Identification and Characterization of *Sejongia lycomia* JJC, a Unique Isolate from Loyalsock Creek

Presented to the faculty of Lycoming College in partial fulfillment
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By:
Tyler M. Marcinko
Lycoming College
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Abstract:

In lotic ecological systems (as is the case with Loyalsock Creek), bacteria fill a variety of roles, such as decomposing organic material, existing in biofilm communities, nutrient cycling, and/or in commensal relationships with stream organisms. Identifying a novel bacteria species from the Loyalsock Creek can provide more insight into the microbial communities that exist within that ecosystem. The aim of this study is to identify and characterize a potentially novel organism that was isolated from the Loyalsock Creek in Lycoming County, Pennsylvania (41° 15' 00.53" N, 76° 56' 09.63" W). Our hypothesis is tha t *Sejongia lycomia* JJC, one of the organisms that was isolated for Lycoming College's Microbiology course, is indeed a novel species based upon preliminary studies utilizing Bergey's Manual of Determinative Microbiology as well as comparison and alignment of the partial 16S rRNA sequence. *Sejongia lycomia* JJC is a novel organism belonging to the Sejongia genus in the Flavobacteriaceae family based upon 16S sequence homology of less than 97% and a unique membrane fatty acid composition. *Sejongia lycomia* is an aerobic, gram-negative, mucoid, yellow-pigmented, rod-shaped organism. *Sejongia lycomia* optimally grows at approximately 30ºC. Exoenzyme actvitity includes lecithinase and amylase. Produces acid aerobically from D-Xylose, D-Glucose, and Maltose. Does not reduce nitrate or produce indole. Predominant fatty acids include 13:0 iso, 15:0 iso, 15:0 anteiso, 10 Methyl 16:0, and 17:0 iso 3OH.

Introduction:

The discovery and identification of novel organisms is significant because of the properties and uses of the microbes in industry and scientific research. In this age of advanced molecular techniques, microbes have many properties that can be manipulated and utilized. For example, *Corynebacterium glutamicam* can be used to produce L-glutamic acid in industrial quantities. Approximately 1.5 million tons of L-glutamic acid is produced using Coryneform bacteria per year, which can be used as a food supplement (Hermann 2003).

Another example comes from bacteria that are used to produce alternate fuel sources. These include species of bacteria that can metabolize hemicelluose or cellulose, as these bacteria are in high demand for developing alternatives to fossil fuels (Patel, *et al*. 2006). Bioremediation is another important function as well. Bacteria are used for their metabolic properties to remove contaminants in environments to return them to their natural state. For example, Ayyasama and his colleagues used Pseudomonas sp. RS-7 to remove excess nitrates from groundwater (2007).

Identification of novel microbes also has important implications in the field of medicine. Infections with no apparent cause could be linked to undiscovered bacteria The identity and characteristics of these bacteria could lead to possible treatments of these infections. For Gram-negative nonfermentative rods (such as *Sejongia lycomia* JJC), trovafloxacin in conjunction with other agents appear to be very effective in killing the organisms (Visalli, *et al*. 1997). The molecular composition of the cell walls of

bacteria is important as well, as antibiotics such as penicillin attack the cell wall to kill microorganisms.

Identifying a novel bacteria species from the Loyalsock Creek can provide more insight into the microbial communities that exist within that ecosystem. Experts suggest that fewer than 1% of microbes on the planet have been discovered and described (Amann, *et al*. 1995). Thus, more can be discovered about the interactions and the role that the bacteria have within the Loyalsock Creek community. In lotic (flowing water) ecological systems bacteria primarily fill a variety of roles, such as decomposing organic material, existing in biofilm communities, nutrient cycling, and/or in commensal relationships with stream organisms. Biofilms are natural heterogeneous aggregations of bacteria that are stabilized by a protective and adhesive secreted matrix. In addition, bacterial biofilm populations have been noted to cycle based on the dynamics of seasons (Brümmer, *et al*. 2000). Most importantly, bacteria are vital to decomposing organic material in streams, as bacterial biofilms tend to accumulate on and degrade floating leaf material (Das 2007). If a novel bacterium has a particularly important role to play, then this could impact the environmental system.

A common technique used to identify bacteria quickly is to sequence the 16S rRNA gene (Tang, *et al*. 2000), which can show relationships between members of the same family, such as Flavobacteriaceae (Weisburg, *et al* 1985). This technique was primarily pioneered by Carl Woese and his colleagues (Woese and Fox, 1977, Woese, *et al*. 1990). rRNA refers to ribosomal RNA, which is an untranslated component of ribosomes. The 16S refers to the smaller of the two subunits of the ribosome. Although

the exact base pair-length of the 16S rRNA gene can differ slightly (between 1450-1500 base pairs), the reason why the 16S rRNA gene is important is because it is under selective pressure to remain conserved over time (Zhang, *et al* 2004). A mutation in the ribosome would most likely be deleterious, so selection would not favor mutants. This means that the 16S rRNA gene can be used to differentiate as well as examine the phylogenetic or evolutionary relationships between different prokaryotic organisms.

In Lycoming College's Microbiology course, partial 16S rRNA sequencing was utilized to identify the bacteria that were isolated. Generally speaking, when two 16S rRNA sequences are compared (such as aligning them using the National Center for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool (BLAST)), a base-pair similarity score is calculated. If the base pairs are over 97% identical, the bacteria can reasonably be considered to be the same species. According to current scientific standards, a score under 97% may suggest that the species is novel.

The lipid composition of a cell membrane can also serve to differentiate organisms. Bacteria have unique lipid compositions of their membranes, and this factor can distinguish organisms from one another. For example, Veys, *et al*. found 23 different types of fatty acids that could distinguish between non-fermenting bacteria using gas chromatography (1989). The type and relative amounts of each lipid make up a profile, and the probable identity of the organism is determined by comparing lipid profiles (Puech, *et al*. 2000). This procedure is accomplished by isolating the lipids from the membrane, transesterifying them into methyl esters, and then analyzing the mixture using gas chromotography and mass spectroscopy to identify the bacteria (Methods for

General and Molecular Bacteriology 634-635).

In this study, a proposed novel bacterium was identified and characterized that belongs to genus Sejongia, which is part of family Flavobacteriaceae. It is a relatively new genus, as the first two members of the genus *(Sejongia jeonii and Sejongia antarctica)* were characterized by Yi and his colleagues (2005). More recently, Lee, *et al*. described *Sejongia marina*, which was isolated from Antarctica seawater (2007). *Sejongia jeonii* and *Sejongia antarctica* were noted for their yellow pigment, gram-negative stain, rod morphology, non-motility, predominant fatty acids of 15:0 iso, 15:0 anteiso, and 17:0 iso omega-9c, and psychrotolerant growth (Yi, *et al.* 2005).

Psychrotolerance refers to the adaptation of organisms to be able to survive and reproduce at cold temperatures. An organism capable of this type of existence is known as a psychrophile. Psychrotolerance is significant because two of the three described species (*Sejongia jeonii* and *Sejongia antarctica*) are defined as psychrophiles. In order to demonstrate that *Sejongia lycomia* is indeed a separate species that exists in temperate environments, there must be a difference between the optimal growth rates of the three species.

These strains were initially found in terrestrial and moss samples that were taken near King George Station in Antarctica. Because genus Sejongia has been observed to exist in such a low-temperature environment, members described thus far are considered to be psychrotolerant. In terms of sampling these psychrotolerant organisms, Walker and his colleagues found that they could increase the yield of psychrotolerant bacteria by subjecting samples to further freeze/thaw cycles to kill off

nonpsychrotolerant species (2006). Although Yi, *et al*. does not specifically state their sampling technique, this is certainly one way to obtain such psychrotolerant species.

After analysis of the nearly complete16S rRNA sequence, it was revealed that *Sejongia lycomia* JJC is very similar to both *Sejongia antarctica* and *Sejongia jeonii*, but different enough to be a novel species based on our data. Phylogenetically, it falls within the same areas of the Flavobacteriaceae tree, and some of the most closely related genera include *Chryseobacterium*, *Reimerella*, *Bergeyella*, *Kaistella*, and *Elizabethkingia*.

The aim of this study is to identify and characterize a potentially novel organism that was isolated from the Loyalsock Creek in Lycoming County, Pennsylvania. **Our hypothesis is that *Sejongia lycomia* JJC, one of the organisms that was isolated for Lycoming College's Microbiology course, is indeed a novel species, based upon preliminary studies utilizing Bergey's Manual of Determinative Microbiology as well as comparison and alignment of the 16S rRNA sequences.** Identification and characterization was accomplished through a series of genetic, molecular, structural, and biochemical procedures in order to define the organism, its phylogeny, and the differences from other members of the Sejongia genus.

Methods:

Gram Stain, Morphology Analysis

The first step in this study was to successfully retrieve and reculture stocks of *Sejongia lycomia* JJC that had been stored in refrigeration. Once cultured onto Trypticase Soy Agar (TSA) media plates, colonies appeared to be yellow-pigmented, flat, irregularly shaped, and of a mucoid texture.

For gram staining, an inoculation originally from the -20℃ stock culture TSA plate was placed into 15 µL of deionized H_2O (dH_2O) on a microscope slide, and was then spread in order to both dilute the culture and dry it out. The slide was then heat-fixed using a Bunsen burner. Following the heat-fixing, the gram staining procedure was then carried out . The smear was then covered with crystal-violet stain for 1 minute, and was then washed off with dH_2O. Gram's Iodine was then flooded onto the slide, and allowed to react for 1 minute before being washed off with dH_2O. The slide was then angled at 45 degrees, and decolorizer (ethanol) was applied dropwise. The slides were then washed with dH_2O. Finally, the slide was then counter-stained with Safranin for one minute before being rinsed with dH_2O and blotted dry on a bibulous pad (Microbiology Lab Manual).

This slide was then observed using a light microscope at 40X, 100X, 400X, and then under oil immersion at 1000X magnification. The color stain that the bacteria retained as well as the general morphology of the culture was then recorded.

16S rRNA Sequencing

Following gram staining, the next procedure carried out in this study was sequencing the 16S rRNA gene of *Sejongia lycomia* JJC. Two Sejongia cultures were initially used (TSA 9/23/07 and TSA 9/17/07). One colony was taken from each Trypticase Soy Agar media plate, and was suspended in 100 mL of dH_2O. These tubes two underwent a freeze/thaw cycles. The significance of this procedure was to lyse the bacterial cells so that the DNA is freed. The samples were placed into a -70℃ block for 2 minutes, and then transferred to a 70℃ block for two minutes.

Sample tubes for the polymerase chain reaction (PCR) procedure were then prepared. 1 µL of freeze/thawed bacterial cells were then added to each sample tube. 12.5 µL 2x ExTaq Premix (Takara), 4 µL of 5 uM 27f primer (Takara) 4 µL of 5 uM 1492r primer (Takara), 3.5 µL of dH_2O were then added to each PCR tube. 30 µL of mineral oil was layered on top of the sample in order to prevent evaporation during the PCR procedure. These tubes were then loaded into the Thermocycler, and the rRNA.fl program was used. rRNA.fl (Microbiology Lab Manual), as depicted below, ran for a total of 35 cycles.

	Stage 1	
Step 1	94C for 3 minutes	
Step 2	50C for 1 minute	Initial Denaturation
Step 3	72C for 2 minutes	
	Stage 2	
Step 1	94C for 1 minute	Standard Denaturation
Step 2	50C for 1 minute	Primer Annealing
Step 3	72C for 2 minutes	Primer Extension
	Stage 3	
Step 1	94C for 1 minute	Primer Extension

Step 2	50C for 1 minute
Step 3	72C for 10 minutes

It was then necessary to isolate and visualize the PCR products. This was done via gel electrophoresis. A 1% agarose gel was prepared by adding 0.5 g of agarose powder to 50 mL of dH2O. This mixture was then microwaved for 1.5 minutes, swirled, and microwaved for an additional 30 seconds. 1.0 mL of 1X TAE buffer was then added, along with 12.5 µL of ethidium bromide, which would make the bands of DNA fluoresce in the presence of UV light. This mixture was then poured into a gel tray and allowed to solidify.

The agarose gel and tray were placed into an electrophoresis chamber. The chamber was then filled with 1X TAE buffer. 25 µL of each PCR product was transferred into the gel lane from the sample tube and mixed with 2 µL of bromophenol blue dye. The combined dye and product was then placed in wells in the agarose gel. The PCR products were run alongside a Lambda phage DNA marker for size comparison.

The presence of fluorescent bands in the agarose gel confirmed the success of the PCR procedure. Now that the PCR products had been isolated, the bands of DNA had to be purified. This involved cutting the bands of DNA out of the gel manually. Once isolated, the gel slices needed to be purified. With the DNA embedded in the band, 3 volumes of Buffer QG (according to mass of the gel slice) were added to the gel slice and were allowed to incubate for 10 minutes at 50℃. This step dissolved the agarose gel, freeing the DNA molecules, and suspending them back into the buffer

solution. The sample was then added to a QIAquick spin column, and was centrifuged for 1 minute at 10,000 RPM. The flow-through was then discarded. 500 µL of Buffer QG was then added to column in order to remove residual agarose. This was then centrifuged for 1 minute at 10,000 RPM, and the flow-through was discarded. 750 µL of Buffer PE was then added to the spin column. This was centrifuged twice for 1 minute at 10000 RPM in order to ensure the removal of all of the PE buffer. The QIAquick spin column was then removed and added to a 1.5 mL tube. 50 µL of Buffer EB was then added, and the tube was centrifuged for 1 minute at 10,000 RPM. The flow-through from this final step contained the PCR products suspended in EB buffer.

In order to be sequenced, the DNA concentration in the PCR products was then quantified. 5 µL of the purified DNA samples were mixed with 2 µL of bromophenol blue, and were run on 1% agarose gels against a Lambda phage DNA marker. The presence of bands here suggested the successful purification of DNA. The location of the bands versus the Lambda phage DNA allowed for the estimation of the sequence size. The concentration of DNA could be estimated by observing the brightness of fluorescent signal given off by the band versus the known concentration of the Lambda marker.

For the sequencing of the 16S rRNA gene, AgenCourt, Inc. (http://www.agencourt.com/) was used. For sequencing, the sample was prepared with three different primers. It was determined to be unnecessary to sequence both Sejongia samples, so only one was sent. In the tube that was sent off for sequencing, 10 µL of DNA/buffer was added along with 2 µL of the respective primers, and 8 µL of

dH_2O.

16 rRNA Primer (5'-3')	**Sequence**
27f	AGAGTTTGATCMTGGCTCAG
1492r	TACGGYTACCTTGTTACGACTT
785f	GGATTAGATACCCTGGTAGTCC

AgenCourt sequenced the 16S rRNA gene three separate, with each primer. This meant that there were three different 16S sequences. In order to form one sequence, all three sequences were loaded into SeqMan, which is part of LaserGene's software suite. In addition to our three sequences, the rRNA2 sequence from the Microbiology course was added in order to improve sequence quality. Once all four sequences were uploaded to the program, a consensus (or contiguous) sequence was assembled. SeqMan does this by choosing the bases that occur with the most frequency across the uploaded sequences, thus refining the sequence and eliminating errors or false bases.

The consensus sequence was then used to search through the Ribosomal Database Project (RDP), which is maintained by Michigan State University (http://rdp.cme.msu.edu/). This takes the entered sequence and compares it to other 16S rRNA sequences in the database. Using the Type strain search only returns those species with submitted sequences and published papers. Once the best (highest identity) matches were found, two sequences (*Sejongia lycomia* JJC and the highest match) were compared with NCBI's BLAST tool. This aligns the two sequences and gives a percent similarity match. If the percentage of similarity is under 97%, it is reasonable to hypothesize that the bacteria is a novel species.

The 16S rRNA sequence of *Sejongia lycomia* was submitted to GenBank (http://www.ncbi.nlm.nih.gov/Genbank/). The accession number for this sequence is EU523664.

Phylogenetic Analysis

Once the 16S rRNA sequence was assembled and its closest relatives were identified, phylogenetic trees could be constructed. This was done using MegAlign, another component of the LaserGene software suite. Along with the 16S sequence for *Sejongia lycomia*, all of the 16S sequences for the type species for the Flavobacteriaceae family were gathered via NCBI's CoreNucleotide search function. All of these sequences were loaded into MegAlign, and then aligned using the Clustal W method (Hickson, *et al* 2000). A cladogram of the aligned sequences could then be viewed. In addition, the 16S sequences from the most closely related species were also assembled into a tree.

Biochemical Characterization

Following these analyses, it was essential to characterize the organism based upon its phenotypic qualities. In order to test for these capabilities, specialized media were then prepared. For general growth purposes, Tripticase Soy Agar (TSA) plates were incubated at various temperatures in order to find the tolerance limits for growth. In addition, MacConkey Agar was also used. MacConkey agar is a medium containing bile salts and crystal violet designed to grow gram-negative bacteria and stain them for

lactose fermentation.

In order to test for carbohydrate metabolism, liquid media were prepared with phenol red as a pH indicator to indicate the production of acids as a result of successful carbohydrate breakdown. The carbohydrates prepared were: D-Arabinose, Ethanol, D-Fructose, Glycerol, Lactose, Maltose, D-Mannitol, D-Xylose, D-Glucose, Sucrose, D-Cellobiose, and L-Rhamnose. In order to test for exoenzyme production, Lipase and Starch plates were prepared, as well. An oxidase test was also administered.

For nitrogen and amino acid metabolism, Hydrogen Sulfide/Indole/Motility (SIM) media was prepared. These tests were scored based upon either a positive or negative result after 48 hours of incubation in a shaking 30℃ incubator. For temperature growth, bacterial colony formation on the plate was scored as positive result. For the carbohydrates, observed growth plus a change in pH of the media denoted a positive result.

In the case of the lipase plates, a zone of clearing had to be observed for a positive result. For starch analysis, iodine was added to the plates. If starch was still present, iodine would react and produce a deep violet color. In the case of SIM media, the presence of the cysteine desulfhydrase enzyme was tested for via the presence of hydrogen sulfide, which is produced as a byproduct of the enzyme's activity. In addition, tryptophanase activity was tested for via the presence of indole in the media, which is a byproduct of the enzyme's activity.

Growth Curve Analysis

Growth curve analysis was carried out in order to determine the optimal temperature at which *Sejongia lycomia* JJC grows. Bacterial growth can be measured using a Spectronic -20D+ spectrophotometer to determine the amount of 600 nm light that the bacterial culture absorbs in the liquid TSB media solution. From growth curve analysis, doubling time as well as growth rate can be determined. For this analysis, *Sejongia lycomia* was inoculated in 5 mL of liquid TSB media overnight at 30℃ in a shaking incubator. The next morning, 3 mL of liquid TSB was aseptically transferred to two 13 x 100 tubes, one being the recipient of overnight bacterial culture and the other a blank tube. Depending on the amount of overnight growth, enough liquid culture was added to achieve an approximate absorbance reading of 0.050 A on the spectrophotometer.

After the initial reading, the bacterial TSB cultures were then placed back into the shaking incubator at the desired temperature for the duration of the procedure. Every hour, the cultures were taken out of the incubator, vortexed, and then readings were taken. The optimal time for calculating the doubling time for the organism was between 0.300 and 0.600 absorbance. The doubling time is the amount of time it takes for the culture to go from 0.300 A to 0.600 A. The growth rate can be calculated by taking the reciprocal of the doubling time, with units in generations/hour.

In addition to standard growth, growth under variable salt conditions was also carried out. The salt content in liquid TSB was altered (0%, 0.1%, 0.2%, 0.5%, 1%, and 5%) and growth was measured in the same manner as a standard growth curve at 30℃

using a Spectronic 20-D to monitor growth.

FAME Analysis

Fatty Acid Methyl Ester (FAME) analysis was then carried out on the organism. FAME analysis seeks to identify as well as quantify the composition of the fatty acids found on the bacterial membrane. FAME analysis was carried out by Microbial ID, Inc. of Newark, Delaware utilizing the MIDI system. Fatty acids were freed from the membrane via a saponification reaction, and then underwent methylation. The methyl esters were then extracted, and the organic solution containing the methyl esters was then separated using gas chromatography and mass spectroscopy. This allowed for the identification of the different types of fatty acids in the membrane, as well as the relative amounts. The total of all the fatty acids present added up to 100%.

Results:

Figure 1: Photograph of *Sejongia lycomia* on TSA plate

Figure 1 is a photograph of *Sejongia lycomia* on a Tripticase Soy Agar plate incubated at 30℃ for 48 hours. Note the yellow pi gmentation and the mucoid quality of the colonies. Morphological characteristics are some of the first things examined when characterizing a species. The colonies of *Sejongia lycomia* JJC grown on TSA plates are similar to *Sejongia antarctica, Sejongia jeonii* and *Sejongia marina* that were described by Yi, *et al*. (2005) and Lee, *et al*. (2007). All three share the same distinct yellow pigmentation. In addition, all three share the quality of being gram-negative rods,

suggesting a thin layer of peptidoglycans on the cell wall surface (Figure 2).

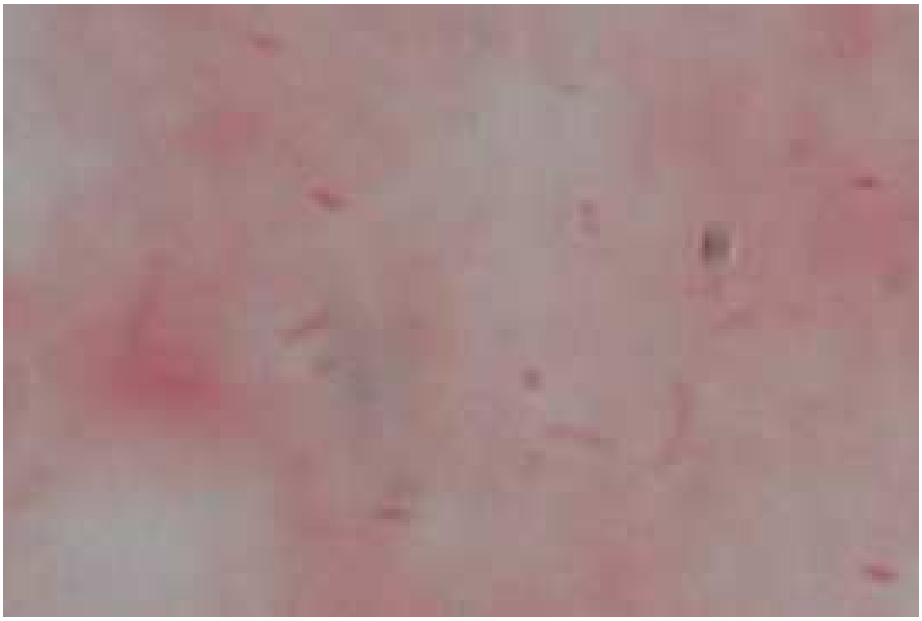


Figure 2: Gram Stain of -20C culture stock of *Sejongia lycomia* JJC (1000X oil)

Figure 2 is a photograph taken through a light microscope under oil immersion at 1000X magnification. This was a culture of *Sejongia lycomia* JJC after undergoing the gram staining treatment. Although faint (due low concentration of bacteria), gram-negative rods were observed. All members of the *Sejongia genus* all share the quality of being gram-negative rods, suggesting a thin layer (or no layer at all) of peptidoglycans on the cell wall surface

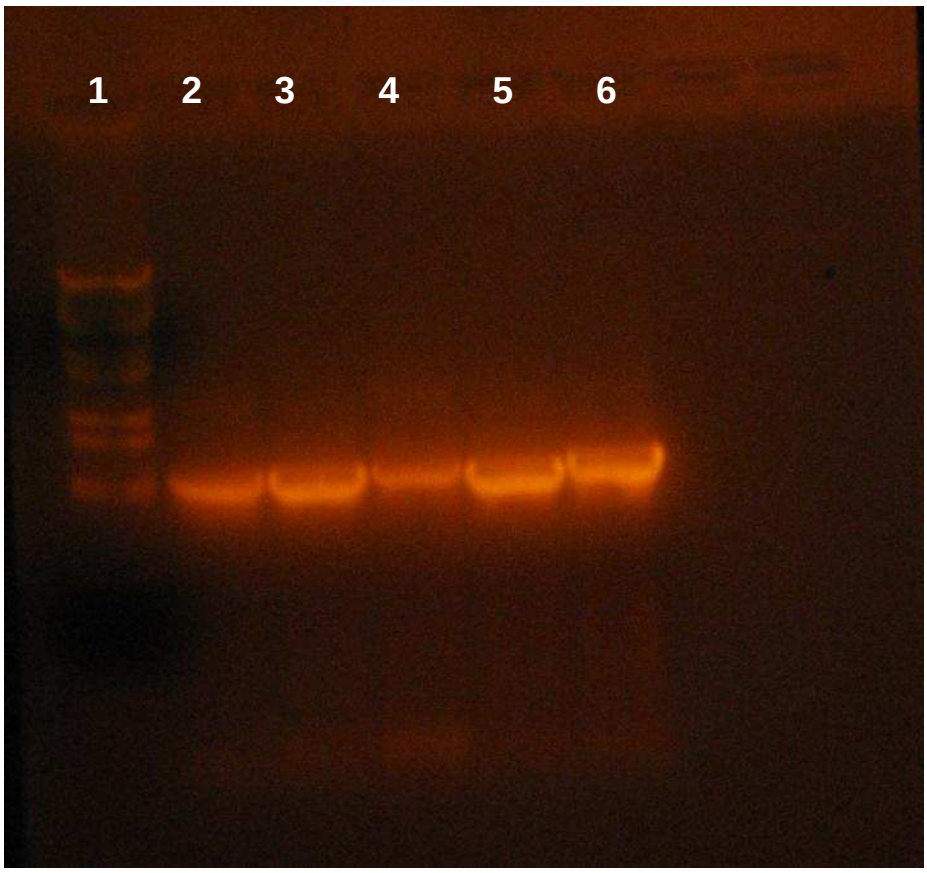


Figure 3: Photograph of 1% Agarose Gel containing crude PCR products under ultraviolet light. Lane 1 contains the Lambda phage DNA marker, Lane 2 contains *Sejongia lycomia* JJC TSA 9/23/07, Lane 3 contains *Sejongia lycomia* JJC TSA 9/17/07, Lane 4 contains *Carnobacterium lycomia* AJR 12/06, Lane 5 contains *Flavobacterium novum* AMR, and Lane 6 contains *Enterococcus novum* JB 11/06.

Figure 3 presents a photo of the agarose gel following completion of the gel electrophoresis of PCR products. Isolation appeared to be successful, as strong (in terms of fluorescence) bands were present in all lanes. The brightness of the bands refers to concentration, which suggests a high yield for the procedures. In addition, only single bands were found on the gels in the 1500 base pair fragment range in comparison to the lambda marker. This further supports the successful isolation, as the

16S rRNA gene is approximately 1500 bp, depending on species.

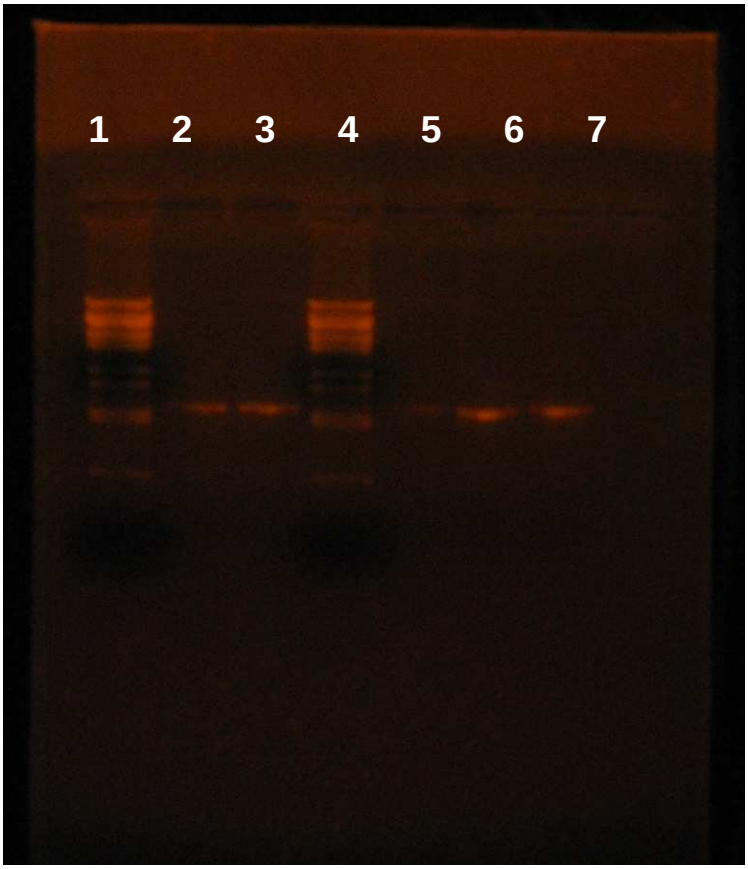


Figure 4: Photograph of 1% Agarose Gel containing purified PCR products used for quantification. Lane 1 contains Lambda Phage DNA marker, Lane 2 contains *Sejongia lycomia* JJC TSA 9/23/07, Lane 3 contains *Sejongia lycomia* JJC TSA 9/17/07, Lane 4 contains another Lambda DNA marker, Lane 5 contains *Carnobacterium novum* AJR 12/06, Lane 6 contains *Flavobacterium novum* AMR, and Lane 7 contains Enterococcus *novum* JB 11/06.

Figure 4 is a photograph of the agarose gel following gel electrophoresis of the purified PCR products. The presence of the bands in the right range suggests that the purification procedure was successful. Based upon the brightness of the bands, the concentration of the DNA was estimated to be 20 ng/mL, which is within range of being able to be sequenced.

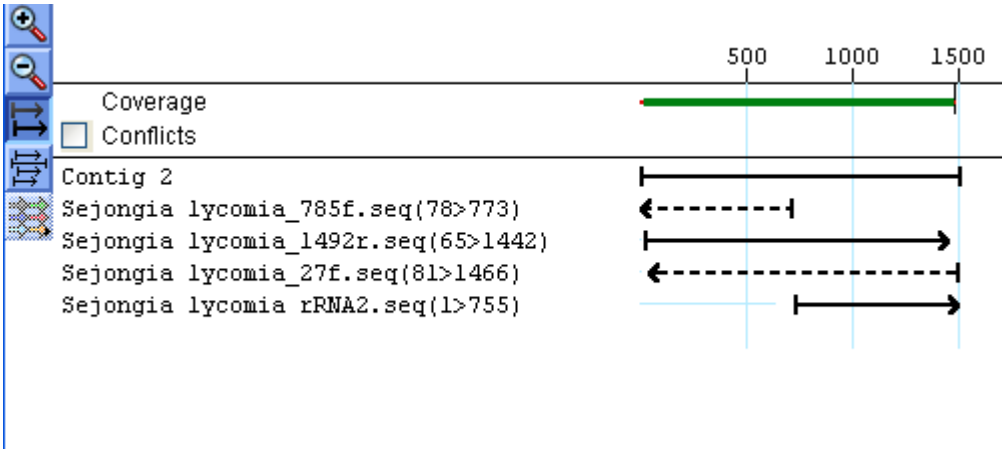
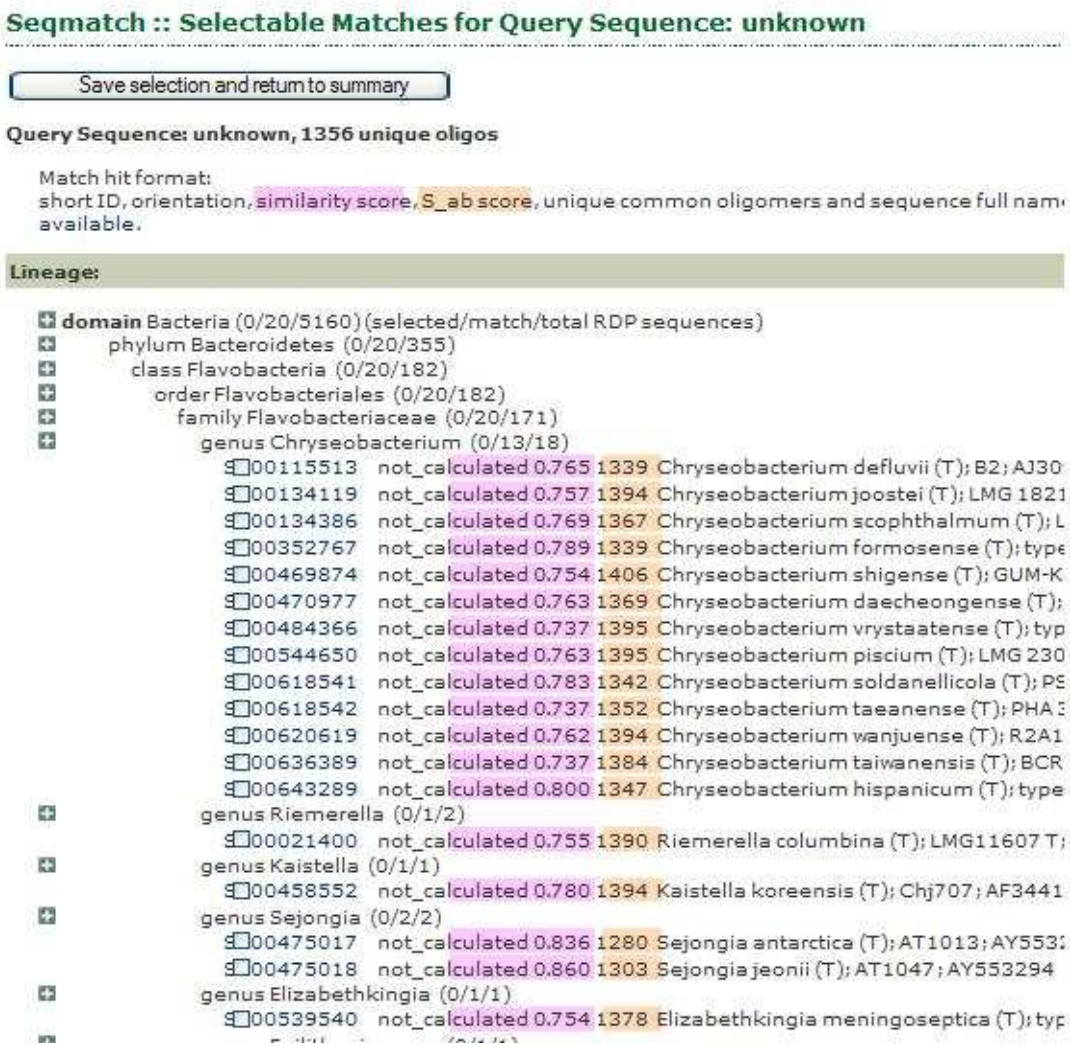


Figure 5: Strategy View of Sequence Coverage of 27f, 1492r, 785f, and rRNA2

Figure 5 is a representation of sequence coverage by the different primer sequences. The sequencing using primers 27f, 1492r, and 785f were carried out in this study, while the sequence data from rRNA2 was from Jarrod Chipp and his work in the Microbiology course. Each of the arrows denote the direction the primer moved in relation to their binding. Both the top and bottom strands were sequenced twice, giving a total of 4 sequences that went into the Contig sequence.

Figure 6: Contiguous Assembled 16S rRNA sequence of *Sejongia lycomia* JJC in EditSeq

Figure 6 is a screenshot of the assembled 16S rRNA sequence of Sejongia lycomia JJC. This sequence is a result of entering four different sequences (27f, 1492r, 785f, and rRNA2) to SeqMan and using the Contig function. The final trimmed sequence is 1469 base pairs long. Note that the W, M, and N codes present are IUPAC Nucleic Acid codes, and stand for T, U, or A, C or A, and Any base, respectively.



Seqmatch :: Selectable Matches for Query Sequence: unknown

Save selection and return to summary

Query Sequence: unknown, 1356 unique oligos

Match hit format:
short ID, orientation, similarity score, S_ab score, unique common oligomers and sequence full nam
available.

Lineage:

domain Bacteria (0/20/5160) (selected/match/total RDP sequences)
phylum Bacteroidetes (0/20/355)
class Flavobacteria (0/20/182)
order Flavobacteriales (0/20/182)
family Flavobacteriaceae (0/20/171)
genus Chryseobacterium (0/13/18)

S00115513	not_calculated	0.765	1339	Chryseobacterium defluvii (T); B2; AJ30
S00134119	not_calculated	0.757	1394	Chryseobacterium joostei (T); LMG 1821
S00134386	not_calculated	0.769	1367	Chryseobacterium scophthalmum (T); L
S00352767	not_calculated	0.789	1339	Chryseobacterium formosense (T); type
S00469874	not_calculated	0.754	1406	Chryseobacterium shigense (T); GUM-K
S00470977	not_calculated	0.763	1369	Chryseobacterium daecheongense (T);
S00484366	not_calculated	0.737	1395	Chryseobacterium vrystaatense (T); typ
S00544650	not_calculated	0.763	1395	Chryseobacterium piscium (T); LMG 230
S00618541	not_calculated	0.783	1342	Chryseobacterium soldanellicola (T); PS
S00618542	not_calculated	0.737	1352	Chryseobacterium taeanense (T); PHA 3
S00620619	not_calculated	0.762	1394	Chryseobacterium wanjuense (T); R2A1
S00636389	not_calculated	0.737	1384	Chryseobacterium taiwanensis (T); BCR
S00643289	not_calculated	0.800	1347	Chryseobacterium hispanicum (T); type

genus Riemerella (0/1/2)

S00021400	not_calculated	0.755	1390	Riemerella columbina (T); LMG11607 T;

genus Kaistella (0/1/1)

S00458552	not_calculated	0.780	1394	Kaistella koreensis (T); Chj707; AF3441

genus Sejongia (0/2/2)

S00475017	not_calculated	0.836	1280	Sejongia antarctica (T); AT1013; AY553
S00475018	not_calculated	0.860	1303	Sejongia jeonii (T); AT1047; AY553294

genus Elizabethkingia (0/1/1)

S00539540	not_calculated	0.754	1378	Elizabethkingia meningoseptica (T); typ

Figure 7: Ribosomal Database Project Search Results

Figure 7 displays the Type strain Seqmatch search that was conducted with the assembled contiguous sequence of *Sejongia lycomia* JJC. *Sejongia lycomia*'s sequence did not share 100% identity to any organisms in the database. The most relevant matches include (in order of similarity): *Sejongia jeonii*, *Sejongia antarctica*, and *Chryseobacterium hispanicum*.

```
 Score = 2355 bits (1225),  Expect = 0.0
 Identities = 1323/1367 (96%), Gaps = 2/1367 (0%)
 Strand=Plus/Plus

Query  42    CGAGCGGTATTTCTTCTTCGGAAGAGAGAGAGCGGCGCACGGGTGCGTAACACGTGTGCA  101
             ||||||||||||| |||||||||| |||||||||||| ||||||||||||||||||||||
Sbjct  1     CGAGCGGTATTTATTCTTCGGAATAGAGAGAGCGGCGTACGGGTGCGTAACACGTGTGCA  60

Query  102   ACCTACCTTTATCTGGGGGATAGCCTTTCGAAAGGAAGATTAACACCCCATAATATATTA  161
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Query  162   GATGGCATCATTTGATATTGAAAACTCCGGTGGATAAAGATGGGCACGCGCAAGATTAGA  221
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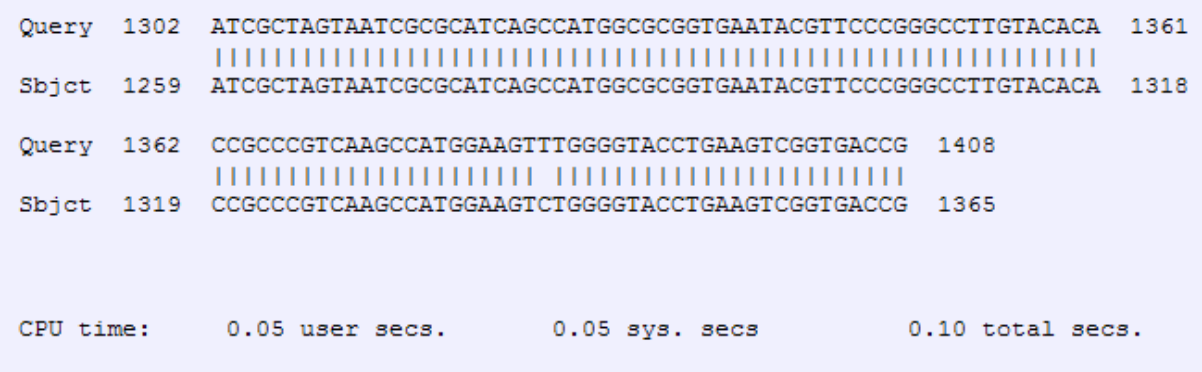

Figure 8: BLAST Alignment of 16S rRNA sequences of *Sejongia jeonii* and *Sejongia lycomia* JJC

Figure 8 exhibits the BLAST alignment of the 16S rRNA sequences of *Sejongia lycomia* JJC and its closest relative (according to 16S rRNA sequencing), *Sejongia jeonii*. From the diagram, the entire sequence is compared, and 1323 out of 1367 base pairs were found to be identical. This gives a percentage of approximately 96.78%. This figure is under the threshold of 97%, so the organism is still considered to be a novel species.

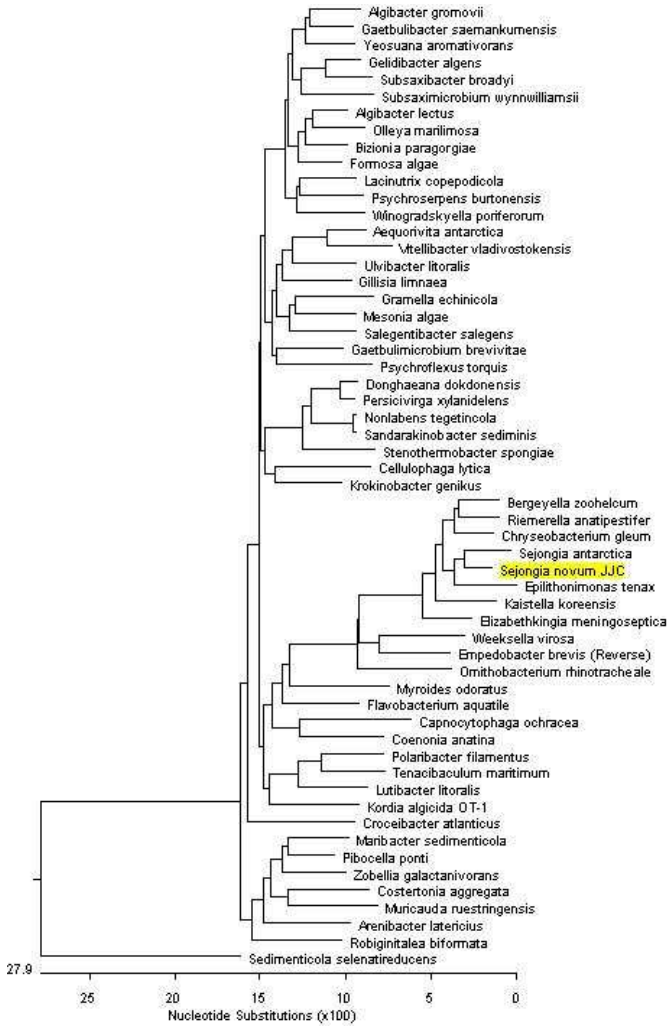

Figure 9: Phylogenetic Tree of *Sejongia lycomia* JJC and type species of Genera within family Flavobacteriaceae

Figure 9 is a phylogenetic cladogram of *Sejongia lycomia* JJC and the type species of the genera belonging to the Flavobacteriaceae family. Highlighted in yellow is *Sejongia lycomia* JJC, coming off of a branch of *Sejongia antarctica*. This tree was assembled based upon aligning 16S rRNA sequences for each organism using the Clustal W method.

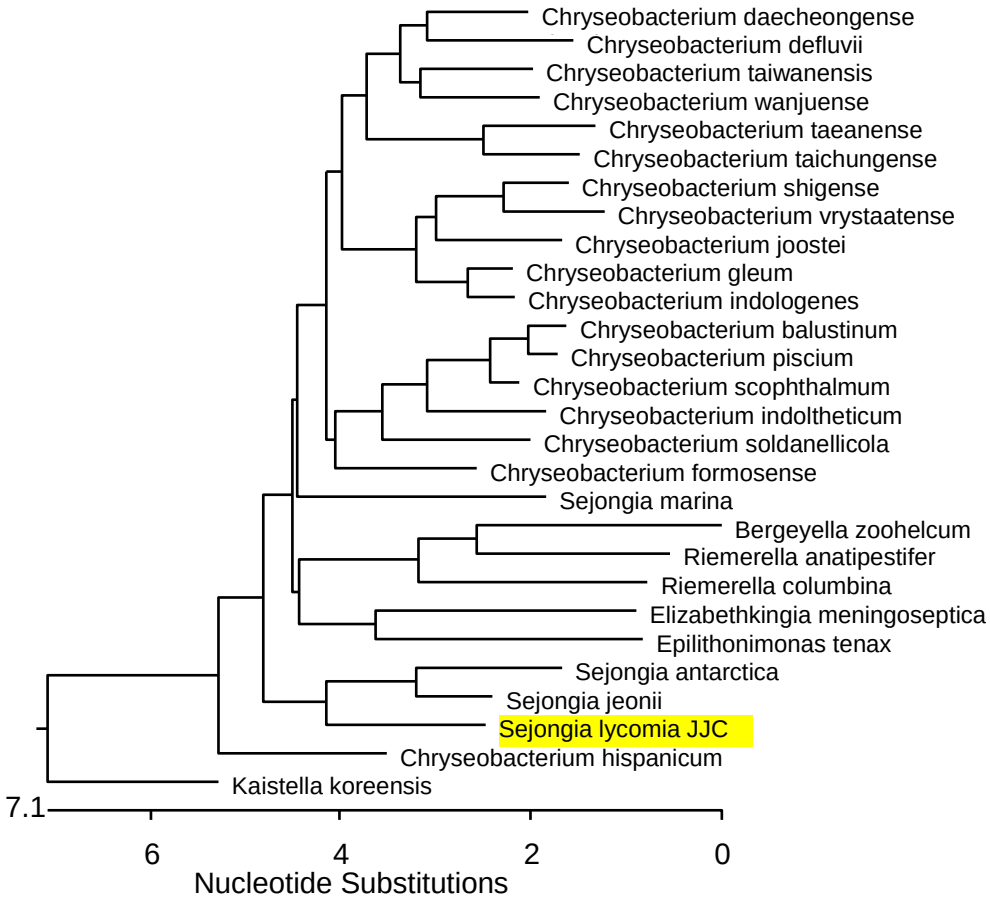

Figure 10: Phylogenetic Tree of All Known Closely Related Organisms to *Sejongia lycomia* JJC

Figure 10 is a phylogenetic tree of *Sejongia lycomia* JJC and the most closely related organisms. Highlighted in yellow is *Sejongia lycomia*. The tree was constructed in the same manner as Figure 9.

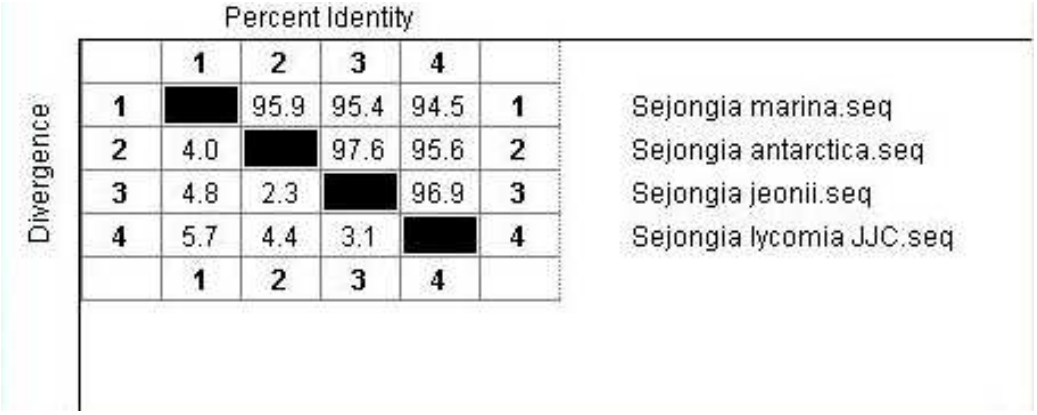

Figure 11: Pair Distances of *Sejongia lycomia* JJC, *Sejongia antarctica*, and *Sejongia jeonii*

Figure 11 is a Pair Distance Matrix based upon 16S rRNA sequences. This is tabulated using the Pair Distance function in MegAlign. The top row is the most important, as it contains the comparison data between *Sejongia lycomia*, *antarctica*, *jeonii,* and *marina*. From the matrix, *Sejongia lycomia* JJC and *Sejongia antarctica* are share 95.6% identity, *Sejongia lycomia* JJC and *Sejongia jeonii* share 96.9% identity, and *Sejongia lycomia* JJC and *Sejongia marina* share 94.5% identity.

Table 1: Compiled Table of Phenotypic Biochemical Data for members of genus *Sejongia* V represents a variable result, while ND stands for not determined or not present in the literature

	Sejongia lycomia	*Sejongia antarctica*	*Sejongia jeonii*	*Sejongia marina*
Growth on MacConkey Agar	-	-	-	-
Growth at 5°C	-	+	+	-
Growth at 30°C	+	V	V	+
Growth at 37°C	-	-	-	-
Growth at 42°C	-	-	-	-
Growth at ~22°C	+	ND	ND	ND
Capable of Nitrate Reduction	-	-	-	+
Capable of Nitrite Reduction	-	-	-	-
Capable of Starch Hydrolysis	+	+	+	+
Presence of Oxidase	+	+	+	-
Presence of Lipase	+	+	+	+
Production of Hydrogen Sulfide	-	-	-	ND
Production of Indole	-	+	+	-
Production of Indole 2	-	N/A	N/A	N/A
Observed Motility	+	ND	ND	ND
Methyl Red	-	ND	ND	ND
Vogues-Proskauer	-	ND	ND	ND
Aerobic Acid Production from…				
D-arabinose	+	ND	ND	ND
Ethanol	-	ND	ND	ND
D-fructose	-	-	-	ND
Glycerol	-	ND	ND	ND
Lactose	-	-	-	ND
Maltose	+	+	+	ND
D-mannitol	-	-	-	ND
D-xylose	-	-	-	ND
D-glucose	+	+	+	-
Sucrose	-	-	-	ND
D-cellobiose	-	-	-	ND
L-rhamnose	-	-	-	ND

Table 1 details the results of the growth conditions, exoenzymatic activity, carbohydrate metabolism, amino acid metabolism, and nitrogen metabolic tests. Data for *Sejongia lycomia* JJC was gathered at Lycoming College, but data for *Sejongia jeonii*

and *Antarctica* are from Yi, *et al* (2005) and *Sejongia marina* from Lee, *et al* (2007). The Indole 2 test was a second retesting performed on *Sejongia lycomia* only to reaffirm results of the first test and thus other species are N/A.

Each type of test had different parameters for judging positive and negative results. The carbohydrate tests used phenol red as a pH indicator that would be a sign of acid production as a result of successful carbohydrate metabolism. Growth on varying temperature plates was scored based on presence of bacterial growth or not. In nitrogen metabolism, the reduction of nitrates to nitrites, and the denitrification to nitrogen gas was tested for using special media. Lipid and Starch plates tested for the presence of lipase and amylase enzymes, and positives were scored based on zones of clearing around the bacteria. The SIM media tested for the presence of enzymes that could break down the nitrogenous compounds that were present in the medium.

Notably, *Sejongia lycomia* did not reduce nitrate or denitrify nitrite. Unlike *Sejongia jeonii* and *Sejongia Antarctica*, it did not grow at 5℃. It also produced acid aerobically from D-Arabinose. In comparison to other species, *Sejongia lycomia* had lipase, oxidase, and starch hydrolysis activity.

Table 2: Significant Phenotypic Differences between *Sejongia lycomia, Sejongia antarctica, Sejongia jeonii,* and *Sejongia marina*

Parameter	*Sejongia lycomia*	*Sejongia antarctica*	*Sejongia jeonii*	*Sejongia marina*
Growth at 5ºC	-	+	+	ND
Growth at 30ºC	+	var	var	-
Presence of Lipase	+	+	+	+
Capable of Nitrate Reduction	-	-	-	+
Capable of Nitrite Reduction	-	-	-	ND
Hydogren Sulfide Production	-	-	-	ND
Indole Production	-	+	+	-
Acid Produced from Maltose	+	+	+	ND
Acid Produced from D-Xylose	+	-	-	ND
Acid Produced from D-Glucose	+	+	+	-

Table 2 displays some of the phenotypic differences between the four *Sejongia* species. Var denotes a variable result and ND is nondetermined as described in the literature. Data for *Sejongia jeonii* and *Sejongia antarctica* is from by Yi, *et al*. (2005). Data for *Sejongia marina* is from Lee, *et al*. (2007). This table is a summary of some of the results noted by this study and others that can serve to differentiate the different species based upon phenotype.

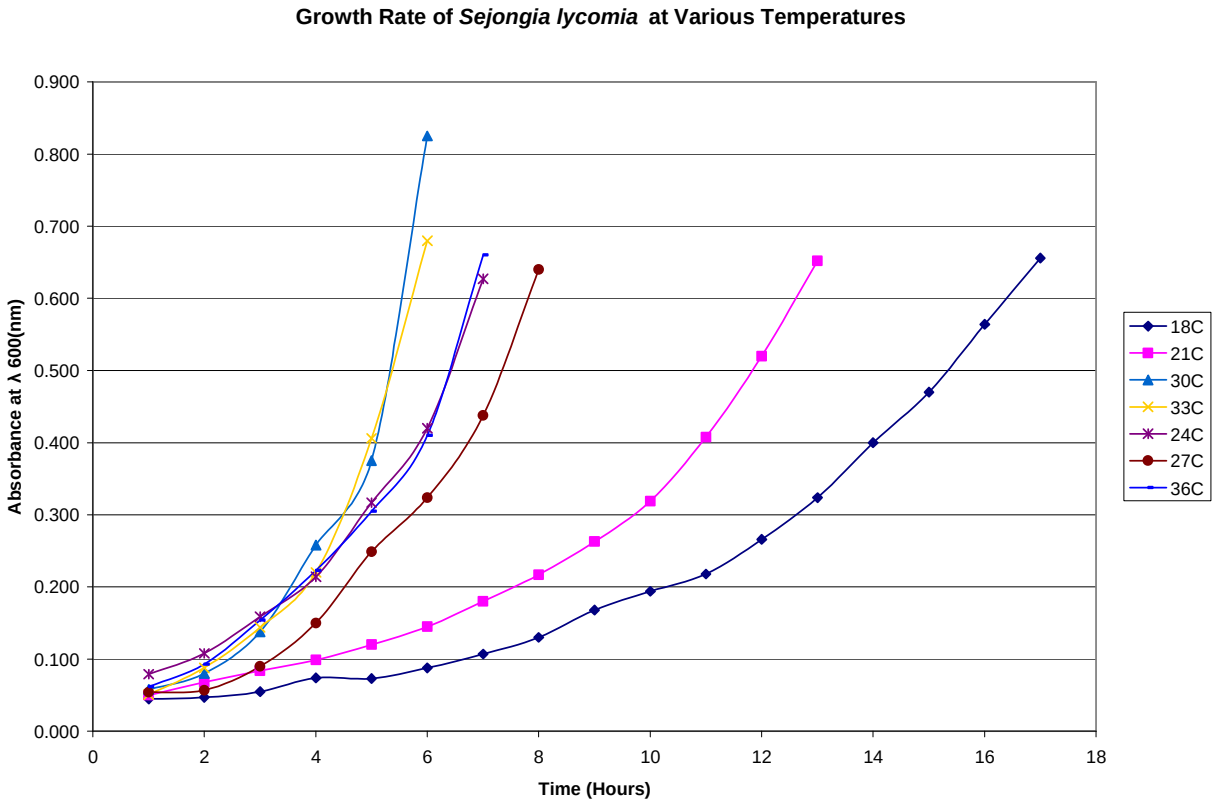

Figure 12: Graph of Absorbance versus Time under varying Temperatures

Figure 12 is a graph of the changes in absorbance over time at varying temperatures. The rate of change of light absorbance is the growth rate of the organism. Each curve represents a different temperature trial.

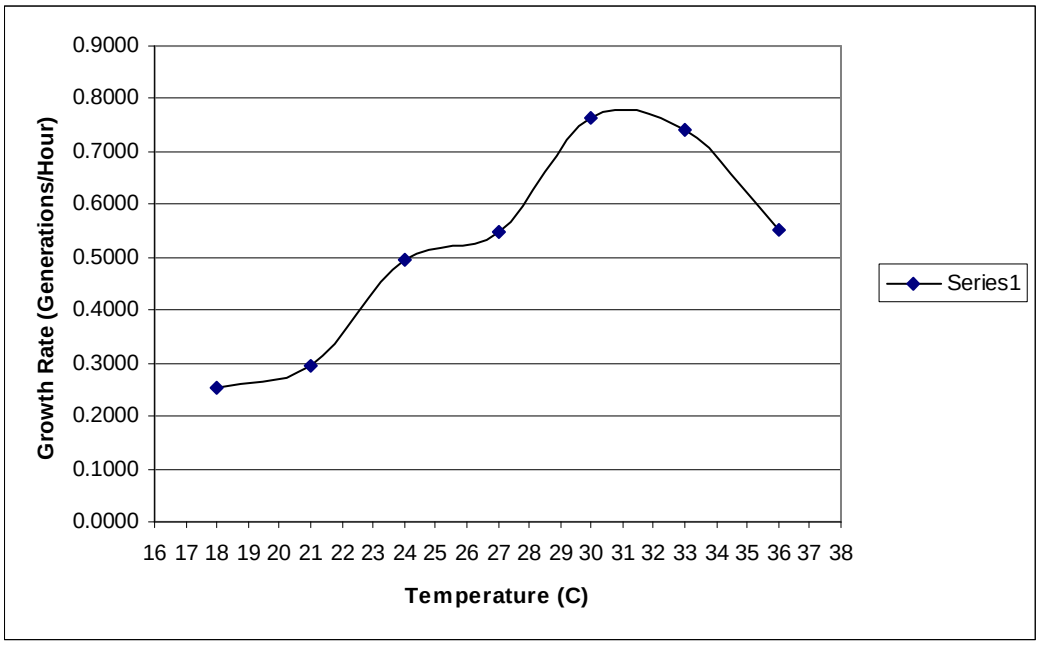

Figure 13: Growth Rate Analysis of *Sejongia lycomia* JJC

Figure 13 is a graph of the growth rates of *Sejongia lycomia* JJC under varying temperatures. Each point on the graph denotes a different incubation temperature. The X-axis represents the time in hours, while the Y-axis represents the growth rate. From the graph, it is evident that *Sejongia lycomia* JJC grows the slowest at 18℃. From the data, it appears that 30℃ is the optimum due to th e most rapid growth occurring there.

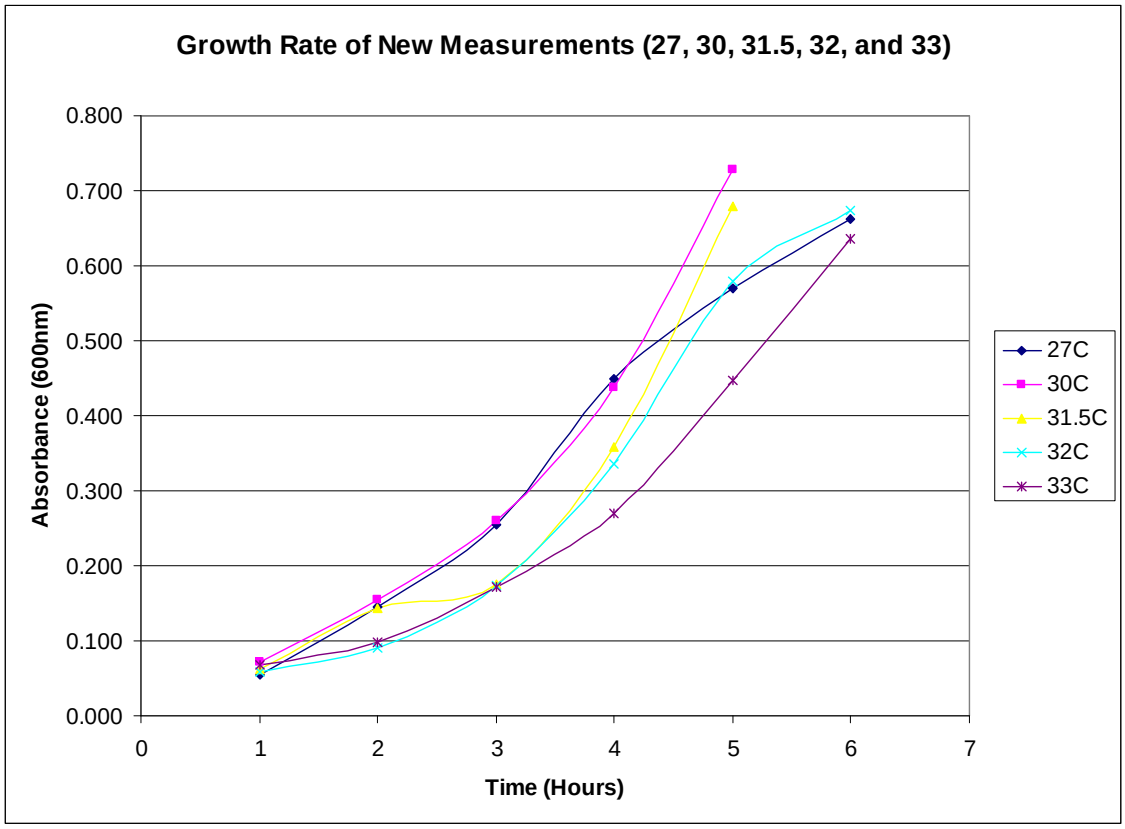

Figure 14: Growth Curves with new repeated points

Figure 14 is a graph of the changes in absorbance over time again, with each curve representing a different temperature. These procedures were done in order to further pinpoint the exact optimum temperature by focusing between 30°and 33℃.

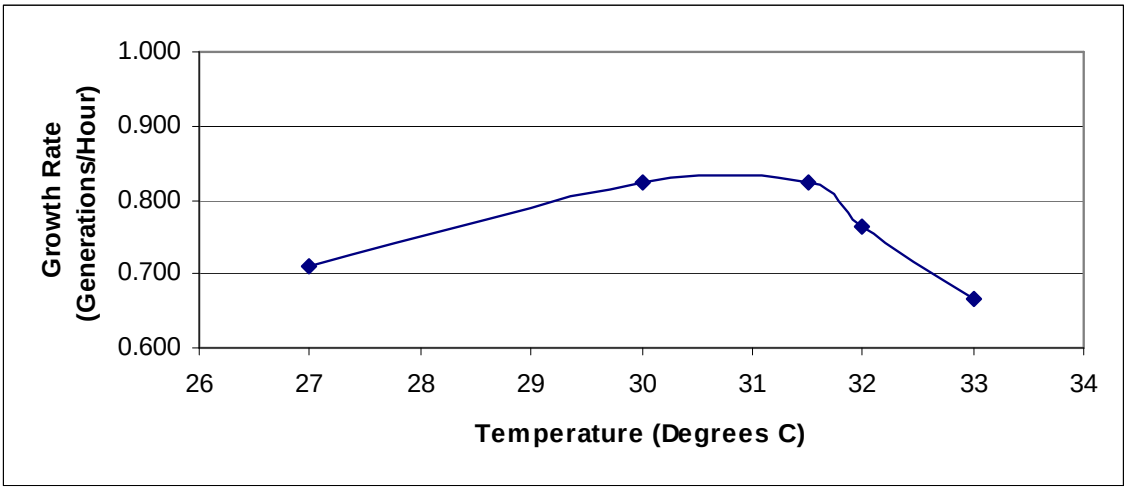

Figure 15: Growth Rate of *Sejongia lycomia* under varying Temperature Conditions

Figure 15 is a graph of growth rate in generations per hour versus temperature for *Sejongia lycomia.* Each point denotes a different temperature trial. From the graph, the growth rate is greatest in the region between 30℃ and 31.5℃, suggesting that the true growth optimum likely lies in that region.

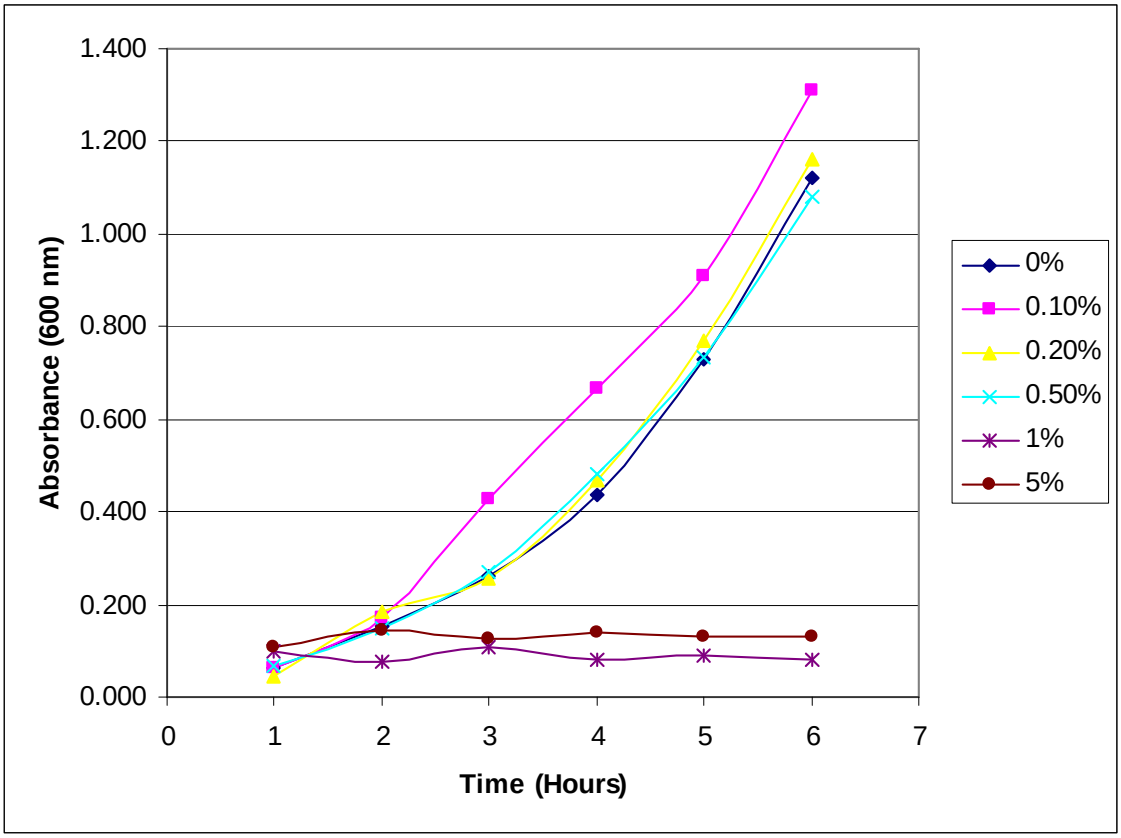

Figure 16: Growth of *Sejongia lycomia* under varying [NaCl] at 30°C

Figure 16 contains several growth curves of *Sejongia lycomia* that were incubated at 30℃ in different TSB media solutions that contained varying amounts of dissolved NaCl. Because *Sejongia marina* was isolated from Antarctic seawater, it was relevant to test *Sejongia lycomia'*s salt growth optima. It appears that *Sejongia lycomia'*s growth optimum for salt is most likely between 0% and 0.1%.

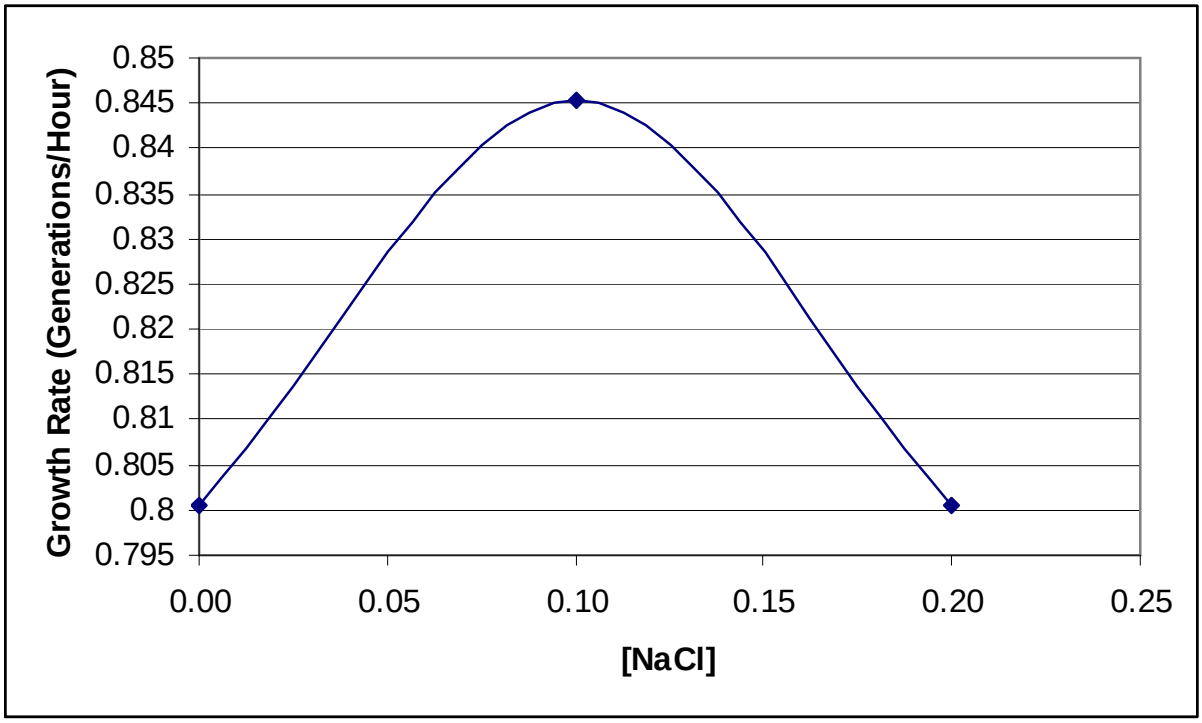

Figure 17: Growth Rates of *Sejongia lycomia* versus salt concentration. Data for 1% and 5% NaCl are not represented.

Figure 17 displays the growth rates of *Sejongia lycomia* under varying NaCl concentrations. From the graph, the optimal NaCl for growth is approximately 0.10%. Data for 1% and 5% NaCl are not represented on the graph, as there was no measureable growth in either of the cultures and thus had a growth rate of zero.

Table 3: Dominant Fatty Acids found in *Sejongia lycomia, Sejongia antarctica, Sejongia jeonii*, and *Sejongia marina*

Fatty Acid	*Sejongia lycomia*	*Sejongia antarctica*	*Sejongia jeonii*	*Sejongia marina*
13:0 iso	8.92	2.50	2.90	0.60
13:0 anteiso	0.76	3.20	3.60	0.50
15:0 iso	39.01	13.60	12.20	17.01
15:0 anteiso	8.42	15.20	24.20	33.80
15:0 iso 3OH	4.21	1.00	1.30	3.20
15:1 anteiso A	0.00	6.60	0.00	0.00
16:0 iso	0.36	2.80	5.70	0.60
16:0 10-Methyl	15.79	0.00	0.00	0.00
16:0 iso 3OH	0.00	5.10	5.70	2.90
16:1 ω7c/16:1 ω6c	6.57	2.70	2.60	0.00
16:1 iso H	0.00	3.60	9.10	2.30
17:0 iso 3OH	8.86	5.60	4.40	5.00
17:1 iso ω9c	0.00	21.30	8.60	8.80
17:1 anteiso ω9c	0.00	2.50	1.90	2.30

Table 3 displays the Fatty Acid profile of *Sejongia lycomia* as determined by Microbial ID, Inc. The dominant (>2%) fatty acids are displayed. Each number represents a percent of the total fatty acids found in each organism. The significant differences in species are highlighted in yellow. Data for *Sejongia jeonii* and *Sejongia antarctica* is from Yi, *et al*. 2005 while data for *Sejongia marina* is from Lee, *et al*. 2007.

Discussion of Results:

The highest percentage matching Ribosomal Database Project search results of the 16S rRNA sequence belonged to *Sejongia antarctica* and *Sejongia jeonii* (Figure 7). RDP queries can be limited to only include type strains. Type strains are published and have a corresponding paper, making the RDP far more reliable than other search engines for 16S rRNA sequences (such as GenBank). Currently, *Sejongia* marina is not yet included in the RDP.

The data corroborates what Jarrod Chipp originally found in the Microbiology course. Although he only used one sequence (rRNA2), a total of four different sequences (including Chipps's initial sequencing data) were assembled in this study. Figure 6 displays the coverage of the four different sequences and its assembled contiguous sequence. As a result, the 16S rRNA sequence data is very refined, as there is excellent coverage of the entire sequence with two separate sequences for each strand. Although sequence data at the ends of the gene can sometimes be of lesser quality, there are at least two high quality reads at each position. Therefore, there is confidence of the data, as assembling the contiguous sequence refines the sequence by eliminating errors in bases by selecting the base that appears in the majority. In total, 1469 bases were obtained, after trimming and manually refining the sequence, which is nearly the entire 16S gene. Yi's 16S rRNA sequencing of *Sejongia jeonii* and *Sejongia antarctica* only yielded 1365 base pairs.

The sequence distance matrix that was calculated by MegAlign gives a more precise (to three significant figures) of the differences in percent identity between the

organisms (Figure 11). The sequence distance data supports what was found during the RDP search in that *Sejongia lycomia* has less than 97% identity to its closest relative, *Sejongia jeonii* (96.9%). It is also notable that *Sejongia jeonii* and *Sejongia antarctica* share a 97.6% identity. Although this is over the commonly accepted 97% value, they are considered two different species due to several other key differences and are accepted as such.

The phylogenetic tree of closely related species constructed here (Figure 10) resembles that found in Yi *et al*., with closely related *Chryseobacterium*, *Riemerella*, and *Bergeyella* (2005). Given that *Sejongia lycomia* JJC is an outbranching from the sharp monophyletic clade formed by *Sejongia jeonii* and *Sejongia antarctica*, it is evident that the three organisms are very closely related (Figure 10).

Sejongia marina appears to be out of place on Closest Related tree (Figure 10). It appears to be coming off of the *Chryseobacterium* group, rather than the *Sejongia* group. It is currently unknown why this behavior is observed, although the sequence was aligned multiple times and in reverse compliment in an effort to try to resolve this problem, but nothing settled the anomaly.

In comparing the biochemical data, members of genus Sejongia share certain characteristics as well as have some differences (Table 1, 2). *S. lycomia, S. antarctica,* and *S. jeonii* did not grow at high temperatures (37℃, 42℃), on MacConkey agar, did not reduce nitrates/nitrites, produce hydrogen sulfide, and produce acid aerobically from many of the carbohydrates (Table 1). *S. lycomia, S. antarctica,* and *S. jeonii* did grow at 30℃, break down lipids, perform starch hydrolysis, metabolize maltose, and metabolize

glucose. The four species also did have some differences. Unlike the psychrotolerant *Sejongia jeonii* and *Sejongia antarctica*, *Sejongia lycomia* did not grow at 5℃. In addition, it did not produce indole, which was tested twice. *Sejongia lycomia* also had oxidase activity, meaning that it has cytochrome c as a part of its electron transport chain and uses oxygen as a final electron acceptor.

For the growth curve analysis, *Sejongia lycomia* displayed normal exponential growth during the period of measurement. Unlike *Sejongia jeonii* and *Sejongia antarctica*, *Sejongia lycomia* does not appear to be psychrotolerant. Yi, *et al*. found that *Sejongia jeonii* and *Sejongia antarctica* optimally grew at approximately 20℃ (2005). However, growth at even 18℃ for *Sejongia lycomia* was very slow, taking over 16 hours to reach the optimal area (Between 0.300 and 0.600 A) for calculating rates and had an overall growth rate of only 0.2519 generations per hour (Figure 12).

In comparison to 18℃, *Sejongia lycomia* grew very quickly at both 30℃ and 33℃, reaching optimal range for calculation in app roximately 5 hours (Figure 12). However, the optimal temperature for growth appears to be in between the values of 30℃ and 33℃, as 30℃ appears to have more growth at the same endpoint.

Figures 13 and 14 display the data from temperature trials that were performed in order to obtain a more accurate growth optimum. This was accomplished by taking more precise measurements in the previously described optimal range (30°-33℃). *Sejongia lycomia* was observed to have the fastest growth at both 30℃ and 31.5℃. This suggests that the true optimum for growth most likely is near 31℃. This high growth optimum, in comparison to the psychrotolerant *Sejongia jeonii* and *Sejongia*

antarctica, demonstrates the mesophilic qualities of the organism, making this data integral in differentiating *Sejongia lycomia* from the other species.

The higher growth optimum temperatures of *Sejongia lycomia* are not unexpected, as *Sejongia jeonii* and *Sejongia antarctica* were initially isolated from terrestrial and moss samples on King George Island in Antarctica (Yi, *et al*. 2005). In contrast, the Loyalsock Creek does not even come close to reaching Antarctic temperatures, and thus *Sejongia lycomia* would not be expected to be adapted for that extreme environment.

The FAME analysis revealed that there were several differences between the composition of the fatty acids found in the membranes of the four *Sejongia* species (Table 3). Differences in fatty acids found in the membranes could be expected, as this might result from the temperature at which the organisms are adapted to live at.

For example, one might expect to find more unsaturated fatty acids in the membrane of the psychrotolerant species in order to keep their membranes more fluid-like in response to the cold temperatures. From the analysis in this study, there are several different fatty acid species that demonstrate this concept. The fatty acids 16:1 iso H, 17:1 ω9c, and 17:1 anteiso ω9c all appear in larger proportions in the membranes of the psychrotolerant species, while *Sejongia lycomia* has either lesser amounts or has none at all. These unsaturated fatty acids are more likely to be found in organisms adapted to colder temperatures due to the structural “kink” that the *cis* double bond provides to the hydrocarbon chain. Unsaturated fatty acids are unable to pack as closely as saturated fatty acids, and thus confer fluidity to the membrane. An

organism adapted to a colder environment would need more unsaturated fatty acids to keep its membrane from freezing and becoming immobile.

Hydroxyl groups (such as on 15:0 Iso 3OH) found on fatty acids are thought to help stabilize membranes structures through hydrogen bonds and strengthen interactions with membrane proteins. Methylated fatty acids are an alternative strategy to using double bonds, as the extra methyl groups help increase fluidity of the membrane and are more resistant to oxidation.

Overall, no match could be found in the MIDI database for the fatty acid composition of *Sejongia lycomia*, further suggesting the novel identity of this organism.

Concluding Remarks

The concept of a species has been a constant problem in biology since its inception. There are many different ways to define a species, and each definition has its advantages and disadvantages. In this study, a polyphasic approach was utilized in order to establish the novel identity of *Sejongia lycomia* and establish it as a member of genus *Sejongia.* More specifically, *Sejongia lycomia* was demonstrated to be a genetically different species from other members of genus *Sejongia* through 16S rRNA sequencing, phenotypically different using biochemical and growth tests, and morphologically unique using FAME analysis.

In light of all of the above evidence, it is concluded that *Sejongia lycomia* is a unique species belonging to genus *Sejongia* in family *Flavobacteriaceae*. The basis for this lies in the molecular, phenotypic, and taxonomic data that was collected during this

study. *Sejongia lycomia* is named in honor of Lycoming College, located in Williamsport, PA

.**Description of Species**

Sejongia lycomia (Se.jong.i.a. ly.com.i.a.) is an aerobic, gram-negative, mucoid, yellow-pigmented, rod-shaped organism. *Sejongia lycomia* optimally grows at approximately 31ºC. Metabolizes lecithinase and starch. Produces acid aerobically from D-Xylose, D-Glucose, and Maltose. Does not reduce nitrate or produce indole. Predominant fatty acids include 13:0 iso, 15:0 iso, 15:0 anteiso, 10 Methyl 16:0, and 17:0 iso 3OH. The strain was initially isolated from Loyalsock Creek near Montoursville, Pa (41°15’00.53” N, 76°56’09.63” W)

Acknowledgements

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Appendix I: GenBank Record for *Sejongia lycoma* JJC (EU523664)

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ACCESSION   EU523664
VERSION     EU523664.1  GI:170674450
KEYWORDS    .
SOURCE      Flavobacteriaceae bacterium JJC
  ORGANISM  Flavobacteriaceae bacterium JJC
            Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales;
            Flavobacteriaceae.
REFERENCE   1  (bases 1 to 1469)
  AUTHORS   Marcinko,T.M. and Newman,J.D.
  TITLE     Identification and Characterization of Sejongia lycomia, a Unique
            Isolate from Loyalsock Creek
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1469)
  AUTHORS   Marcinko,T.M. and Newman,J.D.
  TITLE     Direct Submission
  JOURNAL   Submitted (27-FEB-2008) Biology, Lycoming College, 700 College
            Place Box 767, Williamsport, PA 17701, USA
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       61 ggaagagaga gagcggcgca cgggtgcgta acacgtgtgc aacctacctt tatctggggg
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      181 gaaaactccg gtggataaag atgggcacgc gcaagattag atagttggtg aggtaacggc
      241 tcaccaagtc aatgatcttt aggggtcctg agagggagat cccccacact ggtactgaga
      301 cacggaccag actcctacgg gaggcagcag tgaggaatat tggacaatgg gtgaaagcct
      361 gatccagcca tcccgcgtga aaggatgacg gtcctatgga ttgtaaactt cttttgtaca
      421 gggataaacc tactctcgtg agggtagctg aaggtactgt acgaataagc acccggctaa
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      541 taaagggtcc gtaggcgggc ccgtaagtca gtggtgaaat ctcatagctt aactatgaaa
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      661 gtgaaatgca tagatattac ttagaacacc aattgcgaag gcaggtcact aagatttaac
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      841 gttagccacc tggggagtac gttcgcaaga atgaaactca aaggaattga cgggggcccg
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APPENDIX II: Lipid Index

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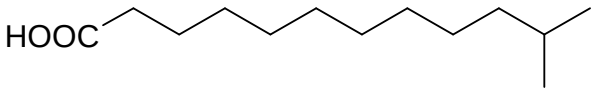

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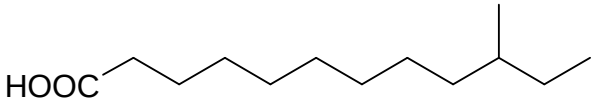

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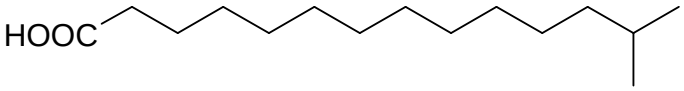

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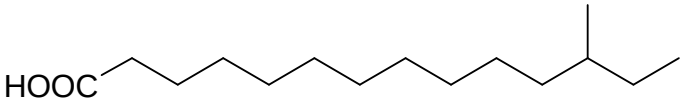

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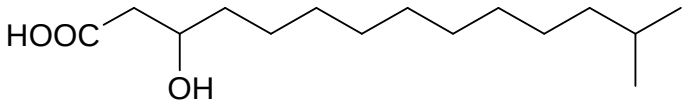

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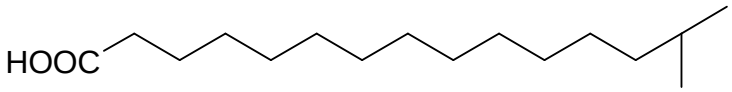

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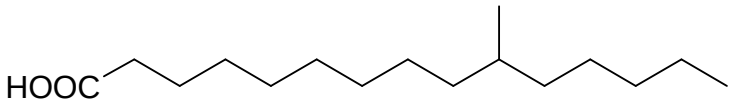

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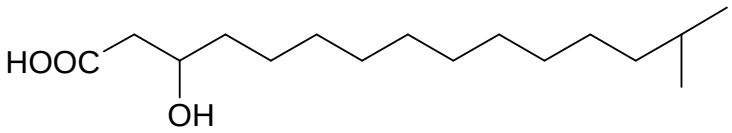

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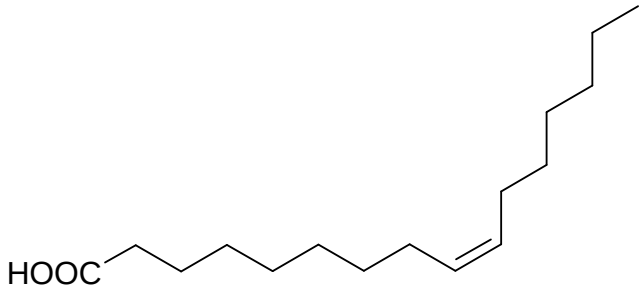

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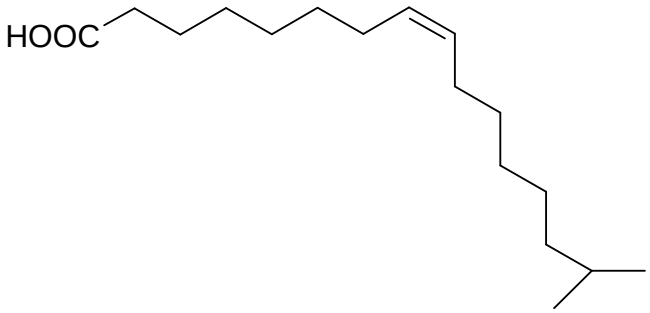

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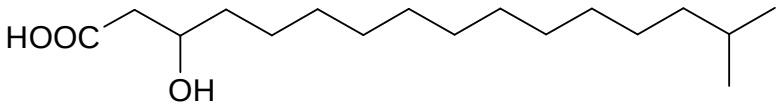

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