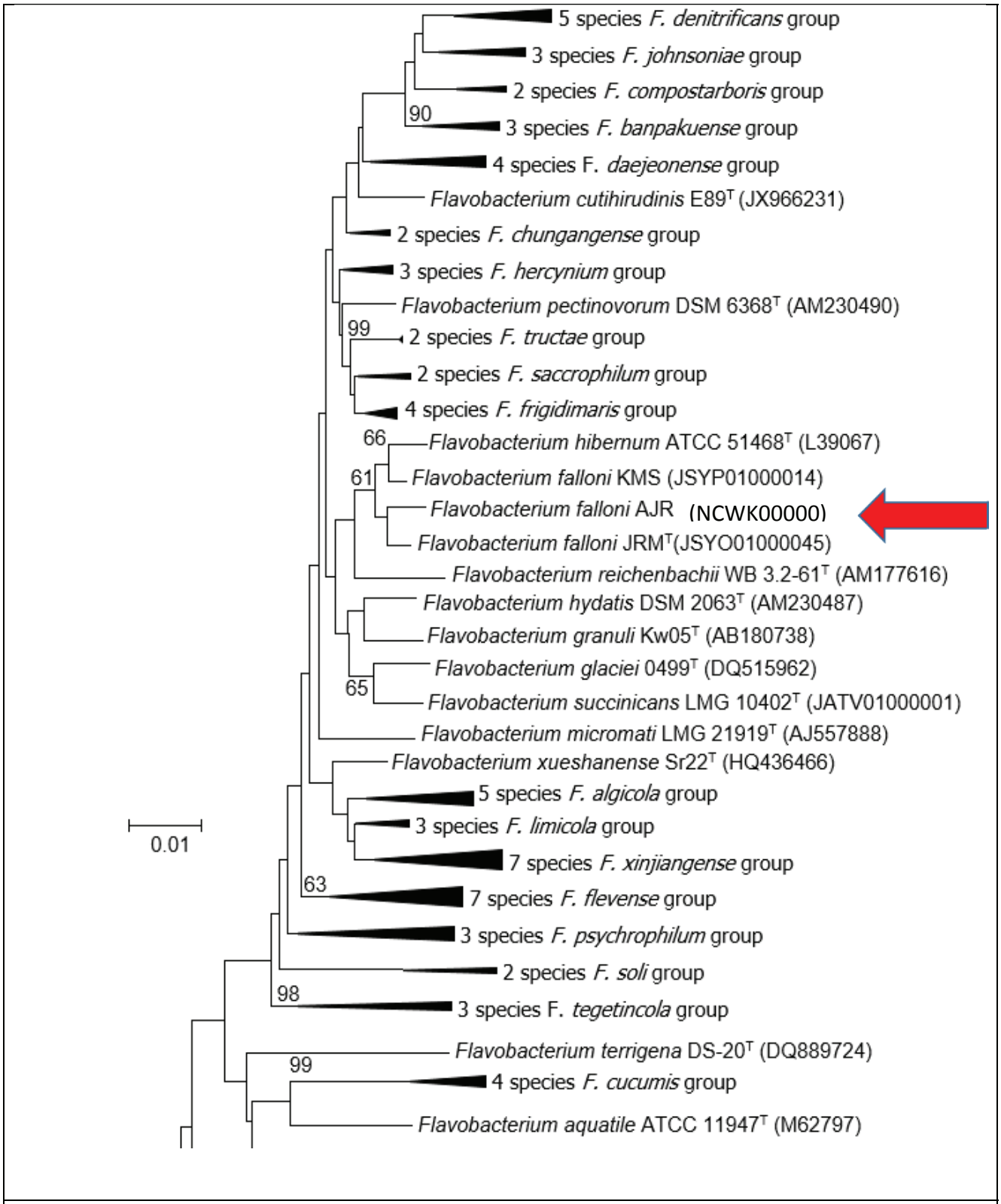


Figure 5. Evolutionary Relationships of Taxa using a Neighbor-Joining Phylogenetic Tree.

Genome Sequencing- The genome of *Flavobacterium fallonii sp. JRM* had 826,175 total reads and was assembled into 169 total contigs. The genome of *Flavobacterium fallonii sp. KMS* had 725,291 total reads and was assembled into 58 total contigs. The genome of *Flavobacterium fallonii sp.* AJR had 1,031,047 total reads a final contig count of 204. *Flavobacterium aquatile* had a total number of 7 contigs. A lower assembled contig number is also an indication of a higher quality of the sequence. These parameters indicate a higher quality sequence than *Flavobacterium fallonii sp. JRM* with a total number of contigs of 169. More manual editing can be performed to combine contigs and make a better and more complete sequence. The sequence of *Flavobacterium fallonii sp. JRM* was contaminated with *Corynebacterium.* To remove these contaminated sequences, each contig was sorted based off of average coverage and then Blasted in NCBI and compared in RAST. One of the most striking comparisons of the genomes based on genome sequencing is the difference in the sizes of the genomes. *Flavobacterium aquatile* is the type species for *Flavobacterium* and has a genome of 3,490,856bp while *Flavobacterium fallonii JRM* has a genome of 5,380,719bps long. *Figure 6a* is an example of the format that a submitted genome in the Whole Genome shotgun looks like in GenBank. This is a valid publication of a genome that can be downloaded and used by labs all over the world.

Genome Sequencing Statistics (MiSeq v3 2X300 PE)

	Flavobacterium fallonii sp. JRM	*Flavobacterium fallonii sp. KMS*	*Flavobacterium fallonii sp. AJR*	*Flavobacterium hydatis*	*Flavobacterium hibernum*	*Flavobacterium aquatile*
Total Reads	826,175	725,291	1,031,047	1,489,773	2,865,011	2,034,788
Contigs	169	58	204	99	29	7
Average Coverage	27X	28X	33X	54X	109X	161X
Assembly Length	5,380,719 bp	5,620,217 bp	5,401,825 bp	5,877,671 bp	5,283,662 bp	3,490,856 bp
Accession	JSYO01	JSPY01	NCWQ01	JRHH01	JPRK01	JRHH01

Figure 6. Genome Sequencing Statistics MiSeq v3 2X300 PE Table

Figure 6a. Whole Genome Shotgun Publication of Flavobacterium sp. JRM in GenBank

Full genome analysis was performed using the Genome-to-Genome Distance Calculator (Meier-Kolthoff et al., 2013) and the Newman lab Average Amino Acid Identity (AAI) tool http://lycofs01.lycoming.edu/~newman/OrthologyScore.html (*Figure 7)*. *Flavobacterium fallonii JRM* had a eDDH value of 74.2 when compared to *Flavobacterium fallonii KMS*, and a value of 73.5 when compared to *Flavobacterium fallonii AJR*. As these values lie above 70%, this indicates that all three of these isolates represent the same species. *Flavobacterium hydatis* had an eDDH value of 41.7 when compared to *Flavobacterium fallonii JRM*, 40.8 when compared to *Flavobacterium fallonii KMS*, and 41.2 when compared to *Flavobacterium fallonii AJR.* These values are well below the cut-off of 70%. *Flavobacterium hibernum* had eDDH values below 23.4 in comparison to all three isolates of *Flavobacterium fallonii.* Despite being the type strain for the genus *Flavobacterium*, *Flavobacterium aquatile* had eDDH values below 20.2 in comparison to *Flavobacterium fallonii* as well as the other reference organisms.

Average Amino Acid Identity has a species delineation threshold of ~95.0% (Thompson et al., 2013). *Flavobacterium fallonii JRM* had AAI values of 97.8% with *Flavobacterium fallonii KMS* and 97.3 with *Flavobacterium fallonii AJR (Figure 8)*. These values lie above 95.0 indicating that they are the same species. *Flavobacterium fallonii JRM* had an AAI value of 90.5 when compared to *Flavobacterium hydatis*. This value lies under the 95.0 species cut-off. However, the AAI value of 93.1 indicates a high level of similarity supporting the use of *Flavobacterium hydatis* as a reference organism. The AAI between *Flavobacterium hydatis* and the other two isolates of *Flavobacterium fallonii* were 92.5 and 92.7 respectively. *Flavobacterium hibernum* had AAI values of 79.5, 79.2, and 79.3 when compared to *Flavobacterium fallonii JRM, KMS, and AJR. Flavobacterium aquatile* had AAI values below

67.9 with all isolates of *Flavobacterium fallonii* and the other reference organisms. This indicates a low level of genomic similarity with these other species of *Flavobacterium* despite being the type strain for the genus.

		Estimated DNA-DNA hybridization (eDDH)					
		1	2	3	4	5	6
Flavobacterium fallonii sp. JRM	1		74.2	73.5	41.7	23.4	20.1
Flavobacterium fallonii sp. KMS	2	97.8		74.6	40.8	23.3	19.7
Flavobacterium fallonii sp. AJR	3	97.3	97.7		41.2	23.4	20.2
Flavobacterium hydatis DSM 2063	4	93.1	92.5	92.7		23.3	20.1
Flavobacterium hibernum DSM 12611	5	79.5	79.2	79.3	79.6		20.1
Flavobacterium aquatile LMG 4008	6	67.7	67.9	67.6	67.5	67.7	
		Average Amino Acid Identity (AAI)					

Figure 7. Genomic Comparison using the Genome-to-Genome Distance Calculator from the DSMZ and AAI using the Newman lab AAI Calculating Tool.

In order to compare *Flavobacterium fallonii JRM* to other species of *Flavobacterium,* a large comparison was performed using the Newmanlab AAI Calculator. In this evaluation, *Flavobacterium fallonii JRM* was compared using AAI to 53 other species of *Flavobacterium*. The AAI values cover a range of 61.74 with *Flavobacterium akiainvivens* to 79.5 with *Flavobacterium hibernum*. All named species of *Flavobacterium* in RAST were used. *Flavobacterium hydatis* was excluded from this table as it is the most genetically similar and data can be found in *Figure 8.* The average AAI value for the 53 species of *Flavobacterium* was 71.46. The type species for *Flavobacterium* is *Flavobacterium aquatile* which has an AAI value of 68.06. Other species included in this data set were from the genus *Chryseobacterium*. Organisms in this genus had AAI values between 49.98 and 50.8. An organism from the genus

Elizabethkingia and an organism from *Epilithonomonas* was also included in the data set. All of those 59 organisms evaluated fall into the family Flavobacteriaceae. *Escherichia coli* was included in the data set to show the difference in AAI values between organisms in the same family and then in different phyla.

Flavobacterium fallonii JRM- AAI vs Flavobacteriaceae				
	AAI Value			AAI Value
Flavobacterium aquidurense	78.88		Flavobacterium hibernum	79.5
Flavobacterium chilense	78.67		Flavobacterium piscis	79.15
Flavobacterium denitrificans	78.14		Flavobacterium sacchrophilum	78.76
Flavobacterium araucananum	78.02		Flavobacterium resistens	77.96
Flavobacterium cutihirudinis	77.45		Flavobacterium pectinovorum	77.92
Flavobacterium defluvii	77.34		Flavobacterium johnsoniae	77.1
Flavobacterium chungangense	77.2		Flavobacterium reichenbachii	76.9
Flavobacterium glaciei	76.3		Flavobacterium limicola	76.25
Flavobacterium granulie	75.34		Flavobacterium xueshanense	75.62
Flavobacterium fryxellicola	74.77		Flavobacterium xanthum	74.82
Flavobacterium degerlachei	74.19		Flavobacterium micromati	74.11
Flavobacterium frigoris	73.98		Flavobacterium segetis	73.58
Flavobacterium fluvii	72.99		Flavobacterium seoulense	72.41
Flavobacterium frigidarium	72.72		Flavobacterium succinicans	72.36
Flavobacterium gillisiae	72.3		Flavobacterium psychrophilum	69.13
Flavobacterium glycines	72.16		Flavobacterium soli	68.22
Flavobacterium daejeonense	71.7		Flavobacterium saliperosum	67.59
Flavobacterium aquatile	68.06		Flavobacterium suncheonense	67.02
Flavobacterium dankookense	67.6		Flavobacterium tegetincola	66.59
Flavobacterium antarcticum	67.55		Flavobacterium limnosediminis	66.39
Flavobacterium cauense	67.13		Flavobacterium sasagense	65.59
Flavobacterium enshiense	67.06		Flavobacterium indicium	63.8
Flavobacterium caeni	67.02		Flavobacterium rivuli	62.94
Flavobacterium branchiophilum	66.45		Flavobacterium subsaxonicum	62.76
Flavobacterium cheniae	66.32		Chryseobacterium greenlandense	51.08
Flavobacterium filum	65.74		Chryseobacterium hispalense	50.8
Flavobacterium cucumis	65.46		Elizabethkingia miricola	50.26
Flavobacterium gelidilacus	64.18		Chryseobacterium haifense	50.2
Flavobacterium columnare	63.95		Chryseobacterium gleum	49.98
Flavobacterium beibuense	63.76		Epilithonomonis lactis	49.5
Flavobacterium akiainvivens	61.74		Escherichia coli	38.09

Figure 8. AAI Table comparing *Flavobacterium fallonii JRM* to multiple species of *Flavobacterium* and other species in the family of Flavobacteriaceae and also with E. coli. AAI was calculated using RAST files and the Newmanlab AAI Calculator.

Average-Nucleotide-Identity (ANI) was calculated to compare the isolates of *Flavobacterium fallonii* and the reference organisms. An ANI value of 95% has been shown to correlate to a 70% DDH value (Konstantinidis and Tiedje, 2005). Using the ANI Calculator by the Kostas Lab, when compared to each other, *Flavobacterium fallonii JRM, KMS,* and AJR all had values that were above 96.9%. The ANI value between *Flavobacterium hydatis* and *Flavobacterium fallonii JRM* was 93.1 whereas it was 90.2 and 90.4 % respectively to the other two isolates. In comparison to the OrthoANI values, the values calculated by the Kostas Lab calculator are mostly comparable. The larger difference in value between ANI and OrthoANI came between *Flavobacterium hibernum* and *Flavobacterium aquatile.* For ANI, this value was 67.7% and for OrthoANI it was 73.4%. The values calculated by OrthoANI appear more uniform across each calculation. Shown in *Figure 10,* a heatmap was generated through OAT software shows the OrthoANI values when comparing the novel isolates of *Flavobacterium* to the reference organisms. The values coordinate to the scale on the right side of the figure. The OrthoANI values between all isolates of *Flavobacterium fallonii* have values over 97.1%. This is over the ~95-96% cutoff for species differentiation. The values between all *Flavobacterium fallonii* isolates and *Flavobacterium hydatis* lie between 90.6 and 90.8. These values are still less than 95%. The OrthoANI values comparing *Flavobacterium hibernum* and the three strains of *Flavobacterium fallonii* fall between 78.9 and 79.1%. The OrthoANI values comparing *Flavobacterium aquatile* and the three strains of *Flavobacterium fallonii* were between 73.2 and 73.3%.

		OrthoANI generated with OAT Software					
		1	2	3	4	5	6
Flavobacterium fallonii sp. JRM	1		97.17	97.07	90.80	79.10	73.32
Flavobacterium fallonii sp. KMS	2	97.0		97.25	90.54	78.92	73.22
Flavobacterium fallonii sp. AJR	3	96.9	97.38		90.62	78.98	73.17
Flavobacterium hydatis DSM 2063	4	90.5	90.2	90.4		79.02	73.14
Flavobacterium hibernum DSM 12611	5	78.4	81.6	81.3	81.3		73.48
Flavobacterium aquatile LMG 4008	6	77.8	77.7	78.2	77.8	78.2	
		Average Nucleotide Identity (Original ANI)					

Figure 9. Table Comparing OrthoANI using OAT software and the Original ANI algorithm from the ANI Calculator from the Kostas Lab.

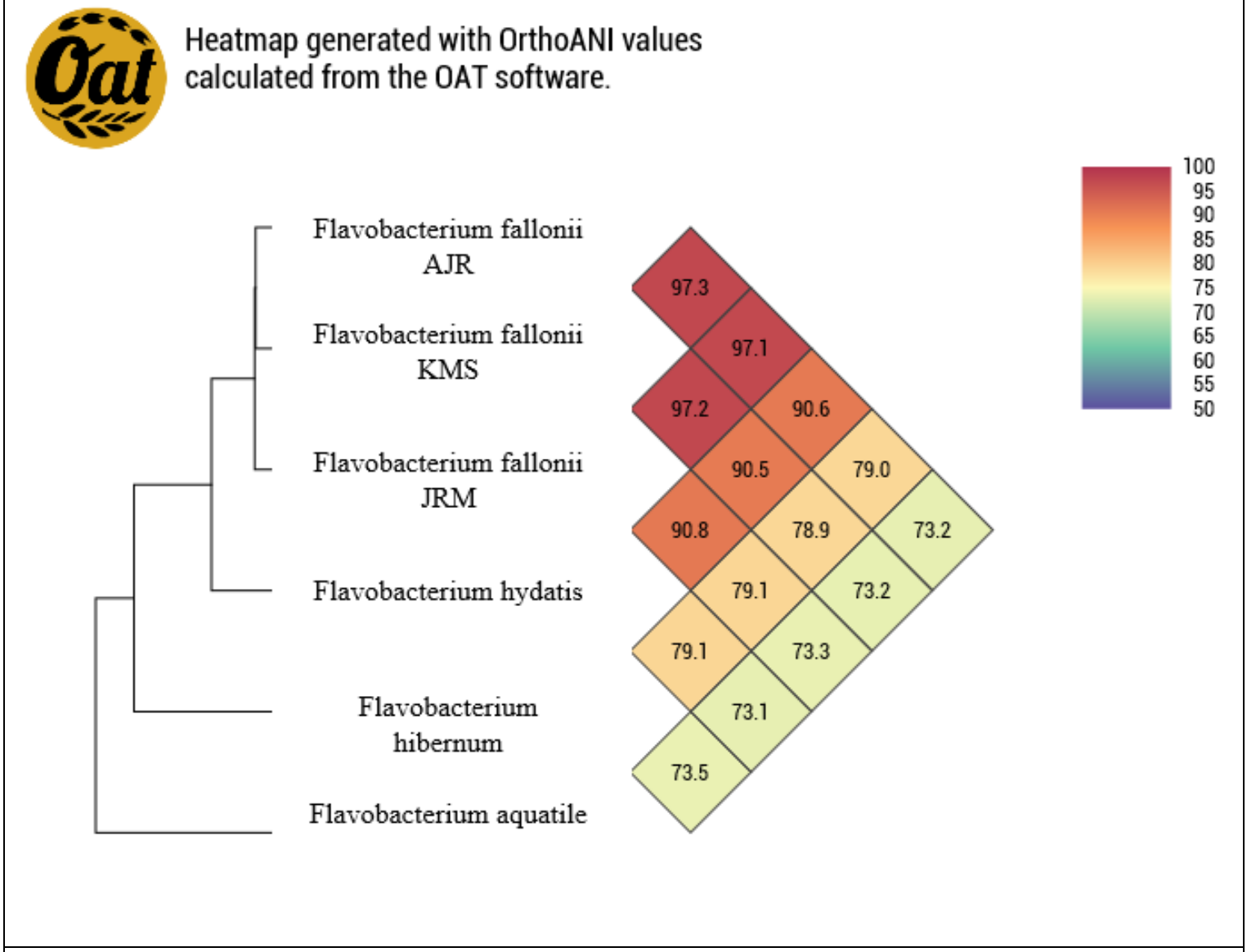


Figure 10. Heatmap of OrthoANI calculated using OAT Software (Lee et al., 2016)

Comparison of the unique and shared genes was performed using the Venn Diagram Tool developed by Tom Sontag ’14 and Andrew Gale ’15 in the Newman lab (*Figure 11-12)*. The Venn Diagram Tool uses the genome comparison function in RAST to determine the number of unique and shared genes amongst other organisms. Some key data points from the Venn Diagram are that all strains of *Flavobacterium fallonii* and the reference organisms share a core set of 2,784 genes. In comparison to the reference organisms *Flavobacterium hydatis* and *Flavobacterium hibernum, Flavobacterium fallonii* has 179 unique genes shared amongst all three isolates. Strain *Flavobacterium fallonii JRM* has 302 unique genes. Strain *Flavobacterium fallonii KMS* has 297 unique genes. Strain *Flavobacterium fallonii AJR* has 259 unique genes. *Flavobacterium fallonii JRM* and *Flavobacterium fallonii KMS* have 70 unique genes shared and *Flavobacterium fallonii JRM* and *Flavobacterium fallonii AJR* have 29 unique genes shared. *Flavobacterium fallonii KMS* and *Flavobacterium fallonii AJR* share 180 unique genes. This makes a gene count of 1,316 unique genes found in *Flavobacterium fallonii* in comparison to the reference organisms when looking at all combinations of the three isolates of *Flavobacterium fallonii.* The most genetically similar reference organism according to the genomic matrix from *Figure 7* was *Flavobacterium hydatis*. This is apparent when looking at the unique gene count from all the isolates of *Flavobacterium fallonii* and *Flavobacterium hydatis* as they share 731 unique genes not found in *Flavobacterium hibernum.*

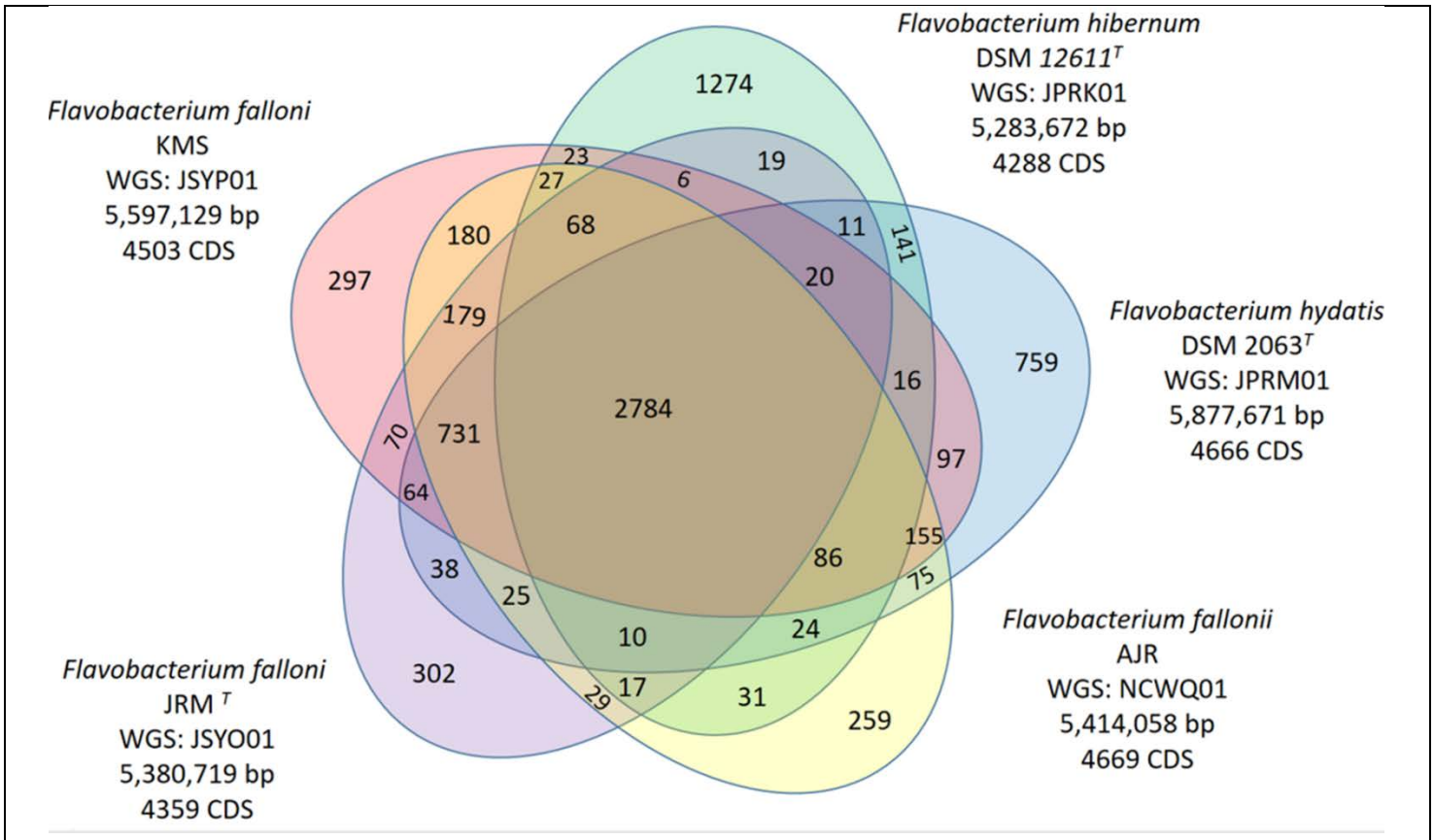


Figure 11. Venn Diagram of unique and shared genes between *Flavobacterium fallonii* and the reference organisms using Reciprocal Genome Comparisons by Rapid Annotation using Subsystems Technology (RAST). Venn Diagram was made using the Venn Diagram tool developed by the Newmanlab.

Another comparison of the unique and shared genes was performed using the Venn Diagram Tool developed by the Newman lab. This Venn Diagram differs from *Figure 10* as *Flavobacterium aquatile* was included in the comparison instead of *Flavobacterium fallonii AJR* in order to view genes in relationship to the type strain of the genus *Flavobacterium*. Some key data points from the Venn Diagram are that all organisms share a core set of 1,903 genes. This value is nearly 1,000 genes lower than the core set of genes found from the organisms in *Figure 10.* This indicates a lower number of total shared genes between the bacterial species used. *Flavobacterium fallonii JRM* has 321 unique genes in comparison to the reference organisms.

Flavobacterium fallonii KMS has 405 unique genes. The two strains of *Flavobacterium fallonii* share 295 unique genes. *Flavobacterium aquatile* has 849 unique genes in comparison to the reference organisms. There are 942 genes found in all of the compared organisms that are shared by all except for *Flavobacterium aquatile*..

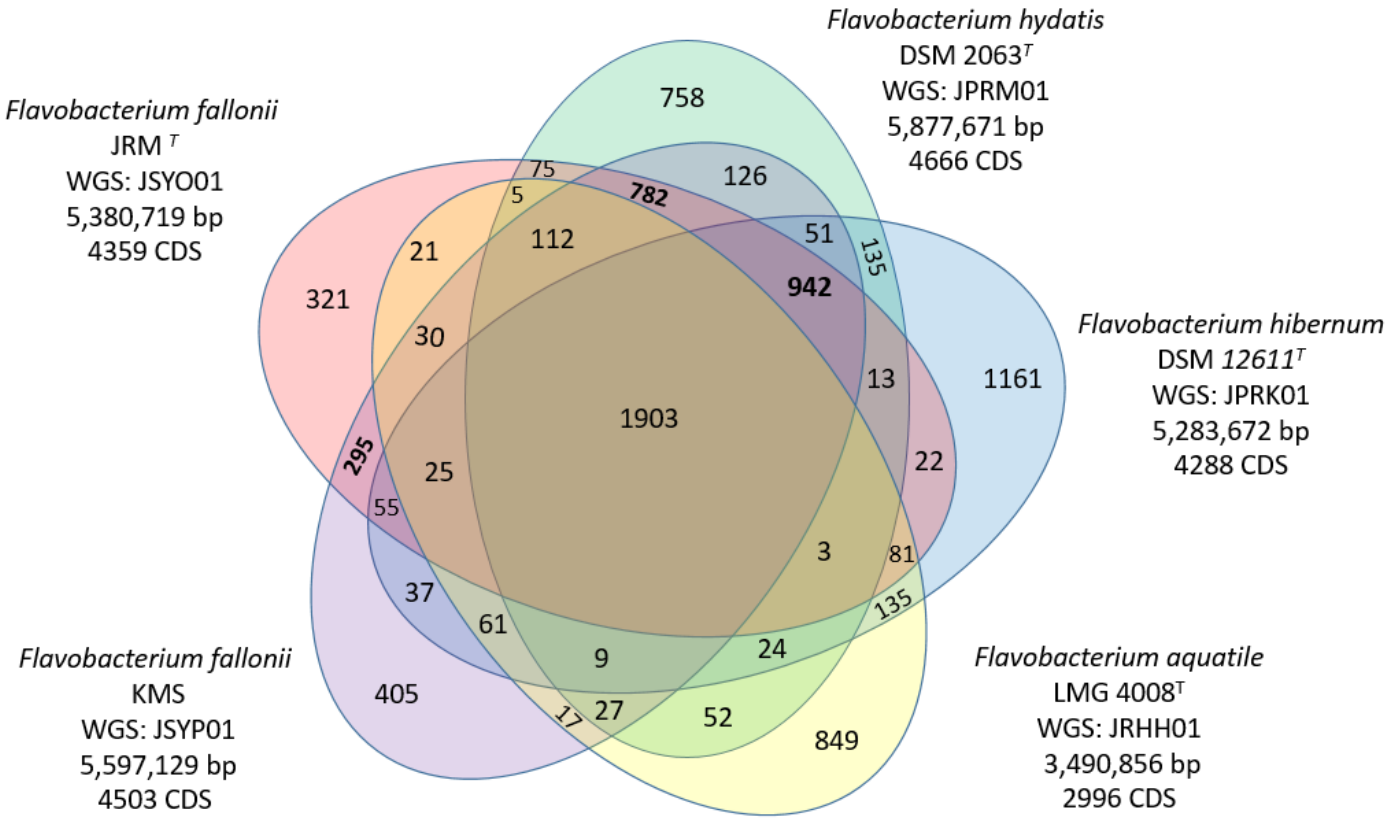


Figure 12. Venn Diagram of unique and shared genes between *Flavobacterium aquatile* and the reference organisms using Reciprocal genome comparisons by Rapid Annotation using Subsystems Technology (RAST). Venn Diagram was made using the Venn Diagram tool developed by the Newmanlab.

Phenotypic Tests

Biolog-Growth in Biolog GenIII plates was completed to quantitatively compare phenotypic data between *Flavobacterium fallonii* and the reference organisms grown on Bug+ Blood. The values seen in *Figure 13* are assigned by the computer according to how well the organism grew in that well. Numbers are given from 0-100 with 100 being maximum growth and 0 being no growth. Each value has been color coded to better visualize the differences in growth. Green colored cells indicate growth values near 100 while red colored cells indicate poor growth values near 0. Differences in growth can be seen between *Flavobacterium fallonii* and the reference organisms by comparing the color coded cells. Key differences from the Biolog results have been further compared in *Figure 13d*. Some differences in the growth patterns between the three isolates of *Flavobacterium fallonii* are that *Flavobacterium fallonii KMS* was able to utilize sucrose as a carbon source whereas the other strains of *Flavobacterium fallonii* did not show positive growth. Another difference between the three isolates was that *Flavobacterium fallonii KMS* was also able to utilize D- raffinose as a carbon source while *Flavobacterium fallonii JRM* and *AJR* did not show growth under this condition. With D-fructose as the metabolite, *Flavobacterium fallonii AJR* had a growth value of 11.0. However, *Flavobacterium fallonii JRM* and *KMS* both had positive growth under that condition. In the presence of nalidixic acid, *Flavobacterium fallonii JRM* and *KMS* both had low growth with values of 44.5 and 51.5 respectively whereas *Flavobacterium fallonii AJR* had a growth value of 13.0. *Flavobacterium fallonii AJR* also had a growth value of 98.0 with D- cellbiose as a metabolite and the other two isolates showed no growth. In comparison within all of the reference organisms, *Flavobacterium hibernum* was the only to utilize N-acetyl neuraminic acid, L- fucose, L-rhamnose, β-methyl-D-

glucoside, and D-salicin as carbon sources. *Flavobacterium hydatis* was the only organism not to grow with D-trehalose but also the only organism to utilize glycerol as a carbon source.

Growth Condition	Flavobacterium JRM	Flavobacterium KMS	Flavobacterium AJR	Flavobacterium hydatis	Flavobacterium hibernum
neg control	23.5	24.0	20.0	24.0	23.3
dextrin	99.0	99.0	99.0	99.0	98.3
D-maltose	98.5	97.5	98.0	98.0	98.0
D-trehalose	98.5	98.0	98.0	12.5	69.0
D-cellobiose	13.5	14.5	98.0	18.0	72.0
gentiobiose	99.0	98.0	97.0	98.0	97.7
sucrose	12.5	97.5	10.0	13.5	70.0
D-turanose	13.5	12.5	9.0	13.5	11.3
stachyose	12.0	23.5	10.0	14.5	26.3
pos control	97.5	97.5	97.0	98.5	97.7
pH 6	96.5	97.5	96.0	97.0	97.0
pH 5	40.5	17.0	12.0	14.0	15.0
D-raffinose	13.5	93.5	14.0	18.0	69.7
α-D-lactose	10.5	11.0	13.0	15.0	18.7
D-melibiose	12.0	13.5	14.0	16.5	13.0
β-methyl-D-glucoside	9.5	10.5	10.0	15.0	68.7
D-salicin	8.5	9.5	9.0	10.5	69.0
N-acetyl-D-glucosamine	97.5	96.5	96.0	98.0	96.3
N-acetyl-β-D-mannosamine	10.0	9.5	9.0	13.5	13.0
N-acetyl-D-galactosamine	98.5	97.5	98.0	98.0	95.3
N-acetyl neuraminic acid	12.0	11.0	7.0	7.5	66.0
1% NaCl	51.5	58.0	55.0	61.0	72.3
4% NaCl	11.0	11.5	9.0	9.0	10.7
8% NaCl	15.5	14.0	20.0	15.5	10.7
α-D-glucose	98.5	98.0	99.0	99.0	97.3
D-mannose	97.5	97.5	99.0	98.0	96.7
D-fructose	47.5	96.5	11.0	74.5	93.0
D-galactose	98.0	97.5	99.0	97.5	96.7
3-methyl glucose	31.0	9.0	10.0	10.0	9.0
D-fucose	10.5	9.5	11.0	8.5	8.7
L-fucose	20.5	11.5	17.0	15.5	65.7
L-rhamnose	10.5	11.0	10.0	12.0	66.7

Figure 13a. Biolog results comparing all strains of *Flavobacterium fallonii* and the reference organisms.

Growth Condition	Flavobacterium JRM	Flavobacterium KMS	Flavobacterium AJR	Flavobacterium hydatis	Flavobacterium hibernum
inosine	51.0	14.5	7.0	11.0	8.7
1% Na-lactate	48.0	51.0	87.0	37.0	91.7
fusidic acid	10.5	12.0	9.0	10.0	9.3
D-serine	12.5	19.0	10.0	13.0	14.0
D-sorbitol	13.5	15.0	11.0	18.0	13.7
D-mannitol	9.5	11.5	11.0	13.0	9.7
D-arabitol	10.0	10.0	6.0	11.0	8.0
myo-inositol	10.5	11.5	8.0	10.5	8.0
glycerol	46.5	47.0	8.0	94.0	37.0
D-glucose-6-PO4	24.0	23.5	28.0	39.0	48.0
D-fructose-6-PO4	22.5	30.5	36.0	34.5	43.7
D-aspartic acid	8.5	10.0	5.0	6.5	7.3
D-serine	8.0	8.0	5.0	8.0	6.0
troleandomycin	9.5	10.0	9.0	10.5	9.3
rifamycin SV	94.5	94.5	93.0	95.5	95.0
minocycline	14.5	15.0	12.0	14.5	13.3
gelatin	99.0	87.0	97.0	99.0	98.3
glycyl-L-proline	97.5	97.5	95.0	97.0	92.7
L-alanine	88.0	86.0	32.0	61.5	53.7
L-arginine	90.0	89.0	80.0	92.5	74.7
L-aspartic acid	97.5	95.5	96.0	98.5	94.7
L-glutamic acid	98.0	97.0	98.0	98.5	96.0
L-histidine	46.5	67.5	41.0	46.0	43.3
L-pyroglutamic acid	12.0	11.5	14.0	11.5	12.3
L-serine	94.0	93.0	92.0	94.5	80.3
lincomycin	91.0	50.0	91.0	46.5	37.0
guanidine HCl	47.5	50.5	10.0	48.0	51.3
niaproof 4	13.0	15.0	15.0	17.0	13.0
pectin	48.5	80.0	23.0	19.5	53.3
D-galacturonic acid	98.0	97.5	98.0	98.5	96.3
L-galacturonic acid lactone	53.5	53.5	11.0	10.0	38.3
D-gluconic acid	13.0	12.5	17.0	11.0	9.3

Figure 13b. Biolog results comparing all strains of *Flavobacterium fallonii* and the reference organisms.

Gowth Condition	Flavobacterium JRM	Flavobacterium KMS	Flavobacterium AJR	Flavobacterium hydatis	Flavobacterium hibernum
D-glucuronic acid	16.0	15.5	72.0	25.0	44.3
glucuronamide	16.0	14.0	24.0	20.0	19.7
mucic acid	11.0	10.0	15.0	9.5	7.7
quinic acid	13.0	12.0	19.0	12.0	14.3
D-saccharic acid	13.0	11.0	14.0	10.5	8.7
vancomycin	95.0	93.5	95.0	96.0	94.0
tetrazolium violet	99.0	99.0	99.0	99.0	79.3
tetrazolium blue	99.0	99.0	99.0	99.5	97.0
p-hydroxy-phenylacetic acid	14.5	14.0	12.0	15.0	11.3
methyl pyruvate	12.0	12.5	15.0	12.0	62.0
D-lactic acid methyl ester	15.5	15.5	19.0	13.5	15.3
L-lactic acid	10.0	9.5	10.0	9.5	10.0
citric acid	14.5	14.0	31.0	18.0	14.3
α-keto-glutaric acid	11.5	11.5	19.0	12.0	9.7
D-malic acid	13.0	12.0	19.0	14.0	12.0
L-malic acid	61.5	53.0	19.0	87.5	41.7
bromo-succinic acid	13.0	11.5	6.0	8.0	9.7
nalidixic acid	44.5	51.5	13.0	13.5	13.7
LiCl	10.5	12.0	9.0	14.0	9.7
K-tellurite	16.5	19.5	20.0	19.0	19.0
tween-40	77.5	84.5	89.0	95.0	89.0
γ-amino-butyric acid	13.5	13.0	18.0	16.5	15.3
α-hydroxy-butyric acid	12.5	11.0	15.0	14.0	11.7
β-hydroxy-D,L-butyric acid	13.5	12.5	16.0	13.0	11.7
α-keto-butyric acid	9.0	7.5	11.0	7.0	6.7
acetoacetic acid	60.5	61.0	89.0	55.5	50.3
propionic acid	8.5	13.0	9.0	12.5	9.3
acetic acid	94.0	92.0	98.0	97.5	72.0
formic acid	10.5	11.0	10.0	11.0	10.0
aztreonam	94.0	95.0	96.0	56.0	95.0
Na-butyrate	12.5	13.5	14.0	15.5	12.3
Na bromate	13.5	13.5	15.0	14.0	11.3

Figure 13c. Biolog results comparing all strains of *Flavobacterium fallonii* and the reference organisms.

Growth Condition	Flavobacterium JRM	Flavobacterium KMS	Flavobacterium AJR	Flavobacterium hydatis	Flavobacterium hibernum
D-trehalose	98.5	98.0	98.0	12.5	69.0
D-cellobiose	13.5	14.5	98.0	18.0	72.0
sucrose	12.5	97.5	10.0	13.5	70.0
pH 5	40.5	17.0	12.0	14.0	15.0
D-raffinose	13.5	93.5	14.0	18.0	69.7
β-methyl-D-glucoside	9.5	10.5	10.0	15.0	68.7
D-salicin	8.5	9.5	9.0	10.5	69.0
N-acetyl neuraminic acid	12.0	11.0	7.0	7.5	66.0
D-fructose	47.5	96.5	11.0	74.5	93.0
L-fucose	20.5	11.5	17.0	15.5	65.7
L-rhamnose	10.5	11.0	10.0	12.0	66.7
glycerol	46.5	47.0	8.0	94.0	37.0
lincomycin	91.0	50.0	91.0	46.5	37.0
guanidine HCl	47.5	50.5	10.0	48.0	51.3
pectin	48.5	80.0	23.0	19.5	53.3
D-glucuronic acid	16.0	15.5	72.0	25.0	44.3
methyl pyruvate	12.0	12.5	15.0	12.0	62.0
L-malic acid	61.5	53.0	19.0	87.5	41.7
nalidixic acid	44.5	51.5	13.0	13.5	13.7

Figure 13d. Biolog results comparing all strains of *Flavobacterium fallonii* and the reference organisms highlighting main differences in growth patterns.

Antibiotic Sensitivity Testing- The three isolates of *Flavobacterium fallonii* and the reference organisms were grown in the presence of antibiotics to test their growth under generally inhibitory conditions (*Figure 14)*. The zones of inhibition (ZOI) were measured from the center of the disc to the end of the inhibitory region surrounding the disc. Some key difference in resistance to antibiotics can be found with erythromycin. *Flavobacterium fallonii KMS* was susceptible to the inhibitory actions of erythromycin and had a ZOI greater than 20mm. *Flavobacterium fallonii AJR* was resistant to this antibiotic and had a ZOI less than 10mm. *Flavobacterium hibernum* was susceptible to erythromycin while *Flavobacterium hydatis* was resistant. In the presence of nalidixic acid, the growth of *Flavobacterium fallonii AJR* was inhibited, whereas the other two isolates were only slightly inhibited and had ZOIs between 10mm and 20mm. All of the organisms tested were susceptible to clavulanic acid and resistant to the penicillin class of antibiotics with the exception being *Flavobacterium hibernum* which was slightly inhibited by ampicillin.

Antibiotics	F. sp JRM	F. sp KMS	F. sp AJR	F. hydatis	F. hibernum
Kanamycin	R	R	R	R	R
Erythromycin	I	S	R	R	S
Penicillin	R	R	R	R	R
Nalidixic acid	I	I	S	S	S
Streptomycin	R	R	R	R	R
Rifampicin	I	I	I	I	S
Tetracycline	R	R	R	R	I
Amoxicillin	R	R	R	R	R
Carbenicillin	R	R	R	R	R
Ampicillin	R	R	R	R	I
Chlortetracycline	I	I	S	I	I
Chloramphenicol	R	R	I	I	I
Clavulanic Acid	S	S	S	S	S

	ZOI
Resistant	0-10mm
Intermediate	10-20mm
Susceptable	>20mm

Figure 14. Antibiotic Sensitivity Testing by modified Kirby-Bauer Method

FAME Analysis- In order to compare the fatty acid composition of the bacteria, FAME was performed. Seen on *Figure 15* each value corresponds to the percentage of the total fatty acid composition made up of that particular fatty acid. While highly similar, there are some key differences in the fatty acid compositions. *Flavobacterium aquatile* had a 14.3% of iso-C 16:0 whereas none of the other organisms tested had percentages higher than 2.2% for that fatty acid. *Flavobacterium hydatis* had a 17.9% Summed Feature 3 percentage which is more than 5% higher than any isolate of *Flavobacterium fallonii* and more than 12% more than *Flavobacterium*

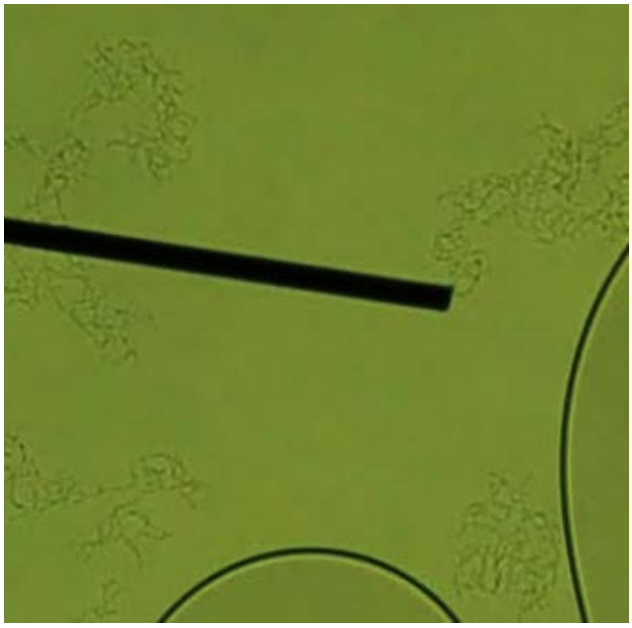
aquatile. Flavobacterium fallonii AJR had a 10.1% of iso-C 17:0 3-OH in comparison to *Flavobacterium fallonii JRM* with 7.3% and *Flavobacterium fallonii KMS* with a 6.9% composition.

Fatty Acid	JRM	KMS	AJR	hydatis	hibernum	aquatile
iso-$C_{12:0}$	1.6	0.0	0.3	1.6	1.5	0.5
iso-$_{13:0}$	1.7	1.5	0.5	1.2	1.2	0.3
$C_{13:0}$	0.6	0.5	0.3	1.0	0.4	0.0
iso-$C_{14:0}$	2.6	2.5	1.0	2.0	2.4	3.8
isoG-$C_{15:1}$	5.2	5.7	4.3	5.5	4.0	8.7
iso-$C_{15:0}$	22.6	22.5	25.8	22.7	26.1	21.6
anteiso-$C_{15:0}$	2.3	2.3	1.4	1.9	1.5	3.2
$C_{15:1}$ω6c	5.1	6.9	5.5	6.5	7.2	2.6
$C_{15:0}$	0.0	0.0	0.0	0.0	0.0	0.0
isoH-$C_{16:1}$	1.8	1.5	0.7	0.5	0.9	6.5
iso-$C_{16:0}$	2.2	1.6	1.3	0.7	1.4	14.3
Summed Feature 3	12.8	12.5	11.9	17.9	13.2	5.8
$C_{16:0}$	2.9	2.6	3.1	3.2	3.3	1.8
iso-$C_{15:0}$3-OH	7.6	7.7	8.7	7.1	8.1	4.2
Summed Feature 9	6.4	6.4	4.9	4.5	6.8	4.0
iso-$C_{17:0}$	0.8	0.8	0.3	0.4	1.0	0.4
$C_{17:1}$ω8c	1.4	1.8	0.9	1.7	1.3	0.9
$C_{17.1}$ω6c	4.7	5.9	3.3	5.8	4.5	1.0
iso-$C_{16:0}$3-OH	1.7	1.5	2.2	1.1	1.7	6.5
$C_{16:0}$3-OH	1.9	1.8	3.1	3.1	1.7	0.7
iso-$C_{17:0}$3-OH	7.3	6.9	10.1	6.2	7.7	5.1

Figure 15. FAME Analysis using the MIDI FAME Standard Method.

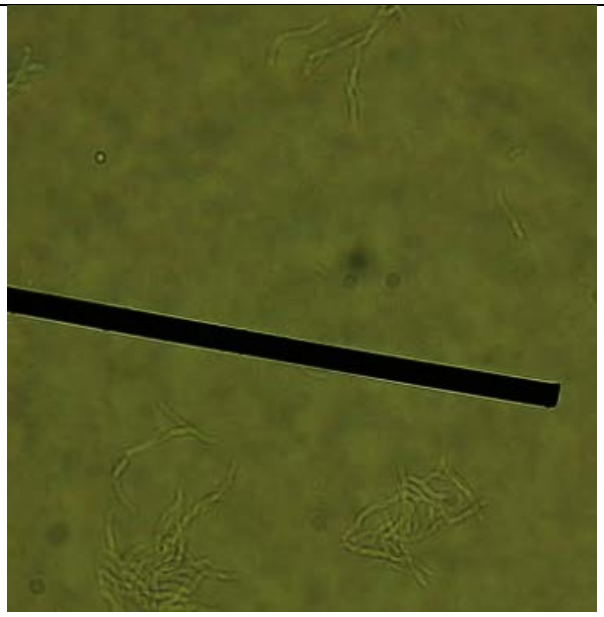
Immunological Tests

Agglutination Assay-An agglutination assay was performed to examine the cross reactivity of the anti-JRM antibodies with the reference organisms. The images used in *Figure 14* were taken of slides using 5 uL of the antibody solution at a concentration of 1:500 and 5 uL of cell samples at a 1:10 dilution. The images were taken with either the 10X or 40X objectives and a 10X eyepiece for a total magnification of either 100X or 400X. Agglutination is the clumping of cells that occurs when an antigen is mixed in the presence of its corresponding antibody. Under the microscope, the culture of *Flavobacterium fallonii JRM* clearly shows agglutination of the cells. Even at the 10X, the cells visibly appear in large clumps, indicating the presence of cross-linking by the antibodies. *Flavobacterium fallonii KMS* also indicated cross-linking by antibodies as the cells appeared in large clumps. The clumping of cells was also present in the slide containing *Flavobacterium fallonii AJR*. The *Flavobacterium hydatis* cells did not agglutinate in the presence of the anti-JRM antibody. Under the microscope, the cells appear either solo or in short chains. The *Flavobacterium hibernum* culture also did not appear to agglutinate in the presence of anti-JRM antibodies. The cells are distinctly separate or seen in short chains. For a negative control, *Escherichia coli* was also viewed under the microscope in the presence of anti-JRM antibodies. Upon examination, the cells did not react as they are seen as solo short rods.

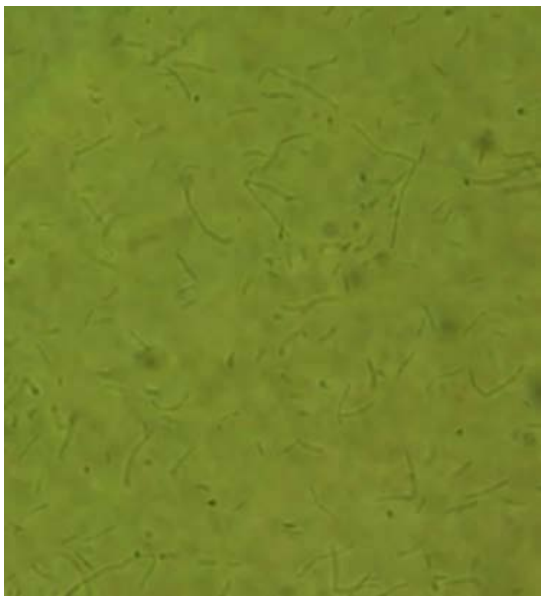


Flavobacterium fallonii JRM (10X obj)

Flavobacterium fallonii KMS (40X obj)

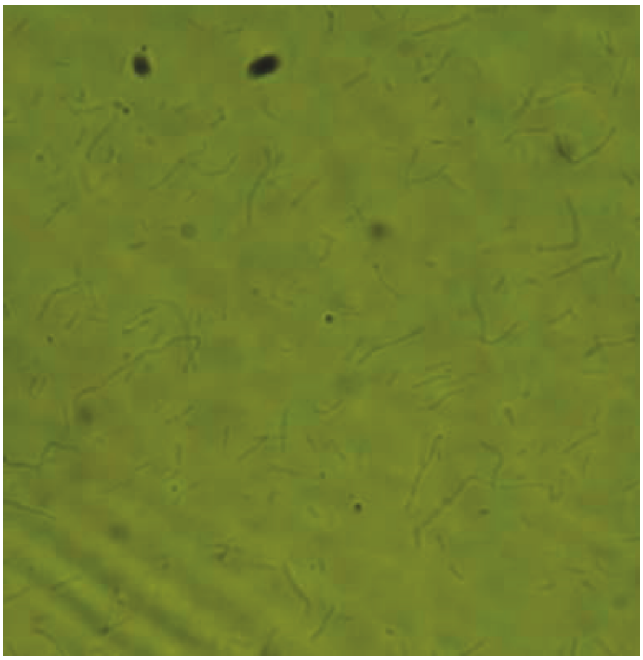


Flavobacterium fallonii AJR (40X obj)



Flavobacterium hydatis (40X obj)

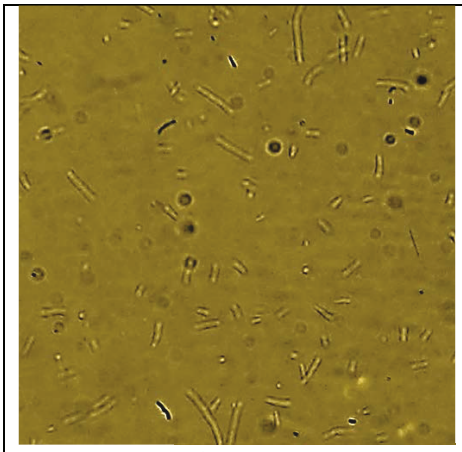
Figure 16a. Agglutination Assay viewed with the 40X unless otherwise specified. Overnight cell cultures in TSB diluted 1:10 and Day 56,58 Anti-JRM at a 1:500 dilution. To improve visibility of the images, the contrast of each image was increased by 40%.



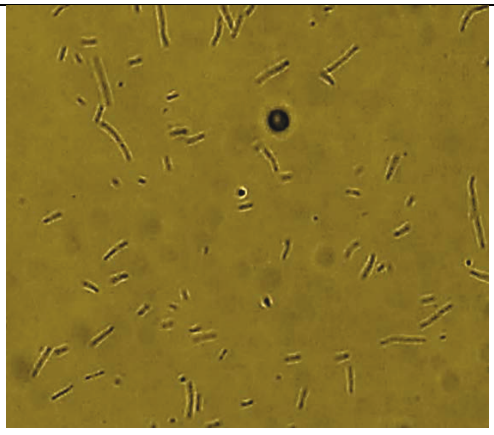
Flavobacterium hibernum (40X obj)

Escherichia coli (40X obj)

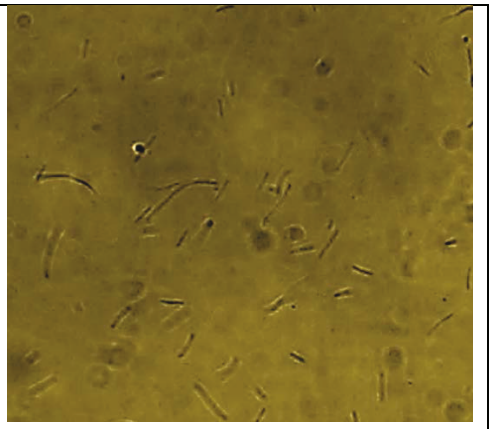
Figure 16b. Agglutination Assay viewed with the 40X unless otherwise specified for a total magnification of 400X. Overnight cell cultures in TSB diluted 1:10 and Day 56,58 Anti-JRM at a 1:500 dilution. To improve visibility of the images, the contrast of each image was increased by 40%.



1



2



3

Figure 16c. Negative Control- 1. *F.JRM. 2. F. KMS*. 3. *F. AJR* with a total magnification of 400X, no Ab added.

Western Blot and Gel Stains- The BioRad Coomassie Stained Protein gel shown in *Figure 15* was the result of the protocol found in the methods section. The gel dried out during its staining process which resulted in the hazy background blue coloration upon rehydration. However, protein can be seen in the gel. Each band represents a specific size of protein. From analyzing this gel, *Flavobacterium fallonii KMS* and *Flavobacterium hydatis* had the cleanest separation of protein bands. Although not nearly as distinct as the lanes containing *Flavobacterium fallonii KMS* and *Flavobacterium hydatis*, *Escherichia coli* appeared to have a similar protein composition to the species of *Flavobacteria*. The lane that contained *Flavobacterium fallonii JRM* does not show very clear separation of bands. The negative control of *Escherichia coli* LPS did not show any bands in the gel indicating that the LPS prep sample for *Escherichia coli* did not have any protein in it. Conclusions from Gel 1 from *Figure 15* were that the protein extractions were successful as there were proteins found in the gel. Although being covered in a blue background haze, all protein lysates from each reference organism have a similar profile. *Figure 16* shows Gel 2 after electrophoresis. This gel contained LPS and was not stained with Coomassie. It was washed in a methanol, acetic acid, and dH20.

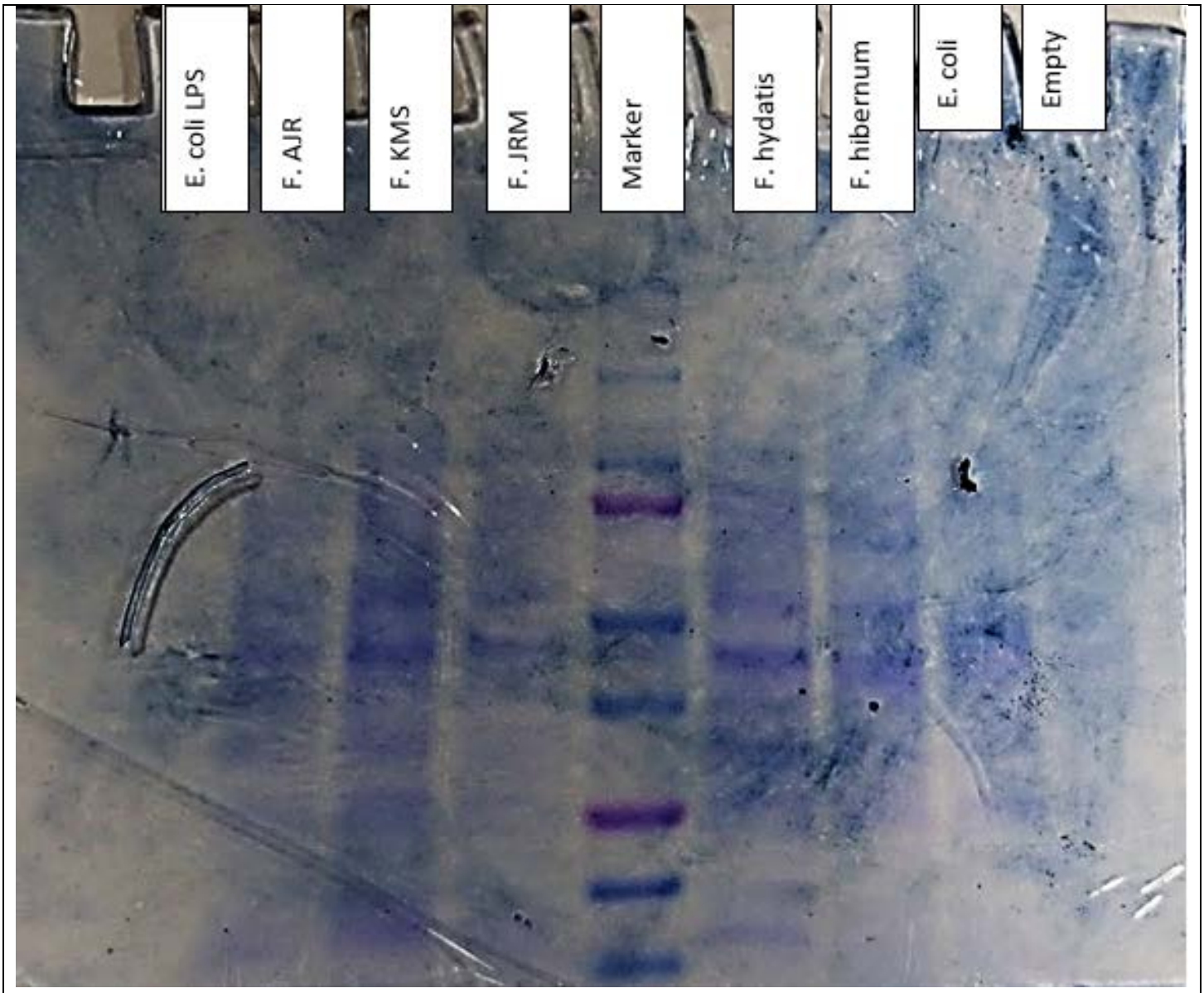


Figure 17. Western Blot- Protein Gel 1. BioRad Coomassie Stain

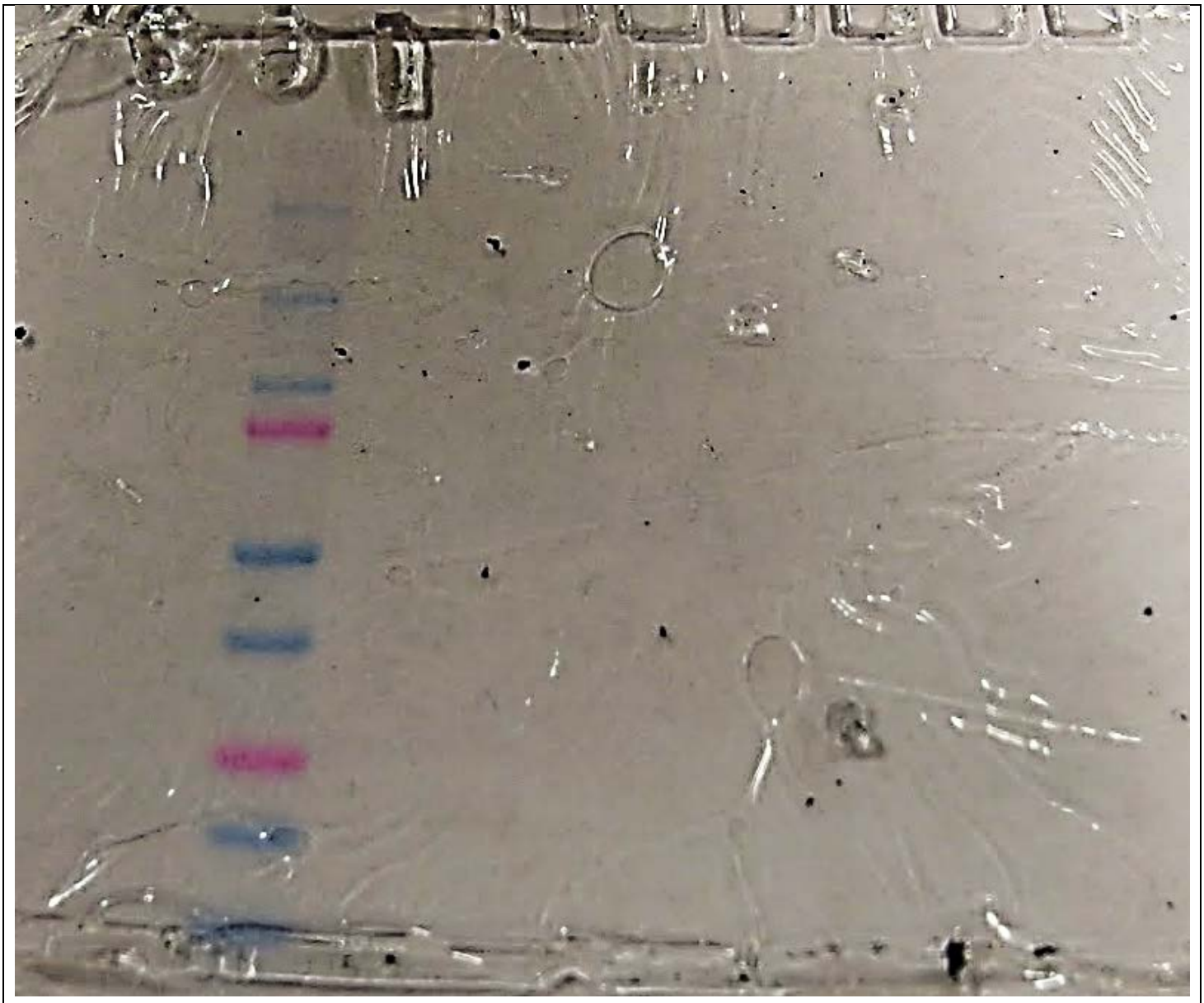


Figure 18. Western Blot-LPS Gel 2 with no stain.

Figure 19 shows the Western Blot membrane from Gel 3. The membrane itself reacted with the substrate in the final step as the integrity of the membrane was compromised. However, the Western Blot indicated that anti-JRM antibodies bind to many of the same proteins found in the three *Flavobacterium fallonii* isolates and the reference organisms. However, although being hard to distinguish and blocked by the folds of the membrane, *Flavobacterium fallonii JRM* does appear to have a lower massed protein that reacted strongly with the anti-JRM antibodies. This can be seen towards the bottom of the *Flavobacterium fallonii JRM* lane in a deep purple streak. The other species of *Flavobacterium fallonii* did not show a strong reaction in that area of the membrane and neither did the other reference organism. *Escherichia coli* did not react with the anti-JRM antibody as no purple pigmentation can be seen in the *E. coli* lane.

Figure 20 shows the Western Blot membrane from Gel 4. The membrane itself also reacted negatively with the substrate in the final step. Gel 4 contained the LPS extractions. The positive control for the membrane was the *Flavobacterium fallonii JRM* protein extract in lane 8. This control appeared with little distinction between individual bands of protein, but indicated that along with the marker, there were products from electrophoresis on the membrane. The difference in the protein and the LPS patterns showed that there was not protein in the LPS isolations. In each lane with LPS extracts, there was a purple band near the bottom of the membrane. This would translate to a molecular of approximately 15 kDa. When treated with SDS and heat, LPS usually has a molecular mass of 50-100kDa and a molecular mass of 10-20 kDa when intact (Macphee, 2010). However, *Flavobacterium fallonii JRM* did have the most purple bands out of any of the lanes. Around the universal purple band towards the bottom of the membrane, the JRM lane has a purple streak where the other lanes do not. There may be also be

small purple mark in the middle of the *Flavobacterium fallonii JRM* lane, but with the quality of the membrane, no conclusions can be made.

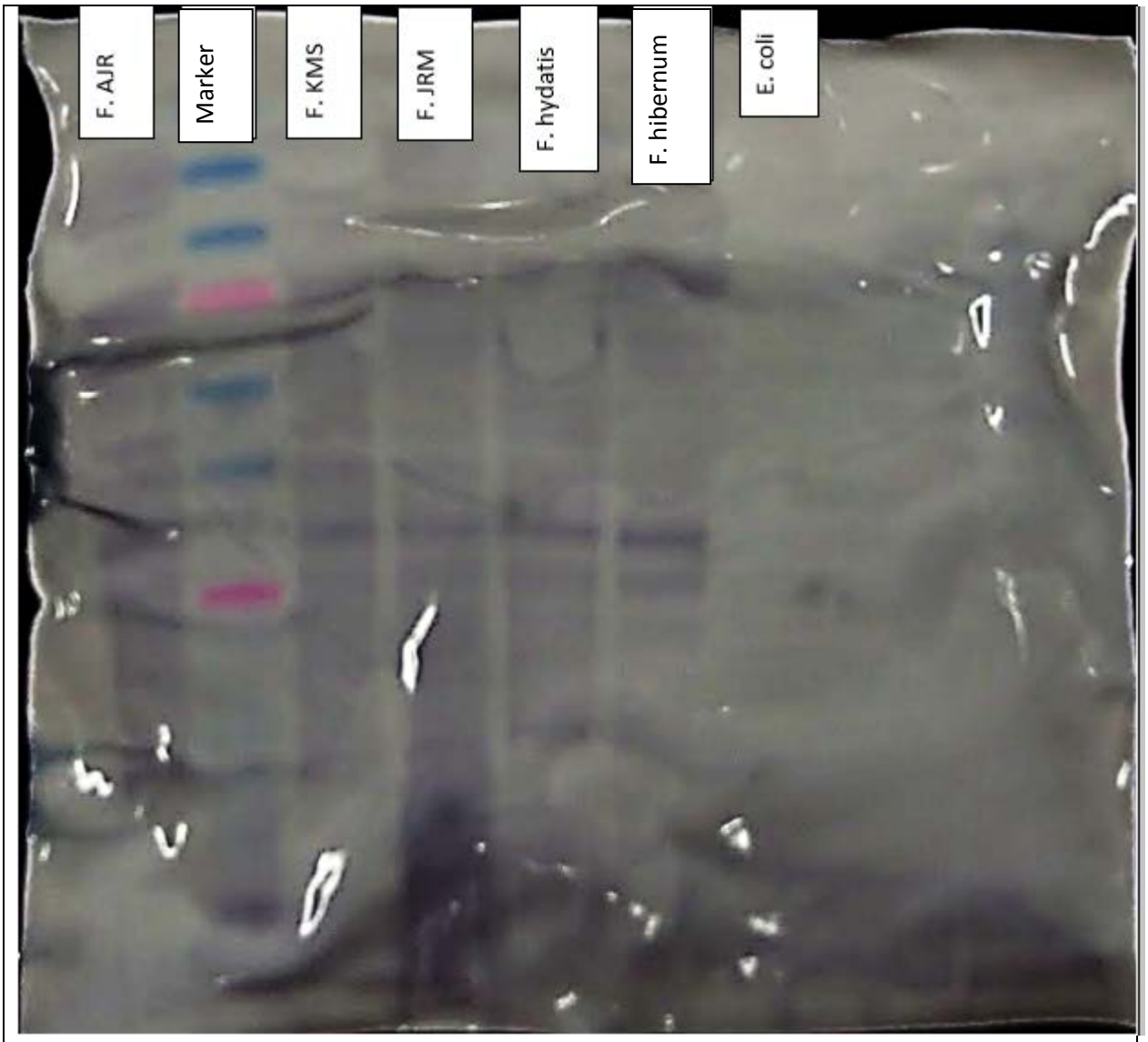


Figure 19. Western Blot-Protein Gel 3

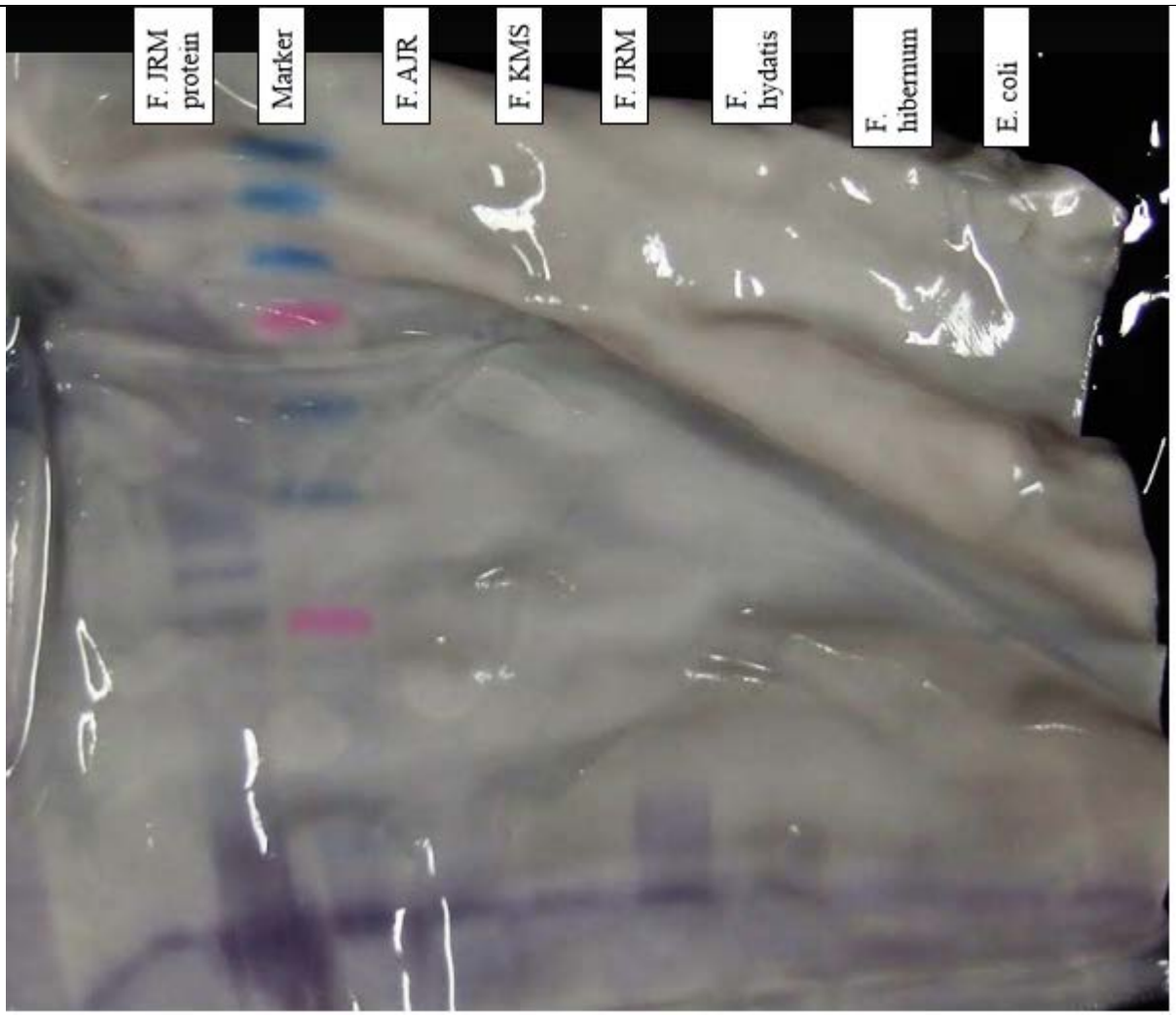


Figure 20. Western Blot – LPS Gel 4

Figure 21 shows the UGF202 stains using one of the immunology LPS gels. No fluorescence can be seen on the image indicating that there was either no LPS found on the gel, or that the staining procedure did not work.

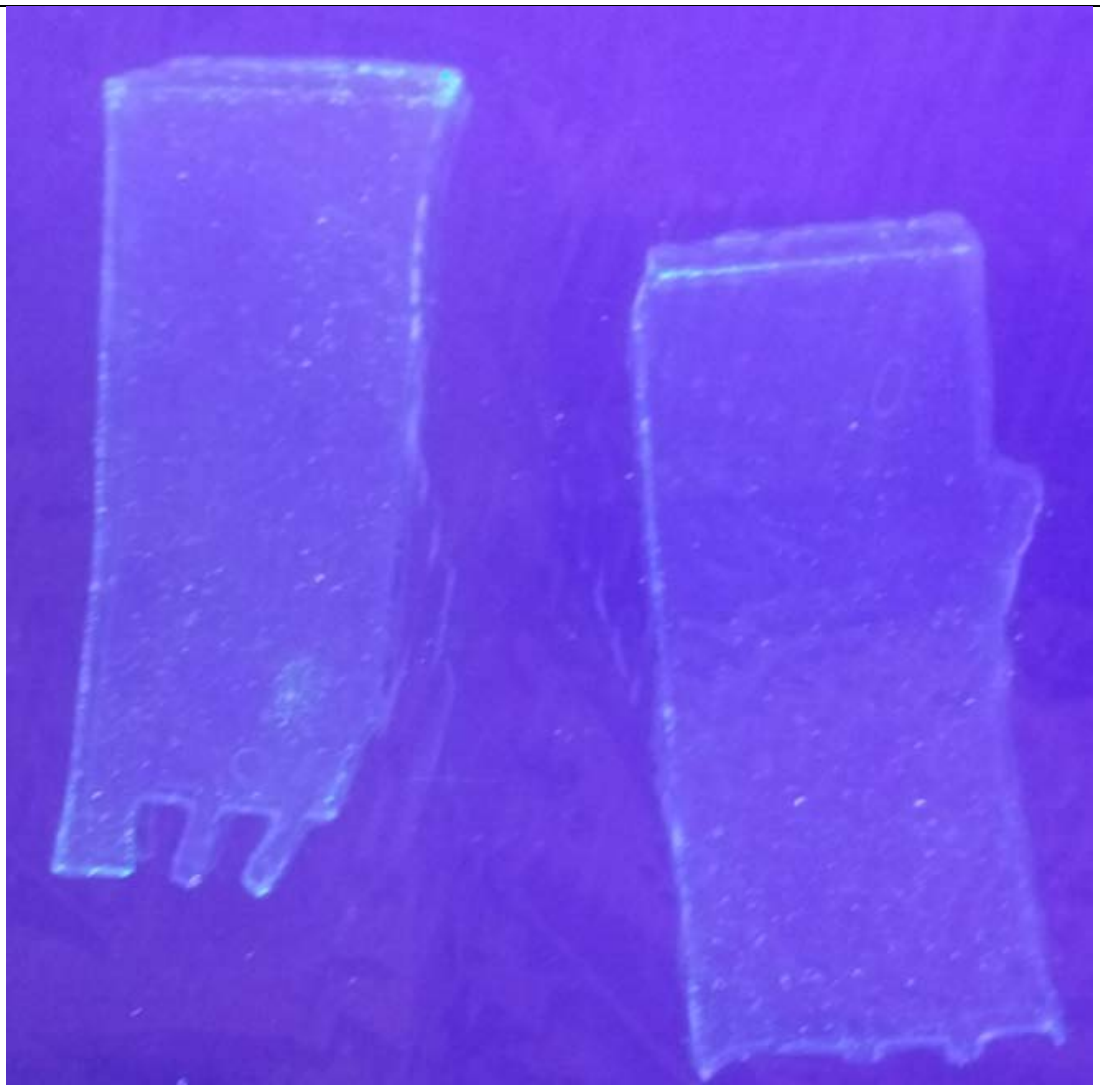


Figure 21. UGF202 LPS Stain viewed on Immunology LPS Gel on a UV Platform

ELISA- Results from the ELISA using Day 0 serum from rabbit 227 and Day 56,58 serum from rabbit 227 can be seen in *Figure 22* below. The numbers are color coded to show relationships. The numbers are the values read by the plate reader at 450nm. The plate reader uses a camera to check the intensity of the color of the substrate in each well. The intensity of the substrate in the well should be correlated to how well the primary antibody was bound to whatever antigen was in the cell. The results are inconclusive. *Escherichia coli* was used as a negative control. All results from the Day 0 ELISA with *Escherichia coli* yielded positive results. In well A for *Escherichia coli*, there was no primary antibody present. While cells were fixed to that well, only the secondary antibody and the substrate were added to that well and it still converted the substrate and yielded an optical density value of 0.545. Well H for *Escherichia coli* did not contain any fixed cells, but did receive both the primary antibody, secondary antibody, and the substrate. It had a value of 0.843 despite not having any fixed cells. Across the plate in row A, the substrate was visualized with values of 0.314 or higher for each well despite not having any primary anti-JRM added to those wells. Well H for *Flavobacterium fallonii JRM* and *Flavobacterium aquatile* both did not contain any cells and only received PBS controls and had values of 0.766 and 0.844 respectively. For the Day 56,58 ELISA, the negative control containing no cells and PBS had a value of 0.799. If looking at the average of the wells from this ELISA, *Flavobacterium fallonii JRM* had the strongest overall reaction according to the values indicated by the plate reader. However, this value is not significant. *Flavobacterium johnsonsiaea* had a response in well G with a value of 1.241. This was the highest individually recorded value on this ELISA. In both ELISAs there was little distinction between the anti-JRM dilutions and the values do not follow the predicted statistical increase at the lower dilutions.

ELISA Day 0 Rabbit 227

Anti-JRM		E. coli	F. hib	F. hyda	-	F. JRM	F. KMS	F. AJR	-	F. aqua	F. jon	C. bac	-
A	PBS	0.545	0.384	0.428	0.033	0.426	0.314	0.387	0.036	0.39	0.372	0.39	0.034
B	1:32k	0.68	0.373	0.498	0.035	0.428	0.443	0.378	0.036	0.392	0.359	0.29	0.035
C	1:16k	0.591	0.445	0.514	0.032	0.606	0.551	0.408	0.042	0.433	0.37	0.381	0.035
D	1:8k	0.7	0.481	0.546	0.033	0.522	0.426	0.445	0.034	0.423	0.305	0.406	0.035
E	1:4k	0.758	0.482	0.68	0.034	0.499	0.424	0.495	0.034	0.441	0.42	0.42	0.034
F	1:2K	0.795	0.546	0.593	0.034	0.503	0.548	0.409	0.034	0.482	0.436	0.378	0.034
G	1:1k	0.96	0.738	0.693	0.036	0.632	0.703	0.565	0.034	0.497	0.529	0.339	0.036
H	1:1K	0.843	0.043	0.402	0.036	0.766	0.041	0.402	0.035	0.844	0.041	0.04	0.036
		0.73	0.44	0.54	0.03	0.55	0.43	0.44	0.04	0.49	0.35	0.33	0.03

ELISA Day 56,68 Rabbit 227

Anti-JRM		E. coli	F. hib	F. hyda	-	F. JRM	F. KMS	F. AJR	-	F. aqua	F. jon	C. bac	-
A	PBS	0.332	0.26	0.295	0.036	0.3	0.246	0.268	0.057	0.31	0.061	0.38	0.035
B	1:32k	0.516	0.496	0.55	0.036	0.694	0.417	0.211	0.027	0.384	0.059	0.51	0.035
C	1:16k	0.475	0.415	0.629	0.045	0.783	0.517	0.519	0.035	0.426	0.33	0.548	0.036
D	1:8k	0.572	0.569	0.79	0.036	0.927	0.562	0.696	0.035	0.533	0.467	0.472	0.036
E	1:4k	0.508	0.543	0.809	0.035	0.992	0.68	0.732	0.035	0.653	0.703	0.406	0.037
F	1:2K	0.541	0.574	0.8	0.033	0.906	0.737	0.756	0.035	0.667	0.676	0.454	0.032
G	1:1k	0.437	0.945	0.977	0.034	1.013	0.72	0.805	0.036	0.549	1.241	0.57	0.034
H	1:1K	0.799	0.042	0.041	0.037	0.042	0.042	0.042	0.035	0.402	0.042	0.402	0.035

Figure 22. ELISA comparing *Flavobacterium fallonii* isolates with reference organisms from various genera.

Immunofluorescent Staining- *Figure 22a* shows an Alexa488 stain of *Flavobacterium fallonii JRM* observed using the 40X objective. The cells appear outlined in green fluorescence which would support the presence of anti-JRM binding to the outside of the cells. *Figure 22b* shows the *E. coli* slide under the blue emission filter to observe the Hoescht 33258 DNA stain.

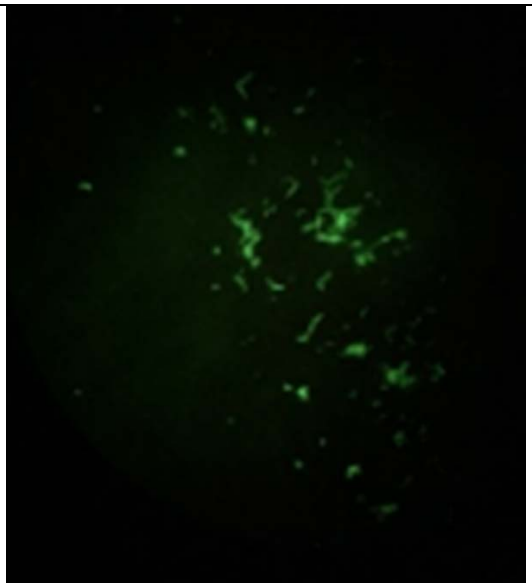


Figure 23a. Alexa488 Stain of *Flavobacterium fallonii JRM* under the 40x objective on a Nikon E200 fluorescent microscope.

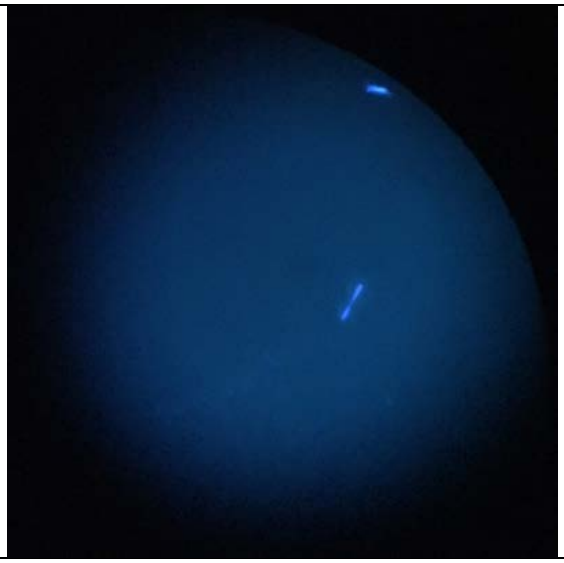


Figure 23b. Hoechst 33258 stain of *Escherichia coli* with the 40X objective on a Nikon E200 fluorescent microscope.

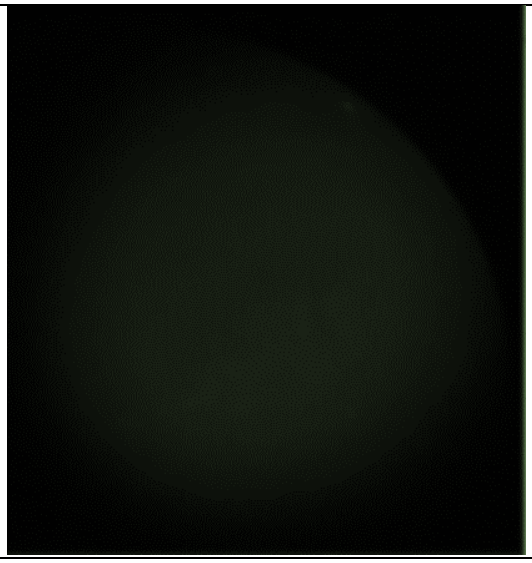


Figure 23c. Alexa488 Stain of *Escherichia coli* with the 40X objective on a Nikon E200 fluorescent microscope.

Genome Analysis

Genome Analysis- Genome comparisons were made using a number of different methods. The Venn Diagram comparisons as shown in *Figures 11-12* were generated using the comparative sequence based tool as shown in *Figure 24*. The RAST sorting uses the full annotated genomes to sort by bidirectional and unidirectional best hits. *Figure 25* shows the sorted genes unique to *Flavobacterium fallonii* in comparison to *Flavobacterium hydatis* and *Flavobacterium hibernum* as seen in the Venn Diagram Data Generator. There were 179 unique genes to *Flavobacterium fallonii*. 92 of the genes did not have an annotation and appeared under the name "hypothetical protein". These proteins were omitted from the figure. Key proteins were highlighted in yellow. *Figure 26* shows the list of genes unique only to *Flavobacterium fallonii JRM* when compared to the other isolates, *Flavobacterium hydatis,* and *Flavobacterium hibernum.* Using this comparison, *Flavobacterium fallonii JRM* had 302 unique genes. After omitting the hypothetical proteins from the final list, there were 58 annotated unique genes only found in this organism. *Figure 27* shows the list of genes unique only to *Flavobacterium fallonii KMS* when compared to the other isolates, *Flavobacterium hydatis,* and *Flavobacterium hibernum.* Using this comparison, *Flavobacterium fallonii KMS* had 297 unique genes. After omitting the hypothetical proteins from the final list, there were 68 annotated unique genes only found in this organism. *Figure 28* shows the list of genes unique only to *Flavobacterium fallonii AJR* when compared to the other isolates, *Flavobacterium hydatis,* and *Flavobacterium hibernum.* Using this comparison, *Flavobacterium fallonii AJR* had 258 unique genes. After omitting the hypothetical proteins from the final list, there were 52 annotated unique genes only found in this organism. *Figure 29* outlines a key set of highlighted unique genes to *Flavobacterium fallonii KMS* and *Flavobacterium hibernum*.

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Welcome to the SEED Viewer - a read-only browser of the curated SEED data.
For more information about The SEED please visit theSEED.org.

»Navigate »Help

Result

change organism selection

You chose to compute data for the following organisms:

Reference	Flavobacterium sp. JRM (1566022.5)
Comparison Organism 1	Flavobacterium hibernum DSM 12611 (37752.6) BlastDotPlot
Comparison Organism 2	Flavobacterium hydatis DSM 2063 (991.10) BlastDotPlot
Comparison Organism 3	Flavobacterium sp. AJR (6666666.222921) BlastDotPlot
Comparison Organism 4	Flavobacterium sp. KMS (1566023.5) BlastDotPlot

Percent protein sequence identity

	100	99.9	99.8	99.5	99	98	95	90	80	70	60	50	40	30	20	10
Bidirectional best hit																
Unidirectional best hit																

export table | clear all filters

display 30 items per page

«first «prev displaying 2 - 31 of 4359 next» last»

percent identity 37752.6	<	**percent identity 991.10**	<
percent identity 6666666.222921	<	**percent identity 1566023.5**	<

1566022.5			37752.6			991.10			6666666.222921			1566023.5		
Contig	Gene	Length	Hit	Contig	Gene	Hit	Contig	Gene	Hit	Contig	Gene	Hit	Contig	Gene
all			all	all		all	all		all	all		all	all	
1	2	222	bi	2	380	bi	31	3608	bi	94	2268	bi	37	4645
1	3	337	uni	2	339	bi	31	3609	bi	94	2267	bi	37	4646
1	4	265	bi	7	1814	bi	31	3610	bi	94	2266	bi	37	4647
1	5	377	bi	7	1815	bi	31	3611	bi	175	3517	bi	3	1137
1	6	281	bi	2	336	bi	31	3612	bi	175	3518	bi	3	1136
1	7	1352	bi	7	1816	bi	31	3613	bi	175	3519	bi	3	1135
1	8	893	-			-			bi	175	3520	bi	3	1134
1	9	460	bi	7	1817	bi	31	3614	bi	175	3521	bi	3	1133
1	10	115	bi	7	1819	-			bi	175	3522	bi	3	1132
1	11	476	bi	2	69	uni	47	4426	bi	175	3523	bi	3	1131
1	12	629	bi	7	1820	bi	31	3615	bi	175	3524	bi	3	1130
1	13	1050	bi	7	1821	bi	31	3616	bi	175	3525	bi	3	1129
2	14	354	bi	5	1236	bi	15	2210	bi	114	2511	bi	11	2681
2	15	507	bi	5	1235	bi	15	2209	bi	114	2512	bi	11	2682
2	16	263	bi	5	1234	bi	15	2208	bi	114	2513	bi	11	2683
2	17	199	bi	5	1233	bi	15	2207	bi	114	2514	bi	11	2684
2	18	465	bi	5	1232	bi	15	2206	bi	114	2515	bi	11	2685
2	19	140	-			bi	15	2205	bi	114	2516	bi	11	2686
2	20	1514	bi	5	1231	bi	15	2204	bi	114	2519	bi	11	2689
2	21	308	bi	5	1230	bi	15	2203	bi	114	2520	bi	11	2690
2	22	106	bi	5	1195	bi	15	2202	bi	114	2521	bi	11	2691
2	23	855	bi	5	1194	bi	15	2201	bi	114	2522	bi	11	2692
2	24	313	bi	5	1193	bi	15	2200	bi	114	2523	bi	11	2693
2	25	276	bi	5	1191	bi	15	2199	bi	114	2524	bi	30	4430
2	26	449	bi	5	1190	bi	15	2198	bi	114	2525	bi	30	4431
2	27	351	bi	5	1189	bi	15	2197	bi	114	2526	bi	30	4432
2	28	530	bi	5	1188	bi	15	2196	bi	114	2527	bi	30	4433
2	29	167	bi	5	1161	bi	15	2195	bi	114	2528	bi	30	4434
2	30	128	bi	5	1162	bi	15	2194	-			bi	30	4435
2	31	159	bi	5	1163	bi	15	2193	bi	55	1390	bi	30	4436

«first «prev displaying 2 - 31 of 4359 next» last»

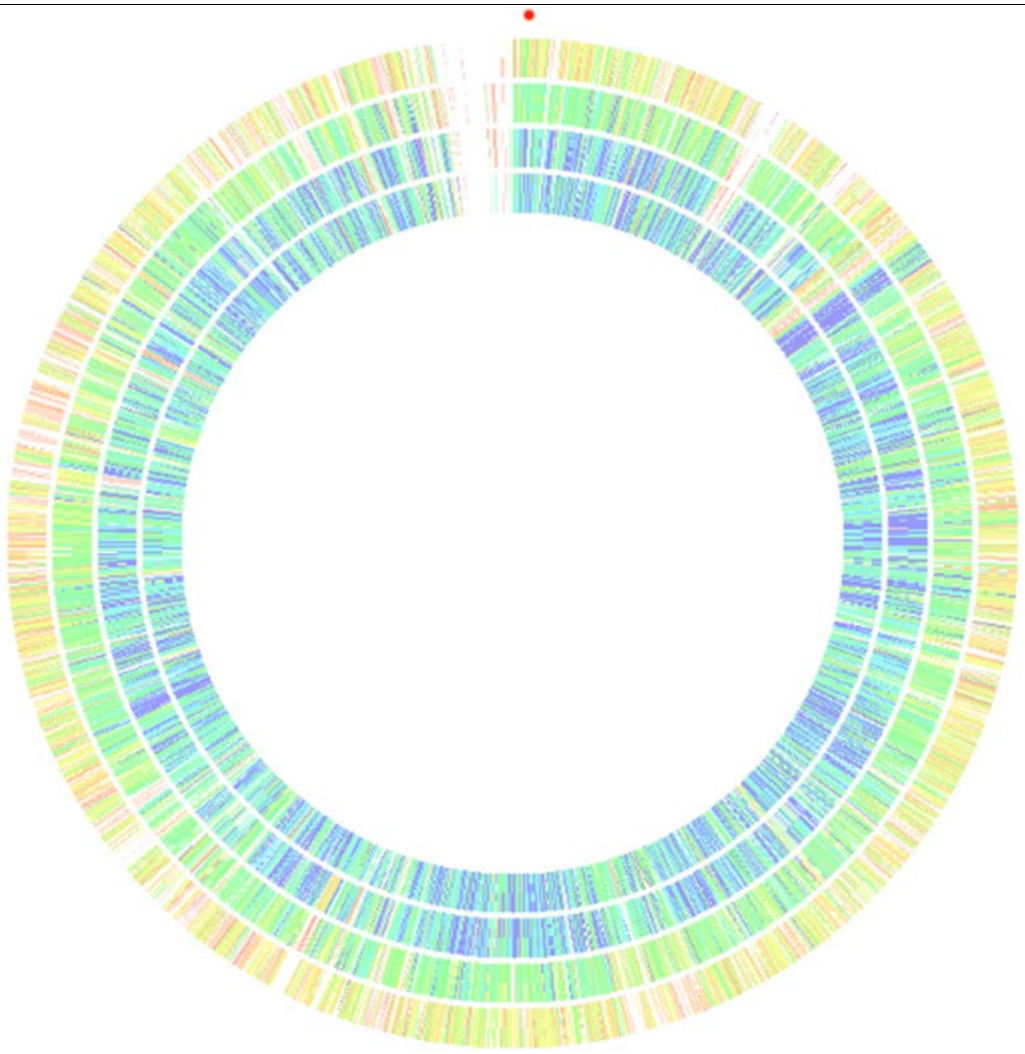

Figure 24. RAST Sequence Based Comparison viewed in the RAST Seed Viewer, sorted by Bidirectional Best Hits and Unidirectional Best Hits

Unique Genes to *Flavobacterium fallonii* in comparison to reference organisms	
Mobile element protein	2-amino-3-ketobutyrate coenzyme A ligase (EC 2.3.1.29)
Multidrug resistance protein D	acetyltransferase, GNAT family
N-acetylglucosamine-1-phosphate uridyltransferase (EC 2.7.7.23) / Glucos	Alcohol dehydrogenase (EC 1.1.1.1)
NADPH:quinone oxidoreductase	Alkanal monooxygenase alpha chain (EC 1.14.14.3)
Nicotinamidase family protein YcaC	Alpha-D-GlcNAc alpha-1,2-L-rhamnosyltransferase (EC 2.4.1.-)
nodulin 21-related protein	Alpha-galactosidase (EC 3.2.1.22)
Oligo alginate lyase	Anaerobic C4-dicarboxylate transporter
Oligosaccharide repeat unit polymerase Wzy	Arginase (EC 3.5.3.1)
Outer membrane lipoprotein omp16 precursor	Arylsulfatase regulator (Fe-S oxidoreductase)
Phosphoenolpyruvate carboxykinase [ATP] (EC 4.1.1.49)	Aspartate racemase (EC 5.1.1.13)
Phytoene desaturase, neurosporene or lycopene producing (EC 1.3.-.-)	Biotin synthesis protein BioC
Pirin	bll2395; hypothetical protein
Polysaccharide export lipoprotein Wza	cAMP-binding proteins - catabolite gene activator and regulatory sul
Probable multidrug resistance protein norM	Carbohydrate binding family 6
Probable transmembrane protein	Carbonic anhydrase (EC 4.2.1.1)
putative acetyltransferase	Catalase (EC 1.11.1.6) / Peroxidase (EC 1.11.1.7)
putative dioxygenase	Cobalt-zinc-cadmium resistance protein CzcA; Cation efflux system p
putative integral membrane protein	D-alanyl-D-alanine carboxypeptidase (EC 3.4.16.4)
putative outer membrane protein, probably involved in nutrient binding	DNA-binding response regulator
RagB/SusD domain protein	Dodecin
RhtB family transporter	Excinuclease ABC, C subunit-like
Rod shape-determining protein MreB	FIG00638667: hypothetical protein
short chain dehydrogenase	FIG00649072: hypothetical protein
SIGNAL-TRANSDUCTION SENSOR PROTEIN-PAS/PAC domain	FIG00693309: hypothetical protein
sodium/hydrogen exchanger	FIG01092758: hypothetical protein
Thiamin biosynthesis lipoprotein ApbE	FIG048548: ATP synthase protein I2
Thioredoxin Disulfide Isomerase	Genes
Thioredoxin reductase (EC 1.8.1.9)	Glucan endo-1,3-beta-glucosidase A1 precursor (EC 3.2.1.39) ((1->3)-
TonB-dependent receptor	glycosyl transferase, group 1
Transcriptional regulator	Glycosyltransferase (EC 2.4.1.-)
Transcriptional regulator, AraC family	Isocitrate dehydrogenase [NADP] (EC 1.1.1.42)
Transcriptional regulator, AraC family	Hydrolase, carbon-nitrogen family
Transcriptional regulator, AraC family	Histone acetyltransferase HPA2 and related acetyltransferases
Transcriptional regulator, AraC family	GNAT family acetyltransferase Bsu1853 (YoaA)
transcriptional regulator, Crp/Fnr family	Laminarinase (EC 3.2.1.39)
Transcriptional regulator, TetR family	Lanthionine biosynthesis cyclase LanC
transposase	Major facilitator family transporter
Two-component sensor histidine kinase	major facilitator superfamily MFS_1
Type IV fimbrial biogenesis protein PilY1	membrane protein, putative
Tyrosine-protein kinase Wzc (EC 2.7.10.2)	Methyl-accepting chemotaxis protein
Von Willebrand factor type A domain protein	Mobile element protein

Figure 25. Unique Genes for *Flavobacterium fallonii* compared to *Flavobacterium hydatis* and *Flavobacterium hibernum*. Venn Diagram Data Generator was used to sort the genes. Genes are arranged in alphabetical order, with hypothetical proteins omitted. Interesting genes were highlighted.

Flavobacterium fallonii JRM Unique Genes	
abortive infection protein, putative	Integrase/recombinase
Aminopeptidase N	internalin, putative
Ankyrin domain protein	internalin, putative
Archaeal DNA polymerase I (EC 2.7.7.7)	Membrane protein
Arylsulfatase (EC 3.1.6.1)	export of O-antigen, teichoic acid lipoteichoic acids
ATPase involved in DNA repair	membrane protein, putative
ATP-dependent DNA helicase UvrD/PcrA	Mll6838 protein
Beta-1,3-glucosyltransferase	Mobile element protein
Beta-lactamase class C and other penicillin binding proteins	Mobile element protein
Biofilm-associated protein	Mobile element protein
Biofilm-associated protein	Mobile element protein
Capsular polysaccharide synthesis enzyme Cap5F	Mobile element protein
Chaperone protein HtpG	Mobile element protein
Deoxyadenosine kinase (EC 2.7.1.76) / Deoxyguanosine kinase (EC 2.7.1.113)	O-antigen flippase Wzx
Deoxycytidylate deaminase	Phage protein
Deoxyuridine 5'-triphosphate nucleotidohydrolase (EC 3.6.1.23)	Phage protein
Dihydroflavonol-4-reductase (EC 1.1.1.219)	Phage tail fiber protein
DNA primase/helicase, phage-associated	Phosphoribosylglycinamide synthetase, ATP-grasp (A) domain protein
DNA-directed RNA polymerase subunit A' (EC 2.7.7.6)	protein of unknown function DUF323
DNA-directed RNA polymerase subunit B' (EC 2.7.7.6)	Putative exported protein precursor
Endonuclease V (EC 3.1.21.7)	Ribonucleotide reductase of class Ia (aerobic), beta subunit (EC 1.17.4.1)
Endonuclease V (EC 3.1.21.7)	serine/threonine kinase
enzyme; Degradation of DNA	Subtilase family protein
Flap structure-specific endonuclease (EC 3.-.-.-)	ThiJ/PfpI family protein
Genes	Thiol:disulfide interchange protein
Glucosamine-6-phosphate deaminase (EC 3.5.99.6)	TolA protein
Glycosyl transferase, group 1 family protein	two-component system sensor histidine kinase/response regulator,
Il-IS_2, transposase	Type III restriction enzyme, res subunit:DEAD/DEAH box helicase
Il-IS_2, transposase	UDP-glucose 4-epimerase (EC 5.1.3.2)
Il-IS_2, transposase	UDP-N-acetylglucosamine 4,6-dehydratase (EC 4.2.1.-)

Figure 26. ***Flavobacterium fallonii JRM*** **Unique Gene list in comparison to the other two isolates,** ***Flavobacterium hydatis,*** **and** ***Flavobacterium hibernum*****.**

Unique Genes to Flavobacterium fallonii KMS	
2-dehydro-3-deoxygluconate kinase (EC 2.7.1.45)	Export of O-antigen, teichoic acid lipoteichoic acids
3-oxoacyl-[acyl-carrier protein] reductase (EC 1.1.1.100)	Mobile element protein
4-hydroxy-2-oxoglutarate aldolase (EC 4.1.3.16) @ 2-dehydro	Mobile element protein
acyltransferase 3	Mobilization protein BmgA
best DB hits: PFAM: PF01909; Nucleotidyltransferase domain;	NADH:ubiquinone oxidoreductase subunit 5 (chain L)/Multisubunit
Chitinase (EC 3.2.1.14)	Outer membrane lipoprotein omp16 precursor
Chitinase (EC 3.2.1.14)	oxidoreductase
conserved hypothetical protein	Pentapeptide repeat protein
D-alanyl-D-alanine carboxypeptidase (EC 3.4.16.4)	phage tail assembly-like protein
demethylmenaquinone methyltransferase	Plasmid stabilization system protein
DNA double-strand break repair Rad50 ATPase	Polysaccharide deacetylase, caspase activity
DNA primase (EC 2.7.7.-)	possible DNA helicase
DNA-binding response regulator, AraC family	Probable poly(beta-D-mannuronate) O-acetylase (EC 2.3.1.-)
dTDP-4-dehydrorhamnose 3,5-epimerase (EC 5.1.3.13)	Prolidase (EC 3.4.13.9)
Enoyl-[acyl-carrier-protein] reductase [FMN] (EC 1.3.1.9)	putative carboxypeptidase
EpsK domain protein	putative esterase
Excinuclease ABC, C subunit-like	putative glycosyltransferase - possibly involved in cell wall
FIG00656499: hypothetical protein	putative glycosyltransferase - possibly involved in cell wall
FIG01047370: hypothetical protein	Putative insecticidal toxin complex
FIG01123853: hypothetical protein	Putative mobilization protein BF0133
Gluconate dehydratase (EC 4.2.1.39)	putative protein phosphatase
Gluconolactonase (EC 3.1.1.17)	Putative pyrogenic exotoxin B
Glycosaminoglycan attachment site	putative type II restriction endonuclease
Glycosyl transferase, group 1	Restriction modification enzyme
Glycosyl transferase, group 2 family protein	Serine/threonine protein kinase PrkC, regulator of stationary phase
Glycosyltransferase	SSS sodium solute transporter superfamily
Glycosyltransferase (EC 2.4.1.-)	TonB family protein / TonB-dependent receptor
Glycosyltransferase (EC 2.4.1.-)	Transcriptional regulator, AraC family
Glycosyltransferase (EC 2.4.1.-)	transposase
Helix-turn-helix motif	tRNA pseudouridine synthase C (EC 4.2.1.70)
Hypothetical protein. HMMPfam hit to PF03028	Type II restriction enzyme HphI (Endonuclease HphI) (R.HphI)
internalin, putative	type IV secretion protein Rhs
Internalin-like protein (LPXTG motif) Lmo0331 homolog	UDP-4-amino-4-deoxy-L-arabinose--oxoglutarate aminotransferase
Lmo0471 protein	unknown

Figure 27. ***Flavobacterium fallonii KMS*** **Unique Gene list in comparison to the other two isolates,** ***Flavobacterium hydatis*****, and** ***Flavobacterium hibernum*****.**

Flavobacterium fallonii AJR Unique Genes	
ABC transporter ATP-binding protein	Phage DNA binding protein
Adenylate kinase (EC 2.7.4.3)	Phage DNA replication protein
Bifunctional acetyl transferase/isomerase	Phage external scaffolding protein #Protein D
Chitinase (EC 3.2.1.14)	Phage major capsid protein
Coenzyme F420-dependent oxidoreductase	Phage major spike protein
Conserved domain protein	Phage minor capsid protein - DNA pilot protein
D-alanyl-D-alanine carboxypeptidase (EC 3.4.16.4)	Phage tail fiber protein
EA59 gene protein	Phage-associated homing endonuclease
FIG00821895: hypothetical protein	Phophatidylinositol-4-phosphate 5-kinase (EC 2.7.1.68)
FIG01210424: hypothetical protein	Poly(glycerol-phosphate) alpha-glucosyltransferase (EC 2.4.1.52)
FIG028593: membrane protein	Possible surface protein, responsible for cell interaction
Genes	Probable transposase
glycosyl transferase, group 1	Putative cytoplasmic protein
Glycosyltransferase	putative exported protein
Hep_Hag	putative glycosyltransferase
Lj965 prophage protein	Putative phosphatase
Maltose O-acetyltransferase (EC 2.3.1.79) / Chorismate mutase	Putative predicted metal-dependent hydrolase
Methyltransferase (EC 2.1.1.-)	Recombinational DNA repair protein RecT (prophage associated)
Mobile element protein	Rossmann fold nucleotide-binding protein Smf
Mobile element protein	Sulfate transport system permease protein CysT
Mobile element protein	Superfamily I DNA helicase
Mobile element protein	Tlr1343 protein
Outer membrane protein YfgL, lipoprotein	Transcriptional regulator, AraC family
ParD protein (antitoxin to ParE)	transferase, putative
ParE toxin protein	Utilization protein for unknown catechol-siderophore X
PE-PGRS virulence associated protein	Very-short-patch mismatch repair endonuclease (G-T specific)
Peptidoglycan-binding domain 1	WzxE protein

Figure 28. ***Flavobacterium fallonii AJR*** **Unique Gene list in comparison to the other two isolates,** ***Flavobacterium hydatis,*** **and** ***Flavobacterium hibernum*****.**

Flavobacterium fallonii KMS and *Flavobacterium hibernum* gene comparison			
1765	Fructokinase (EC 2.7.1.4)	fig|1566023.5.peg.1766	fig|37752.6.peg.622
1766	putative outer membrane protein, probably involved in nutrient binding	fig|1566023.5.peg.1767	fig|37752.6.peg.625
1767			
1768	Sucrose-6-phosphate hydrolase (EC 3.2.1.B3)	fig|1566023.5.peg.1769	fig|37752.6.peg.627
1769	D-xylose proton-symporter XylE	fig|1566023.5.peg.1770	fig|37752.6.peg.628
1770	DNA-binding response regulator, AraC family	fig|1566023.5.peg.1771	fig|37752.6.peg.629

Figure 29. Unique Genes shared between ***Flavobacterium fallonii KMS*** **and** ***Flavobacterium hibernum.***

V. Discussion:

Genome Comparison Review

These experiments support the hypothesis that *Flavobacterium fallonii* is genetically different enough from *Flavobacterium hydatis* and the other reference species to be recognized as a different species and the three isolates are distinctly different enough from each other to be considered separate strains. The five *in silico* comparative genomic methods yielded values below the cut-off level for species delineation in comparing all strains of *Flavobacterium fallonii,* especially the type strain *Flavobacterium fallonii JRM.* 16S rRNA values between *Flavobacterium fallonii JRM* and its closest matches were all below 98.7% (Kim et al., 2014). The GGDC calculated eDDH to be below 70%, with the highest value coming with *Flavobacterium hydatis* at only 41.7%. When compared to each other, all isolates of *Flavobacterium fallonii* had eDDH values higher than 73.5%. When examining *Flavobacterium fallonii, Flavobacterium hydatis,* and *Flavobacterium hibernum,* none of them had eDDH values higher than 20.2% when compared to *Flavobacterium aquatile*. This indicates that despite being the type species for the genus *Flavobacterium, Flavobacterium aquatile* shares a low genetic similarity with these other species.

Based on eDDH values, *Flavobacterium aquatile* is a poor type species for the genus and reclassification is needed within the genus. Average Amino Acid Identity values when comparing the isolates of *Flavobacterium fallonii* were above 97.3% which lies above the species cut-off of ~95-96% (Thompson et al., 2013). *Flavobacterium hydatis* had an AAI value of 90.5% when matched with *Flavobacterium fallonii JRM*. This value is close to the species cut-off value, but not higher, indicating that it is the most genetically similar species of *Flavobacterium* currently published based on AAI. The two algorithms to calculate ANI both

yielded results indicating that *Flavobacterium fallonii* is genetically different enough to be a new species. The OrthoANI values between all species of *Flavobacterium fallonii* were above 97.07% which is greater than the ~95-96% species threshold indicating that all three isolates are the same species (Lee et al., 2016). In comparison to the reference organisms, *Flavobacterium fallonii* shares the highest ANI value with *Flavobacterium hydatis* at 90.80%, which is well below the proposed cut-off for species delineation of ~95-96% (Konstantinidis and Tiedje, 2005). Although slightly different on some of the values, ANI values calculated by the Kostas Lab ANI Calculator are comparable to the OrthoANI values. Values amongst the novel isolates lie above 96.9% and all ANI values with the reference organisms are below 90.5%.

While all of the comparative genetic methods indicated that *Flavobacterium fallonii* should be classified as a novel species, they also highlight the disconnect in the world of bacterial taxonomy. As stated above, according to the Bacteriological Code, if the 16S rRNA sequence is above 97% similar, another form of genetic comparison is needed. One of the first problems with that is that 16S rRNA is not the most accurate method for determining whether a species is novel. When examining the 16S rRNA data for the three novel isolates of *Flavobacterium*, just based on 16S sequences, all matched differently with reference organisms. When the list of full *Flavobacterium* 16S rRNA sequences was uploaded to MEGA6 (http://www.megasoftware.net/) to create a neighbor joining tree, reference organisms were picked according to proximity on the tree. As seen in *Figure 4*, based on 16S sequences, the most related organism to *Flavobacterium fallonii* was *Flavobacterium hibernum*. While *Flavobacterium hibernum* was one of the more related species to *Flavobacterium fallonii*, after further comparative studies (*Figure 7&9)* , *Flavobacterium hydatis* has a much higher degree of

genetic similarity. Some organisms have nearly identical 16S sequences, but have an extremely low level of similarity when it comes to whole genome sequences (Goris et al., 2007).

Another pitfall of the 97% cutoff of 16S similarity, is that according to the Bacteriological Code, organism can be published as a novel species if its highest pairwise match is below 97%. While it is more than likely that if an organism has a 16S sequence below 97% it is novel, the fact that it can be published without further genetic testing is a major roadblock in setting a new standard for species delineation. With the cost and accessibility of full genome sequencing, to publish an organism without a sequenced genome is also slowing down the progression of the outdated practice of bacterial taxonomy.

Although other methods are becoming more accepted, the official way to determine if an organism is a novel species is still DNA-DNA hybridization. As stated, this method is not financially practical, it is time consuming, requires specialized equipment, and results are not always reproducible (Goris et al., 2007). There is also no way to catalogue the information given by DNA-DNA hybridization into a public database. While there are algorithms like the GGDC to calculate DNA-DNA hybridization using full genome sequences and computers, this method predicts how likely the DNA is to hybridize, not necessarily how similar the sequences are. Average Nucleotide Identity (ANI) compares how similar the nucleotides are between organisms and not just their ability to hybridize, making it a better indicator for species delineation (Konstantinidis and Tiedje, 2005).

ANI may be accepted as the new standard for separating prokaryotic species, but it also is not a flawless system. For this study, multiple algorithms were used for determining the ANI of the novel isolates and reference organisms. While their results were similar, the fact that there are multiple ways to calculate the same value indicates there should be a standardized ANI

calculator for use in the publication of new species. ANI is a good method for comparing organisms at the species level, but as stated, cannot accurately calculate ANI values below 60% as this value is insignificant on account such a low percentage of the genomes being compared (Konstantinidis and Tiedje, 2005). This limits the practicality of comparing organisms that are not closely related. This makes ANI impractical for examining organisms of different taxa. While not as popular as ANI, Average Amino Acid Identity (AAI) does have the ability to compare organisms that are more distantly related than ANI making it more practical for comparing different genera or families of bacteria (Thompson et al., 2013). AAI compares sequences based on the amino acid composition and not just the nucleotide sequence.

In conclusion, there are many different methods for comparing full genome sequences. Each algorithm has its own separate pros and cons, but describing one of the already published methods or publishing a new algorithm for genome comparison as a new standard for the Bacteriological Code would help the modernization of the field of bacteria taxonomy. The Newman lab has developed a tool known as the Reciprocal Orthology Score Average (ROSA) as a method for comparing full genome sequences. ROSA has the ability to compare both conserved and non-conserved regions of a genome, accounts for differences in the size of the two genomes, and can be used to compare bacteria across a large range of taxa. The tool factors in the similarity of orthologs at the protein level like AAI and also looks at the percentage of the genome that is composed of orthologs or the BBH%. However, this tool is still in the process of being published. Until then, a combination of all of the phylogenetic metrics should be used to compare the genomes of novel species to reference organisms. The procedure of using multiple genetic comparisons improves the classification of the novel species.

Phenotypic Test Review

Having access to a full genome, especially with annotation, permits the comparisons of organisms by looking at unique genes. However, not every gene has an annotation, and many genes code for hypothetical proteins. This is why classification by phenotype is also an important aspect of bacteria taxonomy. Based on laboratory and genetic testing, the three strains of *Flavobacterium fallonii* are independent isolates and not clones of the same organism. Along with the genetic comparisons, wet lab experiments were also performed to examine these different phenotypic properties of the isolates of *Flavobacterium* in correspondence with the Bacteriological Code.

Biolog GenII- Biolog is a really practical way to examine many different growth conditions, provide a quick genus classification, and build a database for individual species. The speed and ability to test 96 different metabolites or growth conditions makes Biolog an important instrument in bacteria classification. The Biolog results, these results were compared to other wet lab experiments. For example, in the Biolog results, *Flavobacterium fallonii JRM* and *KMS* both had positive growth values (40-50s) in the presence of nalidixic acid whereas *Flavobacterium fallonii AJR* only had a growth value of 13.0. In comparison to the antibiotic sensitivity testing as seen in *Figure 14*, *Flavobacterium fallonii AJR* was susceptible to nalidixic acid, whereas the other two strains were only slightly inhibited. Another validation that the three strains are *Flavobacterium fallonii* are not clones and are independent isolates can be seen from the D-fructose utilization. *Flavobacterium fallonii AJR* was not able to use D-fructose as a carbon source whereas the other strains were. The strong growth of *Flavobacterium fallonii KMS* in sucrose and D-raffinose while the other two isolates were inhibited also supports this hypothesis.

Immunological Test Review

Agglutination Assay- The agglutination assay performed was successful in that it was the preliminary to test the function of the rabbit sera. Results from the agglutination assay supported the hypothesis that antibodies against *Flavobacterium fallonii JRM* would react with the other two isolates of the species. This could be seen by the agglutination of the bacterial cells under the microscope. The clumping observed with isolates of *Flavobacterium fallonii* was not observed with *Flavobacterium hydatis, Flavobacterium hibernum* or *Escherichia coli*. An agglutination assay is a simple and easy test to do to examine the cross reactivity of antibodies. However, it is a qualitative test and does not provide any qualitative data on the comparative binding affinity between strains.

Western Blot and Gel Staining Review- In review, the results from this Western Blot could have been better and should be re-done in future work. The results of the BioRad Coomassie stain as seen in *Figure 17* indicate that the protein extractions were successful. The blue haze background resulted from the fact that the gel became dried out while in the staining solution during a weekend stain. During the staining process, the staining trays on the shaker were not adequately covered. The background smudges from the staining solution were improved by carefully rubbing the gels in hazy areas with gloved fingers. This did slightly improve the overall quality of the gel and allowed for a better image of the gel to be taken. The residual SDS could also be a reason for the blue haze on the gel. The gel stained the protein in the gel and conclusions based on the visible protein bands indicate that the crude protein lysates from all of the tested organisms was very similar.

Figure 18 was the LPS Gel and was not stained with BioRad Coomassie stain. For future work, a Coomassie stain on the LPS gel would be beneficial as it would show if there was any protein contamination in the LPS extractions. Another stain that should be performed in future work would be to reevaluate the LPS stain for the LPS gel. While there was no protein contamination of the LPS preps, there was also no confirmation that there was LPS in the LPS sample either. For this study, the experimental UGF202 fluorescent stain was unsuccessful as indicated by *Figure 21*. If re-attempting the UGF202 stain, an updated procedure should be followed when synthesizing the UGF202 compound. Contracting this step out to an organic chemist might be the most effective way to improve the success rate of the stain. Also, standardizing conditions to visualize the gel under continuous light at 532nm would improve the probability of a successful stain. The more conventional ways to visualize LPS in SDS-PAGE are to use silver staining. Silver staining was initially avoided for the study as original silver stain procedures used chemicals that were not user friendly in an undergraduate laboratory. However, further background research has provided new procedures for silver staining. For future work, the protocol described by the Fomsgaard lab may be a more practical silver staining procedure to follow (Fomsgaard, Freudenberg, and Galanos, 1990).

While minor conclusions could be drawn from the Western Blots, the condition of the membrane severely limits the ability to gather data. The Western Blot Gel 3 as seen in *Figure* 19 did not show a reaction with the negative control of E. coli. The darker purple coloration at the bottom of the F. JRM lane may also indicate that the strongest reaction came from *Flavobacterium fallonii JRM* with anti-JRM as the primary antibody. The LPS gel showed a universal band in all lanes that may or may not be LPS. The predicted kDa for the LPS region did not show any purple bands. For future work, new Western blots for the LPS preparations and

protein preparations should be performed. The transfer from the gel to the membrane appeared to be successful, but during the incubation of the membranes in the substrate, the membranes began to dissolve. Unknown percentages of substrate were used during this step as the bottles of substrate did not have percentages of chloronaphthol provided. Dilutions were guessed on and the membranes reacted poorly, probably as a result of the overall viscosity of the substrate solution, and the high concentration of ethylene glycol. While the bands did become darker, this did not occur until after the substrate was removed and washed with PBS multiple times in attempts to save the membranes. The membranes appeared to dissolve, and folded and hardened into the conformations seen in *Figure 17* and *Figure 18.*

ELISA Review- The ELISA results were non-conclusive as all wells containing cells, and wells only containing PBS were positive. Multiple factors could have contributed to these results. Non-specific binding of either the primary antibody or the secondary antibody would yield these results. Nonspecific binding of the primary antibody could occur with a flaw in the blocking step. 100uL of BSA was pipetted into each well after the bacteria samples were fixed to the wells. If the BSA did not block all remaining regions of the wells, the primary antibodies may have bound nonspecifically to the wells. A potential fix for this step for future work would be to fill the well with BSA to coat the entire well. This may help nonspecific binding. To help prevent nonspecific binding of the secondary antibody, longer and more numerous washing steps could be added to the procedure. Contamination of the wells could also have led to the inconclusive data and positive results for wells that did not contain bacterial cells or specific antibodies. The process of pipetting amounts 96 times was tedious and error prone due to its monotonous action. For future work, the multi-channel pipette would speed up these steps and

probably encourage less user error. Another potential area for contamination was during the inversion of the plates during washes. The mixing of well contents during washes probably occurred. Residual splashes from the discard vessel was apparent which could have caused the contamination. A plate washer might also be utilized during the washing steps for more thorough and regulated washing steps. Dilutions of the secondary antibody also could be changed for future work. The recommended working dilution for the Goat anti-rabbit antibody conjugated to Horseradish peroxidase is 1:120,000. For these ELISAs, the dilution used was only 1: 50,000 as a result of user error.

Immunofluorescence Review- While images were collected under the fluorescent microscope; the quality of the images was not very good. A 5-megapixel camera was used to take the pictures of the slides. Other images were taken of various conditions, but the quality was too poor to visualize anything of value. To improve this test, more time needs to be devoted to looking at the images under the microscope and a camera of higher quality must be used. Also, the florescent stains faded rather quickly under the fluorescent lights in the molecular and microscope lab, making long term visualization One of the other problems with this particular lab was how difficult it was to locate the bacteria were fixed onto the slides. The bacteria samples had a small diameter and were not easily observed under the microscope. For future work, marking the regions on the slide with a glass etcher may make it easier to visualize where on the slide the bacteria samples were placed.

Genome Annotation Analysis

The comparisons of the annotated genomes of each organism is an informative way to draw conclusions and make predictions about phenotypic traits. There are 174 genes exclusive to *Flavobacterium fallonii* when compared to the most genetically similar species. Each of the three isolates then has over 259 genes unique to each individual strain despite being compared to the same genetic species and the closest genetic relatives. One of the unique genes to the species *Flavobacterium fallonii* is for phytoene desaturase (EC 1.3.99). This enzyme is lycopene producing and is involved in carotenoid metabolism. These are pigment compounds, indicating that in comparison to the reference organisms, *Flavobacterium fallonii* has a unique pigment. From *Figure* 26 one of these unique genes to *Flavobacterium fallonii KMS* is prolidase (EC.3.4.13.9). This unique metallopeptidase degrades dipeptides with a proline or hydroxyproline in the C-terminal end of the protein (Namiduru, 2016). From a metabolic standpoint, this enzyme can break down dipeptides into single peptides which can then be utilized as an energy source.

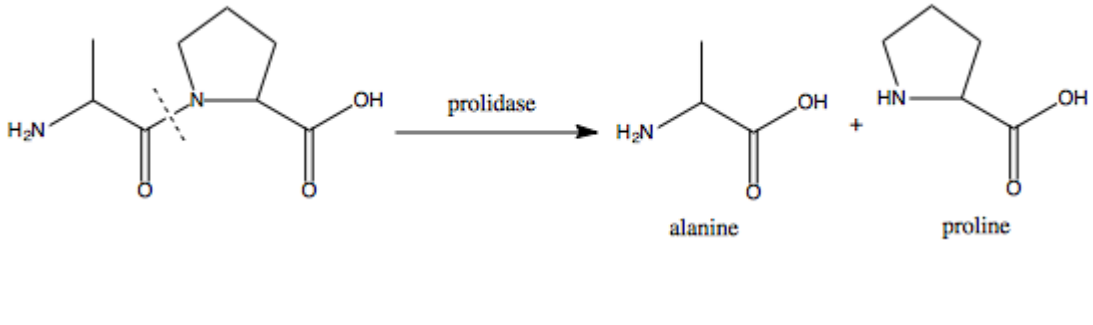

Figure 29. Schematic of Prolidase (EC.3.4.13.9) cleaving a dipeptide.

This enzyme allows for the utilization of generally unusable dipeptides for energy. Biolog results indicate that *Flavobacterium fallonii KMS* can utilize alanine as an energy source which could make having prolidase an important enzyme for survival. This enzyme is also of particular

interest as it has been utilized in multiple biotechnical applications. For example, this enzyme has the ability to degrade organophosphorus compounds that can act as damaging nerve agents (Theriot, Tove, and Gruden, 2009). In humans, this enzyme is critical for the maintenance of collagen metabolism. Prolidase deficiency is a rare autosomal disorder that interferes with the body's ability to maintain collagen and connective tissues. Enzyme replacement therapies have used recombinant enzymes from bacterial species as potential treatments (Theriot, Tove, and Gruden, 2009). These recombinant prolidases have also been researched as an anti-cancer strategy as pro-drugs have been researched to stimulate prolidase activity in melanoma patients (Kitchener and Grunden, 2012).

Comparing phenotypic results such as Biolog to the genome can give answers as to why some organisms grow under certain conditions and others do not. Biolog results indicate that *Flavobacterium fallonii KMS* is able to utilize sucrose, raffinose, and fructose as carbon source whereas the other strains of *Flavobacterium fallonii* did not demonstrate strong growth under these conditions. As seen in *Figure 28, Flavobacterium fallonii KMS* and *Flavobacterium hibernum* share this set of genes that the other strains of *Flavobacterium fallonii* do not. The enzymes of importance are fructokinase (EC 2.7.1.4) and sucrose-6-phosphate hydrolase (EC 3.2.1.26). By examining the function of the unique set of genes and comparing to the metabolic pathway, conclusions were drawn as to why *Flavobacterium fallonii KMS* grew under these conditions and the other strains did not. Sucrose-6-phosphate hydrolase is a necessary enzyme in the conversion of sucrose-6-phosphate into D-glucose-6-phosphate (Kanehisa et al., 2017). Fructokinase is a transferase that is used in both sucrose and fructose metabolism. With ATP, fructokinase transfers a phosphate group onto the 6-position on the fructose molecule (Kanehisa

et al., 2017). These pathways can be seen in *Figure 30.* Raffinose is a trisaccharide composed of galactose, glucose, and fructose. This molecule can be hydrolyzed to sucrose by α-galactosidase. In *Figure 24,* unique genes in all three isolates of *Flavobacterium fallonii,* α-galactosidase (EC 3.2.1.22) can be found. Although all of the strains of *Flavobacterium fallonii* have this gene, as seen, neither *Flavobacterium fallonii JRM* or *Flavobacterium AJR* has sucrose-6-phosphate hydrolase (EC 3.2.1.26) to be able to utilize sucrose. This phenotypic trait can be seen on the Biolog as only *Flavobacterium fallonii KMS* showed growth in the well containing D-raffinose.

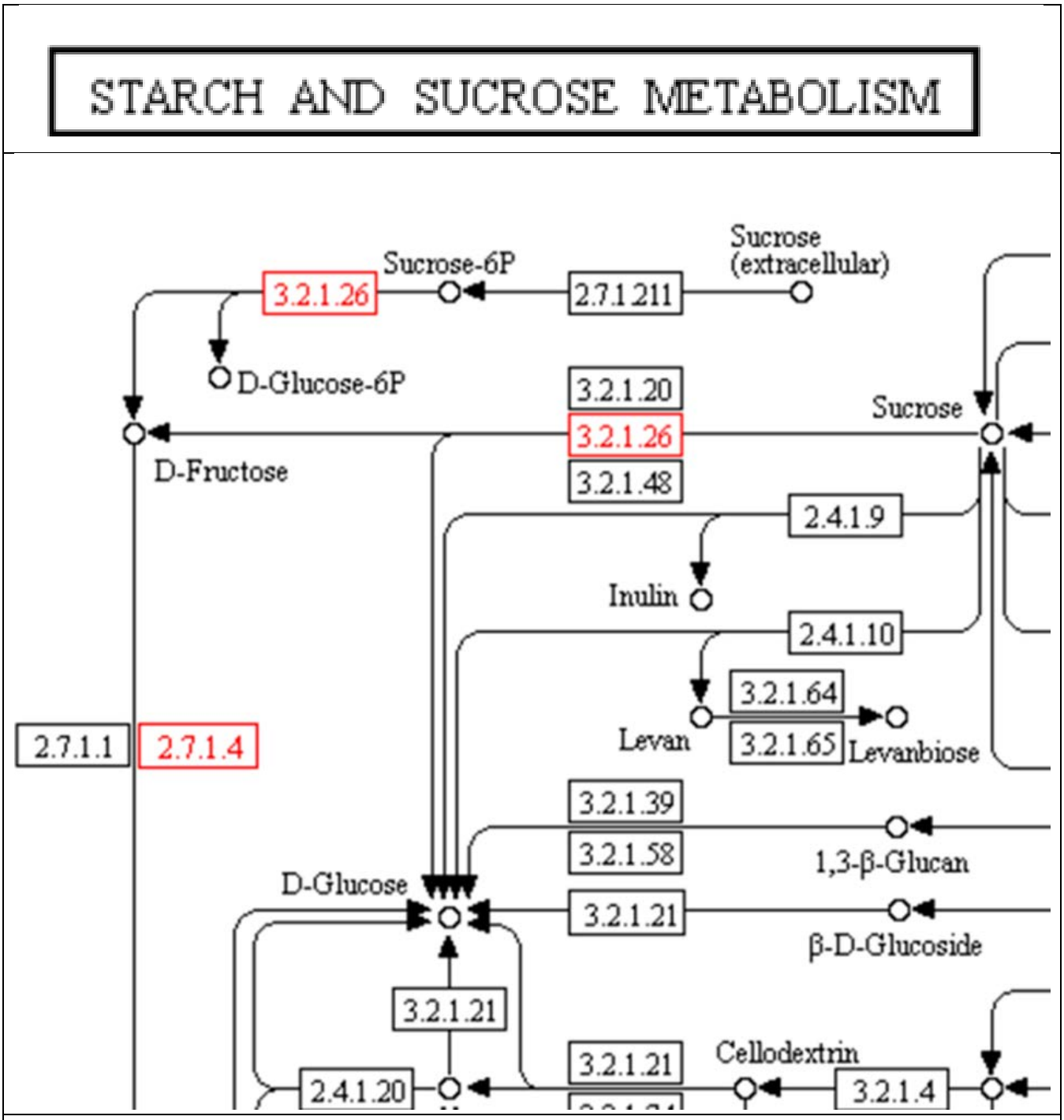

Figure 30. Starch and Sucrose Metabolic Pathway as shown in the KEGG Orthology Metabolic Pathway Browser. Fructokinase (EC 2.7.1.4) and sucrose-6-phosphate hydrolase (EC 3.2.1.26) (Kanehisa et al., 2017)

In conclusion, *Flavobacterium fallonii* represents a novel species of *Flavobacteria* based on both the genetic and phenotypic differences. 16S rRNA, eDDH, ANI, OrthoANI, and AAI results supported that *Flavobacterium fallonii* represents a new species, and that all three isolates of *Flavobacterium fallonii* represent the same species. However, the lack of effective standards for comparing whole prokaryotic genomes needs to progress to better accommodate the ease of access to genomes. The Newman lab's work with novel species and comparing phenotypic data to the annotated genome represents an effective method for classifying bacteria. For future work, based on the work of this study, lab experiments can be performed and improved for use in course lab work as well as independent student research. While experiments for further immunological comparisons must be performed, based on the agglutination results, anti-JRM cross linked *Flavobacterium fallonii* isolates and not the reference organisms.

VI. References:

Auch, A.F., Klenk, H.-P., Göker, M. (2010). Standard operating procedure for calculating genome-to-genome distances based on high-scoring segment pairs. *Standards in Genomic Sciences* 2:142-148, .

Aydin, S. (2015). A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA. *Peptides*, 72, 4-15. doi:10.1016/j.peptides.2015.04.012

Bernardet, J., Holmes, B., & Nakagawa, Y. (2002). Proposed minimal standards for describing new taxa of the family Flavobacteriaceae and emended description of the family. *International Journal of Systematic and Evolutionary Microbiology, 52*(3), 1049-1070. doi:10.1099/00207713-52-3-1049

Biolog, Inc. (2006). GEN III MicroPlate: Instructions for use. Biolog, Inc.

Brenner, Don J., George R. Fanning, Adrian V. Rake, and Karl E. Johnson. (1969). Batch Procedure for Thermal Elution of DNA from Hydroxyapatite." *Analytical Biochemistry* 28 : 447-59. Web

Burnette WN. (1981). Western blotting: electrophoretic transfer of proteins from dodecyl sulphate–polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem* 112: 195–203.

Fomsgaard, A., Freudenberg, M. A., & Galanos, C. (1990). Modification of the silver staining technique to detect lipopolysaccharide in polyacrylamide gels. *Journal of Clinical Microbiology*, *28*(12), 2627–2631.

Goris, Johan, Joel A. Klappenbach, Peter Vandamme, Tom Coenye, Konstantinos T. Konstantinidis, and James M. Tiedje. (2007). DNA-DNA Hybridization Values and Their Relationship to Whole-genome Sequence Similarities. I*nternational Journal of Systematic and Evolutionary Microbiology* 57.1 : 81-91. Web

Gram HCJ (1884). Uber die isolirte Farbung der Schizomyceten in Schnitt- and Trockenpraparaten. *Fortschritte der Medizin* 2:185–189

Harlow, Ed, and David Lane. (1999). Antibodies: A Laboratory Manual. New York: Cold Spring Harbor Laboratory, Print.

Huang, X. and Madan, A. (1999). CAP3: A DNA Sequence Assembly Program. *Genome Research*, 9(9), 868-877. doi:10.1101/gr.9.9.868

Jan P. Meier-Kolthoff · Markus Göker · Cathrin Spröer · Hans-Peter Klenk. (2013). When Should a DDH Experiment be Mandatory in Microbial Taxonomy? *Arch Microbiol.* 195 (6):413-8

Janda, J. M., & Abbott, S. L. (2007). 16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls. *Journal of Clinical Microbiology*, 45(9), 2761-2764. doi:10.1128/jcm.01228-07

Janeway CA Jr, Travers P, Walport M, et al. (2001). Immunobiology: The Immune System in Health and Disease. 5th edition. New York: Garland Science;

Jensen, E. C. (2012), The Basics of Western Blotting. *Anat Rec*, 295: 369–371. doi:10.1002/ar.22424

Kanehisa, Furumichi, M., Tanabe, M., Sato, Y., and Morishima, K.; (2017). KEGG: new perspectives on genomes, pathways, diseases and drugs. *Nucleic Acids Res*. 45, D353-D361

Kim OS, Cho YJ, Lee K, Yoon SH, Kim M, Na H, Park SC, Jeon YS, Lee JH, Yi H, Won S, Chun J. (2012). Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *International Journal Systematic Evolution Microbiology*. 62, 716-721

Kim, M., Oh, H., Park, S., & Chun, J. (2014). Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. *International Journal of Systematic and Evolutionary Microbiology*, 64(Pt 2), 346-351. doi:10.1099/ijs.0.059774-0

Kitchener, R., & Grunden, A. (2012). Prolidase function in proline metabolism and its medical and biotechnological applications. *Journal of Applied Microbiology, 113*(2), 233-247. doi:10.1111/j.1365-2672.2012.05310.x

Kohler, G., and C. Milstein. (1975). Continuous Cultures of Fused Cells Secreting Antibody of Predefined Specificity. *Nature* 256.5517 : 495-97. Web.

Koichiro Tamura, Daniel Peterson, Nicholas Peterson, Glen Stecher, Masatoshi Nei, and Sudhir Kumar .(2013). MEGA6: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. 28, 2731-9.

Konstantinidis, K. T., & Tiedje, J. M. (2005). Genomic insights that advance the species definition for prokaryotes. *Proceedings of the National Academy of Sciences of the United States of America*, 102(7), 2567–2572.

Lee, I., Kim, Y. O., Chun, J., & Park, S. (2016). OrthoANI: An improved algorithm and software for calculating average nucleotide identity. *International Journal of Systematic and Evolutionary Microbiology, 66*(2),

Lindstrom, P., & Wager, O. (1978). IgG Autoantibody to Human Serum Albumin Studied by the ELISA-Technique. *Scandinavian Journal of Immunology*, 7(5), 419-425. doi:10.1111/j.1365-3083.1978.tb00472.

Lipman, N. S., L. R. Jackson, L. J. Trudel, and F. Weis-Garcia. (2005). Monoclonal Versus Polyclonal Antibodies: Distinguishing Characteristics, Applications, and Information Resources. *ILAR Journal* 46.3 : 258-68. Web

Macphee, D. J. (2010). Methodological considerations for improving Western blot analysis. *Journal of Pharmacological and Toxicological Methods*, 61(2), 171-177. doi:10.1016/j.vascn.2009.12.001

Meier-Kolthoff, J.P., Göker, M., Klenk, H.-P. (2014). Taxonomic use of DNA G+C content and DNA-DNA hybridization in the genomic age. *Int J Syst Evol Microbiol* 64:352-35.

Namiduru, E. S. (2016). Prolidase. *Bratislava Medical Journal, 117*(08), 480-485. doi:10.4149/bll_2016_093

Newman J.D, Krebs, J.E., Gale, A.N., Anspach, T.J., Kirk, K.E., Sontag, T.C., Keyser, V.K., and Peluso, E.M. (2013). Integration of Average Amino Acid Identity (AAI) and Percentage of Orthologous Genes in a Single Phylogenomic Metric, the Reciprocal Orthology Score Average (ROSA). Manuscript in Preparation.

Parija, Subhash Chandra. Textbook of Microbiology and Immunology. Haryana, India: Elsevier, 2009. Print

Raetz, C. R. H., & Whitfield, C. (2002). Lipopolysaccharide Endotoxins. *Annual Review of Biochemistry*, 71, 635–700.

Ross Overbeek et al., (2014). The SEED and the Rapid Annotation of Microbial Genomes Using Subsystems Technology (RAST) *Nucleic Acids Res* 42, 206–214.

Rosselló-Móra, R., & Amann, R. (2001). The species concept for prokaryotes. *FEMS Microbiology Reviews*, 25(1), 39-67.

Rosselló-Móra, R., Mercedes Urdiain, Arantxa López-López. (2011). DNA–DNA Hybridization, In: Fred Rainey and Aharon Oren, Editor(s), *Methods in Microbiology*, Academic Press, Volume 38, Pages 325-347

S. A. McCammon, B. H. Innest J. P. Bowman, P. D. Fanzrnann, S. J. Dobson,l P. E. Holloway, J. H. Skerratt,' P. D. Nichols and L. M. Rankin. (1998). Flavobacterium hibernum sp. nov., a lactose-utilizing bacterium from a freshwater Antarctic lake

International Journal of Systematic Bacteriology. 48, 1405-1410

Samantha J. Stropko, Shannon E. Pipes and Jeffrey D. Newman. (2014). Genome-based reclassification of Bacillus cibi as a later heterotypic synonym of Bacillus indicus and emended description of Bacillus indicus *International Journal of Systematic and Evolutionary Microbiology* 64, 3804–3809

Sasser, M. (2001). Identification of Bacteria by Gas Chromatography of Cellular Fatty Acids. MIDI, Inc.

Schildkraut, Carl L., Julius Marmur, and Paul Doty. (1961). The Formation of Hybrid DNA Molecules and Their Use in Studies of DNA Homologies. *Journal of Molecular Biology* 3.5 : n. pag. Web

Shicheng Chen, Michael G. Kaufman, Michelle L. Korir, and Edward D. Walker. (2014). Ingestibility, Digestibility, and Engineered Biological Control Potential of Flavobacterium hibernum, Isolated from Larval Mosquito Habitats. *Appl. Environ. Microbiol.* 80 no. 3 1150-1158.

Silhavy, T. J., Kahne, D., & Walker, S. (2010). The Bacterial Cell Envelope. *Cold Spring Harbor Perspectives in Biology*, 2(5),

Stackebrandt, E., Garrity, G. M., Traper, H. G., Whitman, W. B., Grimont, P. A., Nesme, X., . . . Swings, J. (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *International Journal of Systematic and Evolutionary Microbiology*, 52(3), 1043-1047. doi:10.1099/00207713-52-3-1043

Stefan R. Henz, Daniel H. Huson, Alexander F. Auch, Kay Nieselt-Struwe, Stephan C. Schuster.(2005). Whole-genome prokaryotic phylogeny. *Bioinformatics* (; 21 (10): 2329-2335. doi: 10.1093/bioinformatics/bth324

Thompson, C. C., Chimetto, L., Edwards, R. A., Swings, J., Stackebrandt, E., & Thompson, F. L. (2013). Microbial genomic taxonomy. *BMC Genomics,* 14(1), 913. doi:10.1186/1471-2164-14-913

Tindall, B. J., Busse, H., Ludwig, W., Rossella-Mara, R., & Kampfer, P. (2010). Notes on the characterization of prokaryote strains for taxonomic purposes. I*nternational Journal of Systematic and Evolutionary Microbiology*, 60(1), 249-266. doi:10.1099/ijs.0.016949-0

Theriot, C. M., Tove, S. R., & Grunden, A. M. (2009). Chapter 3 Biotechnological Applications of Recombinant Microbial Prolidases. *Advances in Applied Microbiology,*99-132. doi:10.1016/s0065-2164(09)01203-9

Towbin H, Staehelin T, Gordon J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proc Nat Acad Sci USA* 76: 4350–4354

Wang, X., Zhou, A., Cai, W., Yu, D., Zhu, Z., Jiang, C., & Jin, L. (2015). Highly sensitive fluorescent stain for detecting lipopolysaccharides in sodium dodecyl sulfate polyacrylamide gel electrophoresis. *Electrophoresis, 36*(15), 1795-1800.

Wayne, L. G., Moore, W. E., Stackebrandt, E., Kandler, O., Colwell, R. R., Krichevsky, M. I., . . . Moore, L. H. (1987). Report of the Ad Hoc Committee on Reconciliation of

Approaches to Bacterial Systematics. *International Journal of Systematic and Evolutionary Microbiology*, 37(4), 463-464. doi:10.1099/00207713-37-4-463

Woese, C. R. (1987). Bacterial evolution. *Microbiological Reviews,* 51(2), 221–271.

Woo, P.C.Y., Lau, S.K.P., Teng, J.L.L., Tse, H., & Yuen, K.-Y. (2008). Then and Now: Use of 16S rDNA Gene Sequencing for Bacterial Identification and Discovery of Novel Bacteria in Clinical Microbiology Laboratories. *Clin Microbiol Infect* 14, 908-34

Yarza, P., Yilmaz, P., Pruesse, E., Glackner, F. O., Ludwig, W., Schleifer, K., . . . Rossella-Mara, R. (2014). Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. *Nature Reviews Microbiology, 12*(9), 635-645. doi:10.1038/nrmicro3330