Abstract:

Yersinia novum MAC was isolated from Loyalsock Creek, a freshwater stream in northeastern Pennsylvania. The microbe can be distinguished from other species of *Yersinia* through biochemical differentiation as it possesses dissimilar metabolic and enzymatic profiles. The strain is differentiated from closely related species by high concentrations of 18:1 w7c and 19:0 cyclo w8c and low concentrations of 16:1 w7c, 16:1 w6c, and 17:0 cyclo. *Yersinia* novum MAC was susceptible to most antibiotics. 16S rRNA gene sequence analysis revealed pairwise similarities of 98.46%, 98.39%, and 98.32% to *Yersinia mollaretii, Yersinia rohdei,* and *Yersinia aldovae*, respectively. Further 16S rRNA gene sequence analysis revealed a pairwise similarity of 99.9% to an unpublished strain labeled *Yersinia* MH-1. Multi-Locus Sequence Typing examining the glnA, gyrB, recA, and Y-HSP60 housekeeping genes generated pairwise similarities of less than 95% to all published *Yersinia* species. Pairwise similarities of above 95% were generated when comparing *Yersinia* novum MAC and *Yersinia* MH-1. The current study proposes that *Yersinia* novum MAC and *Yersinia* MH-1 constitute a novel species of bacteria.

Introduction:

Yersinia novum MAC was isolated from Loyalsock Creek, a small stream that drains into the Susquehanna River. Preliminary 16S rRNA gene sequencing, which was performed by Melissa Cashner (MAC) in Lycoming College's Spring 2009 Microbiology course, suggested the strain is a novel species belonging to the genus

Yersinia. The current study tested the hypothesis that *Yersinia* novum MAC is a novel microbe.

With the exception of *Yersinia pestis*, *Yersinia* species are ubiquitous organisms. They are distributed globally in a wide range of environments. Typically, isolates are recovered from soil samples, surface water, and human and canine feces (Brenner et al. 2005). These organisms are found virtually everywhere. Research examining *Yersinia* novum MAC will provide information on the species' possible niche in a local environment. Additionally, all known *Yersinia* species are potentially pathogenic. Three species produce disease in humans. *Yersinia pestis* is the causative agent of the plague, while *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* cause diarrhea, mesenteric lymphadenitis, and septicemia. All other *Yersinia* species are opportunistic pathogens, meaning they cause infection only in immunocompromised individuals (Brenner et al. 2005). An investigation of *Yersinia* novum MAC will provide a basis for future research into the potential pathogenicity of this organism. Moreover, there is a lack of extensive research on *Yersinia enterocolitica*-like organisms, a category that includes all *Yersinia* species except *Yersinia pestis* and *Yersinia pseudotuberculosis*. Considerable research has not been performed on these organisms because they were misclassified as Biogroups of *Yersinia enterocolitica* (Aleksic et al. 1987). Recently, research has reclassified these organisms as separate species (Aleksic et al. 1987). Therefore, an investigation of *Yersinia* novum MAC will benefit the microbiology community by extending the knowledge surrounding *Yersinia enterocolitica*-like species.

The gold standard for the characterization of a novel species, as outlined by the International Journal of Systematic and Evolutionary Microbiology, is centered upon

polyphasic taxonomy. This system entails the completion of many diverse analyses in order to obtain as complete a description of the novel microbe as possible. While morphological and biochemical studies are critical parts of the characterization process, offering key distinguishing features, the foundation of determining the novelty of a species is molecular analysis (Ebers & Stackebrandt 2006).

Three types of molecular analysis exist for the characterization of novel microbes: DNA hybridization, 16S rRNA gene sequencing, and Average Nucleotide Identity. DNA hybridization, the oldest of the three techniques, studies microbial similarity based on DNA relatedness. Double-stranded DNA from the two alleged species is heated to high temperatures, causing denaturation of the helices into two single strands. The DNA is then cooled, allowing new double helices to form at areas of complementary sequence. Less than 70% DNA relatedness indicates a novel species (Doolittle 2006). This method has been used extensively in past *Yersinia* research (Bercovier et al. 1984). However, this is a relatively outdated procedure as only two species can be analyzed at a time and no real insight into evolutionary relationships is provided. Moreover, the current study did not employ the technique due to cost restrictions. Lycoming College does not possess the instruments needed to perform DNA-DNA hybridization, so samples would have had to be sent to another facility for analysis. Instead, 16S rRNA gene sequencing and a form of Average Nucleotide Identity were used.

Currently, 16S rRNA gene sequencing serves as the basis of most research characterizing novel species. The technique analyzes the 16S rRNA gene, which serves as the structural component of ribosomes. Ribosomes are sites of protein production. The gene is highly conserved, having an extremely low mutation rate. As a

result, the16S rRNA gene serves as a molecular clock, enabling the differentiation of species based on differences in sequence (Chun et al. 2007). Sequences are compared using the EzTaxon server to perform a BLAST (Basic Local Alignment Search Tool) search, which assesses the pairwise similarity of the entered sequence to a curated collection of all bacterial type strains in the National Center for Biotechnology Information (NCBI) GenBank. Recent research has overturned the old cut-off value of 97% when determining whether or not a species in novel. Studies have shown that species can have 16S rRNA gene sequence similarities above 99%, while possessing DNA-DNA hybridization reassociation values below 70% (Ebers and Stackebrandt 2006). As a result, less than 98.5% 16S rRNA gene sequence similarity suggests a novel species (Ebers and Stackebrandt 2006). However, when comparing the 16S rRNA gene sequences of any two *Yersinia* species, pairwise similarity never falls below 97.4%, with most above 99% (Kotetishvili et al. 2005). As a result, Average Nucleotide Identity analysis was also employed.

Like 16S rRNA gene sequencing, Multi-Locus Sequence Typing (MLST), a form of Average Nucleotide Identity, determines interspecies relationships based on gene sequences. However, while 16S rRNA gene sequencing analyzes a highly conserved gene, MLST studies genes with higher mutation rates such as those of the glnA, gyrB, recA, and Y-HSP60 loci (Kotetishvili et al. 2005). These genes have higher mutation rates because they are protein coding, allowing for differences in the nucleotide sequence at DNA wobble positions (third nucleotide of a codon) without changing the protein sequence. Conservative substitutions, where one amino acid is substituted for another with similar properties, also contribute to these genes higher mutation rate.

Conversely, the 16S rRNA gene codes for a functional RNA, limiting the mutation rate. The glnA, gyrB, recA, and Y-HSP60 genes are known as housekeeping genes because they are needed for the regular maintenance of the cell. GlnA codes for glutamine synthase, while gyrB codes for DNA gyrase. RecA is involved in DNA repair and Y-HSP60 codes for a heat-shock protein. A disadvantage of MLST is the possibility of horizontal gene transfer, a process where bacteria exchange and incorporate genetic material. When using MLST, less than 95% gene sequence similarity indicates a novel organism (Kotetishvili et al. 2005). Recent research has shown MLST to be superior to both 16S rRNA gene sequencing and DNA hybridization when differentiating *Yersinia* species. 16S rRNA gene sequencing is less effective because the 16S rRNA gene is extremely conserved within the genus *Yersinia*. DNA hybridization does not provide the information necessary for the determination of evolutionary relationships (Kotetishvili et al. 2005). Thus, MLST is a more appropriate technique when characterizing *Yersinia* species.

While molecular studies serve as the foundation for determining novel species, morphological and biochemical analyses generate the data necessary for the complete characterization of a microbe. Morphological investigations uncover basic colony and cellular structure, while biochemical tests provide the organism's enzymatic and metabolic profiles. API test strips are especially helpful in this process as they provide a reliable degree of standardization not found in other types of biochemical testing (Poh & Loh 1988). Three API test strips were used: 50CH, 20E, and ZYM. The results for each strip are determined based on color changes. API 50CH strips test for the fermentation of 49 different carbohydrates. During incubation, the fermentation process

produces acids, lowering the pH. An indicator detects this pH change, causing a color change (*api 50 CHB/E* 2005). API 20E strips test for metabolic activity common to *Enterobacteriaceae*. Inoculation of the strip's cupules containing dehydrated substrates activates the media. Metabolism of these substrates during incubation causes color changes (*api 20 E* 2007). API ZYM strips test for enzymatic activity. The cupules of this strip contain enzymatic substrates and their buffers. After incubation, the metabolic products are revealed through color changes upon the addition of two ZYM reagents (*api ZYM* 2004). Comparison of results to those of closely related species reveals key distinguishing features of the novel microbe. For example, acid production from L-rhamnose and negative reactions for fermentation of sorbose, cellobiose, and melibiose differentiates *Yersinia aldovae* from all other *Yersinia* species (Bercovier et al. 1984). Biolog GenIII plates were employed to further assess *Yersinia* novum MAC's metabolic profile. GenIII plates test for the utilization of various carbon sources. Positive reactions are indicated by a colorless substrate changing to a purple end product. The pattern of purple positive reactions and colorless negative reactions produced in the wells of the GenIII plate can then be compared to the Biolog database. This database is capable of identifying over 1900 species of aerobic and anaerobic bacteria, yeasts, and fungi based on their ability to utilize various carbon sources (*Biolog* 2007). The comparison of OmniLog results enabled additional biochemical differentiation of *Yersinia* novum MAC from closely related species. A MIDI Sherlock Microbial Identification System (MIS) was used as well. The Sherlock MIS is able to identify over 1500 species of bacteria based on their fatty acid composition using gas chromatography (*MIDI* 2010). By comparing the fatty acid profiles of *Yersinia* novum

MAC and closely related species, further biochemical differentiation is possible. Morphological and biochemical analyses are critical components of polyphasic taxonomy.

The characterization of *Yersinia* novum MAC will provide the background knowledge needed for future studies into the environmental role of the microbe. Moreover, as a *Yersinia enterocolitica*-like species, describing *Yersinia* novum MAC will offer information concerning a relatively unstudied group of organisms.

Methods:

The current study employed a polyphasic approach, meaning that numerous different analyses were used in an attempt to attain as thorough a characterization as possible. Morphological testing was completed to note colony and cell structure and size. Enzymatic and metabolic profiles were assessed through biochemical tests. Molecular analysis was performed to determine interspecies genetic similarities and evolutionary relationships.

Morphological:

A Gram stain and wet mount were completed according to Gerhardt et al. 1994. To assess colony morphology, a TSA plate was streaked and incubated at 30˚C for 24 hours. Colonies were observed at this time and morphology was determined. A hemocytometer was used to note cell size. To analyze growth at various pHs, the pH of TSB medium was adjusted using HCl and NaOH and recorded with a pH meter. The tubes of various pHs were cultured to an absorbance of 0.05-0.1. The turbidity of the

pH tubes (incubated at 30˚C with shaking) was measured every hour using a Spectronic 20 set to 600nm for 12 hours or until absorbance reached 0.6.

Biochemical:

The API 50CH, API 20E, and API ZYM test strip procedures were completed using the manufacturer's instruction manuals and the methods of Laughon et al. 1982. In all cases, distilled water was inoculated until an absorbance (OD_{600}) of approximately 0.7 was obtained. The cupules of each test strip were then inoculated and incubation at 30˚C followed. The 50CH strip was observed after 24 hours and 48 hours of incubation for a yellow color, which indicates a positive result. Results for the 20E strip were determined after 24 hours using the manufacturer's reading table. After 4 hour incubation of the ZYM strip, a drop of both ZYM A reagent and ZYM B reagent was added to each cupule. Results were then collected using the manufacturer's reading table. All media (agars and broths) were prepared using the procedures of Difco Laboratories (1984). Subsequent biochemical tests were performed according to Gerhardt et al. 1994. Antibiotic susceptibility tests were completed using the methods of Bauer et al. 1966. An inoculated TSA plate was placed in an anaerobic chamber and incubated at 30˚C for 48 hours to determine the oxygen requirements of *Yersinia* novum MAC. OmniLog testing was completed using the manufacturer's instruction manual. In preparation, a blood agar plate was inoculated and incubated at 30˚C for 24 hours. From this plate, a tube of inoculating fluid A was cultured to a transmittance of 90-98%. The culture was then used to inoculate the wells of a GenIII plate. The GenIII plate was placed in the Biolog, which incubates at 30˚C, for 24 hours. After this period, results were determined using a MicroLog. MIDI fatty acid analysis was performed according

to the manufacturer's instruction manual. In preparation, a TSA plate was inoculated and incubated at 30˚C for 24 hours. From this plate, a heavy inoculum was obtained and transferred to a MIDI vial. MIDI reagent I was added and followed by 15 seconds of vortexing. MIDI reagent II was added and followed by 5 seconds of vortexing. MIDI reagent III was then added. The resulting organic layer was transferred to a second MIDI vial, which was then analyzed using the MIDI Sherlock Microbial Identification System.

Molecular:

16S rRNA gene sequencing:

In order to isolate DNA, *Yersinia* novum MAC cells were placed in blocks at -70˚C for 2 minutes, 70˚C for 2 minutes, -70˚C for 2 minutes, and 70˚C for 2 minutes to lyse the cells. Polymerase Chain Reaction (PCR) was completed according to Newman et al. 1998.

3 minutes at 94˚C, 1 minute at 53˚C, 2 minutes at 72˚C for 1 cycle
1 minute at 94˚C, 1 minute at 53˚C, 2 minutes at 72˚C for 35 cycles
1 minute at 94˚C, 1 minute at 53˚C, 10 minutes at 72˚C for 1 cycle

Primer	Sequence
16SrRNA27f	AGAGTTTGATCMTGGCTCAG
16SrRNA1492r	TACGGYTACCTTGTTACGACTT

Gel electrophoresis was used to estimate DNA concentrations following PCR, ensuring the presence of sufficient product. The PCR product was then sent to Agencourt for sequencing using the 1100r primer. Using the methods of Huang and

Madan (1999), the sequence was analyzed with previously collected sequences from 27f, rRNA1, 785f, 1492r, and rRNA2 primers to create a consensus sequence using the CAP3 Assembly Program. To determine which species are mostly closely related to *Yersinia* novum MAC, the consensus sequence was analyzed using EzTaxon 2.1. This procedure was performed using the methods of Chun et al. 2007. A phylogenetic tree was constructed with MEGA 4.0 according to Tamura et al. 2007. Sequence alignment was completed using the Clustal *w* system. The phylogenetic tree was constructed using a neighbor-joining system illustrating p-distance. A bootstrap value of 1000 was employed. A BLAST 2 search was completed comparing *Yersinia* MH-1 and *Yersinia* novum MAC.

Multi-Locus Sequence Typing:

DNA for MLST Polymerase Chain Reaction was isolated using a Qiagen DNeasy Blood and Tissue kit according to the manufacturer's handbook. First, cells were lysed using proteinase K. A series of buffers was then added to the sample, ensuring the DNA would bind to the DNeasy Mini spin column. The sample was centrifuged, allowing the DNA to bind to the DNeasy membrane while contaminants passed through. Remaining impurities were removed with two wash steps. The DNA was then eluted using a final buffer (Qiagen 2006). MLST PCR was completed using the methods of Kotetishvili et al. 2005.

5 minutes at 94˚C

45 seconds at 94˚C, 45 seconds at 51˚C, 1 minute at 72˚C for 35 cycles

5 minutes at 72˚C

Gene	Primer	Sequence (5'→3')
Glutamine Synthase	glnA-f	CGATTGGTGGCTGGAAAGGC
	glnA-r	TTGGTCATRGTRTTGAAGCG
DNA Gyrase, Subunit B	gyrB-f	CGGCGGTTTGCAYGGYGTRGG
	gyrB-r	CAGSGTRCGRGTCATYGCCG
RecA Protein	recA-f	GGGCCAAATTGAAAARCARTTCGG
	recA-r	CGCCRATYTTCATRCGRATYTGGT
Heat Shock Protein 60 (chaperonin)	Y-HSP60-f	GACGTNGTAGAAGGTATGYAG
	Y-HSP60-r	CGCCGCCAGCCAGTTTAGC

Gel electrophoresis was used to estimate DNA concentrations following PCR, ensuring the presence of sufficient product. The PCR products were then sent to Agencourt for sequencing using the glnA, gyrB, recA, and Y-HSP60 primers. Using the methods of Huang and Madan (1999), the CAP3 Assembly Program was used to find consensus sequences by correlating the forward and reverse sequences. A BLAST search was completed for each of the consensus sequences to find the closest matches in NCBI GenBank. Orthologous sequences for each *Yersinia* species were then retrieved from the NCBI GenBank. Using the collected sequences, a phylogenetic tree for each primer was constructed using MEGA 4.0 according to the methods of Tamura et al. 2007. Sequence alignment was completed using the Clustal *w* system. The phylogenetic tree was constructed using a neighbor-joining system illustrating p-distance. A bootstrap value of 1000 was also employed. For each primer, Blast 2 searches were completed comparing *Yersinia* novum MAC sequences to those of *Yersinia* MH-1, *Yersinia rohdei,* and *Yersinia mollaretii*.

Insect Pathogenicity:

In preparation for testing insect pathogenicity, stock cultures of *Drosophila melanogaster* (fruit fly) were initiated by adding flies to jars containing a *Drosophila*

culture medium consisting of an oatmeal mixture activated by water. Once a healthy fly population was available, a *Yersinia* novum MAC culture, *Escherichia coli* culture, and TSB (tryptic soy broth) medium were incubated at 37˚C for 48 hours. *Escherichia coli* and the uninoculated TSB served as negative controls. Fly cultures were then created by adding *Drosophila* medium to 8 glass jars. The medium was activated using water. 1mL of *Yersinia* novum MAC TSB culture was added to 3 jars, 1mL of *Escherichia coli* TSB culture was added to 3 jars, and 1mL of uninoculated TSB was added to 2 jars. In order to transfer flies from the stock cultures to the test cultures, the flies were anesthetized using ethyl ether. Under a dissecting microscope, the flies were sexed based on size, genitalia apparatus, and dorsal coloring. Five females and 3 males were transferred to each of the 8 test jars. The fly test cultures were maintained at a temperature of 21˚C. Once the cultures completed one life cycle, the number of flies was determined for 3 days over a span of 4 days. Counted flies were removed from the test cultures each day.

Results:

Morphological Results:

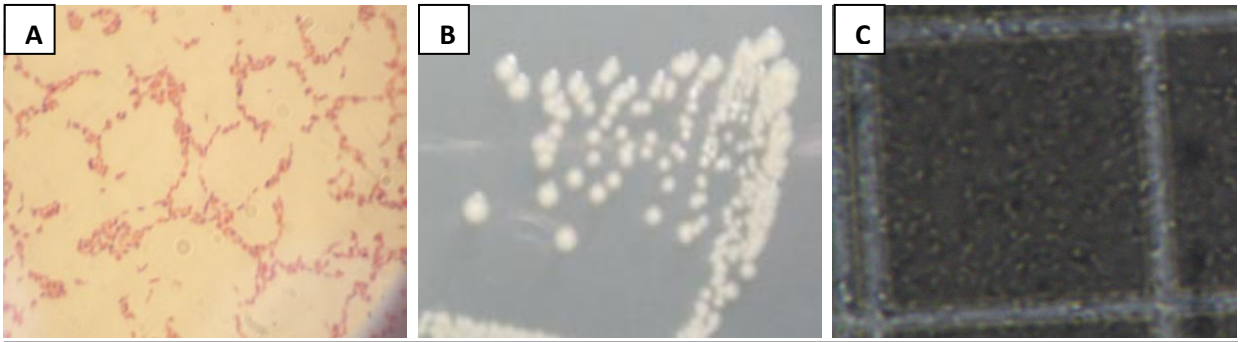

Figure 1: **A**. Gram stain (1000X magnification). **B**. TSA plate after 24 hour incubation (30˚C). **C**. Hemocytometer (square= 50x50µm)

From a Gram stain, it was determined that *Yersinia* novum MAC is a Gram-negative bacillus (short rods) (Figure 1A). TSA inoculation showed *Yersinia* novum MAC colonies to be opaque, mucoid, and approximately 1.0 mm in diameter after 24 hour incubation. Cell size was estimated to be 2.5µm in length using a hemocytometer. A wet mount revealed motility.

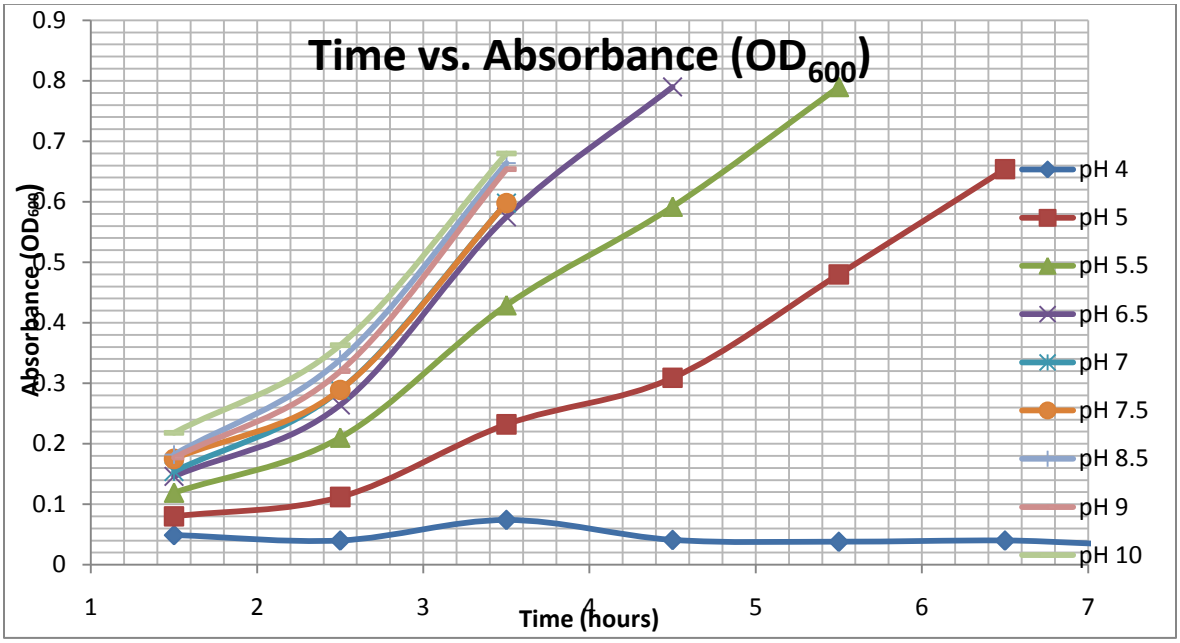

Figure 2: Growth in TSB medium at pH 4.0-10.0.

Compared to growth at pH 7 or above, *Yersinia* novum MAC did not grow as well at pHs ranging from 5 to 6.5. The microbe was unable to grow at pH 4.

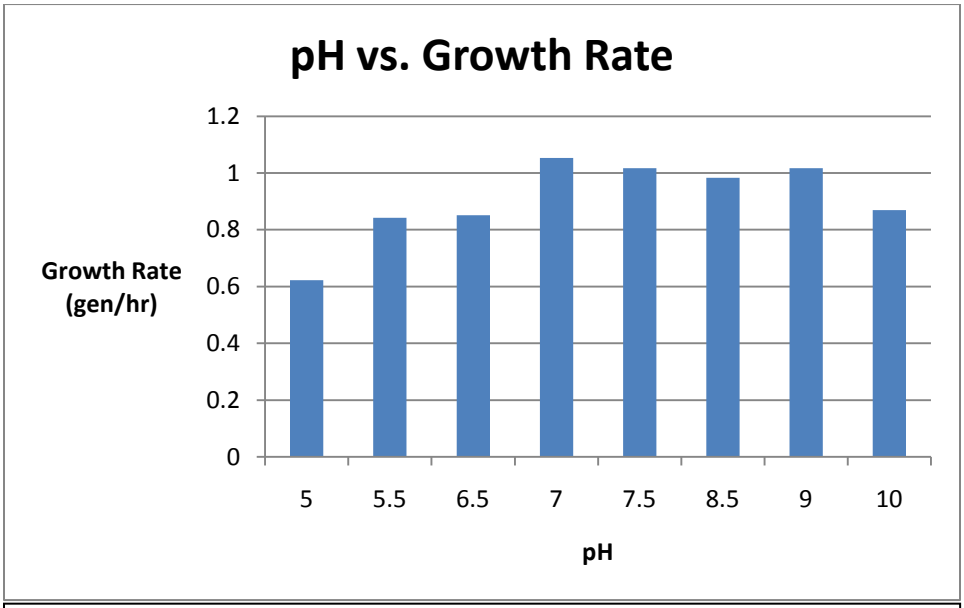

Figure 3: Growth rate (based on doubling time) at various pH.

The highest growth rate was observed at pH 7, with a near maximal growth rate continuing until pH 9. Decreased growth rates were noted at pHs of less than 7 and greater than 9. Based on growth at a range of pH, it was determined that *Yersinia* novum MAC prefers a neutral to slightly alkaline environment.

Biochemical Results (incubation at 30˚C):

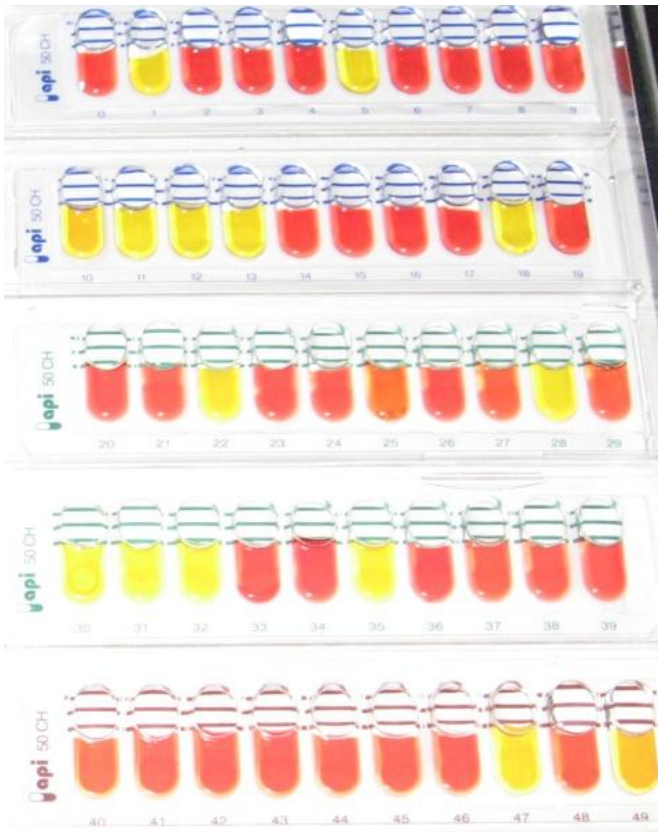

Figure 4: API 50CH – Positive result for carbohydrate utilization indicated by yellow colored cupule.
*Results read after 24 hour and 48 hour incubation at 30˚C.

Yersinia novum MAC produced acids during the fermentation of glycerol, D-ribose, D-galactose, D-glucose, D-fructose, D-mannose, D-mannitol, N-acetyl glucosamine, D-maltose, D-melibiose, D-saccharose, D-trehalose, D-raffinose, potassium gluconate, and potassium 5-ketogluconate.

Figure 5: API 20E – Positive result for substrate utilization in wells 1-11 indicated by variable color change in cupule. Positive result for carbohydrate utilization in wells 12-20 indicated by yellow colored cupule.
*Results read after 24 hour incubation at 30˚C.

Yersinia novum MAC showed positive results for having β-galactosidase, ornithine decarboxylase, and the fermentation/oxidation of glucose, mannitol, saccharose, and melibiose.

Figure 6: API ZYM – Positive result for enzymatic activity indicated by variable color change.
*Results read after 4 hour incubation at 30˚C.

Yersinia novum MAC provided positive results for having leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, and N-acetyl-β-glucosaminidase.

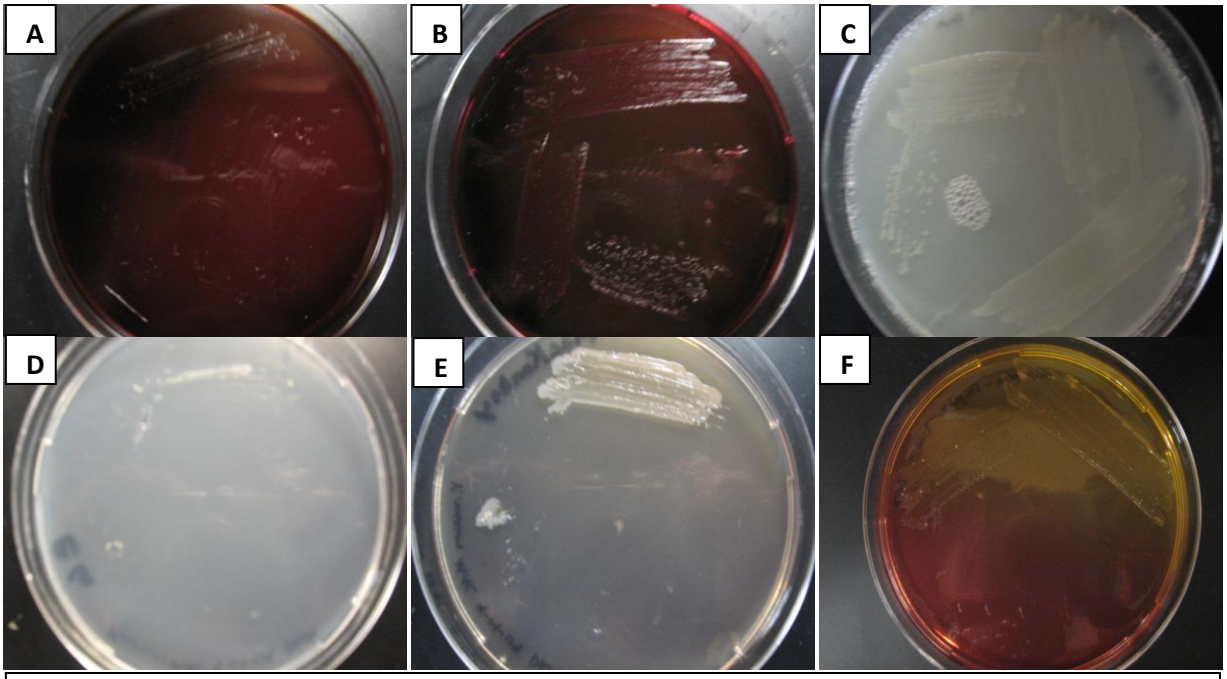

Figure 7: **A**. Blood agar **B**. Eosin Methylene Blue agar **C**. Milk agar
D. EG(minimal nutrient) agar **E**. MacConkey agar **F**. Salmonella-Shigella agar

Growth on blood agar without producing any clear zones indicates *Yersinia* novum MAC is not hemolytic. The lack of black colonies on the Eosin Methylene Blue agar, colorless colonies on the MacConkey agar, and clear colonies on the Salmonella-Shigella agar indicate *Yersinia* novum MAC is unable to ferment lactose. The lack of clear zones on the milk agar suggests *Yersinia* novum MAC cannot utilize casein. *Yersinia* novum MAC's ability to grow on the EG agar indicates the microbe can grow with minimal nutrients, as EG agar contains only glucose and inorganic salts.

Additional biochemical tests were also performed using available media. *Yersinia* novum MAC was able to ferment of citrate, dextrose, glucose, and produce acetoin (Voges-Proskauer test). The microbe possesses the catalase enzyme. *Yersinia* novum MAC yielded negative results for the production of mixed acids (Methyl Red test) and possession of phenylalanine deaminase and oxidase. *Yersinia* novum

MAC was able to grow in an anaerobic chamber, suggesting the microbe is a facultative anaerobe as it grows both in the absence and presence of oxygen.

API differentiation of *Y.* novum MAC from closely related published *Yersinia* species					
Test		*Y.* novum MAC	1. *Y. rohdei*	2. *Y. aldovae*	3. *Y. mollaretii*
Methyl Red		-	+	+	+
Urea Hydrolysis		-	+	+	+
Acid Production from:	L-arabinose	-	+	+	+
	D-sorbitol	-	+	+	+
	D-xylose	-	+	+	+
	Melibiose	+	+	-	-
	Lactose	-	+	-	-
	Raffinose	+	+	-	-
Cellobiose		-	+	-	+
Citrate (Simmons)		+	+	-	-

Table 1: Results collected after 24 hour incubation at 30˚C. **1**. Aleksic et al. 1987 **2**. Bercovier et al. 1984 **3**. Brenner et al. 1988

Yersinia novum MAC can be distinguished from closely related species of *Yersinia* by its positive reactions in tests for the fermentation of melibiose, raffinose, and citrate (Simmons) and negative reactions in tests for acid production (methyl red), urea hydrolysis, and the fermentation of L-arabinose, D-sorbitol, D-xylose, lactose, and cellobiose.

Biolog biochemical differentiation of *Y.* novum MAC from *Y. rohdei* and *Y. mollaretii*			
Test	*Y.* novum MAC	*Y. rohdei* (T)	*Y. mollaretii* (T)
D-Sorbitol	-	+	+
D-Arabitol	+	-	-
D-Lactic Acid Methyl Ester	+	-	-
Myo-Inositol	+	-	-
L-Lactic Acid	+	-	-
D-Salicin	+	-	-
3-Methyl Glucose	+	-	-
Gentiobiose	-	+	+
D-Fucose	+	-	-
L-Fucose	+	-	-
Mucic Acid	+	-	-
D-Malic Acid	+	-	-
N-Acetyl-D-Galactosamine	+	-	-
L-Rhamnose	+	-	-
D-Aspartic Acid	+	-	-
L-Pyroglutamic Acid	+	-	-
Quinic Acid	+	-	-
L-Malic Acid	+	-	-
Bromo-Succinic Acid	+	-	-
Formic Acid	+	-	-
Aztreonam	-	+	+
pH 5	+	-	-
D-Raffinose	+	+	-
D-Maltose	+	-	+
D-Melibiose	+	+	-
D-Cellobiose	-	-	+
Citric Acid	+	+	-
Glucuronamide	+	-	+
Acetoacetic Acid	+	-	+
Stachyose	+	+	-
Inosine	+	-	+
D-Serine	+	+	-
4% NaCl	+	-	+
Fusidic Acid	-	-	+
Tetrazolium Violet	+	-	+
Lithium Chloride	+	-	+
Sodium Butyrate	+	-	+
Niaproof 4	+	-	+

Table 2: Results collected using MicroLog after 24 hour incubation in OmniLog at 30˚C.

Yersinia novum MAC can be distinguished from closely related species of *Yersinia* by its positive reactions in tests for the utilization of D-arabitol, D-lactic acid methyl ester, myo-inositol, L-lactic acid, D-salicin, 3-methyl glucose, D-fucose, L-fucose, mucic acid, D-malic acid, N-acetyl-D-galactosamine, L-rhamnose, D-aspartic acid, L-pyroglutamic acid, quinic acid, L-malic acid, bromo-succinic acid, formic acid, D-raffinose, D-maltose, D-melibiose, citric acid, glucuronamide, acetoacetic acid, stachyose, inosine, D-serine, and growth in/at pH 5, 4% NaCl, tetrazolium violet, lithium chloride, sodium butyrate, and niaproof 4. *Yersinia* novum MAC can be distinguished from closely related species of *Yersinia* by its negative reactions in tests for the utilization of D-sorbitol, gentiobiose, D-cellobiose, and lack of growth in fusidic acid and aztreonam.

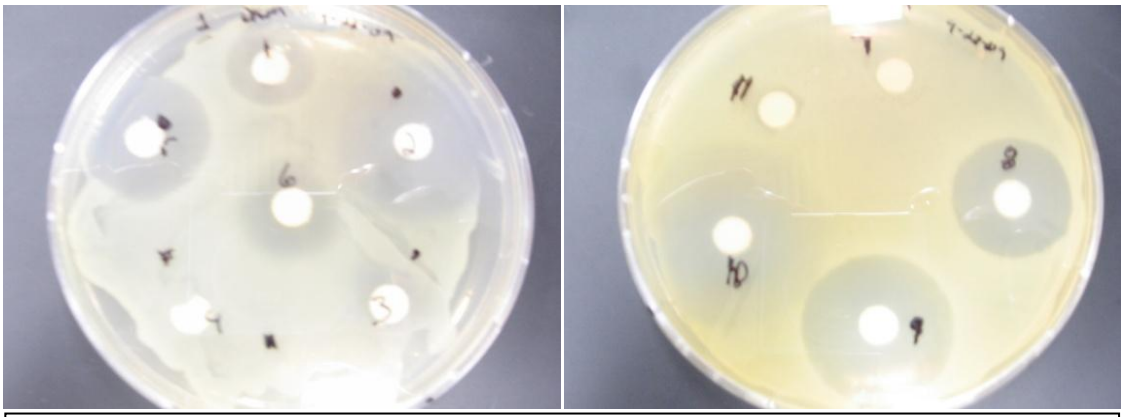

Figure 8: Antibiotic Susceptibility Plates

Antibiotic Susceptibility Results					
			Zone of Inhibition (diameter, mm)		
#	Antibiotic	Amount on Disk (µg/disk)	*Yersinia* novum MAC	*Yersinia* novum Aart-01	*Comamonas* novum *N. cap. B*
1	Carbenicillin	100	19	15	24
2	Chloramphenicol	30	32	11	20
3	Erythromycin	15	12	0	16
4	Penicillin	10	0	0	12
5	Streptomycin	10	27	11	0
6	Tetracycline	5	10	10	15
7	Ampicillin	10	9	0	9
8	Kanamycin	30	24	13	11
9	Nalidixic Acid	30	30	17	15
10	Spectinomycin	100	37	20	16
11	Rifampicin	5	10	0	0

Table 3: 0-5mm zone of inhibition indicates resistance, 5-15mm zone of inhibition indicates moderate susceptibility, 15mm or greater zone of inhibition indicates high susceptibility. *Yersinia* novum Aart-01and *Comamonas* novum *N. cap. B* serve as controls.

Yersinia novum MAC showed resistance to penicillin, moderate susceptibility to erythromycin, tetracycline, ampicillin, and rifampicin and high susceptibility to carbenicillin, chloramphenicol, streptomycin, kanamycin, nalidixic acid, and spectinomycin.

Most Closely Related Species based on Fatty Acid Composition	
Similarity Index	Species
0.791	*Serratia liquefaciens*
0.724	*Pantoea agglomerans*
0.595	*Ewingella americana*
0.591	*Yersinia frederiksenii/entercolitica/intermedia*
0.541	*Yersinia kristensenii*

Table 4: MIDI Sherlock Microbial Identification System

MIDI results analyzing membrane fatty acid composition suggest *Yersinia* novum MAC should not be classified as a *Yersinia* species because a similarity index of only 0.591 was found to the *Yersinia* genus. Much higher similarity indexes (0.791 and 0.724) were obtained to members of the *Serratia* and *Pantoea* genuses.

Differentiation of *Yersinia* novum MAC based on Fatty Acid Composition					
Fatty Acid	MAC	*Y. rohdei*(T)	*Y. mollaretii*(T)	*Y. fred/enter/inter*	*Y. kristensenii*
14:0	1%	2%	1%	1%	2%
16:0	37%	37%	37%	33%	39%
17:0	1%	0%	0%	2%	2%
18:0	1%	0%	0%	1%	1%
Summed Feature 2	2%	2%	2%	2%	2%
Summed Feature 3	45%	49%	50%	49%	47%
Summed Feature 8	13%	10%	10%	12%	8%

Table 5: Fatty acid composition percentages of *Yersinia* novum MAC and closely related *Yersinia* species. Summed Feature 2 includes 16:1 iso I and 14:0 3OH methyl esters. Summed Feature 3 includes 16:1 w7c, 16:1 w6c, and 17:0 cyclo methyl esters. Summed Feature 8 includes 18:1 w7c and 19:0 cyclo w8c methyl esters.

Yersinia novum MAC can be differentiated from *Yersinia* species by a decreased percentage of Summed Feature 3 and increased percentage of Summed Feature 8.

Molecular Results:

16S rRNA gene sequencing:

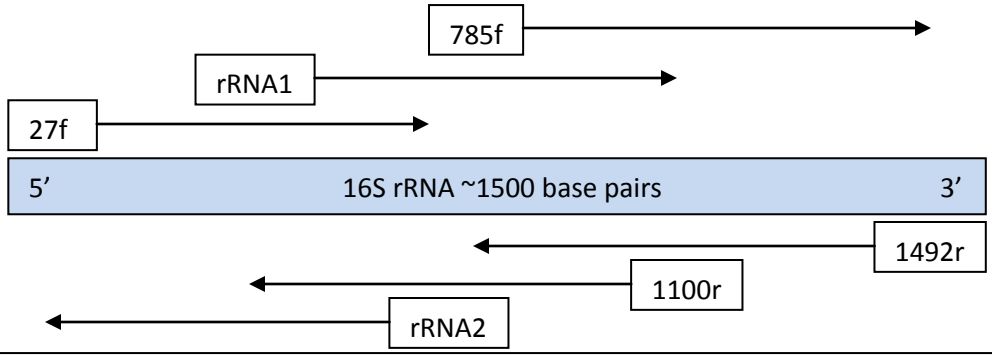

Figure 9: Molecular overlap by primers used for 16S rRNA gene sequencing.

The overlap of sequences selected for by the six primers enabled consensus sequence assembly using the CAP3 Assembly Program (Huang and Madan 1999).

Most Closely Related Published Species				
Rank	Name	Strain	Diff/Total nt	Pairwise Similarity
1	*Yersinia mollaretii*	ATCC 43969(T)	22/1428	98.459
2	*Yersinia rohdei*	ATCC 43380(T)	23/1432	98.394
3	*Yersinia aldovae*	ATCC 35236(T)	24/1428	98.319

Table 6: Results determined using ExTaxon 2.1 server.

Based on 16S rRNA gene sequence results, *Yersinia* novum MAC is most closely related to the published species *Yersinia mollaretii*, *Yersinia rohdei*, and *Yersinia aldovae*. However, all pairwise similarities are less than the 98.5% cut-off value, suggesting *Yersinia* novum MAC is a novel species.

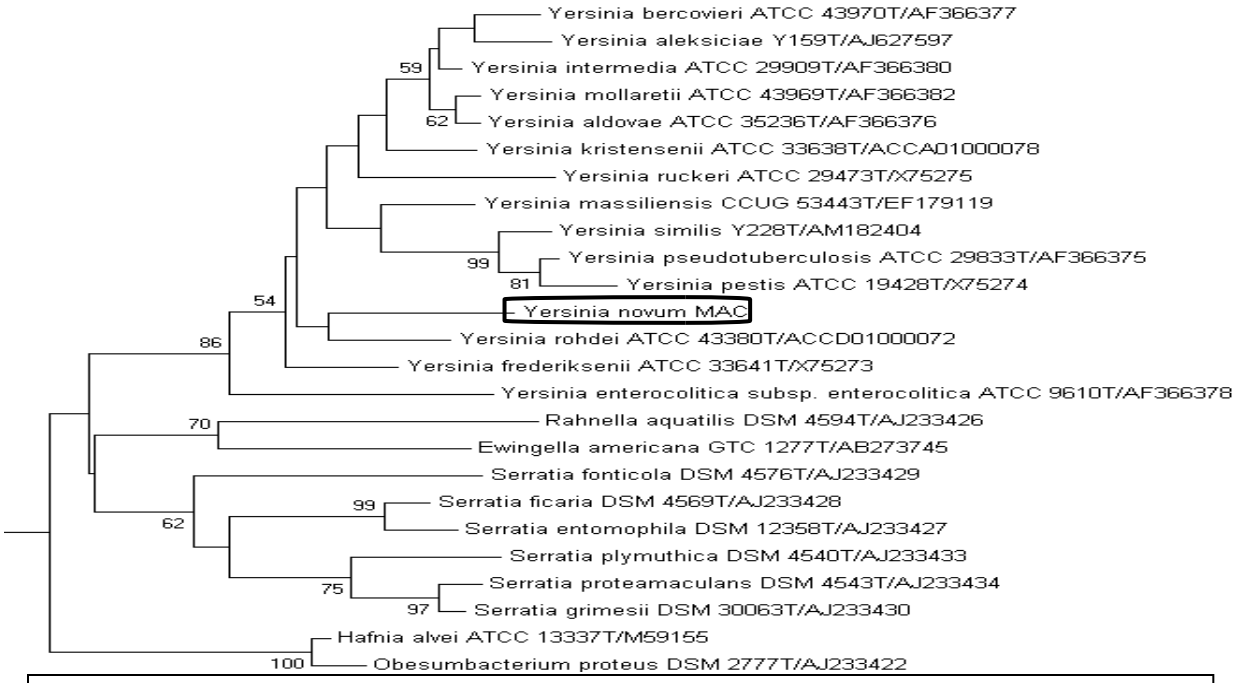

Figure 10: 16S rRNA gene phylogenetic tree constructed with MEGA 4.0. Bootstrap values greater than 50 are shown. The value signifies the percentage of times out of 1000 these nodes were generated.

Yersinia novum MAC is clustered with other species of *Yersinia*, indicating the microbe should be classified in the *Yersinia* genus. Based on direct clustering, *Yersinia rohdei* is the most closely related published species to *Yersinia* novum MAC.

A BLAST 2 16S rRNA gene comparison of *Yersinia* novum MAC and *Yersinia* MH-1 suggests the two microbes are the same species. Of the 1432 nucleotides, only a single nucleotide difference exists, providing a pairwise similarity of 99.9%.

Multi-Locus Sequence Typing:

MLST Sequence Pairwise Similarities				
Species	*Yersinia* novum MAC			
	glnA	gyrB	recA	Y-HSP60
Yersinia rohdei	83.4%	83.8%	84.7%	90.0%
Yersinia mollaretii	83.2%	85.7%	83.2%	90.2%
Yersinia MH-1	99.6%	98.5%	99.4%	99.6%

Table 7: MLST sequence pairwise similarities determined using BLAST 2 searches.

MLST sequence BLAST 2 searches differentiate *Yersinia* novum MAC from closely related published species, *Yersinia rohdei* and *Yersinia mollaretii*, as all pairwise similarities are below the 95% cut-off value. Same searches performed with *Yersinia* MH-1 suggest the two isolates are members of the same species because all pairwise similarities are above the 95% cut-off value.

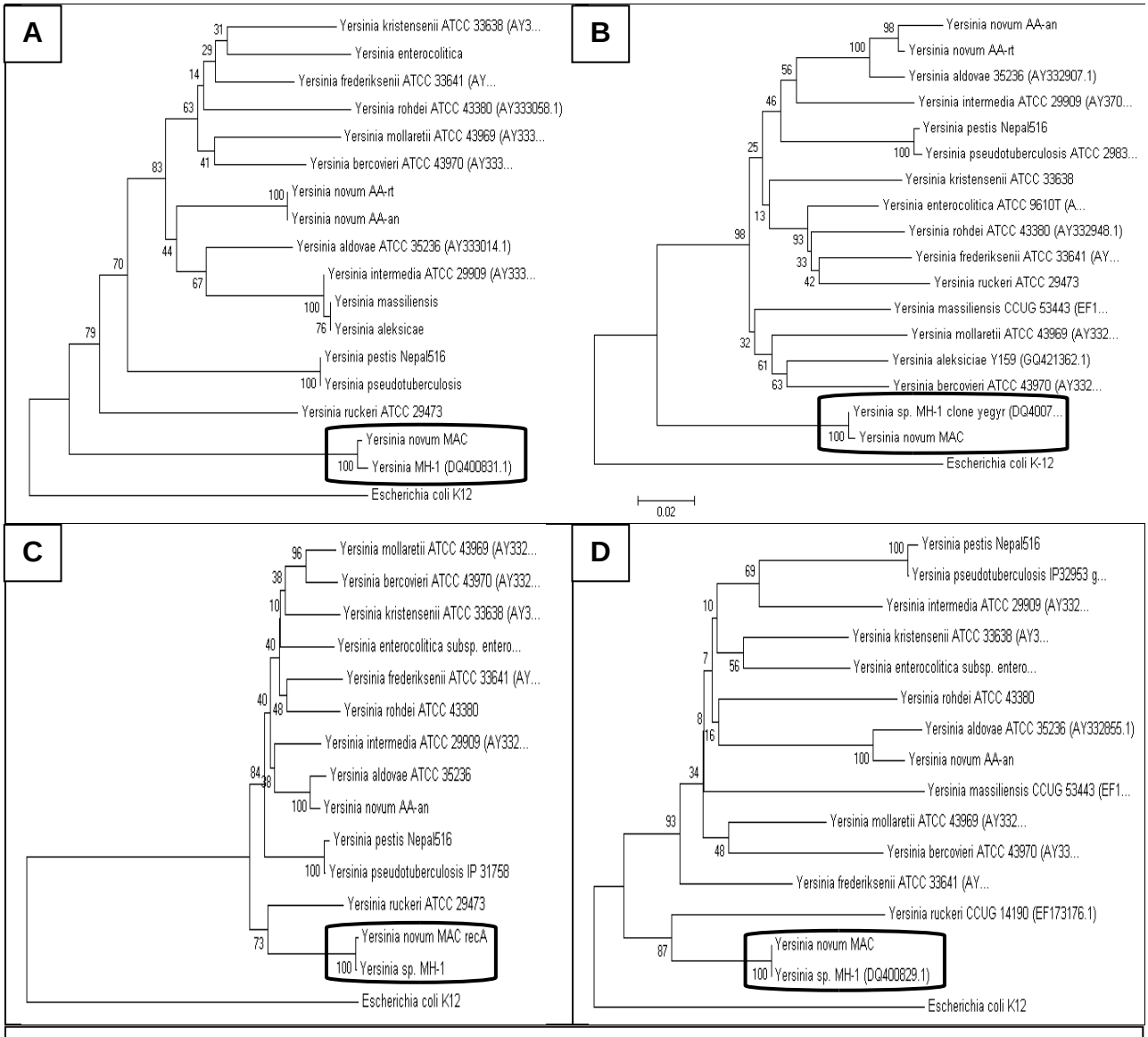

Figure 11: Phylogenetic trees for: **A.** glnA **B.** gyrB **C.** recA **D.** Y-HSP60. Bootstrap values are shown, signifying the percentage of times out of 1000 these nodes were generated.

In all four trees, *Yersinia* novum MAC is not clustered with published *Yersinia* species, suggesting the microbe should not be classified in the *Yersinia* genus. Based on direct clustering in each of the four trees, *Yersinia* novum MAC and *Yersinia* MH-1 are isolates of the same species.

Insect Pathogenicity:

Number of *Drosophila melanogaster*								
Day	TSB 1	TSB 2	MAC 1	MAC 2	MAC 3	*E. coli 1*	*E. coli 2*	*E. coli 3*
3-29	19	5	33	24	16	39	13	15
3-31	84	119	105	103	97	117	86	139
4-1	65	56	60	89	94	111	108	87
Total	168	180	198	216	207	267	207	241

Table 8: The number of *Drosophila melanogaster* counted in each test culture by day.

The data suggests that the growth of the fruit flies was not inhibited by *Yersinia* novum MAC. A greater number of flies was counted each day for the *Yersinia* novum MAC cultures than for the TSB control cultures.

Discussion:

Results collected from morphological, biochemical, and molecular analyses indicate *Yersinia* novum MAC is a novel species. However, results are somewhat conflicting when determining the extent of *Yersinia* novum MAC's novelty. While most findings support *Yersinia* novum MAC being a novel species, Multi-Locus Sequence Typing and MIDI fatty acid data suggest the strain may constitute an entirely new genus.

Results from morphological investigations including Gram-staining, colony morphology, and cell morphology match characteristics common to the *Yersinia* genus, indicating *Yersinia* novum MAC should be classified as a *Yersinia* (Krieg et al 2005).

Conversely, MIDI membrane fatty acid composition data (Table 4) shows *Yersinia* novum MAC to be more closely related to the *Serratia* genus as the microbe shares a 0.791 similarity index with *Serratia liquefaciens*. *Yersinia* novum MAC can be biochemically distinguished from the *Yersinia* genus by its lower concentration of summed feature 3 methyl esters and higher concentration of summed feature 8 methyl esters (Table 2). Further biochemical testing, including API test strip and Biolog analyses, provided the results necessary for metabolic and enzymatic differentiation of *Yersinia* novum MAC from closely related species (Tables 1 & 2). *Yersinia* novum MAC can be distinguished by its positive reactions in tests for the utilization of melibiose, raffinose, D-arabitol, D-lactic acid methyl ester, myo-inositol, L-lactic acid, D-salicin, 3-methyl glucose, D-fucose, L-fucose, mucic acid, D-malic acid, N-acetyl-D-galactosamine, L-rhamnose, D-aspartic acid, L-pyroglutamic acid, quinic acid, L-malic acid, bromo-succinic acid, formic acid, D-maltose, citric acid, glucuronamide, acetoacetic acid, stachyose, inosine, D-serine, and citrate (Simmons) as an energy source. The microbe is differentiated by its ability to grow in 4% NaCl, tetrazolium violet, lithium chloride, pH 5, sodium butyrate, and niaproof 4 as well. *Yersinia* novum MAC can be distinguished by its negative reactions in tests for acid production (methyl red), urea hydrolysis, and fermentation of L-arabinose, D-sorbitol, D-xylose, lactose, cellobiose, and gentiobiose. The strain can also be differentiated by its inability to grow in aztreonam and fusidic acid. The presence of such a large number of biochemically differentiating results strongly suggests *Yersinia* novum MAC is a novel species. Further biochemical analyses should include parallel testing of *Yersinia* novum MAC with *Yersinia* MH-1.

Molecular results are conflicting when determining the extent of *Yersinia* novum MAC's novelty. Molecular results from 16S rRNA gene sequencing support the novelty of *Yersinia* novum MAC as pairwise similarities to published species(Table 6) fall below the 98.5% cut-off value described by Ebers and Stackebrandt (2006). While several pairwise similarities approach the cut-off value, one should remember that due to extreme conservation, 16S rRNA similarities within the *Yersinia* genus never fall below 97.4% with many similarities above 99% (Kotetishvili et al. 2005). In addition, an investigation of evolutionary relationships using MEGA 4.0 to construct a phylogenetic tree clustered *Yersinia* novum MAC within the *Yersinia* genus (Figure 10), indicating the microbe should be classified as a *Yersinia*. A BLAST 2 search comparing the 16S rRNA gene of *Yersinia* novum MAC and unpublished *Yersinia* MH-1 yielded a pairwise similarity of 99.9%, suggesting the two isolates are members of the same species.

In spite of these 16S rRNA gene sequence findings, MLST data suggest *Yersinia* novum MAC should not be classified in the *Yersinia* genus. When comparing *Yersinia* novum MAC's MLST gene sequences to those of *Yersinia rohdei* and *Yersinia mollaretii*, all pairwise similarities fall well below the 95% cut-off value. Conversely, all pairwise similarities to *Yersinia* MH-1 are far above the cut-off value (Table 7). These pairwise similarities suggest *Yersinia* novum MAC and *Yersinia* MH-1 should not be classified as *Yersinia*. Phylogenetic trees constructed using MEGA 4.0 were generated to further analyze the glnA, gyrB, recA, and Y-HSP60 housekeeping genes (Figures 11). In all trees, *Yersinia* novum MAC was clustered outside the *Yersinia* genus with *Yersinia* MH-1, indicating the two isolates constitute a new genus. In the recA and Y-HSP60 trees, *Yersinia ruckeri* is also clustered outside the *Yersinia* genus with *Yersinia* novum MAC

and *Yersinia* MH-1, supporting research completed at the Naval Medical Research Center. In the study, the genomes of all published *Yersinia* species were sequenced. Through a phylogenetic tree analyzing these sequences, the researchers found that *Yersinia ruckeri* was clustered outside the *Yersinia* genus, suggesting the species should not be classified as a *Yersinia* (Chen et al 2010). Because *Yersinia ruckeri* is clustered with *Yersinia* novum MAC and *Yersinia* MH-1 in two of the current study's MLST trees, results suggest that *Yersinia ruckeri* should be classified into a new genus with the two isolates.

MLST phylogenetic tree analysis suggests *Yersinia* novum MAC and *Yersinia* MH-1 are strains of the same species due to direct clustering and minimal branching in all four phylogenetic trees. Pairwise similarities greater than 95% were found between *Yersinia* novum MAC and *Yersinia* MH-1. These findings are supported by the 99.9% 16S rRNA gene sequence similarity between the two strains. Further investigation discovered the MH-1 strain had been isolated in New Zealand by AgResearch, a governmental agricultural research institute. The strain was collected from a diseased grass grub larva in 1996 (Dr. Mark Hurst; personal communication). Studies completed at AgResearch confirmed *Yersinia* MH-1's virulence to insects, specifically to *Locusta migratoria*, a migratory locust responsible for the devastation of crops in Africa, the Middle East, and Australia. *Yersinia* MH-1 showed a high level of pathogenicity in *Locusta migratoria*, killing all insects within 72 hours (McNeill and Hurst 2008). Due to the high MLST and 16S rRNA gene similarities between *Yersinia* novum MAC and *Yersinia* MH-1, tests were completed with *Yersinia* novum MAC in an attempt to determine whether or not the strain is pathogenic to insects. Tests investigating

Yersinia novum MAC's possible pathogenicity in insects indicate the microbe does not possess virulence factors as *Drosophila* melanogaster growth was not inhibited (Table 8). However, more conclusive experimentation should be performed.

Future work regarding *Yersinia* novum MAC should include parallel testing of the strain with *Yersinia* MH-1. Such analysis would enable the determination of whether or not the two isolates are the same species. Additionally, sequencing *Yersinia* novum MAC's entire genome would provide the information necessary for the correct classification of the microbe.

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