IDENTIFICATION AND CHARACTERIZATION OF BACTERIAL ISOLATES FROM SPRING LAKE, TEXAS

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ABSTRACT

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Eight bacterial isolates were retrieved from a biofilm during a homoserine lactone recruitment experiment in Spring Lake, Texas. On the basis of morphology, cellular fatty acid analysis, and 16S-23S intergenic transcribed spacer region sequencing, seven of the eight isolates were identified as *Chromobacterium violaceum.* A polyphasic approach to taxonomy was used to characterize the eighth, potentially novel isolate, A62-14B. The isolate was identified as a gamma proteobacterium, sharing 95-96% 16S rRNA gene sequence similarity to the genera *Rheinheimera* and *Alishewanella*. The 16S rRNA

gene sequence data allowed for a novel species designation for isolate A62-14B, but did not resolve genus level relationships. Fatty acid analysis supported an association at the genus level of isolate A62-14B with *Rheinheimera*. Further physiological and morphological characterization via biochemical tests and electron microscopy revealed that the isolate shared a variety of key phenotypic traits with both related genera. It was concluded that isolate A62-14B represented a novel species, but a generic designation could not be assigned. The characterization of this organism revealed the short comings currently in bacterial taxonomy, including issues with a lack of taxon sampling, inability to compare and fully characterize unculturable organisms, and the lack of stringent control over experimental conditions for the comparison of organisms.

INTRODUCTION

Bacterial cell-to-cell communication known as quorum sensing is facilitated through the expression of various acylated homoserine lactones (AHLs) in many Gram negative bacteria (Fuqua *et al.*, 1994). These molecules, also known as autoinducers, are membrane permeant, extracellular signaling molecules that are regulated in a population density-dependent manner (Fuqua *et al.*, 1996, Kaplan & Greenberg, 1985). Once a threshold concentration of these constitutively released molecules is reached, AHLs bind to and activate target gene transcription factors, inducing expression of various traits from virulence factors to pigment production to increased production of AHLs (Erickson *et al.*, 2002, Mclean *et al.*, 1997). The quorum sensing systems of *Pseudomonas aeruginosa*, for example, contribute to its pathogenesis by regulating expression of virulence factors (exoenzymes and toxins) and by inducing inflammation (Smith *et al.*, 2002). Luminescence by *Vibrio fischeri*, pigment production by *Chromobacterium violaceum* strains, and conjugation in *Agrobacterium tumefaciens*, are other examples of traits regulated by quorum sensing systems (Fuqua *et al.*, 1994, Hwang *et al.*, 1994, Kaplan & Greenberg, 1985, Mclean *et al.*, 1997).

Bacteria are not limited to responding to their own quorum sensing signals since analogous signals can induce transcription of some genes as well (Gray *et al.*, 1994, Mclean *et al.*, 1997, Winson *et al.*, 1995). AHL analogues and other antagonists can competitively inhibit AHLs (Fuqua *et al.*, 2001). Furanone compounds produced by the alga *Delisea pulchra* have been shown to inhibit AHL-controlled processes needed for bacterial colonization (Givskov *et al.*, 1996). This implicates an important connection between cell-to-cell communication and biofilm formation and development.

The basic steps of biofilm formation involve initial colonization, development of biofilm structure, maturation, and finally dispersion of cells (Stoodley *et al.*, 2002). Mature biofilm structure includes microcolonies interspersed with channels that allow the flow of water and metabolites with a matrix of extracellular polymeric substances (EPS) covering the entire structure for stability (Davey & O'toole, 2000, Stoodley *et al.*, 2002). AHL defective strains of *P. aeruginosa* were found to form abnormal biofilms that produced microcolonies one-fifth the normal height and were susceptible to concentrations of sodium dodecyl sulfate by which normal biofilms were not affected (Davies *et al.*, 1998).

Recently, a study was conducted to determine the affect of bacterial colonization in the presence and absence of AHLs (Mclean et al, unpublished). Culture supernatant from *Chromobacterium violaceum* ATCC 31532 (CV31532) containing hexanoyl homoserine lactone (C6-HSL), and supernatant from the

non-AHL producing *C. violaceum* 026 (CV026) were suspended in dialysis tubing and placed into Spring Lake, a pristine spring-fed lake in San Marcos, Texas. Tubing was submerged in the water to a depth of 5 cm, in an area of water depth ranging from 30 cm to 60 cm. Four replicates were exposed for five hours, after which samples were processed. Non-adherent bacteria were removed by gently shaking the tubing in sterile water; tubing-adherent bacteria were sonicated for 10 minutes and serial dilutions on R2A agar, a nutritionally reduced medium, were performed. After incubation for 40 hours, representative colonies were selected and further subcultured onto R2A agar. Pure, isolated colonies were grown in R2A broth and then stored in 12.5% (v/v) glycerol as a cryoprotectant, at -80° C. Eight isolates recovered from this experiment were designated A62-5A, A62-5B, A62-6A, A62-6B, A62-14A, A62-14B, A62-15A, and A62-15B.

The aim of the current study was to characterize the eight unknown isolates recovered from the hexanoyl-HSL (C6-HSL) baiting experiment of (Mclean *et al*, unpublished). First, artifacts of the experiment were assessed by performing a comparative study of the bait strains (CV026 and CV31532) and the isolates. This included culture-based methods of characterization such as cell and colony morphology and metabolic profiling, followed by sequence-based molecular methods. Once isolates were determined not to be CV026 or CV31532, identity at the genus level was determined by comparative 16S ribosomal RNA gene sequence analysis. Potentially novel species were further characterized using methods of higher specificity such as physiological characterization and

chemotaxonomic methods, in a polyphasic approach to taxonomy (Rossello-Mora & Amann, 2001). The phenotypic characterization was then used for comparison with other related organisms, as determined by ribosomal RNA gene sequence analysis, for species identification.

METHODS

Comparative Analysis of Bait Strains and Isolates

Morphology

Strains were initially grown for purity and isolation on R2A agar, at 30 °C for 24 hours. A standard Gram stain was performed on the isolates to determine gross cell morphology and general cell wall composition (Smibert & Krieg, 1994).

Substrate Utilization

To assess substrate utilization the Biolog GN2 Plate system (Biolog Inc, Hayward, CA) was utilized. Two to three replicates were performed for each isolate. To ensure sufficient growth for the assay, isolate A62-14B was subcultured on to R2A twice before inoculation onto Biolog Universal Growth media plus 5% blood (BUG + Blood) (manufacturer's protocol, Biolog). Furthermore, to ensure optimal growth some plates were incubated at both 30 °C and 37 °C. The GN2 Plate system is formulated for Gram negative bacteria and consists of a 96-well microtiter plate supplemented with a variety of nutrient sources. The system is chromogenically based, thus a color change from clear to purple is indicative of substrate utilization. Plates were read as recommended by

the manufacturer at 4, 16, and 24 hours, in some cases plates were incubated and read periodically for up to two weeks. Results from this analysis were entered into a database, which compares metabolic fingerprints of unknown to those of known species and provides similarity values and the probability of identification for a given match (MicroLog GN2 Plate manual, Biolog Inc.).

Fatty Acid Methyl Ester Analysis

A fatty acid methyl ester profile was conducted via gas chromatography at MIDI labs (MIDI Labs Inc, Newark, DE). The process involved harvesting cells grown on trypticase soy agar (TSA) for 24 hours. Preparation for analysis included: cleavage of the fatty acids from the lipids using saponification, an alkaline hydrolysis of fatty acid esters; methylation of the fatty acid to increase volatility in the partially polar column; extraction of the fatty acid methyl esters with hexane and methyl tert-butyl ether, for use with gas chromatography; and finally sample clean up. The profile from the gas chromatography was compared against over 100,000 culture collection and other validated strains in the MIDI Sherlock Microbial Identification System (Sasser, 2001). The baiting strains and three representative isolates, A62-6A, A62-14B, and A62-15B, were analyzed.

Intergenic Transcribed Spacer Region

Intergenic transcribed spacer regions of the isolates were partially amplified and sequenced using the primers G1, 5′- GAAGTGGTAACAAGG-3′ and L1, 5′-CAAGGCATCCACCGT-3′ (Jensen *et al.*, 1993). The thermal cycle

program was as follows; hold at 96 °C for 5 min; 30 cycles of 96 °C for 45s, 44.5 °C for 45s, and 72 °C for 2 min; hold at 72 °C for 7 min, and finally, a hold at 4 °C until PCR clean-up (a rapid thermal ramp, 1°C/second, was programmed between all temperature changes). PCR was performed using a GeneAmp PCR System 2400 (Perkin-Elmer). PCR products were purified using a MinElute Gel Extraction Kit (Qiagen). Sequencing was performed using an Applied Biosystems 377 DNA Sequencer and the protocols of the manufacturer (Perkin-Elmer Biosystems) using the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit v3.0 or v3.1. Unincorporated nucleotides in the cycle sequence product were removed using Centrisep columns (Princeton Separations, Adelphia, NJ).

Novel Species Characterization

Initial data suggested that isolate A62-14B may be a novel isolate. Therefore, the following analyses were undertaken in attempt to identify and characterize isolate A62-14B.

Ribosomal RNA Gene Sequencing and Phylogeny Construction

Pure colonies were isolated on R2A agar from frozen stock cultures then grown for 16-18 hours in R2A broth. DNA was extracted using the DNeasy Tissue Kit (Qiagen) following the manufacturer's protocol for Gram negative bacteria. The following primers were used to amplify fragments from the 16S ribosomal RNA gene: 5′ AGAGTTTGATCATGGCTCAG-3′ (27F) and 5′-TACGGCTACCTGTTACGACTT-3′ (1492R-H2). The PCR program was as

follows; hold 5min at 96 °C; 30 cycles of 96 °C for 45s, 54 °C for 45s, and 72 °C for 2min; hold 7min @ 72 °C, and finally, a hold at 4 °C until PCR clean-up. Sequencing protocols were followed as described previously for ITS sequence analysis. Internal primers were used to allow sequencing of the entire amplified region of the 16S rRNA gene. The internal primers used were, 5′ CCAGACTCCTACGGGAGGCAGC-3′ (355F), and 5′-AGGGTTGCGCTCGTTGC-3′ (1096R). The resulting sequence fragments amplified from the 27F, 355F, 1096R, and 1492R-H2 primers were aligned to form a consensus sequence in Sequencher 4.2 (Gene Codes Corp.). Sequence identities were found by using a BLAST (Basic Local Alignment Search Tool) search through the NCBI website (http://www.ncbi.nlm.nih.gov/) (Altschul *et al.*, 1997). The top BLAST search results were used to construct phylogenies of the isolates for identification (Altschul *et al.*, 1997). Sequences were aligned using Sequencher 4.2.

Phylogenies were constructed in PAUP*4.0b10 (Swofford, 2002). A neighbor joining tree with Kimura-2-parameter (K2P) distance correction , a heuristic maximum parsimony (MP) search with 1000 random addition sequence replicates, a maximum likelihood (ML) heuristic search using trees from the MP search with parameters dictated by MrModeltest2.2 was run, and a Bayesian analysis with parameters also dictated by MrModeltest2.2 were performed as well as the corresponding bootstrap analyses (Felsenstein, 1981, Felsenstein, 1985, Huelsenbeck *et al.*, 2001, Kimura, 1980, Nylander, 2004, Saitou & Nei, 1987).

The best fit model of evolution for the data from MrModeltest2.2 was General Time Reversible (Waddell & Steel, 1997)with a gamma shape parameter and a designated value for the proportion of invariable sites (GTR+I+G), and included the following parameters: base frequencies for A, C, G, and T were set to 0.2384, 0.2357, 0.3160, and 0.2099, respectively; rate matrix values were 0.6147 (A-C), 1.4137 (A-G), 0.9339 (A-T), 0.5342 (C-G), 2.4289 (C-T), and 1.0000 (G-T); proportion of invariable sites (I) = 0.2305; and the gamma distribution shape parameter = 0.3418. For theses analyses an Alpha proteobacteria was selected as, it is differentiated at the highest known taxonomic level from isolate A62-14B, a Gamma proteobacteria. *Beijerinckia derxii* ATCC 49361 (AB119198) was selected as the outgroup with the lineage: Bacteria (superkingdom); Proteobacteria (phylum); Alphaproteobacteria (class); Rhizobiales (order); Beijerinckiaceae (family); Beijerinckia (genus).

In order to find the most appropriate outgroup for the data, other outgroups were also used to construct phylogenies including: Alpha proteobacteria, *Caulobacter leidyi* ATCC 15260 (AJ227812) and *Rickettsia sibirica* ATCC VR151 (D38628); Beta proteobacteria, *Hylemonella gracilis* ATCC 19624 (AF078753) and *Bordetella parapertussis* 12822 (NC_002928); Deinococcus-Thermus bacteria, *Thermus thermophilus* HB27 (NC_005835); Firmicutes, *Staphylococcus aureus* ATCC 29740 (AF015929); and the more closely related Gamma proteobacteria, *Moraxella* ovis ATCC 33078T (AF005186), *Moraxella catarrhalis* ATCC 25238 (U10876), *Alkalimonas delamerensis* (X92130), and *Alkalimonas*

amylolytica (AF250323). For these analyses, neighbor joining with K2P distance correction topologies were created to determine the most suitable outgroup. Bootstrap analyses with 2500 replicates, were performed to support respective topologies.

For selected outgroups, MP and ML phylogenies were constructed. Maximum likelihood trees were created from the MP reconstructions with parameters dictated by Modeltestv3.5 and MrModeltest2.2. The best fit model of evolution for the data when *Moraxella* species were used as the outgroup was Tamara and Nei (TrN+I+G) (Tamura & Nei, 1993). The parameters for the analysis were as follows: base frequencies for A, C, G, and T were set to 0.2589, 0.2227, 0.3044, and 0.2097, respectively; rate matrix values were 1.0000 (A-C), 1.9354 (A-G), 1.0000 (A-T), 1.0000 (C-G), 4.1715 (C-T), and 1.0000 (G-T); proportion of invariable sites (I) = 0.4870; and the gamma distribution shape parameter = 0.6431. From MrModeltest2.2, base frequencies for A, C, G, and T were set to 0.2610, 0.2201, 0.3065, and 0.2124, respectively; rate matrix values were 0.7310 (A-C), 2.0089 (A-G), 0.7794 (A-T), 0.6737 (C-G), 3.5518 (C-T), and 1.0000 (G-T); proportion of invariable sites (I) = 0.5595; and the gamma distribution shape parameter = 0.5918. For *Alkalimonas* outgroup topologies, TrN+I+G was used, base frequencies for A, C, G, and T were set to 0.2550, 0.2236, 0.3115, and 0.2099, respectively; rate matrix values were 1.0000 (A-C), 1.8682 (A-G), 1.0000 (A-T), 1.0000 (C-G), 3.1278 (C-T), and 1.0000 (G-T); proportion of invariable sites (I) = 0.6745; and the gamma distribution shape parameter =

0.9856 . From MrModeltest2.2, Hasegawa-Kishino-Yano (HKY+I+G) (Hasegawa *et al.*, 1985), base frequencies for A, C, G, and T were set to 0.2501, 0.2288, 0.3071, and 0.2141, respectively; the transition to transversion ratio was set at 1.2196; proportion of invariable sites (I) = 0.6900; and the gamma distribution shape parameter = 1.0767.

DNA Base Composition

The DNA base composition of A62-14B was determined using a fluorometric method (Johnson, 1994). The Perkin Elmer Luminescence Spectrometer model LS50B was used to measure absorbance values from ethidium bromide and Hoechst 33825 dye treated DNA. Genomic DNA was purified from cell lysates using phenol-chloroform extractions treated with 4µl (100mg/ml) RNase A (Kirby, 1957, Sambrook & Russell, 2001). First, 0.5ml to 1.5ml of fresh overnight broth culture was pelleted in a microcentrifuge (Eppendorf Centrifuge 5415D) at maximum speed for 1 minute. The supernatant was discarded, the cell pellet was washed in TE buffer pH 8.0 (10mM Tris-HCl pH 8, 1mMEDTA pH 8) once, and then the cells were resuspended in 500µl of TE. Cells were lysed using 25µl Proteinase K (20mg/ml) and 5µl 10% sodium dodecyl sulfate (SDS) then incubated for 2 hours to 24 hours at 55 °C. RNase A was added 5 minutes prior to the phenol: chloroform extraction, the solution was left at room temperature. The following steps were done under a fume hood: 250µl phenol and 250µl chloroform: isoamyl alcohol (24:1) were added to each

cell preparation, mixed, and then centrifuged for 5 minutes at maximum speed. The aqueous layer was moved into a new microfuge tube and an equal part of chloroform was added to extract lingering traces of phenol. This solution was mixed and centrifuged as above. The aqueous solution was again moved into a new tube, sodium acetate pH 5.2 was added to a 0.3M concentration (about 150µl) and isopropanol was added, entirely filling the tube. After centrifugation as above, the isopropanol was discarded, carefully, so as not to disturb the DNA pellet, and the pellet washed twice with 70% (v/v) ethanol, with a centrifugation time of 2 minutes. Excess ethanol was removed and pellets were allowed to air dry. Finally, pellets were redissolved in 20-50µl TE at 4 °C, overnight. DNA was diluted to concentrations of 1:10, 1:100, and 1:1000 in 1X phosphate buffered-standard saline citrate buffer (PB-SSC). A stock solution of 20X PB-SSC was prepared by mixing a 20 fold concentrate of standard saline citrate (0.15 M NaCl, 0.015 M trisodium citrate, pH 7) with 200 mM sodium phosphate buffer (pH 7). Lambda phage DNA was used to create a standard curve for fluorometric quantification of DNA. Concentrations of 2000 ng, 1000 ng, 500 ng, 200 ng, 100 ng, 50 ng, 20 ng, and 0 ng, were also diluted in 500 µl of 1X PB-SSC. Stock solutions of Hoechst dye and ethidium bromide of 10mg/ml were diluted to 2µg/ml in 2X PB-SSC. DNA quantification was carried out using ethidium bromide only, since Hoechst can only be used for quantification when percent AT content of the standard and the unknown are similar. Equal parts of DNA and ethidium bromide solution were mixed and placed into plate reader wells.

Each sample was dispensed into three wells and read twice by the fluorometer. Fluorometer readings were averaged and plotted against DNA concentration. Unknown sample concentrations were assessed through interpolation. Once DNA concentration was known, DNA solutions were diluted to 1µg/ml with 1X PB-SSC as needed. Two parts ethidium bromide/Hoechst dye solution was added to one part DNA solution (1µg/ml) and one part 2X PB-SSC. Samples were dispensed and read as mentioned previously. Excitation and emissions values used for ethidium bromide were 305nm and 615nm, and for Hoechst dye, 365nm and 458nm, respectively (Johnson, 1994). The ratio of Hoechst over ethidium bromide fluorescence was calculated and the ratios were plotted against the known AT% values of the control strains, thereby creating a standard curve. Composition of unknown samples were determined through interpolation and converted to a percent GC value (Bermudez & Hazen, 1988, Johnson, 1994). *Cellulomonas turbata* DSM 20577 (70.5-75% GC), *Escherichia coli B* ATCC 23848 (51.7% GC), and *Bacillus subtilis* ATCC 6051 (43% GC) (Bermudez & Hazen, 1988, Claus & Berkeley, 1986, Lechevalier & Lechevalier, 1986, Orskov, 1984)

Growth Conditions and Biochemical Tests

Optimal growth temperature was assessed by incubating cultures at 4°C, 25°C, 30°C, 37°C, 42°C, and 55°C. To test for salinity tolerance organisms were cultured on 10% (w/v) TSA and full strength R2A agar supplemented with 5, 6.5, 10, 15, and 20 percent (w/v) sodium chloride. The pH growth range was

determined using R2A broth adjusted with phosphate-buffered saline (pH 4.5 – pH 8.7) and tris-hydrochloride buffer (pH 9.0 – pH 11.0), to help ensure toxicity from high molar concentrations of HCl or NaOH, would not skew results (Breznak & Costilow, 1994). Stock solutions of the buffers were made at a concentration of 100mM, and were used at a final concentration of 10mM. Absorbance values read at 600nm (SmartSpec™Plus, BioRad, CA) were taken before incubation of the cultures, and 24 hours after initial incubation, to detect a change in growth. The cultures were read again after 48 hours, optical density readings that did not change or that decreased were not recorded. Atmospheric condition preferences were tested by growth on R2A without CO_2 enrichment, created with BIO-BAG Environmental Chamber (Becton Dickinson, Cockeysville, MD). Inoculated R2A broth was covered with a layer of sterile mineral oil also, to determine oxygen needs. Differential and selective media were used to characterize metabolic traits of the organism as described by Smibert and Krieg including: MacConkey agar (MAC), a media used to detect Gram negative enterics and the ability to ferment lactose; triple sugar iron agar (TSIA) tests an organism's ability to ferment glucose, lactose and or sucrose, and to reduce sulfur to hydrogen sulfide; Simmon's citrate agar, indicates the ability to use citrate as a sole carbon source; sulfur indole motility agar (SIMS); motility agar; 12% gelatin medium, to detect hydrolysis of gelatin; and starch agar (Difco) (Smibert & Krieg, 1994). Glucose, sucrose, and lactose phenol red broth were inoculated as well as methyl red-Vogues Proskauer (MRVP) broth, nitrate broth,

and tryptone broth, for carbohydrate fermentation, nitrate reduction, and tryptophanase activity, respectively (Smibert & Krieg, 1994). In some cases, diluted (1:10, 1:100) versions of these media were used to create a more amiable environment for oligotrophic isolates. Oxidase and catalase tests were also performed. The oxidase test identifies bacteria that utilize cytochrome oxidase to reduce oxygen to water; the presence of catalase allows bacteria to prevent damaging effects of hydrogen peroxide by breaking it down into water and oxygen gas (Smibert & Krieg, 1994). Antibiotic sensitivity was tested using the agar-disk diffusion method, for which small paper disks impregnated with antibiotics are placed on culture inoculated media, here, R2A agar. A clearing, no growth around the disks, indicates susceptibility to the given antibiotic (Smibert & Krieg, 1994). Antibiotics tested included erythromycin, 15 µg; ciprofloxacin, 5 µg; tetracycline, 30 µg, trimethoprim-sulfamethoxazole, 25 µg; bacitracin, 10 µg; ampicillin, 10 µg; and penicillin, 10 µg.

An extensive evaluation of metabolic characteristics including; carbon, nitrogen, phosphate, and sulphate supplements, osmotic sensitivity, pH and growth control, and finally chemical sensitivity, was carried out by the Phenotype Microarray Services at Biolog Inc. (Hayward, CA).

Electron Microscopy

A simple negative stain procedure was used to visualize the presence of flagellae. A 1% (w/v) solution of uranyl acetate was gently mixed with a

bacterial suspension. The solution was dropped on carbon and formvar coated grids, excess solution was wicked up with filter paper after one minute, and the grids were examined for specimen morphology using a JEOL 1200 EX II Electron Microscope (Beveridge *et al.*, 1994).

Phenotypic and genotypic results were compared to data from the closest relatives of A62-14B, to resolve relationships and determine the identity of the isolate.

RESULTS

Comparative Analysis

All strains were found to be Gram negative, rod-shaped, motile, and oxidase positive. Isolates A62-5A, 5B, 6A, 6B, 14A, 15A, 15B and the bait strains were found to be able to utilize dextrin, glycogen, Tween 40, Tween 80, *N*-acetyl-D-glucosamine, D-fructose, α-D-glucose, D-mannose, D-psicose, D-trehalose, pyruvic acid methyl ester, mono-methyl ester acetic acid, formic acid, D-gluconic acid, β-hydroxybuturic acid, propionic acid, succinic acid, bromosuccinic acid, succinamic acid, L-alaninamide, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, D-serine, L-serine, L-threonine, urocanic acid, inosine, uridine, thymidine, glycerol, D,L,α-glycerol phosphate, α-D-glucose-1-phophate, and D-glucose-6-phosphate through the Biolog GN2 Plate (Table 1). The isolates and bait strains did not ferment sucrose, lactose, maltose, or use arabinose, and had varied results for the utilization of citrate, which is common for *C. violaceum* strains (Peel *et al.*, 1999). Other substrates tested that gave negative results were; L-arabinose, α-D-lactose, sucrose and 2, 3-butanediol. Based on these readings,

the Biolog database supported, with 100% probability, that all these strains were *Chrombacterium violaceum* strains.

Plates for A62-14B were incubated at both 30 °C and 37 °C. After 72 hours, a faint color change appeared for wells containing succinic acid mono-methyl ester and succinic acid, at 30 °C. At 37 °C, a faint signal was detected for γ-hydroxybutyric acid and D-mannose. Continued incubation did not result in further changes. These results were likely false positives, due to absence of the reaction in subsequent trails. Overall, the GN2 MicroPlate™ was unable to assess substrate utilization for A62-14B.

Since seven of the 8 isolates appeared to be the same species, two of the seven isolates (A62-6A and A62-15B), the bait strains, and A62-14B were chosen for fatty acid analysis. As suspected the bait strains, A62-6A, and A62-15B had nearly identical profiles (Table 2). Predominant fatty acids were 16:1 ω7c (~ 40%), 16:0 (~27%), and 18:1 ω7c (~14%). Percent composition of minor fatty acids present, were also very similar. Each of these isolates possessed the same fatty acids, except 14:1 ω5c of A62-6A (percent = 0.14). Interestingly, the top matched species for these isolates were *Pseudomonas species*. However, according to MIDI Lab criteria, these were not considered good matches. A similarity value above 0.600 with good separation (at least 0.100) from the next named organism is considered a good match. This was not the case for the isolates; *Chromobacterium violaceum* was third or fourth on the list of similarity index matches and in most cases within 0.100 of the highest similarity match. It is unclear whether the strain

in the MIDI database is a pigment-producing variant of *C. violaceum*. Regardless, the aforementioned isolates and the bait strains share the same fatty acid profile.

The closest match for strain A62-14B was *Pseudoalteromonas nigrifaciens*, with a similarity index of 0.378. Values under 0.400 indicated that the species was not in the database. The closest relatives that were listed do not provide a good indication of the identity of A62-14B, even at low resolution. The official result of the commercial analysis named the isolate as "Gram negative species." Predominant fatty acids were $C_{16\,1}\omega7c$ (38·56%), $C_{16\,0}$ (19·04%), $C_{12\,0}$ 3OH (12·83%), and $C_{18\,1}\omega7c$ (7·70%).

Partial sequencing of the ITS region revealed that isolates A62-5A, 5B, 6A, 6B, 14A, 15A, 15B and the bait strains had the same sequence (Table 3). The ITS region of these seven isolates appeared to be approximately 1500 nucleotides, as was for the control bait strains. Approximately the first 500 nucleotides were sequenced. BLAST results found the nearest relative to be *Chromobacterium violaceum* ATCC 12472 (AE016921) with 90% sequence similarity. Half of the 500 nucleotide sequence matched *C. violaceum* ATCC 12472. The isolates had 100% sequence similarity with the bait strains CV026 and CV31532. The ITS region for isolate A62-14B was approximately 500 nucleotides. About 160 nucleotides or less of the 480 nucleotide sequence of A62-14B was matched with *Pseudomonas fluorescens* (AF148208), *Vibrio mimicus* (AF114744), and *Vibrio cholerae* (AE004319) strains with approximately 90% sequence similarity.

Characterization of Novel Species

Ribosomal RNA Sequencing and Phylogeny Construction

Based on initial analyses, isolate A62-14B was further analyzed as a potentially novel species. With a nearly complete 16S rRNA gene sequence (1447 nucleotides; Table 4), isolate A62-14B was found to have 98% sequence similarity to Gamma proteobacterium F8 (AY07761), a phenotypically uncharacterized organism. A sequence similarity of 96.2% was found for the closest cultured and published isolate, a gamma proteobacterium, *Rheinheimera* sp. HTB082 (AB010842) (Takami *et al.*, 1999). Other *Rheinheimera* species and isolates also shared 95.01% to 96.35% sequence similarity, including *Rheinheimera baltica* (AJ441082) and *Rheinheimera pacifica* (AB073132) and some phenotypically uncharacterized, deep-sea mud *Rheinheimera*: HTB010, HTB109, and HTB021 (95%) (Brettar *et al.*, 2002, Romanenko *et al.*, 2003, Takami *et al.*, 1999). BLAST results gave a 95.93% sequence similarity value for a clinical isolate, *Alishewanella fetalis* (AF144407) (Fonnesbech Vogel *et al.*, 2000). The next closest organisms including *Serratia* sp., *Alkalimonas* sp, and *Photobacterium* sp. shared 90% or less sequence similarity. Distance, parsimony, maximum likelihood and Bayesian phylogenies support the grouping of strain A62-14B in a clade with both the *Rheinheimera* and *Alishewanella* (Fig. 1-5). While A62-14B was grouped in a more terminal clade with *A. fetalis*, the grouping was not well supported by bootstrap values. The normal threshold bootstrap value for significant support of a taxonomic relationship is 70 percent. The distance, MP, and Bayes topologies

supported the grouping of A62-14B with *Alishewanella* with values of 72, 66, and 59; the ML phylogeny displayed an unresolved relationship among the genera with a polytomy for *Rheinheimera, Alishewanella,* and A62-14B. In attempt to resolve the relationship among the genera of interest, various outgroups, from distant to close relatives were analyzed with the ingoup taxa (Fig. 6-8). Neither the Alpha proteobacteria nor the Firmicutes as outgroups were able to resolve the clade of interest, resulting in polytomies. The Beta proteobacteria showed insufficient support for relatedness to *Alishewanella*, with 58 and 56 percent bootstrap values. *Thermus thermophilus* gave very weak support for a grouping with *Rheinheimera* at 51 percent. When the Gamma proteobacteria *Moraxella* was used as the outgroup, a bootstrap for the K2P distance analysis resulted in 83 percent support for an association with *Rheinheimera*, but this was not supported by ML (63%) and MP (57%). The most closely related organisms used as outgroups were the *Alkalimonas* species. *Alkalimonas delamerensis* shared approximately 90% sequence similarity with isolate A62-14B. Two models of evolution were suggested to best fit the data including, HKY+I+G and TrN+I+G. When HKY+I+G was used, a polytomy resulted for the clade of interest. ML and MP supported an association with *Rheinheimera* at 67 and 58 percent, when TrN+I+G was used. However, this was not supported by the distance analysis.

DNA Base Composition

The logarithm of the fluorescence of Hoechst dye divided by ethidium bromide was plotted against the known %AT of the control organisms. Since, the only information for *C. turbata* was a range of 70.5 to 75% guanine-cytosine, both values were plotted. The regression value when 25% AT was used was 0·91, giving an AT content for A62-14B of 51.55215. When a value of 30% was used for *C. turbata*, the regression line was equal to 0·96, and an AT% of 52·12685. The genomic guanine-cytosine content of A62-14B was determined to be approximately 48·16 ± 0·4% (mean ± SD).

Growth Conditions and Biochemical Tests

Strain A62-14B was found to be a non-pigmented, Gram negative, facultatively anaerobic, rod-shaped bacterium. Cell size, as measured from electron micrgraphs, varied from 2.5 μm to 1.25 μm in length and 0.70-0.80 μm in width. Optimal growth occurred between 30 °C and 37 °C. No growth was seen at 4° C or at 42°C and above; at 20-25 °C growth was seen after 48 hours to 7 days. The organism was able to proliferate between pH 6·5 and 9·6, with optimal growth midrange. Strain A62-14B could not grow in the presence of NaCl at a concentration greater than 1%. Carbon sources utilized include *N*-acetyl glucosamine, D-galactose, Tween 20, Tween 40, Tween 80, D-glucose, D-lactose, D-galactose, sucrose, trehalose, methyl pyruvate, β-cyclodextrin, laminarin, glycogen, and palatinose; citrate was not utilized. Strain A62-14B was positive

for starch and gelatin hydrolysis, nitrate reduction, oxidase, and catalase activity. Indole production was not detected, no growth was observed in MRVP broth, hydrogen sulfide was produced in neither SIMS nor TSIA media. Results of antibiotic susceptibility testing included susceptibility to erythromycin, 15 µg; ciprofloxacin, 5 µg; and tetracycline, 30 µg, and resistance to trimethoprim-sulfamethoxazole, 25 µg; bacitracin, 10 µg; ampicillin, 10 µg; and penicillin, 10 µg. The isolate was highly motile, supported by single polar flagellum observed in electron micrographs (Fig. 9). Also, micrographs showed intercellular networks of filaments among cells in close proximity, from an overnight culture.

Comparison of Isolate A62-14B and Related Bacteria

Many of the organisms that showed high sequence similarity to A62-14B were either clones of uncultured bacteria, phenotypically uncharacterized, or unpublished and thus the closest cultured and characterized relatives were chosen for comparison. The type strains chosen for this comparative analysis were: *Rheinheimera baltica* DSM 14885^T (AJ441082), isolated from Baltic sea-water samples; *Rheinheimera pacifica* KMM 1406^T (AB073132), isolated from deep sea sites in the Pacific Ocean; and *Alishewanella fetalis* CCUG 30811^T (AF144407), isolated from a human foetus (Brettar *et al.*, 2002, Fonnesbech Vogel *et al.*, 2000, Romanenko *et al.*, 2003) (Table 5). The DNA mol% G + C content of strain A62-14B was 48·2%. *R. baltica* and *R. pacifica* were reported to have similar mol% G + C values of 48·9 and 49·6, respectively (Brettar *et al.*, 2002, Romanenko *et al.*,

2003). *A. fetalis* also had a similar DNA G + C content of 50·6% (Brettar *et al.*, 2002, Fonnesbech Vogel *et al.*, 2000, Romanenko *et al.*, 2003).

Major fatty acids for A62-14B were $C_{16\,1}\omega7c$ (38·56%), $C_{16\,0}$ (19·04%), $C_{12\,0}$ 3OH (12·83%), and $C_{18\,1}\omega7c$ (7·70%). *Rheinheimera baltica* shared the top four most abundant fatty acids with A62-14B: $C_{16\,1}\omega7c$ (34·8 ± 4·8%), $C_{16\,0}$ (22·1 ± 3·6%), and ($C_{18\,1}\omega7c$) 15·2 ± 5·0%, and $C_{12\,0}$ 3OH (5·6 ± 0·5%) (Brettar *et al.*, 2002). *R. pacifica* also shared a similar profile with $C_{16\,0}$, $C_{17\,0}$, $C_{16\,1}\omega7c$, $C_{17\,1}\omega8c$, and $C_{18\,1}\omega7c$ as the predominant fatty acids (Romanenko *et al.*, 2003). The profile for *A. fetalis* was slightly different than A62-14B and the *Rheinheimera* with $C_{17\,1}\omega8c$ (19·5%), $C_{16\,1}\omega7c$ (19·0%), $C_{17\,0}$ (10·3%), and $C_{16\,0}$ (8·9%), as the dominant fatty acids. $C_{12\,0}$ 3OH represented only 2% of the cellular fatty acids in *A. fetalis* (Fonnesbech Vogel *et al.*, 2000).

All organisms were Gram negative and similar in average length and width. *A. fetalis* and A62-14B were facultatively anaerobic, while the *Rheiheimera* species were strictly aerobic. Isolate A62-14B and the *Rheinheimera* species were flagellated to different degrees. *R. baltica* and A62-14B both have a singular polar flagellum, while *R. pacifica* has bipolar and lateral flagellae, *A. fetalis* was not flagellated. Optimal growth temperature was higher for strain A62-14B, than the *Rheinheimera* species which were able to grow at 4 °C, *A. featlis* had a higher optimal growth temperature with the ability to grow at 42 °C. All organisms had a higher salinity tolerance than A62-14B, two of which were considered halotolerant species. *A. fetalis* could not grow in the absence of NaCl.

Rheinheimera isolates and A62-14B were positive for starch hydrolysis and utilization of *N*-acetyl glucosamine while *A. fetalis* was negative. All organisms were positive for gelatin and Tween 80 hydrolysis, oxidase, catalase, and negative for presence of urease. Differences were found among organisms in ability to utilize glucose, citrate, maltose and cellobiose. *A. fetalis* and A62-14B were able to reduce nitrate, unlike the *Rheinheimera*. A62-14B, *R. pacifica* and *A. fetalis* shared resistance to penicillin. Both A62-14B and *R. pacifica* were resistant to carbenicillin (25µg), but varied in sensitivity to aminoglycoside antibiotics. A62-14B and *A. fetalis* were susceptible to tetracycline (30µg and 10µg, respectively), *R. pacifica* was resistant (10µg) (Fonnesbech Vogel *et al.*, 2000, Romanenko *et al.*, 2003). Antibiotic sensitivity data were not available for *R. baltica*.

DISCUSSION

Based on phenotypic and genotypic data, seven of the eight isolates: A62-5A, 5B, 6A, 6B, 14A, 15A, and 15B were found to be either of the *C. violaceum* (31532 or 026) baiting strains. All isolates had similar metabolic and fatty acid profiles as well as the same sequence for the ITS region. Since ITS sequence analysis is specific to the strain level, it is clear that the isolates were the same, and likely clones of each other (Jensen *et al.*, 1993). Detection of these bait strains may be attributed to individual cells that were extracted in addition to the supernatant from these organisms. Non-sterile supernatant was placed into the dialysis tubing. After placing the tubing in the river for a given time period, the samples were brought back into the lab and the tubing was gently rinsed in sterile water to eliminate non-adherent cells and debris. The tubing was then cut to release the supernatant, and finally, the tubing was placed into scintillation vials with sterile water to remove the adherent bacteria. In this way, any cells that were in the supernatant would be able to contaminate the final samples from which the isolates were collected. A follow up to the AHL bacterial recruitment experiment was recently performed to address the issue of contamination from the supernatant. The supernatant was sterilized prior to placement into the

dialysis tubing. Controls were also conducted to ensure that the sterilization did not destroy the AHLs, whereby commercially obtained AHLs were added to the sterilized supernatant (McLean *et al.*, unpublished). The results of the follow-up experiment have yet to be fully evaluated.

Contrastingly, isolate A62-14B consistently differed in analyses against the baiting strains. The isolate was not able to utilize substrates in the Biolog GN2 Plate, fatty acid profiles were not similar, and the sequence data from the ITS region clearly distinguished A62-14B from the other isolates, with no sequence similarity to *Chromobacterium* species, but low sequence similarity to *Pseudomonas* and *Vibrio* strains.

Further characterization of isolate A62-14B proceeded in a polyphasic approach to taxonomy (Rossello-Mora & Amann, 2001, Vandamme *et al.*, 1996). In order to classify A62-14B to the species level, a combination of phenotypic, genotypic, and phylogenetic data must be utilized in consensus with the accepted prokaryotic species concept. Determining a species concept for bacteria has been a challenge and continues to be controversial. Aside from asexual reproduction and an evolutionarily insignificant fossil record as compared to eukaryotes, difficulties of microbial characterization include: the size of the organisms which makes them invisible for the naked eye; the lack of distinctive and stable morphological characteristics for most of these organisms; and the difficulty of obtaining most of them in pure culture which is necessary to assess physiological traits (Rossello-Mora & Amann, 2001, Vandamme *et al.*, 1996).

Several studies suggest that only up to 1% of all bacteria can be cultured, thus 99% of bacteria in the environment can not be fully characterized in accordance with accepted protocols for identification (Vandamme *et al.*, 1996). As such, currently, bacterial species could be described as "a monophyletic and genomically coherent cluster of individual organisms that show a high degree of overall similarity in many independent characteristics, and is diagnosable by a discriminative phenotypic property" (Rossello-Mora & Amann, 2001).

The small subunit rRNA gene of A62-14B was sequenced to estimate the phylogenetic affiliations of isolate A62-14B. Comparison of 16S rRNA gene sequence data can reliably assign genus level identity using a 95 % sequence similarity borderline (Ludwig *et al.*, 1998). At 97% sequence similarity or above, organisms can be considered of the same species, a delineation that is considered confirmatory in consensus with other data (Ludwig *et al.*, 1998, Rossello-Mora & Amann, 2001, Stackebrandt & Goebel, 1994). These borderline values are not absolute. Organisms with over 98% sequence similarity have been identified as different species (Stackebrandt & Goebel, 1994). Some organisms, however, have multiple RNA (*rrn*) operons, and microhetergeneity (< 1% to 5%) among the encoded gene sequences can hinder differentiation of species (Nubel *et al.*, 1996, Rainey *et al.*, 1996). In this case, the closest characterized relatives of A62-14B had 96% sequence similarity for the 16S rRNA gene. Both *Rheinheimera* and *Alishewanella* were equally similar to A62-14B. Based on the general criteria of

Ludwig *et al.* (1998), A62-14B can be considered a novel species. Also, according to this criterion, A62-14B could belong to either validly classified genus.

Phylogenetic reconstruction using distance, parsimony, maximum likelihood, and Bayesian methods did differntiate A62-14B from its closest relatives supporting a novel species designation, but did not support genus level association of A62-14B with either *Rheinheimera* species or *A. fetalis.* When different outgroups were used in attempt to resolve these clade issues, the results were the same. Regardless of the distance or relatedness of the out group, the 16S rRNA gene sequence data did not provide sufficient signal to place A62-14B in an existing genus classification, nor did it provide sufficient evidence to designate a novel genus classification for A62-14B. The 16S rRNA gene sequence data did not allow definitive generic classification of A62-14B. Therefore, other taxonomic methods of higher specificity were used to asses whether A62-14B belonged to either of its closest related generic groups or to validate a label of novel genus. As more organisms become available, a re-evaluation of all these taxa may be warranted.

Another genotypic method that can resolve generic relationships involves the determination of DNA base composition, usually reported as mole percent guanine and cytosine (mol% G + C) (Johnson, 1984, Sueoka, 1961, Sueoka, 1962). In bacteria, the mean genomic guanine-cytosine content varies from 25%-75% (Johnson, 1984, Sueoka, 1961, Sueoka, 1962). Phylogenetically related bacteria often have similar percent DNA G + C contents (Muto & Osawa, 1987, Sueoka,

1961, Sueoka, 1962). Studies suggest that related genera are within 10 mol% and same species are within 5 mol% DNA G + C content. Furthermore, the biased mutational pressure seems to affect the entire genome unimodally to various degrees (Muto & Osawa, 1987, Sueoka, 1961, Sueoka, 1962). Comparison of A62-14B with *Rheinheimera* species and *A. fetalis,* resulted in percent DNA G + C values too close to differentiate A62-14B as a novel genus or species. While, the percent DNA G + C content can be useful for confirmatory characterization of bacteria, compositional bias can hinder phylogenetic significance. Organisms may be grouped together purely because of similar numbers of genomic guanine and cytosine rather than by true relatedness, since linear sequence arrangement is not considered with this type of analysis (Foster & Hickey, 1999).

Chemotaxonomic methods capitalize on cell composition and constituents to differentiate among groups of microbes. These analyses include cell wall composition and fatty acid analysis, which can help resolve familial to species level relationships (Vandamme *et al.*, 1996). Lipid profiles can be compared and assessed through similarity indices (Kellogg *et al.*, 1996, Rossello-Mora & Amann, 2001). A drawback of this type of analysis is that all organisms must be cultured under the same conditions for comparison. In fact the accuracy of the system depends on using the recommended medium, incubation temperature, and time of incubation, for profile comparison (Haack *et al.*, 1994, Smibert & Krieg, 1994). Environmental factors can greatly affect the presence and composition of fatty acids (Rossello-Mora & Amann, 2001). Here, all organisms except *R. pacifica,*

were analyzed using private or commercial MIDI Inc. systems. However, isolate A62-14B was grown on TSA at 28 °C for approximately 24 hours; *R. baltıca* was grown on half-strength Marine agar at 28 °C for 24 hours; *A. fetalis* was grown on blood agar at 30 °C for 2 to 4 days; and the growth conditions for *R. pacifica* are not clear at this time. It is also unclear why *A. fetalis* was grown at 30 °C when its optimal growth temperature is 37 °C. The Sherlock standard libraries for fatty acid profile comparison are separated into aerobes, anaerobes, and yeast. These categories are further separated by growth temperature, incubation time, and growth medium. Standard conditions for the growth of aerobes include incubation at 28 °C for 24 hours on TSA. Clinical isolates, such as *A. fetalis*, are grown at 35 °C for 24 to 48 hours on blood agar (Sasser, 2001). Deviations from these standard conditions may account for the variation of predominant fatty acids between *A. fetalis* and the *Rheinheimera* and isolate A62-14B. The changes in growth conditions from the standard conditions may make the comparison of profiles invalid. In addition, since clinical isolate profiles are compared against a different database than general aerobes, changes from standard conditions may increase the inability to validly compare the profiles.

Fatty acid analysis was not conclusive for resolving any level of taxonomic rank for isolate A62-14B. In consideration of the current data, dominant fatty acids for relatives determined by 16S rRNA gene sequence data were relatively similar to A62-14B. In fact, *R. baltica* shared similar percent composition values of its most abundant fatty acids with A62-14B. The main difference among major

fatty acids was the percent abundance of $C_{12\,0}$ 3OH. Of the available fatty acid profiles, dominant fatty acid data suggested that A62-14B was more closely related to the genus *Rheinheimera* than *Alishewanella*.

Familial to intraspecific delineation of prokaryotic organisms can be resolved through the use of phenotypic methods. Phenotypic methods for identification of bacteria are based on morphology, growth conditions, and metabolism (Rossello-Mora & Amann, 2001, Vandamme *et al.*, 1996). These techniques can be effective for general identification of known species, but may prove problematic for uncharacterized species (Muto & Osawa, 1987). Some issues encountered with characterization of unknown isolates with phenotypic testing include variability of assays and expressed phenotypes. Controlled conditions must be maintained as gene expression may be dependent on environmental conditions. In addition, lack of differential traits due to phenotypic homogeneity, can lead to incorrect identification or underestimation of diversity (Erickson *et al.*, 2002, Muto & Osawa, 1987). Even through extensive phenotypic testing, isolates can remain unidentifiable (Erickson *et al.*, 2002). Comparison of physiological traits of isolate A62-14B, *Rheinheimera species*, and *A. fetalis* revealed a number of similarities among the organisms and some key differences. The *Rheinheimera* species and A62-14B were motile and flagellated, while *A. fetalis* was non-motile and lacked flagellae. Both *A. fetalis* and A62-14B were facultatively anaerobic and able to reduce nitrate, while the *Rheinheimera* species were strictly aerobic and unable to reduce nitrate. Variation among all

organisms was observed for a number of substrates, however the *Rheinheimera* and A62-14B were able to utilize *N*-acetyl-glucosamine, for which *A. fetalis* gave negative results. Finally, salinity tolerance ranged from salt-dependent growth and halotolerance, to a rather low salt tolerance for A62-14B. These levels of tolerance correlate well with the environments from which the organisms were isolated. Unfortunately, with key differences in atmospheric survivability conditions, morphology, and ability to reduce nitrate, it is difficult to determine whether A62-14B belongs to either genus. Thus far, only the fatty acid analysis has indicated the association of A62-14B with an existent genus. Phylogenetic reconstruction with rRNA data has shown an association with *Alishewanella* however, the association is essentially not supported. DNA base composition of all the organisms were to similar to differentiate them at any taxonomic level. Phenotypic data has not provided sufficient delineation between the genera of interest and the unknown isolate, and data slightly contradicts the closer association to *Rheinheimera* as suggested by fatty acid analysis.

One method that was not used in the characterization of A62-14B was genomic DNA-DNA hybridization. Species in bacterial systematics are defined as a group of strains, including the type strain, sharing 70% or greater whole genomic DNA-DNA relatedness of strains, with a difference in hybrid melting temperature of 5 °C or less (Stackebrandt & Goebel, 1994, Wayne *et al.*, 1987). DNA-DNA relatedness values are not absolute, especially at the recommended 70% threshold. Therefore, DNA-DNA relatedness can be used as rather artificial

value that provides support for species delineation, but cannot stand alone in its ability to differentiate among closely related species (Stackebrandt, 2003). Studies have found a significant relationship between DNA-DNA relatedness values and 16S rRNA gene sequence similarity values (Stackebrandt & Goebel, 1994). DNA-DNA relatedness values have never been shown to be higher than 60%, below 97% 16S rRNA gene sequence similarity (Stackebrandt & Goebel, 1994, Stackebrandt, 2003). Contrastingly, due to the extremely slow rate of evolution of the small subunit ribosomal gene, above 97% sequence similarity, genomic DNA-DNA relatedness can range from 55 to 100% (Stackebrandt, 2003). Considering that the closest relatives to isolate A62-14B shared approximately 96% 16S rRNA sequence similarity, DNA-DNA hybridization would not provide additional information for the classification of A62-14B. The phylogeny of 16S rRNA data supports a species delineation of A62-14B from *Rheinheimera* and *Alishewanella* species. Key phenotypic differences also help establish A62-14B as a novel species. While DNA-DNA hybridization would solidify the argument for species differentiation of A62-14B, it cannot support a genus level classification.

Tentatively, A62-14B could be proposed to belong to a novel genus. Ideally, the proposal of a novel genus should have more than one species, since the variability of genus-specific characteristics can not be summarized by one isolate (Vandamme *et al.*, 1996). This notion was exemplified in the comparison done in this study. Variation in growth temperature, salt tolerance, and utilization of glucose, arabinose, citrate, and cellobiose and slight differences in

fatty acid profiles were observed between *R. baltica* and *R. pacifica*. These details tend to be overlooked in bacterial taxonomy, simply due to a lack of a better system, as often novel genera are proposed with only one isolated species (Fonnesbech Vogel *et al.*, 2000). Also, results of rRNA data tend to be slightly weighted in respect to other genotypic and phenotypic data. In this case, the rRNA similarity values were outside of species limits but within the accepted borderline for genus designation for both *Rheinheimera* and *Alishewanella*. Phenotypic data suggests that A62-14B possesses a combination of distinguishing phenotypes from each related genera.

Based on the uncertainty of where A62-14B belongs, further research is necessary. First a reevaluation of this group of organisms is needed. In part, this study has accomplished this objective however, laboratory analysis of all of the isolates may provide a better basis for comparison in a controlled in environment. Sequence data may need to be re-examined. Since the relatives of A62-14B share similar sequence similarity and cluster together with high support in phylogenetic analyses, these organisms may be apart of the same genus. An approximately 96% sequence similarity between A62-14B and each of its closest known relatives, is within the limits for genus designation. In fact, *R. baltica* and *A. fetalis* share 94.8% sequence similarity, which is rather close to the borderline value, and *A. fetalis* and *R. pacifica* share 95.4% sequence similarity. *A. fetalis* was the first organism of these organisms to be characterized and identified; *R. baltica* followed, and finally, *R. pacifica. R.baltica* was differentiated as a novel genus

from *A. fetalis* predominantly by 16S rRNA gene sequence comparison, fatty acid profile, pigmentation, salt tolerance, optimal growth temperature, and inability to reduce nitrate. Based on the 16S rRNA gene sequence similarity of these organisms, they appear to belong to the same genus. Clearly, deviation from standard conditions set by MIDI Inc. may account for differences in the fatty acid profiles of *A. fetalis, R. baltica, R. pacifica* and isolate A62-14B. Variation in pigmentation, salt tolerance, and growth temperature are not uncommon among generic species, as exemplified between the *Rheinheimera* species. Furthermore, in *Thermus thermophilus*, studies have shown that the presence of nitrate reductase was responsible for strain specific anaerobic growth. *T. thermophilus* is known to be strictly aerobic, however a strain, *T. thermophilus* HB8, was able to grow anaerobically as consequence of a nitrate reductase gene cluster, which was absent in closely related organisms. In facultative anaerobes, nitrate can induce the production of nitrate reductases in anoxic environments allowing for anaerobic respiration (Ramirez-Arcos *et al.*, 1998). The fact that *A. fetalis* and isolate A62-14B are facultatively anaerobic, may be attributed to the possession of nitrate reductase genes. The *Rheinheimera* were strictly aerobic, and neither species was able to reduce nitrate. While more research is needed for this argument, the difference in oxic needs and ability to reduce nitrate, may not be sufficient data to validate separate generic groupings for these organisms.

The shared and dissimilar characteristics of these organisms illustrate the variability across this potential genus. Increased taxon sampling and

characterization of isolates would help to resolve the relationships among these organisms as well as fill in gaps between the organisms. As it may not be difficult to *discover* new bacterial isolates, local sampling may not provide the needed organisms. Especially, considering the diverse environments from which these organisms were isolated. Other options include the use of genetic makers with rates of evolution higher than that of 16S rRNA gene sequence or genes specific to the genera for differentiation (Ludwig *et al.*, 1998, Rossello-Mora & Amann, 2001). Access to all closely related isolates would be needed, since most of the isolates only have 16S rRNA gene sequence available.

Finally, isolate A62-14B can be introduced as a novel species once the fatty acid profiles are re-evaluated and DNA-DNA hybridization comparisons with *A.fetalis* and the *Rheinheimera* species are performed. Currently, genotypic and phenotypic data do not sufficiently support a full classification of A62-14B. In these cases, it is suggested that organisms be designated with numbers rather than non-meaningful taxonomic classification (Rossello-Mora & Amann, 2001). Thus, it is proposed that isolate A62-14B be designated as Gamma proteobacteria A6214B, and be entered into the GenBank database, so that it may be phylogentically classified when more data becomes available.

APPENDICES

Table 1. Biolog GN2 Plate Results for CV31532, CV026, A62-6A, A62-14A, A62-15B, and C. violaceum (Biolog database strain).

A positive reaction for the use of the substrate is designated by (+), a negative reaction (-), a weak reaction (w). Metabolic profiles were very similar, difference were seen for utilization of cis-aconitic acid, citric acid, itaconic acid, α-ketobutyric acid, α-ketoglutaric acid, α-ketovaleric acid, D,L-lactic acid, and succinamic acid. The following substrates gave negative results and are not listed below·α-cyclodextrin, N-acetyl-D-galactosamine, adonitol, L-arabinose, D-arabitol, D-cellobiose, L-fucose, D-galactose, gentiobiose, m-inositol, α-D-lactose, lactulose, maltose, D-mannitol, D-mannose, D-melibiose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, turanose, xylitol, D-galactonic acid lactone, D-galacturonic acid, D-glucosaminic acid, D-glucuronic acid, γ-hydroxybutyric acid, malonic acid, quinic acid, D-saccharic acid, sebacic acid, glucuronamide, L-pyroglutamic acid, D,L-carnitine, γ-aminobutyric acid, phenylethylamine, putrescine, 2-aminoethanol, and 2,3-butanediol.

Substrate	Isolate CV31532	CV026	A62-6A	A62-14A	A62-15B	*C. violaceum*
Dextrin	+	+	+	+	+	+
Glycogen	+	+	+	+	+	+
Tween 40	+	+	+	+	+	+
Tween 80	+	+	+	+	+	+
N-Acetyl-D-glucosamine	+	+	+	+	+	+

i-Erythritol	w	-	-	-	w	-
D-Fructose	+	+	+	+	+	+
α-D-Glucose	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+
β-Methyl-D-Glucoside	v	w	w	-	-	w
D-Psicose	+	+	+	+	w	+
Sucrose	-	-	-	-	-	w
D-Trehalose	+	+	+	+	+	+
Turanose	-	-	-	-	-	+
Pyruvic Acid Methyl Ester	+	+	+	+	+	+
Succinic Acid Mono-Methyl Ester	+	+	+	+	+	+
Acetic Acid	+	+	+	+	+	+
Cis-Aconitic Acid	+	w	w	w	w	w
Citric Acid	+	w	-	-	+	w
Formic acid	+	+	+	+	+	w
D-Gluconic Acid	+	+	+	+	+	+
α-Hydroxybutyric Acid	+	w	+	+	+	+
β-Hydroxybuturic Acid	+	+	+	+	+	+
p-Hydroxyphenylacetic Acid	w	-	-	-	-	-
Itaconic Acid	+	w	w	w	w	-
α-Ketobutyric Acid	+	-	-	w	+	+
α-Ketoglutaric Acid	+	-	+	w	w	w
α-Ketovaleric Acid	+	-	w	w	+	w
D,L-Lactic Acid	+	-	+	-	+	+
Propionic Acid	+	+	+	+	+	+
Succinic Acid	+	+	+	+	+	+
Bromosuccinic acid	+	+	+	+	+	+
Succinamic Acid	+	-	w	+	w	+

L-Alaninamide	+	w	w	+	+	+
D-Alanine	+	+	+	+	+	+
L-Alanine	+	+	+	+	+	+
L-Alanyl-Glycine	+	+	+	+	+	+
L-Asparagine	+	+	+	+	+	+
L-Aspartic Acid	+	+	+	+	+	+
L-Glutamic acid	+	+	+	+	+	+
Glycyl-L-Aspartic Acid	+	+	+	+	+	+
Glycyl-L-Glutamic Acid	+	+	+	+	+	+
L-histidine	+	+	+	+	+	+
Hydroxy-L-Proline	+	+	+	+	+	+
L-Leucine	+	+	+	+	+	+
L-Ornithine	+	+	+	+	+	+
L-phenylalanine	+	+	+	+	+	+
L-Proline	+	+	+	+	+	+
D-Serine	+	+	+	+	+	+
L-Serine	+	+	+	+	+	+
L-threonine	+	+	+	+	+	+
Urocanic Acid	+	+	+	+	+	+
Inosine	+	+	+	+	+	+
Uridine	+	+	+	+	+	+
Thymidine	+	+	+	+	+	+
Glycerol	+	+	+	+	+	+
D,L,α-Glycerol Phosphate	+	+	+	+	+	+
α-D-Glucose-1-Phophate	+	+	+	+	+	+
D-Glucose-6-Phosphate	+	+	+	+	+	+

Table 2. Fatty Acid Profiles for CV026, CV31535, A62-6A, A62-15B, and A62-14B

Peak Name	CV31532	CV026	A62-6A	A62-15B	A62-14B
10:0	NP	NP	NP	NP	1.62
11:0	NP	NP	NP	NP	0 32
10:0 3OH	3.30	3.34	3.42	3.57	0 71
11 0 3OH	NP	NP	NP	NP	1.30
12:0	3.64	3.81	3 88	3.95	2.03
12:0 2OH	1.96	1.91	1 98	2.09	NP
12:0 3OH	3.34	3.25	3 22	3.00	12.83
12:0 iso 3OH	NP	NP	NP	NP	0.27
13 0 3OH/15·1-Iso H	NP	NP	NP	NP	0.26
14:0	2.32	2.65	2.59	2 73	1.54
14:0 3OH/16:1-iso I	NP	NP	NP	NP	0.46
14.1 w5c	NP	NP	0.14	NP	NP
15:0	0.99	0.95	0.78	0.86	0.73
15.0 anteiso	NP	NP	NP	NP	0.32
15:1 w6c	0 20	0.19	0.14	NP	0.21
15.1 w8c	NP	NP	NP	NP	1.05
16.0	27.59	27.78	27 79	27.44	19.04
16:0 iso	NP	NP	NP	NP	0.50
16:1 w7c/w6c	40.08	40.54	40 48	41.36	38.56
16:1 w5c	0 39	0 41	0.41	0.42	NP
17.0	0.21	0.20	0.18	0.17	1 45
17:0 anteiso	NP	NP	NP	NP	0.84
17:0 iso	NP	NP	NP	NP	0 16
17:1 w6c	0.35	0.34	0 29	0 33	NP
17.1 w8c	0 19	0.17	0 15	0 17	3.75
18:0	0.32	0.29	0.29	0 27	2.01
18·1 w7c	15.12	14.16	14.28	13.64	7.70
18:1 w9c	NP	NP	NP	NP	0 29

* NP= not present.

Table 3. Sequences for the 16S-23S rRNA gene intergenic spacer region for experimental isolates and the bait strains (CV026 and CV3153).

CV026 ITS sequence
TAT CGG TTA TTC GCG TTA AGG GCA GCT GGA TCG AAG AGA TTC AGT GCT GAG AAG TTG ACT GGG TTT GTA GCT CAG CTG GTT AGA GCA CTG TGT TGA TAA CGC AGG GGT CGT AGG TTC GAG TCC TAC CAG ACC CAC CAG TTG GGG GAT TAG CTC AGT TGG GAG AGC ACC TGC TTT GCA AGC AGG GGG TCG TCG GTT CGA TCC CGT CAT CCT CCA CCA CTT ACA GTG CAA ACA AAA ACG AAC TTG ATC GAG TTT GAT TCT GTT TGC GTT GTT CGC GTG CAG CGA AAG CTG CTC AGC AAT ACG CCC GAT CTT TAA CAA ACT GAA GAA GCC GAA TAT ATT AAG ACG GCG AAA CAA ACA GCG AGA GTT AAG TCT TTC GTT GAA TGA ATC GTC ATC TTG GGT ATT TGA TTG TAT CAA AGG CTG CGT CGC CAT ATC AAA AGG GGC GGT GCA GTC GTC GCA C
CV31532 ITS sequence
GGC TGG ATC ACC TCC TTT CTA GAG AAG GCG ATC GCC AAG CAC TTA CAG CCT ATC GGT TAT TCG CGT TAA GGG CAG CTG GAT CGA AGA GAT TCA GTG CTG AGA AGT TGA CTG GGT TTG TAG CTC AGC TGG TTA GAG CAC TGT GTT GAT AAC GCA GGG GTC GTA GGT TCG AGT CCT ACC AGA CCC ACC AGT TGG GGG ATT AGC TCA GTT GGG AGA GCA CCT GCT TTG CAA GCA GGG GGT CGT CGG TTC GAT CCC GTC ATC CTC CAC CAC TTA CAG TGC AAA CAA AAA CGA ACT TGA TCG AGT TTG ATT CTG TTT GCG TTG TTC GCG TGC AGC GAA AGC TGC TCA GCA ATA CGC CCG ATC TTT AAC AAA CTG AAG AAG CCG AAT ATA TTA AGA CGG CGA AAC AAA CAG CGA GAG TTA AGT CTT TCG TTG AAT GAA TCG TCA TCT TGG GTA TTT GAT TGT ATC AAA GGC TGC GTC GCC ATA TCA AAA GGG GCG GTG CAG TCG TC
A62-5A, 5B, 6A, 6B, 14A, 15A, and 15B shared the same ITS sequence.
CTG GAT CAC CTC CTT TCT AGA GAA GGC GAT CGC CAA GCA CTT ACA GCC TAT CGG TTA TTC GCG TTA AGG GCA GCT GGA TCG AAG AGA TTC AGT GCT GAG AAG TTG ACT GGG TTT GTA GCT CAG CTG GTT AGA GCA CTG TGT TGA TAA CGC AGG GGT CGT AGG TTC GAG TCC TAC CAG ACC CAC CAG TTG GGG GAT TAG CTC AGT TGG GAG AGC ACC TGC TTT GCA AGC AGG GGG TCG TCG GTT CGA TCC CGT CAT CCT CCA CCA CTT ACA

GTG CAA ACA AAA ACG AAC TTG ATC GAG TTT GAT TCT GTT TGC GTT GTT CGC GTG CAG CGA AAG CTG CTC AGC AAT ACG CCC GAT CTT TAA CAA ACT GAA GAA GCC GAA TAT ATT AAG ACG GCG AAA CAA ACA GCG AGA GTT AAG TCT TTC GTT GAA TGA ATC GTC ATC TTG GGT ATT TGA TTG TAT CAA AGG CTG CGT CGC CAT ATC

A62-14B ITS sequence

TTG GAT CAC CTC CTT ACC AAA AGC GAC ATT CTT TAA CGA AGT GTC CAC ACA GAT GAT TGA TTG TGA TGT AGA GCA AAC AGT AAG AAC CAT TGC GGT TAC GGC TGG TCT GTA GCT CAG GTG GTT AGA GCG CAC CCC TGA TAA GGG TGA GGT CGG TAG TTC GAG TCT ACT CAG ACC AAC CAA TAT CGT CGA TAT GGG GTT ATA GCT CAG CTG GGA GAG CGC CTG CCT TGC ACG CAG GAG GTC AGC GGT TCG ATC CCG CTT AAC TCC ACC ATT CCT TAG GAA TAA ATA CTG TTT GAG TTC AAA TCA GAA TGC ACT GCG GTG TGT TGT GAT TTG AGC TTT ACG CTC ATA CTG CTC TTT AAC AAT TAG GCA AGC TGA TAG AAA GTA AAA AAA CAA GGT AAA CAA TGT TTA TTG TGA ACC TTA ATC ACA ACG TAC GGC ACC AAA GAC AAT TTG GGG TTG TAT GGT TAA GTG ACT AAG CGT ACA CGG T

Table 4. Partial 16S ribosomal RNA gene sequence for isolate A62-14B

A62-14B 16S rRNA gene sequence (1447 nt)

GGCCTAACACATGCAAGTCGAGCGGGGTTTTCGGACCTAGCGGCGGACG
GGTGAGTAATGCGTAGGAAGCTACCCGATAGAGGGGGATACCAGTTGGA
AACGACTGTTAATACCGCATAATGTCTACGGACCAAAGTGTGGGACCTTC
GGGCCACATGCTATCGGATGCGCCTACGTGGGATTAGCTAGTTGGTGAGG
TAATGGCTCACCAAGGCGACGATCCCTAGCTGGTTTGAGAGGATGATCA
GCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGT
GGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGT
GTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTAGGGAGGAAGGGT
GTTGTGTTAATAGCACAGCATTTTGACGTTACCTACAGAAGAAGCACCGG
CTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAAT
CGGAATTACTGGGCGTAAAGCGCACGTAGGCGGTTTTTTAAGTCAGATGT
GAAAGCCCCGGGCTCAACCTGGGAATTGCATTTGAAACTGGAAAACTAG
AGTGTGTGAGAGGGGGGTAGAATTCCAAGTGTAGCGGTGAAATGCGTAG
AGATTTGGAGGAATACCAGTGGCGAAGGCGGCCCCCTGGCACAACACTG
ACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGT
AGTCCACGCCGTAAACGATGTCTACTAGCTGTTCGTGACCTTGTGTCGTG
AGTAGCGCAGCTAACGCATTAAGTAGACCGCCTGGGGAGTACGGTCGCA
AGATTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGC
ATGTGGTTTAATTCGACGCAACGCGAAGAACCTTACCTACTCTTGACATC
TACAGAAGACTGCAGAGATGCGGTTGTGCCTTCGGGAACTGTAAGACAG
GTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCC
GCAACGAGCGCAACCCTTATCCTTAGTTGCCAGCACGTTATGGTGGGAAC
TCTAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGACGACGTC
AAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGTAT
GTACAGAGGGAGGCAAGCCTGCGAGGGTGAGCGGATCTCTTAAAGCATA
TCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGC
TAGTAATCGCAAATCAGAATGTTGCGGTGAATACGTTCCCGGGCCTTGTA
CACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGC
TTAACCTTCGGGAGGGCGCTTACCACTTTGTGATTCATGACTGGGGTGAA
GTCGAACAAGGTAAC

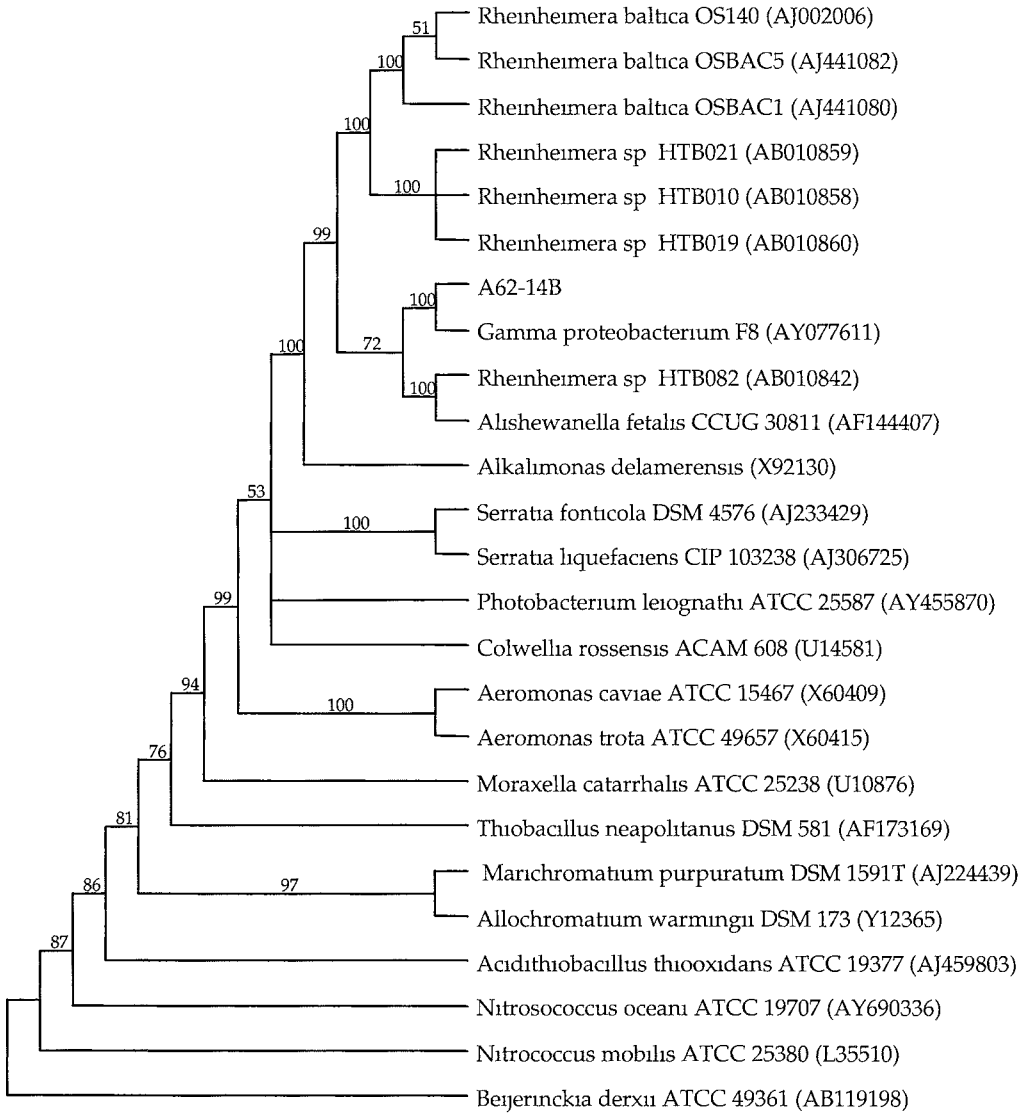

Figure 1. Neighbor joining phylogeny of A62-14B using the K2P distance correction algorithm created in PAUP* 4b10. Percent bootstrap values (2500 replicates) are shown above the branches. *Beijerinkia derxii* has been designated as the outgroup.

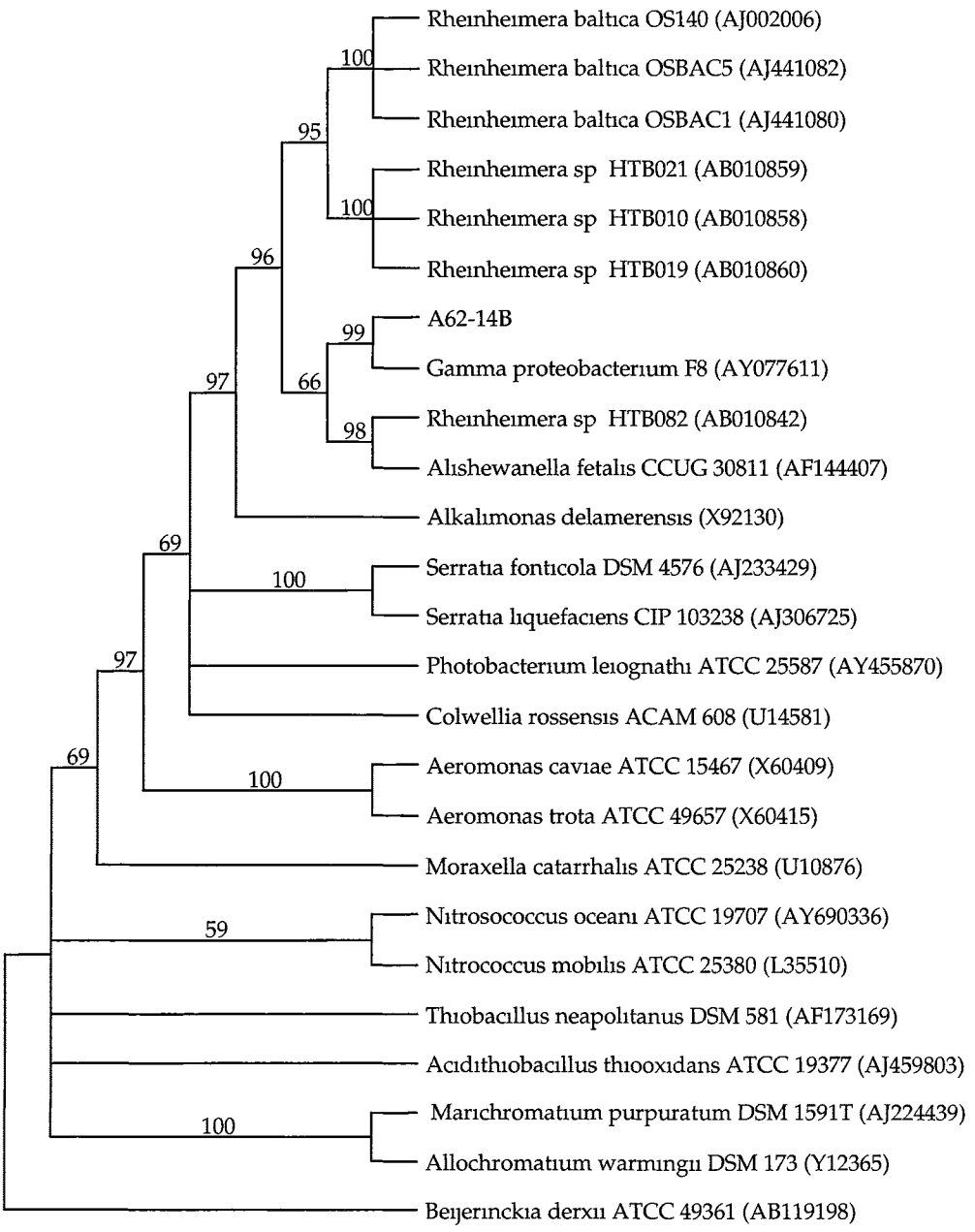

Figure 2. A heuristic search for the maximum parsimony phylogeny of A62-14B resulted in six most parsimonious reconstructions. The bootstrap search was performed with 100 random sequence addition replicates and 1000 bootstrap replicates. Percent bootstrap values are shown above the branches. *Beijerinkia derxii* has been designated as the outgroup.

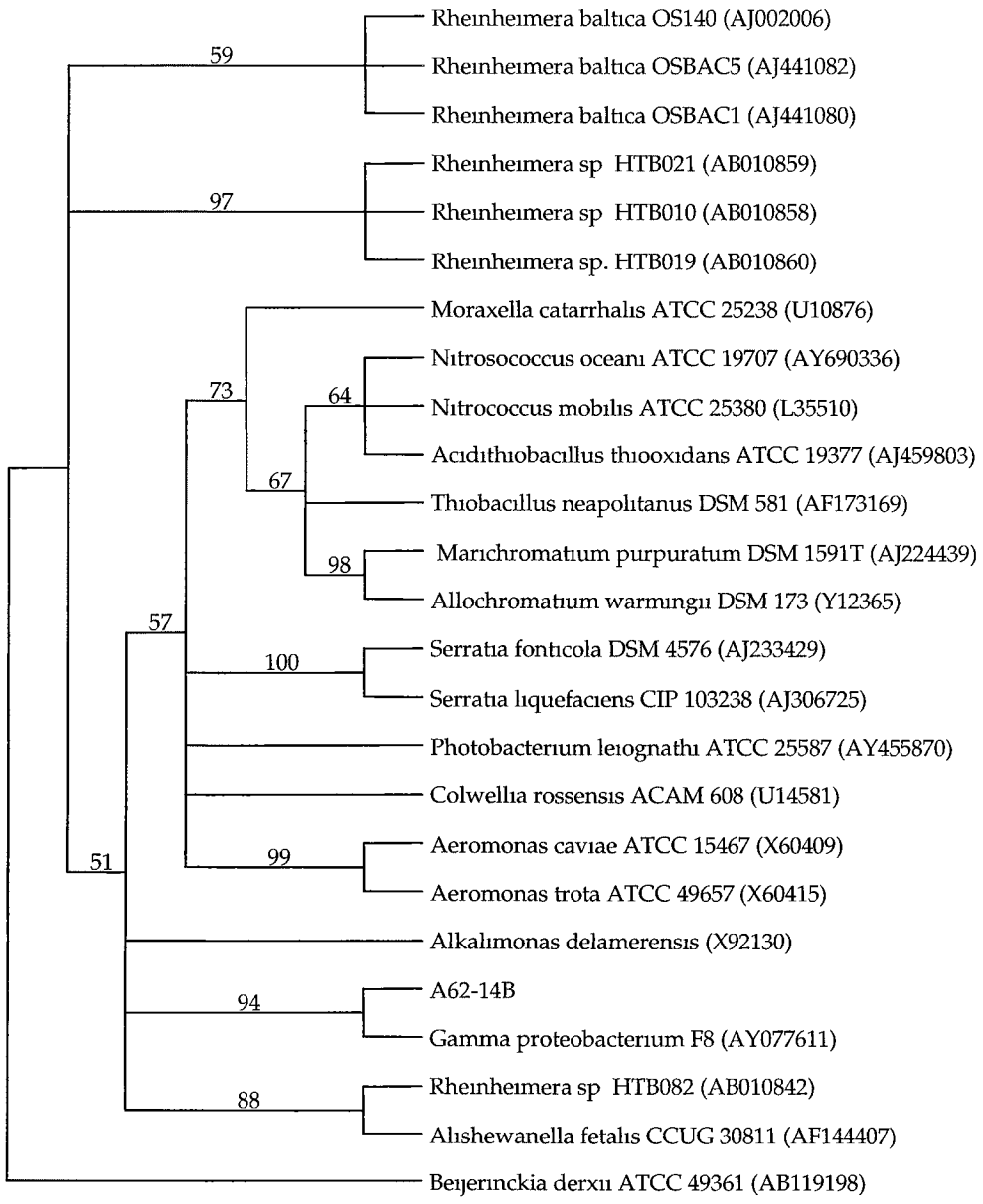

Figure 3. Maximum likelihood phylogeny of A62-14B. The maximum likelihood search was initiated from the heuristic MP search. The model of evolution used was GTR+I+G (I = 0.2305; and g = 0.3418) determined via Modeltestv3.5. Percent bootstrap values, from one random sequence addition for each of 492 replicates, are shown above the branches. *Beijerinkia derxii* has been designated as the outgroup.

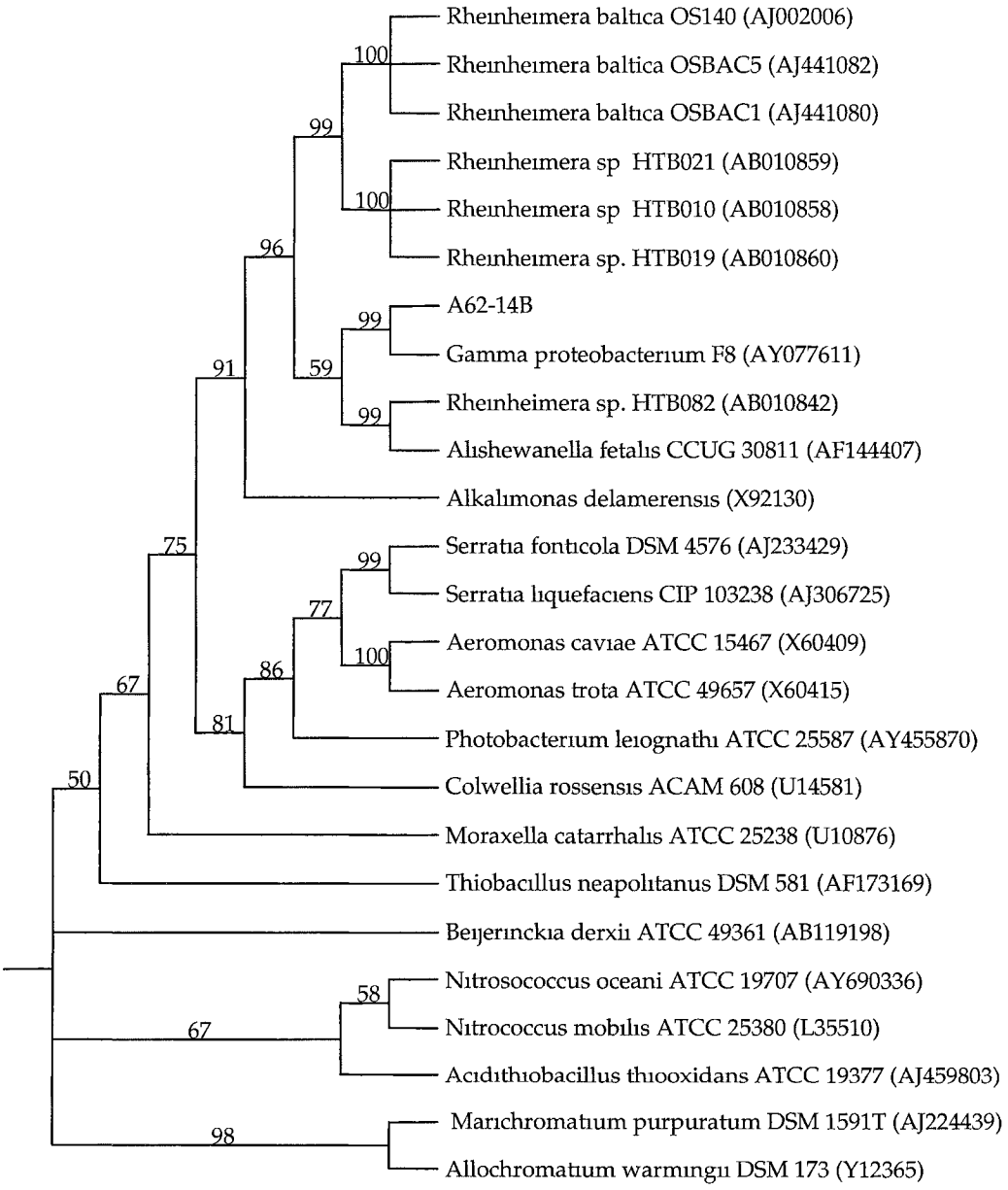

Figure 4. Bayesian phylogeny of A62-14B. A majority rule consensus tree was constructed in PAUP* 4b10 using a 30,000 *a priori* burnin value. Consensus values are shown above the branches. *Beijerinkia derxii* has been designated as the outgroup.

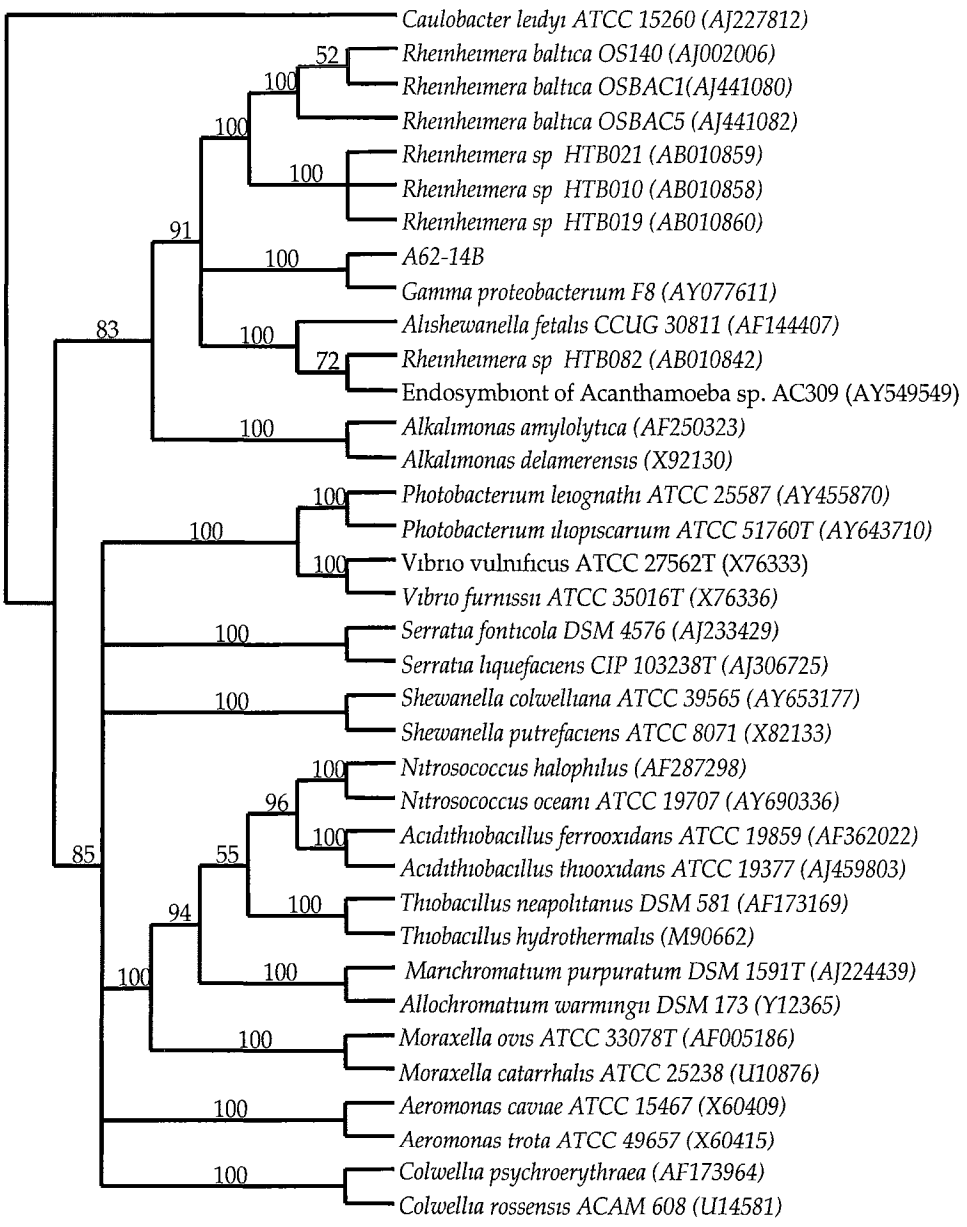

Figure 5. Neighbor joining phylogeny of A62-14B using the K2P distance correction algorithm. Percent bootstrap values (2500 replicates) are shown above the branches. *Caulobacter leidyi* has been designated as the outgroup. A number of outgroups were tested to improve the resolution of the clade of interest. Truncated forms of those phylogenies are shown in following figures. This is an example of the full analysis. Relationships of organism outside the clade of interest (A62-14B) remained relatively stable.

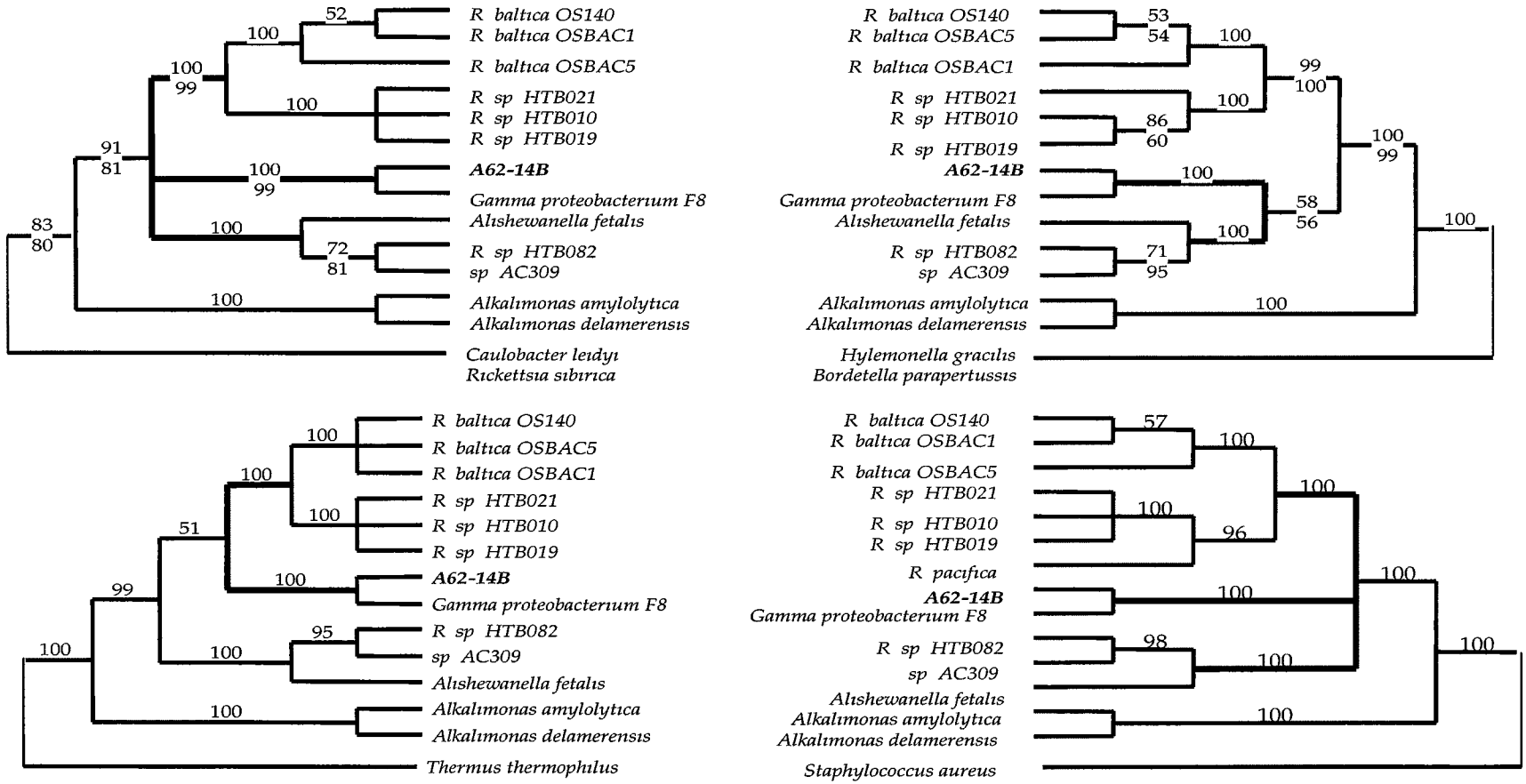

Figure 6. Neighbor joining phylogenies with K2P distance correction for isolate A62-14B using various outgroups. The grouping of A62-14B was not well supported for *Alishewanella* or *Rheinheimera* clades with bootstrap values below the accepted threshold of 70 percent. Tree branch values indicated correspond to percent bootstrap values (2500 replicates); top and bottom values correspond to the respective outgroup listed. Only the clade of interest is represented; see Figure 5 for full set of organisms used in these analyses.

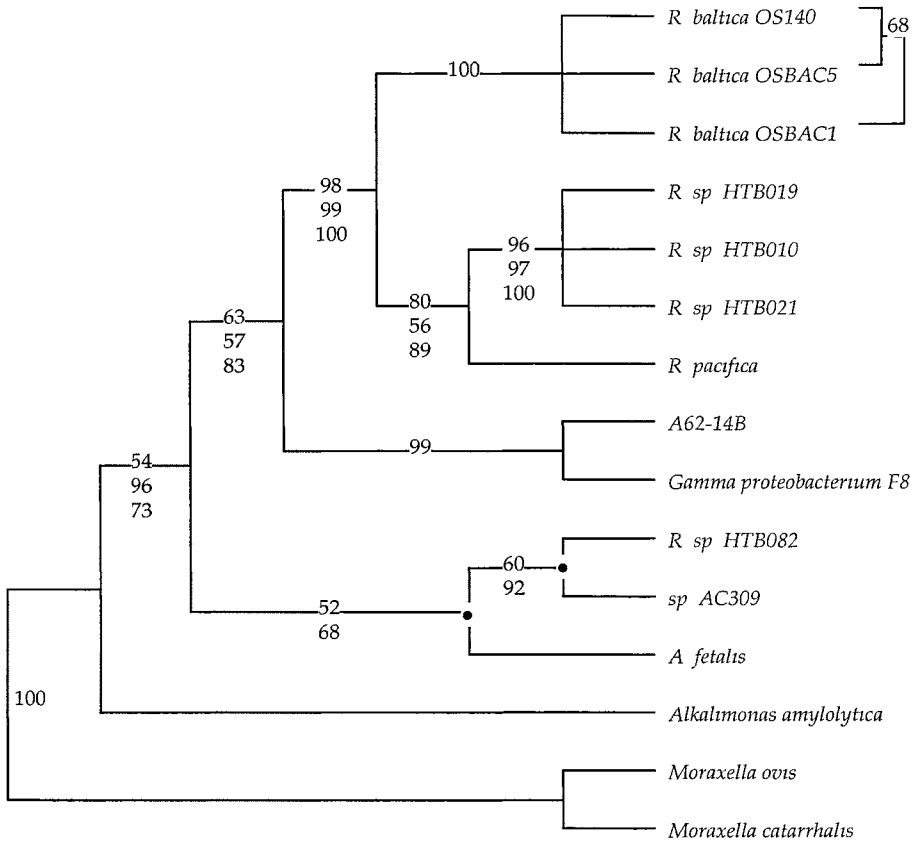

Figure 7. A62-14b phylogeny with *Moraxella* outgroups. Two models of evolution were suggested by ModelTestv3.5 and MrModeltest2.2, TrN+I+G (g= 0.6431; i= 0.4870) and GTR+I+G (g= 0.5918; i= 0.5595). Bootstrap values for ML, parsimony, and distance (K2P) are listed from top to bottom. Minimal differences existed between models, as observed in Figure 8. • = not supported by distance method. Branches opposite to the topology highlight differences in the distance topology.

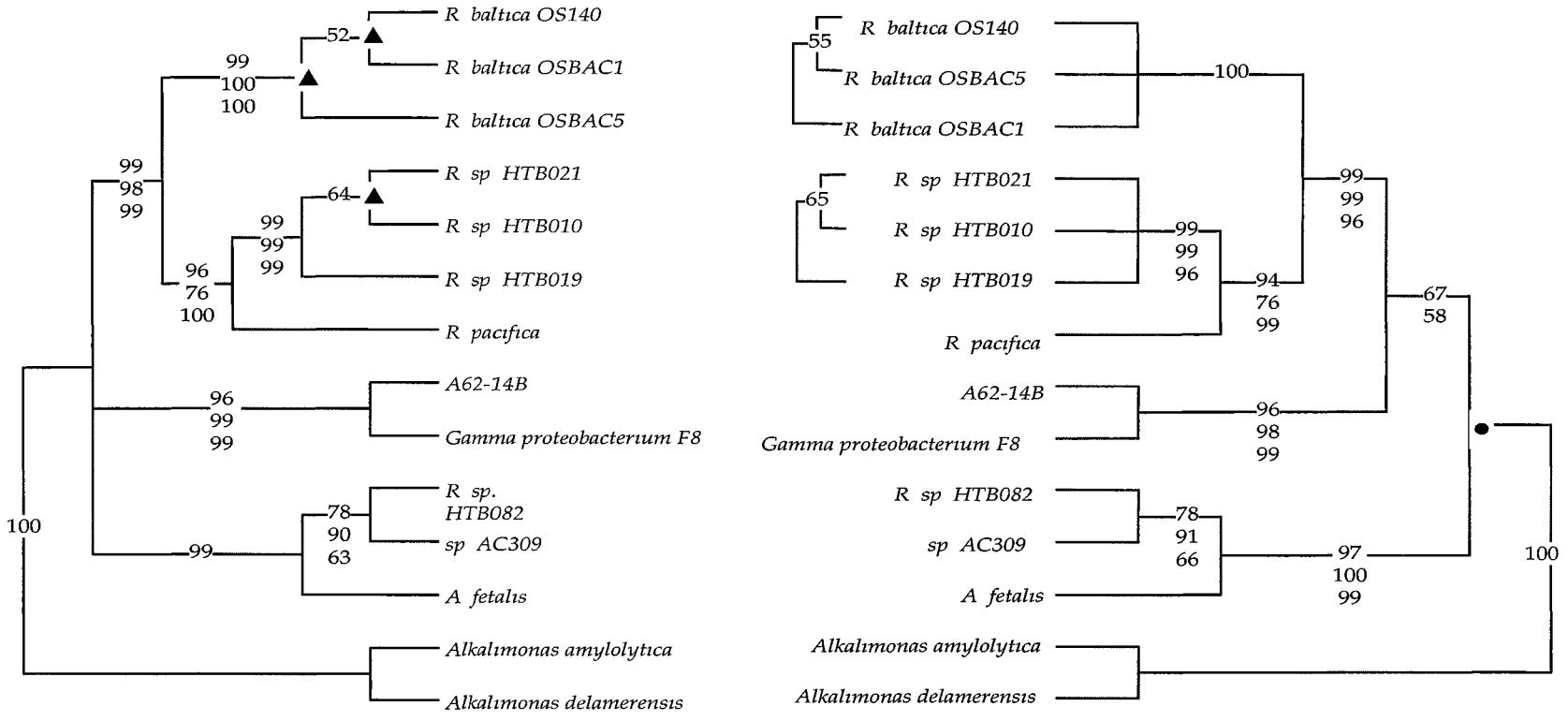

Figure 8. A62-14b phylogeny with *Alkalimonas* outgroups. Two models of evolution were suggested by ModelTestv3.5 and MrModeltest2.2. On the right TrN+I+G (g= 0.9856; i= 0.6745) and to the left HKY+I+G (g= 1.0767; i= 0.6900). Bootstrap values for ML, parsimony, and distance (K2P) are listed from top to bottom. Minimal difference exists between models. ▲= not supported by ML or parsimony; • = not supported by distance. Extra branches on the opposite side of the main tree highlight topology differences of the distance tree.

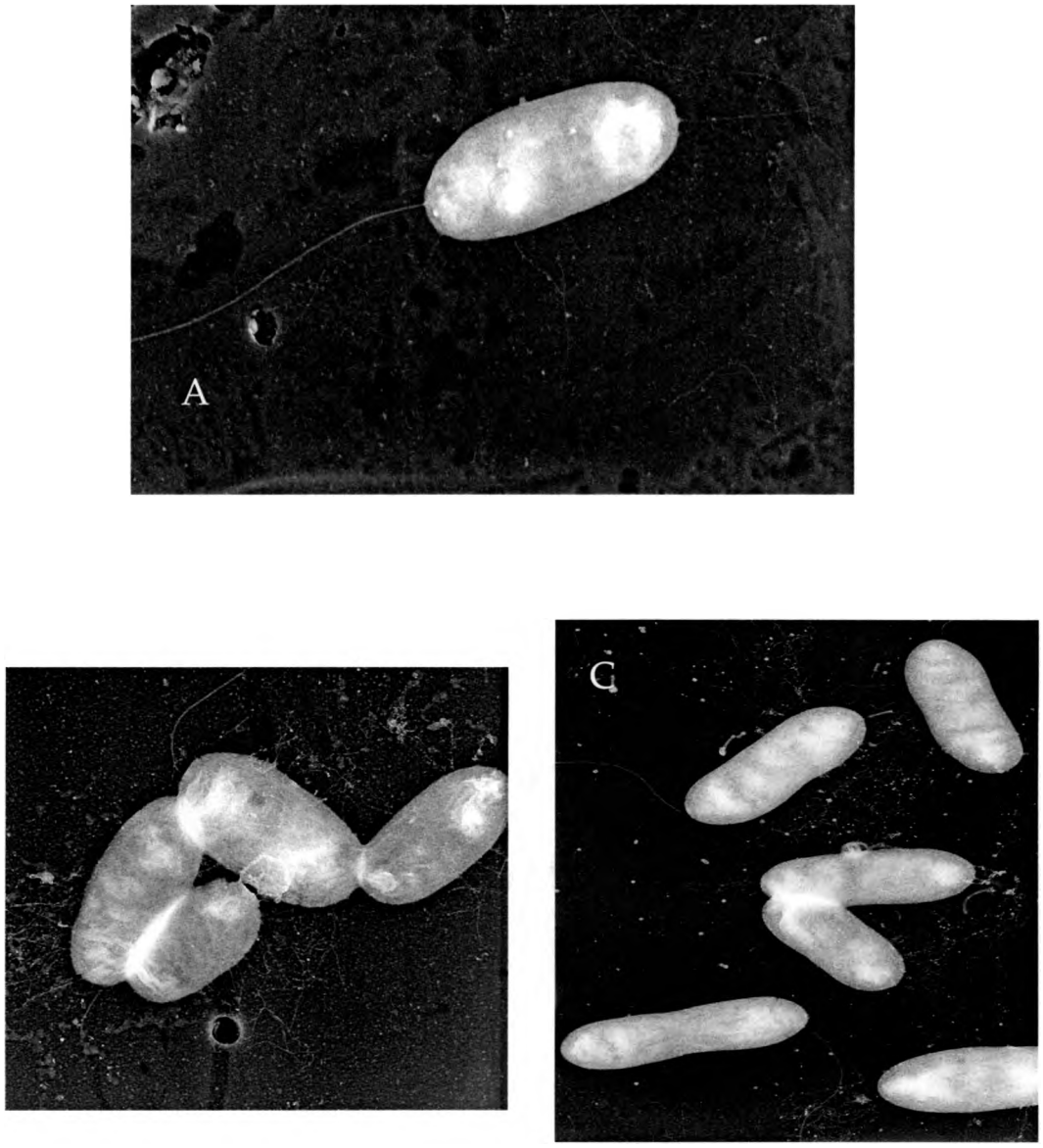

Figure 9. Electron micrographs of A62-14B. A 1% uranyl acetate negative stain was used to visualized the organisms. A) Singular polar flagellum. B & C) Intracellular network of filaments forms among bacteria in close proximity after 24 hour incubation on R2A agar.

Table 5. Phenotypic characteristics of isolate A62-14B and the related bacteria *Rheinheimera baltica* DSM 14885, *Rheinheimera pacifica* KMM 1406, and *Alishewanella fetalis* CCUG 30811.

A positive result is designated by (+), negative (-), weak reaction (w), and ND= not determined. Data are from Brettar et al. (2002), Romanenko et al. (2003), and Fonnesbech-Vogel et al. (2000)

	A62-14B	***R. baltica***	***R. pacifica***	***A fetalis***
	Facultatively anaerobic	Strictly aerobic	Strictly aerobic anaerobic	
Pigment	none	blue	none	none
Size				
(l)	1 25-2 5µm	1 8-2 0µm	0 9-4 5µm	2 0µm
(d)	0 7-0 8µm	0 5-1 5µm	0 6-0 8µm	0 5-1 0µm
Temp °C				
4	-	+	+	-
25	w	+	+	w
37	+	-	+	+
42	-	-	-	+
NaCl (% w/v)				
0	+	+	+	-
3	-	+	+	ND
6	-	-	+	+
8	-	-	+	+
Starch Hydrolysis	+	+	+	-
Gelatin Hydrolysis	+	+	+	+
Tween 80 Hydrolysis	+	+	+	+
Oxidase	+	+	+	+
Catalase	+	+	+	+
Urease	-	-	-	-
Nitrate Reduction	+	-	-	+
Glucose	+	+	-	-
L-Arabinose	+	-	+	-
N-Acetyl-Glucosamine	+	+	+	-
Citrate	-	-	+	-
Maltose	-	+	+	w
Cellobiose	+	+	-	ND
G + C content (mol%)	48 2	48 9	49 6	50 6
Major Fatty Acids	C16 1ω7c C16 0 C12 0 3OH C18 1ω7c	C16 1ω7c C16 0 C18 1ω7c	C16 0 C17 0 C16 1ω7c	C17 1ω8c C16 1ω7c C17 0

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