Acinetobacter piperi MAG sp. nov., isolated from Loyalsock Creek, Williamsport, PA

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By
Kellie M. Cicconi
Lycoming College
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Abstract

Three potentially new species of bacteria were isolated from the Loyalsock Creek in Williamsport, Pennsylvania. The taxonomic status of three gram-negative, non-motile organisms was studied. Full length 16S rRNA gene sequences were obtained from each of the organisms and found to be of the genus *Acinetobacter, Chromobacterium* and *Chryseobacterium.* Preliminary metabolic tests of the *Acinetobacter* showed that the organism was oxidase-negative, catalase-positive and negative for nitrate reduction, characteristics consistent with the genus. The optimal growth temperature for *A. piperi* MAG is 29°C. The 16S rRNA gene sequence indicated that the organism was most closely related to *Acinetobacter johnsonii* X81663, with a similarity of 97%, and to *Acinetobacter bouvetii.* This organism was tenatively named *Acinetobacter piperi* MAG. The 16S rRNA gene sequence of the *Chromobacterium* showed that its sequence did not match any other type strain in the genus by more than 95%, with its closest relative being *Chromobacterium haemolyticum.* The 16S rRNA gene sequence of the *Chryseobacterium* showed that its sequence did not match any other type strain in the genus by more than 94%, with no significantly close relatives. There is enough evidence to suggest that all three of these organisms are new species.

Background

Acinetobacter species are naturally found in soil and water (Seifert et al., 1997). They are commonly found on some raw vegetables, as well as in spoiled meat, milk and cheese (Kampfer et al., 1993). Many uncultured species of *Acinetobacter* have also been found in sewage (Geng et al., 2006). However, over the past 30 years, an increasing number of species have been classified as pathogenic in humans, such as *Acinetobacter baumannii.* Although it is thought that all species of *Acinetobacter* could potentially cause human disease, *A. baumannii* infections currently account for approximately 80% of *Acinetobacter* infections, which are often nosocomial. Infections that can be caused by certain species of *Acinetobacter* include bacteremia, pulmonary infections, and ventilator-associated pneumonias. This genus is also known for being multi-drug resistant (Joly-Guillou, 2005; Paterson, 2006). Despite the increasing concern regarding this organism, little is known about why it is able to assume a pathogenic role. Generally considered a "low-grade pathogen," the pathogenesis of this organism is most likely dependent on a number of different virulence factors (Joly-Guillou, 2005). *Acinetobacter* species are generally not infectious in healthy individuals. However, immunocompromised individuals, such as people with chronic lung disease, diabetes, or hospitalized patients, are at a greater risk for infection.

There are currently 18 type strains of *Acinetobacter.* Although most are quite similar to each other in their sequences and phenotypic characteristics, they each have distinguishing features. The current type strains are as follows: *A. johnsonii, A. radioresistens, A. lwoffi, A. haemolyticus, A. junii, A. baumanii, A. calcoaceticus* (two different DNA groups), *A. ursingii, A. schindleri, A. parvus, A. baylyi, A. towneri, A.*

tjernbergial, A. bouvetii, A. grimontii, A. gerneri, and *A. tandoii. A. baumanii* is currently best known, due to its ability to cause nosocomial infection.

A distinguishing feature of certain isolates of *Acinetobacter* is their resistance to many different antibiotics and to combination antibiotic therapies. This multi-drug resistance is defined as a diminished susceptibility to more than one antibiotic commonly used for treating a specific infection in a clinical setting (Paterson, 2006). *Acinetobacter* infections are often treated with antibiotics such as ampicillin, imipenem, chloramphenicol and gentamicin, among others (Joly-Guillou, 2005). The ability of many species of *Acinetobacter* to become resistant to multiple antibiotics is a phenomenon that is not well understood. This high intrinsic resistance to conventional antibiotic therapy underlines the need for understanding its virulence factors as well as new approaches to combat the pathogens (Rahal, 2006). Because of these antibiotic resistances, *Acinetobacter* infections can cause or contribute to death in very ill patients (Tjernberg, 1990; Hoštacká, 2001).

Chromobacterium species are generally found in soil and in water. They are most common in extreme climates, such as the tropics. There are currently three groups of strains that make up the genus *Chromobacterium* (Leifson, 1956). However, only one species has been named, sequenced and published, a species known as *Chromobacterium violaceum* (Brazilian National Genome Project Consortium, 2003).

Chromobacterium are of the class *Betaproteobacteria*. They are gram negative, straight, often coccoid cells. They are facultative anaerobes with an optimal growth range at about 30-35°C. They generally do not grow in acidic environments. Most strains produce the violet pigment violacein, but strains that produce colonies without

pigment are often encountered. They can be resistant to a number of antibiotics, including benzylpenicillin (Bergey's, 2005).

The genus *Chromobacterium* is important not only to the research community, but also to the medical community because of its ability to cause infection as an opportunistic pathogen. Occasionally, it can cause serious pyogenic or septicemic infections of mammals, including humans. Although the infection can often be treated with tetracyclines, ciprofloxacin, gentamicin, imipenem and trimethoprim-sulfamethazole, the septicemia that often occurs from this infection can be lethal (Bergey's, 2005).

Currently, the genus *Chromobacterium* comprises the following species: *Chromobacterium haemolyticum, Chromobacterium violaceum,* and *Chromobacterium suttsuga.*

The genus *Chryseobacterium* is found in a large variety of locations. These bacteria can be found in soil, fresh water, sewage, marine sediment, and clinical samples (Park et al, 2006). Recently, many new species have been added to this genus, a genus that was recently excluded from the genus *Flavobacterium* due to some phylogenetic discrepancies between the bacteria. Because *Chryseobacterium* strains are so versatile, they can live in a wide variety of temperatures. They have been isolated from diseased fish in different temperatures and types of water. However, there is potential for some species to cause infection in warm-blooded individuals, specifically humans (Michel et al, 2005).

Currently, the genus comprises the following species: *Chryseobacterium balustinum, Chryseobacterium gleum, Chryseobacterium indologenes,*

Chryseobacterium indoltheticum, Chryseobacterium meningosepticum, Chryseobacterium miricola, Chryseobacterium proteolyticum (name not officially published at this time), *Chryseobacterium scophthalmum, Chryseobacterium joostei,* and *Chryseobacterium defluvii* (Young, 2005).

There are several methods that are used to identify and characterize novel bacteria. In order to differentiate a species of bacterium from the other species in the genus, the physical characteristics must first be assessed. Then, 16S rRNA PCR is generally done in order to get a large piece of the gene sequence to compare with the other species.

A series of biochemical metabolism tests were done in order to give information regarding the internal processes of the bacteria, such as the carbohydrates it utilizes and its ability to metabolize in the presence or absence of oxygen. These tests also provided comparative information useful in determining the novelty of the organism. Media preparation was performed as outlined by the *Difco Manual* (Difco Laboratories, 1998). These tests include galactose, glucose, lactose, sucrose, mannitol, and Vogues-Proskauer. Differentiation of bacteria can also be determined if the bacteria in question produce enzymes that metabolize amino acids and nitrogen. Tests include Sulfide-Indole-Motility, lysine decarboxylase, phenylalanine deaminase, urea hydrolysis and nitrate reduction. Because requirements for oxygen and temperature are crucial to the success of the study, it is important to determine the optimum growth conditions early. Examples of these tests incude thioglycollate, GasPak, catalase, oxidase, 20°C, 32°C and 45°C, as well as a series of growth curves.

DNA sequence analysis is generally done using the 16S rRNA segment of the

genome because it is one of the most highly conserved portions of sequence from genus to genus. After the sequences have been received, they are put together to form a single contig, which is entered into Lasergene, a computer program that is designed to compare different sequences. This program provides data about how similar the most closely related bacteria are. The sequences can be compared by means of a multiple sequence alignment. Using this method, the sequence of the bacteria in question is compared in a computer alignment program with the sequences of all other type strains of the same genus. This provides a graphic representation of where the differences are between the different bacteria, as well as a relationship representation in the form of a phylogenetic tree.

In the spring of 2006, the microbiology class at Lycoming College cultured several organisms from Loyalsock Creek. After a series of initial phylogenetic and metabolic tests, some of the organisms were determined to be novel species. Three such organisms were found and studied further into the fall 2006 and spring 2007 semesters. The three organisms are currently known as *Acinetobacter piperi* MAG, *Chryseobacterium lycomii* CTM, and *Chromobacterium loyalsocki* JWM.

The initial testing by the microbiology class consisted of a streak plate to determine the color, size, shape and texture of the colonies, a gram stain, an endospore stain, tests to determine acid production from carbohydrate metabolism and metabolism of amino acids, as well as different growth temperatures and oxygen conditions. 16S rRNA PCR sequence analysis was also done using primers to procure a small, highly conserved segment of the sequence. This sequence was entered into database programs NCBI BLAST and Ribosomal Database Project search in order to compare

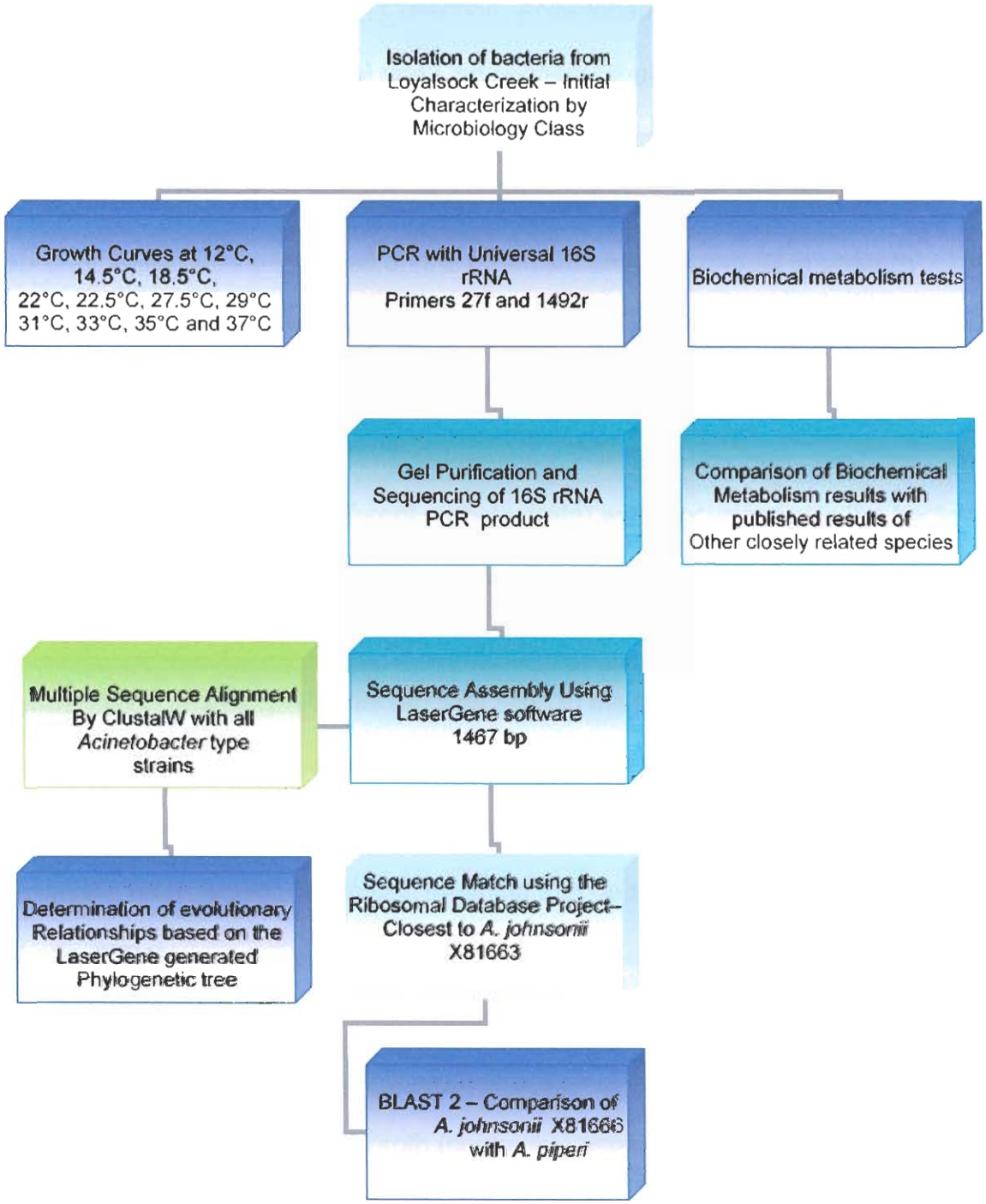
the sequence with other 16S rRNA sequences that have been deposited into the database. This study characterizes *Acinetobacter piperi* MAG and determines where it falls within the phylogenetic tree. *A. piperi* MAG appears to be equally related to *A. johnsonii* and *A. bouvetti,* with a 97.3% similarity. Both *C. lycomii* CTM and *C. loyalsocki* JWM have been determined to be novel, with similarity values of 94% and 95%, respectively, when compared with all other organisms of the same genus in the database.

These three organisms were taken on as an honors project and expanded further to get more specific information about phylogenetic relationships between other related organisms. Longer sequences of approximately 1500 base pairs were procured and compared to all other current type strains from the Ribosomal Database Project. Using Lasergene software, multiple sequence alignments were created by entering the sequences of all other type strains.

To classify and identify *A. piperi* MAG, *C. loyalsocki* JWM, and *C. lycomii* CTM, metabolic testing and genetic methods were used (Nagy et al, 2006). Growth curves determined the optimal growth conditions of the organism. By meticulously identifying this bacterium, its evolutionary relationships to other organisms can be determined, as well as where it falls in the phylogenetic tree. This will also provide insight regarding bacteria's significance in a clinical setting as a human pathogen.

Materials and Methods

Figure 1. Graphical representation of materials and methods.



Organisms. *Pseudomonas fluorescens*, *Acinetobacter johnsonii* BMF-2, from a sequence batch reactor, and *Acinetobacter calcoaceticus* TWM were obtained from the Lycoming College Microbial Strain Collection. *Acinetobacter piperi* MAG, *Chromobacterium loyalsocki* JWM and *Chryseobacterium* CTM were isolated from Loyalsock Creek. Bacteria were grown in TSB medium unless otherwise indicated.

Determination of metabolic, enzymatic and growth properties. Carbohydrate metabolism testing was performed on the organism and the controls in order to determine its metabolic characteristics. Microbes were tested for acid production from glucose, lactose, sucrose, mannitol, and galactose. Phenol red broth had been prepared in separate test tubes each containing a different carbohydrate. Each of the five tubes (each containing a different carbohydrate) was inoculated with the bacteria so that there were twenty tubes tested together. A durham tube was also inverted and placed in each of the phenol red tubes to test for the production of gas (a byproduct of the fermentation process). Phenol red was used in the broth as a pH indicator. When the broth turned acidic, the color of the solution turned from red to yellow, yielding a positive result for acidic byproducts of fermentation. If the results of the test were negative, the broth would remain red, meaning that no acidic byproducts were produced (Methods, 2004).

The organisms were then subjected to methyl-red and Vogues Proskauer tests (MR/VP). The purpose of the methyl red test is also to detect acidic byproducts of glucose in bacteria. The MR/VP broth (containing peptone, glucose, and a phosphate buffer) was mixed before inoculating the broth with the microorganisms. After growth has occurred and fermentation of glucose had taken place, MR had to be added after

incubation. A red color indicated that acidic byproducts have been produced. If none were produced, the broth turned orange or yellow because no acid was present. To perform the Vogues-Proskauer part of the test, Barritt's reagent, a potassium hydroxide (KOH) and alpha naphthol, solution was added. The purpose of the VP test was to detect the presence of 2,3-butanediol, which is a precursor to acetoin. After shaking vigorously and letting the solution sit for about one hour, the results were seen. The presence of a pink to red color was indicative of a positive result, whereas if the solution was yellow or brown, the result of the test was negative (Methods, 2004).

The next biochemical test performed on the known and unknown microorganisms was the citrate utilization test. The purpose of this test was to determine if the bacteria were able to use citrate as their sole carbon source. Citrate agar was aseptically inoculated with the bacteria and the media were incubated (Methods, 2004).

Lysine decarboxylase tests were then performed. Lysine decarboxylase is an enzyme which is used to cleave the carboxyl group from lysine, an amino acid. When this occurs, carbon dioxide and cadaverine are produced. After innoculation and incubation, the media were examined for test results. A purple color was indicative of a positive result for lysine decarboxylase, indicating that the pH of the media had become basic. A yellow color was indicative of a negative result for lysine decarboxylase. The pH indicator used in the media turned yellow when the pH of the media became acidic (Methods, 2004).

The next biochemical test performed on the microorganisms was a phenylalanine deaminase test. The purpose of this test was to determine if the microbes possessed the enzyme phenylalanine deaminase. After aseptic inoculation

and incubation, an iron chloride compound ($FeCl_3$) was added to the media. When $FeCl_3$ and phenylpyruvic acid were mixed, a green color results. Therefore, presence of a green color after the addition of iron (III) chloride was indicative of a positive result. The presence of no color was indicative of a negative result (Methods, 2004).

A urease test was performed to determine if the organisms were able to hydrolyze urea. When urea is hydrolyzed, it is broken down into ammonia and carbon dioxide. Media containing urea, phenol red, and a pH indicator were used. If the color of the media was magenta, the test was positive, a basic result. If the color of the media was orange or yellow, the test was negative, an acidic result (Methods, 2004).

A nitrate reduction test was also performed, which tests for the reduction of nitrate to nitrite. Previously prepared media containing nitrate were inoculated and incubated to allow growth to occur. A red medium was indicative of a positive result, with nitrite being present in the medium. A medium with no color was indicative of a negative result, nitrite not being present in the medium. If no color existed at this point in the test, zinc, an inorganic catalyst, was added to the medium. After the zinc was added, a red medium meant nitrate was not reduced to nitrite. If the medium was now red, the nitrate was reduced to nitrite, which was present in the medium (Methods, 2004).

A thioglycollate broth was used to test for aerotolerance among the microorganisms. It contained a reducing agent that removed oxygen from the broth, which caused the broth to be blue from the indicator present, methylene blue. If oxygen was present, the broth was green, indicating the presence of oxygen. After boiling the thioglycolate broth for approximately five minutes, inoculation of the organisms was

performed and an incubation period took place to allow for growth of the organisms. The level of the broth at which the particular microbes grew in determined how aerotolerant the organisms were. If the organisms grew at the top of the tube, they were considered obligate aerobes. If they grew in the middle of the tube, they were considered facultative anaerobes. If they grew at the very bottom of the tube, they were most likely strict anaerobes (Methods, 2004).

Next, a catalase test was performed on the known and unknown bacteria. Catalase is an enzyme produced by aerobic bacteria. It reverses the reaction $H_2O_2 \rightarrow 2H_2O + O_2$, turning hydrogen peroxide back into water and oxygen gas. Using cotton swabs, samples of the organisms were swiped off of an agar growth plate. The cotton swabs were submerged in hydrogen peroxide. If bubbles appeared, then oxygen gas had formed from the breakdown of hydrogen peroxide by catalase, indicating a positive result. A negative result was interpreted when no bubbles formed. This meant that catalase was not present and therefore could not turn the hydrogen peroxide into water and oxygen gas (Methods, 2004).

After the completion of the above growth tests, the exoenzyme tests were performed on the bacteria. These tests included amylase, caseinase, lipase, and gelatinase. Amylase is an enzyme that hydrolyzes starch, breaking it down into amylose, glucose, and amylopectin. Dextrins are also produced during the hydrolyzation. The dextrins are hydrolyzed, yielding monosaccharides and disaccharides which are used in metabolism. Media containing starch was aseptically inoculated and incubated to allow for growth on the plates to occur. After an incubation period, iodine was added on top of the media. If a clearing of iodine existed around the

bacterial colonies, the test was positive for amylase. The clearing existed because the starch in those places had been broken down by the amylase. If no clearing had existed around the colonies, the test was negative because amylase was not present; therefore, the starch had not been broken down (Methods, 2004).

The next exoenzyme test that the known and unknown microbes were subjected to was a caseinase test. Caseinase is an enzyme that hydrolyzes the large macromolecule casein to form amino acids. The previously prepared media were of an opaque, white color before growth occurred on them. The media were aseptically inoculated and incubated for growth to occur. After bacterial growth had occurred, if the organisms on the plate used caseinase to break down the casein in the media, there was a clearing around the colonies. This loss of whiteness around the bacterial colonies indicated a positive test result. If the test result was negative, there would have been no loss of whiteness around the colonies because caseinase was not used to break down the casein in the media (Methods, 2004).

Next, a lipase test was used to aid in identifying the unknown microorganisms. Lipases are also exoenzymes; they are used in breaking down lipids by cleaving their ester bonds. From the breakdown of lipids come fatty acids and glycerol. The previously prepared media were of an opaque color before growth occurred. The media were aseptically inoculated and incubated. After growth had occurred, if the organisms on the plate used lipase to break down the lipids in the media, there was a loss of opacity around the bacterial colonies. That is, the area around the colonies was more translucent than the rest of the media. This was indicative of a positive result. A lack of

loss of opacity indicated a negative result and meant that lipase was not present, and the lipids were not broken down (Methods, 2004).

The final exoenzyme test that the microbes were subjected to was a gelatinase test. Gelatinase is an enzyme that hydrolyzes gelatin causing the formation of amino acids. The previously prepared gelatin media had solidified. The media were held tightly in order to raise the temperature of the media, liquefying the gelatin. Once the media had liquefied, they were aseptically inoculated and incubated to allow growth to occur. After the incubation period was complete, the media were placed in ice for approximately five minutes. If the gelatin did not resolidify, the test result was positive because the gelatin had been hydrolyzed by gelatinase. If the gelatin did resolidify, the test result was negative because gelatinase was not present, and the gelatin could not be hydrolyzed (Methods, 2004).

After all of the biochemical, growth, and exoenzyme tests were completed, Bergey's Manual of Determinative Bacteriology was used to narrow down the identification of the unknown organisms. Using the results of all of the tests described above, Bergey's Manual was used to compare the experimental results of the unknown bacteria with the characteristics of known bacteria describes in the manual. Best matches were found and the identification of the unknown microbes was narrowed down to a few distinct genus and species of bacteria (Methods, 2004).

Determination of optimal growth temperatures. Cultures of bacteria were grown in TSB at 4°C temperature intervals from approximately 12°C to 40°C.The absorbance of the culture was recorded hourly.

16S rRNA gene sequence analysis. Polymerase Chain Reaction was done using the protocol from Methods for General and Molecular Bacteriology. Braun-Howland et al. and Garciá-Arata et al. have also referenced papers using this method. Cells of the microorganisms were grown overnight in TSB culture at 30°C and centrifuged. The supernatant was decanted and the cells were resuspended in distilled water. The cells were frozen for two minutes in a block at -70°C and then thawed in a heating block at 70°C. This cycle was repeated once more.

The primers that were used for the PCR were
27f (5'AGAGTTTGATCMTGGCTCAG>), 1492r (5'TACGGYTACCTTGTTACGACTT>),
530f (5'GTGCCAGCMGCCGCGG>), 1114f (5'GCAACGAGCGCAACCC>),
356f (5' CGGCCCAGACTCCTACGGGAGGCAGCA.), and
519r (5' GWATTACCGCGGCKGCTG) (Lane, 1991).

PCR was run at the following conditions: Phase 1 (1 Cycle) - 3 min. at 94°C, 1 min. at 50°C, 2 min. at 72°C; Phase 2 (35 Cycles) – 1 min. at 94°C, 1 min. at 50°C, 2 min. at 72°C; Phase 3 (1 Cycle) – 1 min. at 94°C, 1 min. at 50°C and 10 min. at 72°C. The PCR product was then purified using an Invitrogen PCR product purification kit, eluted in 50µL. 5µL of each sample were then run on a gel. Purification of the PCR products in the gel was done using Qiagen's QIAQuick gel extraction kit. The 16S rRNA PCR results was analyzed using Lasergene software. All sequence information that was received was entered into SequenceManager, which took all portions of the sequence and matched them up based on parts of each sequence that overlapped. One single sequence was procured, known as the sequence contig.

Multiple sequence alignment using Lasergene DNA analysis software.

Using the sequences from the PCR analysis, a contig was created in order to obtain the longest sequence read possible (See Figure 2). All type strain sequences of *Acinetobacter* were attained from the Ribosomal Database Project II website (Cole et al., 2007). All sequences were put into the software program Megalign© and compared. They were aligned by the ClustalW method. A phylogenetic tree was then constructed by the program containing all type strain sequences as well as the novel sequence. A similarity table was also constructed based on the top two matching sequences.

Results

Acinetobacter piperi MAG

Phenotypic characteristics. *Acinetobacter piperi* MAG was isolated previously from Loyalsock Creek by the spring 2006 Lycoming College microbiology class. This organism is a gram-negative, non-spore forming, non-motile, rod shaped organism. It forms shiny, yellowish-white colonies.

In order to determine specifics regarding what kind of carbohydrates it metabolizes the best and if it produces gas, a series of metabolic tests were performed. They also provided comparative information useful in determining the novelty of the organism. Metabolic testing showed that *A. piperi* MAG was negative for lactose, sucrose, glucose, galactose and mannitol. It was also negative for gas production in each of these tests. The results were compared with available published results from the two closest relatives, *A. johnsonii* and *A. bouvetii.* (Table 1).

A. johnsonii, A. bouvetii and *A. piperi* MAG were compared due to their sequence similarities. They all grow as yellowish-white colored, circular colonies. All are gram negative and approximately 1.0-1.5 millimeters in size. None produce acid or gas from glucose. They all metabolize catalase. However, some results of the tests were very different from organism to organism. Both *A. bouvetii* and *A. johnsonii* utilize citrate, while *A. piperi* does not. *A. bouvetii* metabolizes gelatinase, while *A. piperi* and *A. bouvetii* do not. *A. piperi* and *A. johnsonii* are both negative for nitrate reduction.

Growth tests were done to determine the optimal growth temperature for the bacteria. Initial testing indicated that *Acinetobacter piperi* MAG grew well at 20°C, 30°C and 32°C. It did not grow at 41°C and 44°C. It grew very little at 37°C. This is similar to

A. johnsonii, as neither grows well at 37°C, and have optimal growth temperatures between 15-30°C.

Test/Characteristic	*A. piperi*	*A. johnsonii* (pub.)	*A. bouvetii* (pub.)
Colony Color	Yellowish-White	Opaque	Opaque
Colony Size	~1.1mm	1.0-1.5mm	1.0-1.5mm
Colony Shape	Circular	Circular	Circular
Colony Texture	Mucoid	N/A	N/A
Gram Stain	Negative	Negative	Negative
Motility	No	No	No
Acid/Gas from Glucose	-/-	-	-
Acid/Gas from Sucrose	-/-	N/A	-
Acid/Gas from Lactose	-/-	N/A	-
Acid/Gas from Mannitol	-/-	N/A	-
Acid/Gas from Galactose	-/-	N/A	-
Citrate	-	+	+
Catalase	+	+	+
Thioglycollate	-	N/A	N/A
Amylase	-	N/A	N/A
Caseinase	-	N/A	N/A
Lipase	+	N/A	N/A
Gelatinase	-	-	+
Nitrate	-	-	N/A

Table 1. Growth characteristics and metabolic tests of *A. piperi*. *A johnsonii* and *A. bouvetii* results were obtained from Bergey's Manual of Determinative Bacteriology and Bergey's Manual of Systematic Bacteriology. Each test of *A. piperi* was done three times. N/A indicates that tests were not published in Bergey's Manual for that organism.

Identification of optimal growth temperatures. Growth curves were done to determine optimum growth temperatures. Tested temperatures included 12°C, 14.5°C, 18.5°C, 25°C, 29°C, 31°C, 33°C, 35°C and 37°C. Doubling times were calculated and graphed (Figure 3).

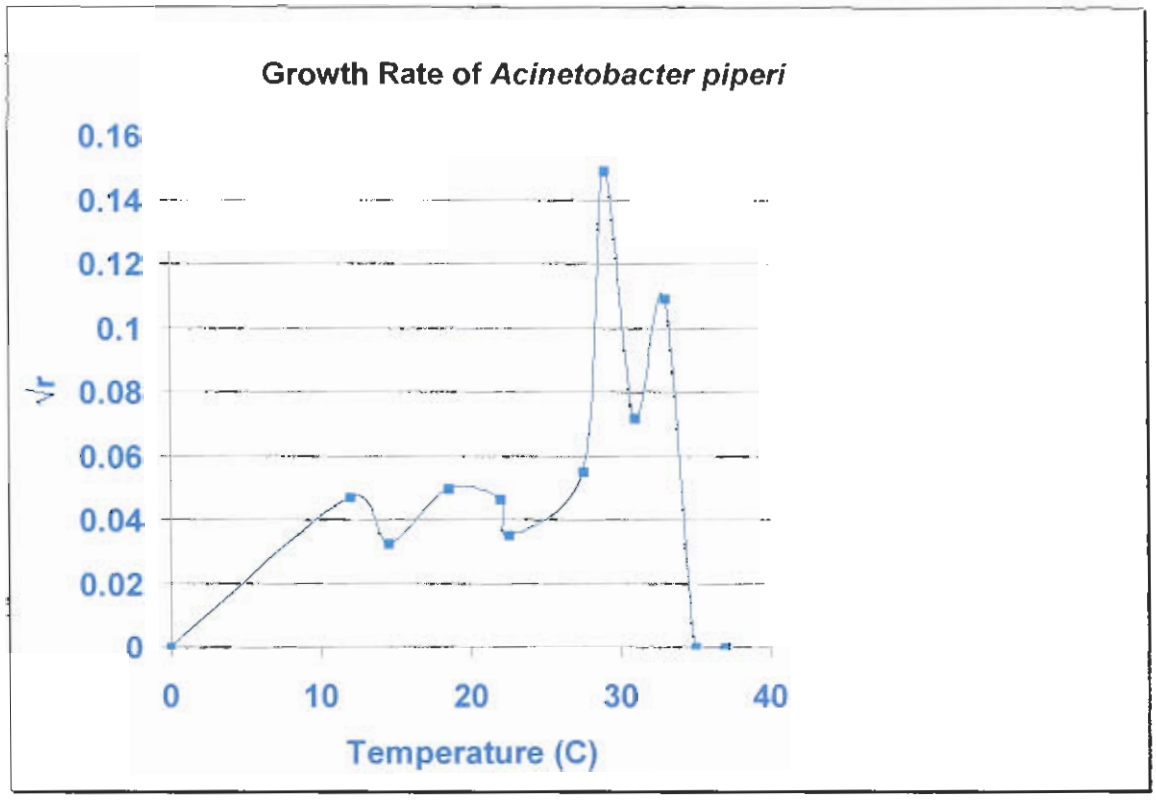
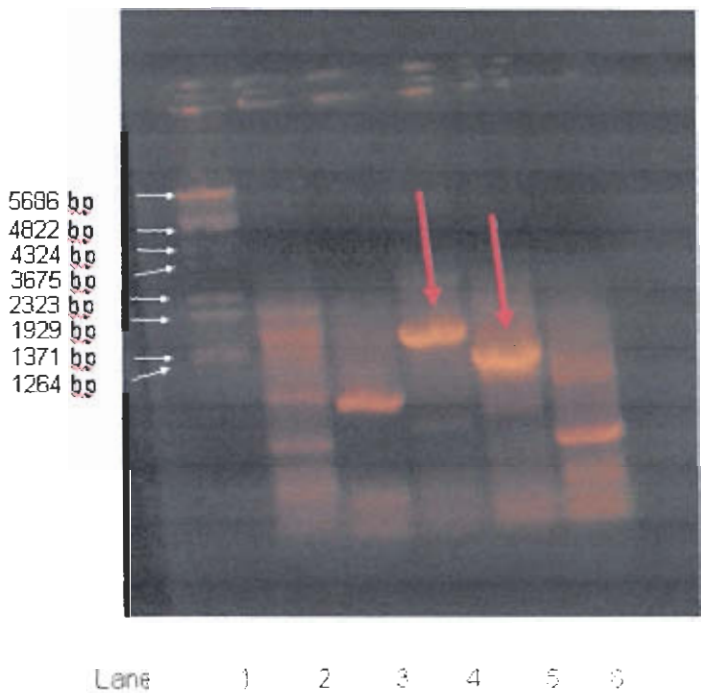


Figure 3. Effect of temperature on *A. piperi* growth rate. √r is the square-root of the reciprocal of the time taken to increase the OD600 from 0.3 to 0.6. Results from 35°C and 37°C were not included, as no doubling time was found for the time period that was investigated.

Determination of phylogenetic characteristics using 16S rRNA sequence analysis. PCR amplification and sequencing of the full length16S rRNA gene sequence of *A. piperi* MAG and controls (*Pseudomonas fluorescens* ATCC 11150 and *Acinetobacter calcoaceticus* ATCC 51432) were done in the initial experiments. The sequence that was obtained from the microbiology class was only a small fragment

detail, making it necessary to obtain the full sequence for more complete comparison. PCR was done again, but only using *A. piperi* MAG. This was done to test the different primers to determine which would be the best to get as much of the 16S rRNA sequence as possible. Results from the gel of the initial trial are shown in Figure 4. The bands from lanes 4 and 5 were purified and sent to Geneway Research, LLC for sequencing.

Figure 4. Electropherogel to analyze 16s rRNA gene PCR products of *A. piperi*. Lane 1 is BstEIII + λ (fragment lengths indicated on the left of the gel photo), Lane 2 is 27f + 519r, Lane 3 is 27f + 785r, Lane 4 is 27f + 1492r, Lane 5 is 356f + 1492r and Lane 6 is 356f + 785r. Bands that were purified and sent for sequencing are in lanes 4 and 5, indicated by red arrows.



The initial sequence of approximately 1134 bases was entered into a BLAST search to determine if the sequence matched any known organisms. *A. piperi* appeared to be closely related to *Acinetobacter johnsonii* and was thought to be a related strain. The data was entered into the Ribosomal Database Project II to determine the closest ATCC strain, which was determined to be *Acinetobacter johnsonii.* A BLAST 2 comparison with *A. johnsonii* X81663 showed a 97% similarity indicated a possible new strain of *A. johnsonii.*

PCR was done with several more sets of primers in order to procure the longest sequence of the organism possible. Electropherogel of the second trial is show in Figure 5.

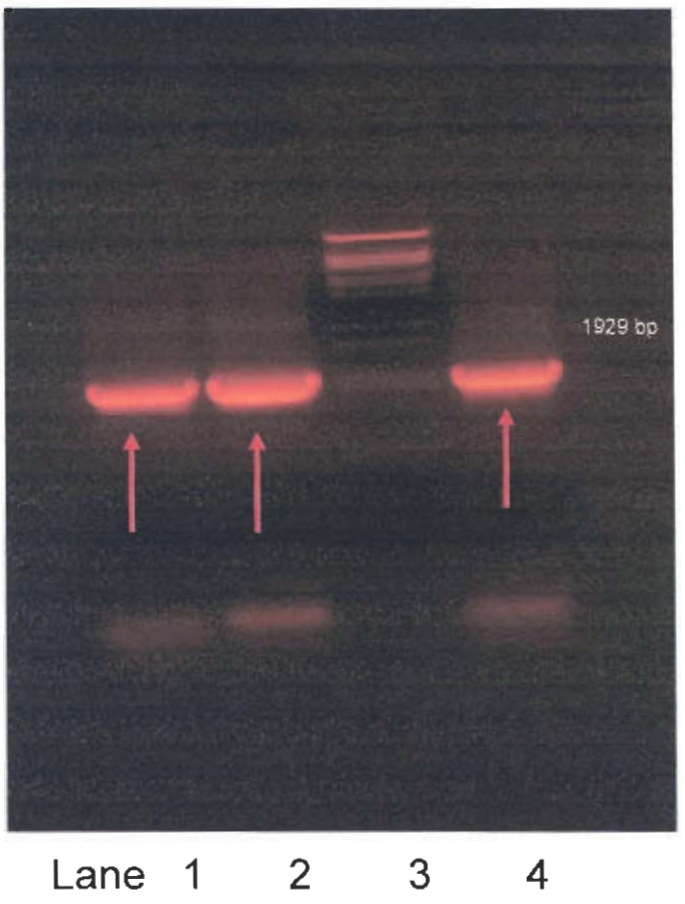


Figure 5. Electropherogel to analyze 16s rRNA gene PCR products of *A. piperi*, *C. loyalsocki* JWM *and C. lycomii* CTM. Primers used for all three organisms were 27f and 1492r. Lane 1 is *A. piperi*. Lane 2 is *C. loyalsocki* JWM. Lane 3 is BstEIII + λ, Lane 4 is *C. lycomii* CTM. Bands that were purified and sent out for sequencing are indicated by red arrows.

The new primers resulted in a much longer sequence, at approximately 1467 bases. New primers used for sequencing included 519r, 356f and 1114f. Sequence from these new primers was combined with the sequences obtained from the initial trials to obtain a more complete 16S rRNA sequence. The strategy view of the sequence is shown in Figure 6.

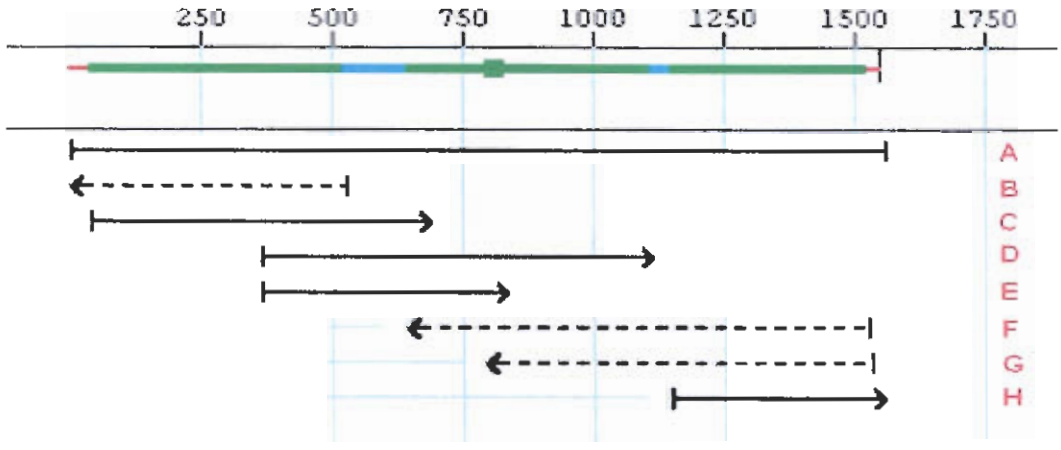


Figure 6. Strategy view of *A. piperi* consensus sequence. Each arrow represents the location and number of bases obtained by each sequencing reaction. The top arrow, **A**, indicates the combination of all the sequences. The primers for each sequence are indicated as follows: **B.** 519r; **C**. 27f; **D.** 356f; **E**. 356f; **F.** 1492r; **G.**1492r; **H**.1114f.

The full sequence was entered into the Ribosomal Database Project II which then compared all the sequences of all current type strains to *A. piperi*. The sequence most closely related to our organism based on just the sequence was *Acinetobacter johnsonii* X81663.

Phylogenetic tree based on multiple sequence with all other type strains. After the sequence of the organism was configured into a complete sequence, the organism was compared in MegAlign © with all other Acinetobacter type strains. The

data that resulted showed that *Acinetobacter piperi* was closely related to *A. bouvetii* and *A. johnsonii*, but was not identical to either. It also showed that it was more closely related to *A. bouvetii*. The data that provided this information was in the form of a phylogenetic tree with all current type strains. The full phylogenetic tree can be seen in Figure 7.

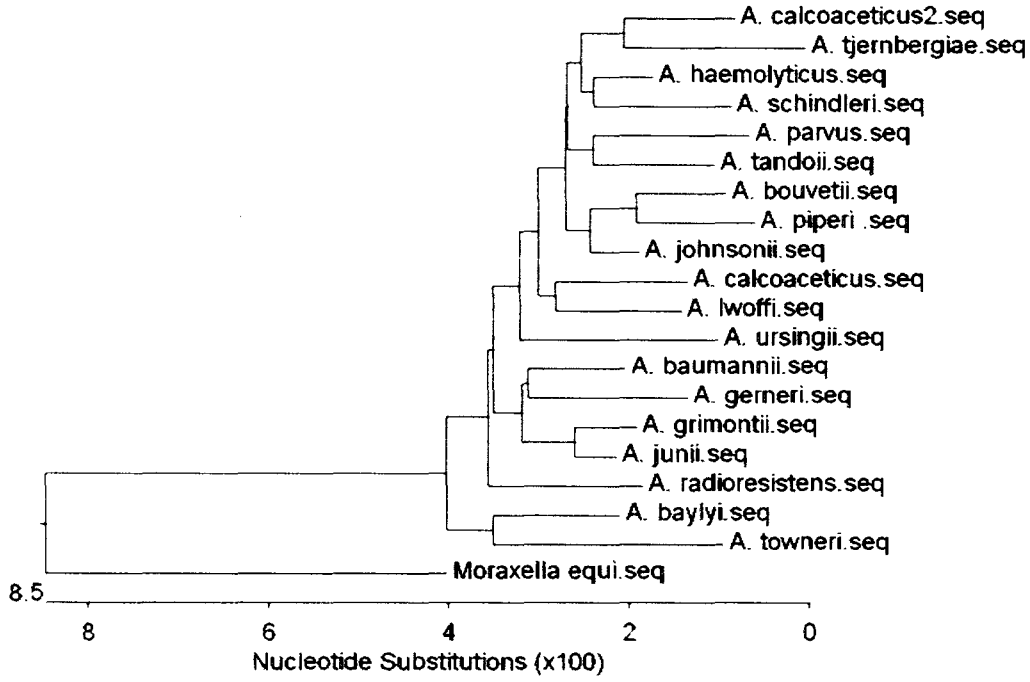


Figure 7. Phylogenetic tree representation of *Acinetobacter piperi* and all *Acinetobacter* type strains. *A. piperi* is shown in the phylogenetic tree grouped with *A. bouvetii* and *A. johnsonii*.

Since the information from the Ribosomal Database Project II presented *A. johnsonii* as the closest relative, but the information from the phylogenetic tree showed *A. bouvetii* as more closely related to *A. piperi*, a multiple sequence alignment was done with the three sequences. To more fully understand the differences between the two

type strains and *A. piperi*, a similarity table was constructed based on the similarity percentage between the three organisms, shown in Figure 8.

Percent Identity

Divergence	1	2	3	
1		97.5	98.3	1
2	2.5		97.3	2
3	1.4	2.4		3
	1	2	3	

A. bouvetii.seq
A. piperi MAG
A. johnsonii.seq

Figure 8. Similarity table of *A. bouvetti, A. piper* MAG, and *A. johnsonii*. Each organism's similarity to one of the others is show in percentage form in the top left boxes. The percent difference between the organisms is shown in the bottom left corner. Based on this table, *A. bouvetii* is most similar to *A. piperi* MAG, with a percent similarity of 97.5%.

The multiple sequence alignment was generated to show a graphical representation of the three organisms and how different the actual sequences were, based on the alignment of the nucleotides. This is shown in Figure 9.

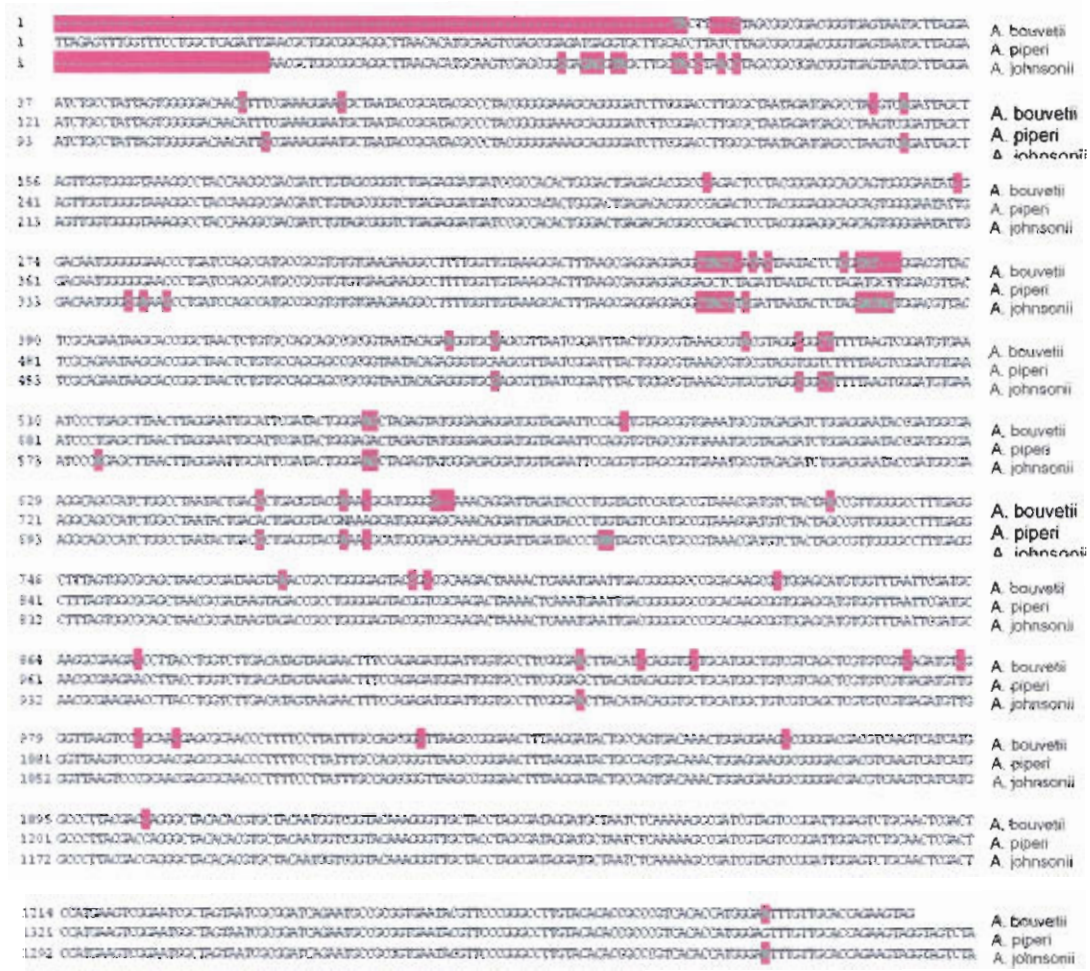


Figure 9. Graphically represented multiple sequence alignment of *A. bouvetii, A. piperi,* and *A. johnsonii.* Differences between *A. johnsonii* and *A. bouvetii* are represented by the magenta boxes. Several differences shown are discrepancies in the type strain sequence. The type strain sequence for *A. bouvetii* is significantly shorter than sequences for the other two, which may account for its similarity percentage with *A. piperi* being lower as compared to *A. johnsonii.*

In this study, a novel species of *Acinetobacter* was isolated from the Loyalsock Creek in North Central Pennsylvania was characterized and tentatively named *Acinetobacter piperi* in honor of Dr. John F. Piper Jr., retiring Dean of Lycoming College. 16S rRNA sequence comparisons and biochemical analyses indicate that this organism

is distinct from its two closest named relatives, *Acinetobacter johnsonii* and *Acinetobacter bouvetii.*

Chromobacterium loyalsocki JWM

Determination of phylogenetic characteristics using 16S rRNA sequence analysis. PCR analysis of the full length16S rRNA gene sequence of *C. loyalsocki* JWM was done to procure the full length sequence. The methods are the same as with *A. piperi* MAG. Results from the gel are shown in Figure 5.

The new primers resulted in a much longer sequence, at approximately 1519 bases. New primers used for sequencing included 27f, 519r, 785r, 356f, 1114f, 530f and 1492f. Sequence from these new primers was combined with the sequences obtained from the initial trials to obtain a more complete 16S rRNA sequence. The strategy view the sequence is shown in Figure 10.

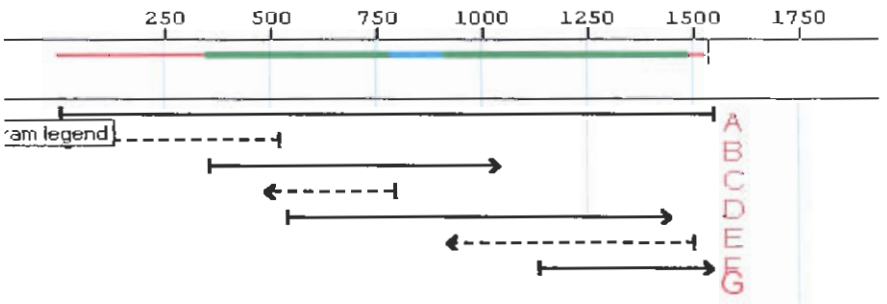


Figure 10. Strategy view of *C. loyalsocki* consensus sequence. Each arrow represents the location and number of bases obtained by each sequencing reaction. The top arrow, **A**, indicates the combination of all the sequences. The primers for each sequence are indicated as follows: **B.** 519r; **C**. 356f; **D.** 785r; **E**. 530f; **F.** 1492r; **G.**1114f.

The full sequence was then put into the Ribosomal Database Project II which then searched the sequences of all current type strains. There were no relevant bacteria that had more than a 95% similarity to *C. loyalsocki* JWM.

Phylogenetic tree based on multiple sequence alignment with all other type strains. After the sequence of the 16S rRNA gene was configured into a complete sequence, the organism was compared in MegAlign with all other *Chromobacterium* type strains. The data that resulted showed that *Chromobacterium loyalsocki* JWM was most closely related to *C. haemolyticum*. The data that provided this information was in the form of a phylogenetic tree with all current type strains. The full phylogenetic tree can be seen in Figure 11.

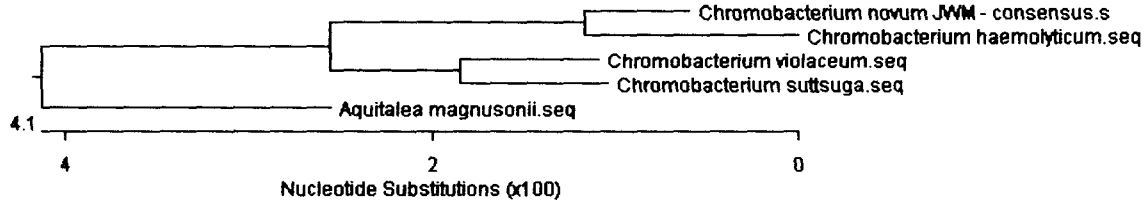


Figure 11. Phylogenetic tree representation of *Chromobacterium loyalsocki* JWM and all *Chromobacterium* type strains. *C. loyalsocki* JWM is clearly more closely related to *C. haemolyticum* than any other type strain. *Aquitalea magnusonii* was added as an outgroup because it is in a different genus, but came up as a similar organism to *C. loyalsocki* when it was put into the Ribosomal Database project.

Because the information from the Ribosomal Database Project II presented *A. magnusonii* as a close match, but the information from the phylogenetic tree showed *C. haemolyticum* as more closely related to *C. loyalsocki* JWM, a multiple sequence alignment was done with the three sequences. To more fully understand the differences between the organisms of the genus *Chromobacterium*, as well as *Aquitalea*

magnusonii, a similarity table was constructed to view the percentage differences, shown in Figure 12.

Percent Identity

Divergence

	1	2	3	4	5	6		
1		95.3	98.0	96.4	94.6	99.9	1	Chromobacterium novum JWM - consensus.s
2	3.5		94.9	97.4	94.6	94.9	2	Chromobacterium violaceum.seq
3	1.7	4.3		96.1	94.6	97.4	3	Chromobacterium haemolyticum.seq
4	3.3	1.6	3.9		95.2	95.9	4	Chromobacterium suttsuga.seq
5	5.2	4.5	5.6	4.8		94.1	5	Aquitalea magnusonii.seq
6	0.0	3.8	1.7	3.7	5.6		6	C.loyalsocki consensus.seq
	1	2	3	4	5	6		

Figure 12. Similarity table of *Chromobacterium loyalsocki* JWM (referred to as *Chromobacterium novum* JWM and *C. loyalsocki* here) and all other Chromobacterium type strains. Each organism's similarity to one of the others is show in percentage form in the top left boxes. The percent difference between the organisms is shown in the bottom left corner. Based on this table, *C. loyalsocki* JWM is most similar to *C. haemolyticum,* with a similarity of 97.4%.

In this study, a novel species of *Chromobacterium* was isolated from the Loyalsock Creek in North Central Pennsylvania was characterized and tentatively named *Chromobacterium loyalsocki* JWM in reference to the location in which it was found. 16S rRNA sequence comparisons and biochemical analyses (not shown here, data from a previous study) indicate that this organism is distinct from its closest relative, *Chromobacterium haemolyticum.*

Chryseobacterium lycomii CTM

Determination of phylogenetic characteristics using 16S rRNA sequence analysis. PCR analysis of the full length16S rRNA gene sequence of *C. lycomii* CTM was done to procure the full length sequence. Results from the gel are shown in Figure 5.

A sequence of approximately 1486 bases was obtained. New primers used for sequencing included 27f, 530f, 356f, 1114f. Sequence from these new primers was combined with the sequences obtained from the initial trials to obtain a more complete 16S rRNA sequence. The strategy view of the sequence is shown in Figure 13.

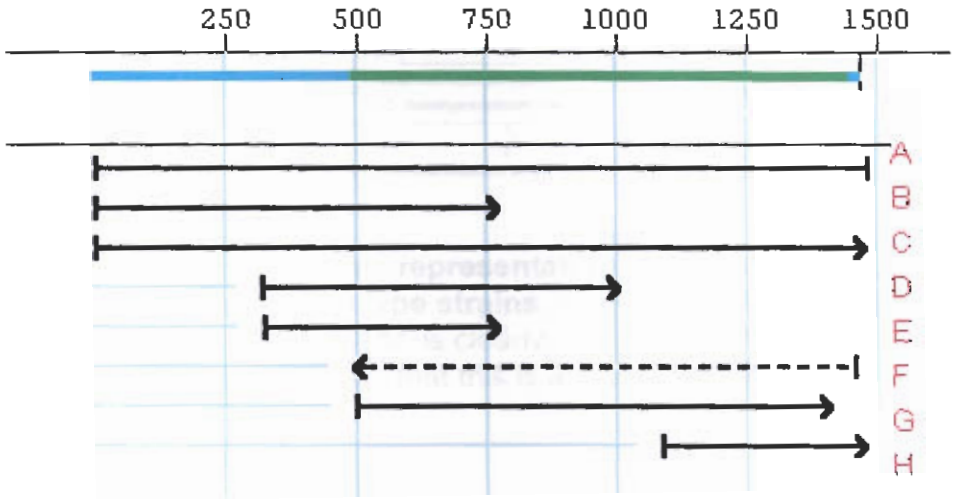


Figure. Strategy view of *C. lycomii* CTM consensus sequence. Each arrow represents the location and number of bases obtained by each sequencing reaction. The top arrow, **A**, indicates the combination of all the sequences. The primers for each sequence are indicated as follows: **B.** 27f; **C**.consensus; **D.** 356f; **E**. 356f; **F.** 1492r; **G.** 530f; **H**.1114f

The full sequence was entered Ribosomal Database Project II which then searched the sequences of all current type strains. There were no type strains that had more than a 95% similarity to *C. loyalsocki* JWM.

Phylogenetic tree based on multiple sequence with all other type strains. After the sequence of the organism was configured into a complete sequence, the sequence of the organism was compared in MegAlign with all other *Chromobacterium*

type strains. The data that resulted showed that *Chromobacterium loyasocki* JWM was most closely related to *C. haemolyticum.* This was clearly seen in the phylogenetic tree. The full phylogenetic tree can be seen in Figure 14.

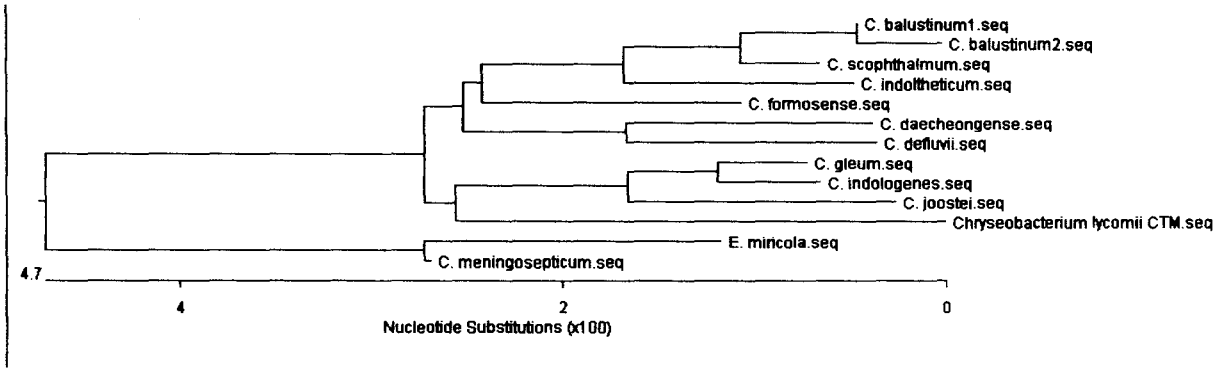


Figure 14. Phylogenetic tree representation of *Chryseobacterium lycomii* CTM and all *Chryseobacterium* type strains. *C. lycomii* CTM, referred to here as *Chryseobacterium novum CTM*, is clearly very different from any other type strain. This information provides evidence that this is a new organism.

In this study, a novel species of *Chryseobacterium* was isolated from the Loyalsock Creek in North Central Pennsylvania was characterized and tentatively named *Chryseobacterium lycomii* CTM in reference to the institution where the research on it was done.16S rRNA sequence comparisons and biochemical analyses (not shown here, data from a previous study) indicate that this organism is distinct from all other type strains.

Discussion

Acinetobacter piperi MAG

A new gram-negative strain of bacteria was isolated from the Lycoming Creek in Williamsport, PA. Phylogenetic analysis classified it within the genus of *Acinetobacter,* tentatively named *Acinetobacter piperi.* Microscopic examination showed that it was a non-motile, rod shaped bacterium. It was gram-negative, oxidase-negative, catalase-positive and nitrate-reduction-negative. These characteristics are consistent with the genus *Acinetobacter* (Bergey's, 1994). This strain cannot utilize glucose, lactose, galactose, sucrose or mannitol. Optimal growth conditions for the organism were determined through a series of growth curves. The cultures were monitored for approximately 30 hours and changes in growth were determined by subsequent increases in absorbance. The bacteria grew optimally at 29°C.

Initial 16S rRNA gene sequence analysis indicated the bacterium was most closely related to *Acinetobacter johnsonii,* with a sequence identity of 97%. Lasergene software assembled and analyzed the sequence data. Using programs such as SequenceManager and MegAlign, the organism was compared to other type strains to determine how closely related the bacteria was to other organisms in the genus. *A. piperi* was most similar to *Acinetobacter johnsonii* X81663 when analyzed through the Ribosmal Database Project II. The similarity to *Acinetobacter johnsonii* X81663 was 97%. When considering an organism as a possible new species, the percent similarity is how closely related it is to other organisms in the database. 97% is considered borderline between a new species and a new strain (Rainey et al, 1994).

When compared in MegAlign, it was shown to be most similar to *A. bouvetii.* We believe this is because of the quality of the *A. bouvetii* sequence in the database. It was approximately 1300 base pairs long, as compared with the *A. piperi* MAG sequence of 1467 base pairs. The sequence in the database also had parts of the sequence where no clear nucleotide appeared, making the sequence less reliable. Although there are similarities between *A. piperi* MAG, *A. bouvetii and A. johnsonii,* there are enough differences for *A. piperi* MAG to be considered a new organism.

Bacteria found in the genus *Acinetobacter* are generally found in soil, water and sewage. However, some species of this genus have been known to occur on the skin and in the respiratory tract, such as *A. baumanii,* and can cause severe infection in humans. Often, these infections are resistant to one or more conventional antibiotics. Because of this, it is important that all new species of this genus be classified with as much accuracy as possible. By finding a new species of *Acinetobacter,* it can be determined what characteristics of the species are of interest, what it may contribute to its environment, and how the human population might respond if ever exposed.

Chromobacterium loyalsocki JWM

A new gram-negative strain of bacteria was isolated from the Lycoming Creek in Williamsport, PA. Phylogenetic analysis classified it within the genus of *Chromobacterium,* presumptively named *Chromobacterium loyalsocki.*

Initial 16S rRNA gene sequence analysis indicated the bacterium was most closely related to *Chromobacterium haemolyticum,* with a sequence identity of 95%.

Initial 16S rRNA gene sequence analysis indicated the bacterium was most closely related to *Acinetobacter johnsonii,* with a sequence identity of 97%.

Lasergene software assembled and analyzed the sequence data. Using programs such as SequenceManager and MegAlign, the organism was compared to other type strains to determine how closely related the bacteria was to other organisms in the genus. *C. loyalsocki* had a clear distinction from the other type strains when compared in the Ribosomal Database Project II. However, a genus came up as distantly related, *Aquitalea magnusonii.* Since this is the only member of this genus, it was difficult to find out what its characteristics were and how it was similar to *C. loyalsocki.* After comparison in MegAlign, it became clear that any relation with this organism was very distant and not significant in determining the novelty of *C. loyalsocki.*

When compared in MegAlign, it was shown to be most similar to *C. haemolyticum.* Based on the phylogenetic tree results as well as the similarity table, there is enough evidence for *C. loyalsocki* to be considered a new organism.

Chryseobacterium lycomii CTM

A new gram-negative strain of bacteria was isolated from the Lycoming Creek in Williamsport, PA. Phylogenetic analysis classified it within the genus of *Chryseobacterium,* tenatively named *Chryseobacterium lycomii* CTM.

16S rRNA gene sequence analysis indicated the bacteria sequence identity of 94% with its nearest match. PCR results were analyzed via Lasergene software. Using programs such as SequenceManager and MegAlign, the organism was compared to other type strains to determine how closely related the bacteria was to other organisms in the genus. *C. lycomii* CTM was not closely related to any of the type strains, according to the phylogenetic tree from each of the organisms. Based on the data, we believe *C. lycomii* CTM to be a new organism.

Description of *Acinetobacter piperi* MAG.

Acinetobacter piperi ('pl.per.'I. adj. *piperi* of Piper, the Dean of Lycoming College in Williamsport, Pennsylvania at the time of the organisms discovery).

Cells are non-spore forming and non-motile. Gram-negative. Does not form spores. Good growth is observed on TSA and nutrient agar at 28-32°C. Grows at 12°C but not at any temperatures above 33°C. Colonies are yellowish, mucoid and shiny. Acid and gas from glucose, sucrose, lactose, mannitol and galactose are not produced. Nitrate is not reduced. Positive for catalase and lipase. Negative for thioglycollate, amylase, and caseinase. Does not utilize citrate. The strain was isolated from the Loyalsock Creek in Williamsport, Pennsylvania.

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