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DOE Contract No. **DE-AC09-96SR18500**

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LIST OF KEY WORDS

Chlorinated ethenes, groundwater, 16S rDNA phylogenetic analysis, BIOLOG, PLFA.

ABSTRACT

Molecular (rDNA), phospholipid fatty acid analysis (PLFA), and substrate utilization (BIOLOG) techniques were used to assess structural and functional differences between groundwater microbial communities from a chlorinated-ethene (CE)-contaminated landfill. Prokaryotic cells were collected from pristine (LFW 43B) and CE-contaminated (LFW 62D) groundwater monitoring wells on 0.2 micron filters, DNA was extracted from the filters, and libraries were prepared. For well LFW 43B, 26 clones were examined by sequencing and restriction endonuclease patterns, and all were found to be closely related to *Pseudomonas gessardii* and *P. libaniensis*. For well LFW 62D, 40 bacterial clones were examined, and 17 ribotypes were found including representatives of type I and II methylotrophs, *Pseudomonas* spp., *Zoogloea* spp., and other proteobacteria. In an archaeal library from well LFW 62D, all 15 of the clones examined were nearly identical and possessed about 89 % sequence similarity to *Cenarchaeum symbiosum*. PLFA analysis revealed that the communities from contaminated groundwater contained primarily gram-negative bacteria, as indicated by the predominance of the biomarker 16:1w7c. The bacteria were in the stationary growth phase as indicated by the abundance of cyclopropyl fatty acids cy17:0 and cy19:0 and their respective precursors 16:1w7c and 18:1w7c. Further, PLFA ratios for 16:1w7t/16:1w7c and 18:1w7t/18:1w7c were greater than 0.1, indicative of increased cellular membrane permeability. Using BIOLOG GN plates, a similar number of substrates were utilized (>0.1 OD590nm) in LFW43B (72) and LFW 62D (63) communities, even though inoculum densities were 2-orders of magnitude greater in LFW 62D. The combination of non-selective characterization techniques was useful to further our understanding of CE-contamination on groundwater microbial communities.

INTRODUCTION

The chlorinated ethenes (CE) tetrachloroethylene (PCE) and trichloroethylene (TCE) are some of the most widely used cleaning and degreasing solvents used in the United States (12). The use and early disposal practices led to CE being among the most frequently-found contaminants at hazardous waste sites. Although degradation of CE by select pure cultures and defined consortia is now well understood, subsurface microbial processes in the complex environment of landfill leachates involve mixtures of compounds and a diversity of microorganisms (37, 40). Groundwater studies have proven that a higher microbial diversity is advantageous for CE bioremediation (25). The diversity and associated subsurface microbial community structure is dependent on key environmental factors including nutrients, groundwater quality, and geological considerations (42).

The sanitary landfill or Non-Radioactive Waste Disposal Facility (NRWDF) at the Savannah River Site (SRS) in Aiken, SC, received sanitary waste, construction material, as well as rags soaked with PCE and TCE for over 20 years (41). Quarterly groundwater monitoring (starting in 1984) of NRWDF leachate indicated PCE, TCE, and microbial transformation products including cis-dichloroethylene (cDCE) and vinyl chloride (VC); however, no other chlorinated ethenes were known to have been disposed of at the NRWDF. The groundwater monitoring data provides convincing evidence that monitored natural attenuation (MNA) of chlorinated ethenes has occurred via reductive dechlorination, including detection of VC down gradient from the PCE/TCE plume. Sections of the NRWDF subsurface are aerobic and others anaerobic. It is now understood that complete biodegradation of CE may occur under aerobic (9), cometabolic (5), or anaerobic (24) conditions given the optimal microbial populations and environmental parameters. Recent microcosm studies with NRWDF groundwater demonstrated that low redox conditions were established but methanogenesis or anaerobic dechlorination was not observed (16). This work suggested a nutrient deficiency, inadequate inoculum, or an inhibitory contaminant such as heavy metals may have contributed to the microcosm inactivity.

We incorporated several direct microbiological analyses to better understand what role microorganisms play in the transformation of PCE and TCE in the NRWDF subsurface. These analyses are part of a larger bioremediation campaign that is currently under consideration as a potential NRWDF clean-up strategy. We hope to better understand the dynamics of environmental microbiology in the NRWDF subsurface and how it relates to the potential degradation and/or metabolism of chlorinated ethenes. The current MNA campaign also includes analysis of physical factors related to remediation of the NRWDF, such as sorption, dilution, and volatilization (39).

In situ subsurface biodegradation is more difficult to study because the subsurface environment is inherently complex and is composed of a diverse microbial community. An *in situ* optimization test in the NRWDF has proven that gaseous nutrient injection stimulates subsurface CE bioremediation by selective biostimulation of methanotrophic bacteria (6). There is presently a biosparge system in operation at the landfill consisting of 400 ft and 600 ft horizontal injection wells (8). Through application of the fatty methyl ester (FAME) technique it was found that the groundwater microbial communities in NRWDF individual wells did not vary over a 6-week interval (18). In contrast, marked differences in both abundance and composition of FAMEs were observed in profiles from spatially distinct wells located 300 m apart.

Because our understanding of the microbial component of the NRWDF subsurface is limited, we sought to provide detailed information about this microbial community using a combination of rapid direct analytical techniques that could discriminate differences between groundwater microbial communities. We also sought to use these data to identify bacteria that have been shown to be capable of the biodegradation of PCE and TCE. Further, we plan to use this research as the foundation for an ongoing bioremediation campaign at the NRWDF.

We report the molecular, PLFA, and metabolic evidence to support the fact that microbial diversity was higher in the CE-contaminated well as compared to the pristine well. Integrated investigations of specific site parameters and microbial community dynamics are needed as a basis for environmental restoration efforts. The microbial characterization is essential for the eventual remedial technology selection. We hypothesize that the NRWDF leachate provides multiple substrates to enhance the

microbial diversity of the groundwater, as we observed at the contaminated well LFW 62D.

MATERIALS AND METHODS

Determination of groundwater microbial biomass and geochemistry. Groundwater was obtained from two permanent groundwater-monitoring wells within the NRWDF located at the Department of Energy Savannah River Site (Aiken, South Carolina, USA). Samples were collected on March 16 and 18, 1999, from monitoring well LFW43B, located in a pristine area with no history of contamination, and from monitoring well LFW62D, located within a chlorinated-ethene solvent plume one-half mile down gradient from LFW 43B (Figure 1). Approximately six well volumes (70L) were purged prior to collecting 15-L samples in autoclaved polycarbonate bottles. Groundwater parameters (temperature, dissolved oxygen, turbidity, conductivity, alkalinity) were measured in the field using precalibrated meters (YSI Inc., Models 6820 and 380, Yellow Springs, Ohio). Samples were stored at 4°C prior to analysis. Gas and ion chromatography analysis was performed as previously described (6). Aliquots (100 mL) of the groundwater were used for microbiological and pH analysis within 4 hours of collection.

Total heterotrophic bacteria densities were determined by serially diluting 1-mL aliquots of groundwater samples in sterile phosphate buffer, spreading the suspension (0.1 mL) on 1% peptone-trypticase-yeast-extract-glucose medium, and incubating for one week at room temperature (2). Acridine Orange Direct Count (AODC) was used to determine total bacterial densities. Total methanotrophic bacteria densities were determined with direct fluorescent antibodies (DFA) as previously described (7).

Molecular analysis. A 10-liter aliquot of groundwater was sequentially passed through 2.7, 1.0, 0.7, and 0.2 μm pore-size cellulose filters (Whatman, England). The 0.2 μm filters contained the highest density of cells (>100 cells/field). Therefore, these filters were utilized in subsequent analyses. The filters were stored at –70 °C for 1 week prior to processing. Filters were crushed with a sterile spatula in a sterile centrifuge tube, combined with lysis solution [2 ml of 0.15M NaCl, 0.1M NaEDTA (pH 8.0), 1.5 mg / ml

of chicken egg white lysozyme (73,000 units/mg protein) and 25 mg/ml PVPP], mixed briefly, and incubated at 37 °C for 1 hour and mixed every 15 minutes. Aliquots (0.5 mL) were transferred to 1.5 ml microfuge tubes and combined with 400 µl of bead-beating solution [0.1M NaCl, 0.5M Tris-HCl (pH 8.0), 10% SDS] and 0.1 g of 0.1 mm glass; the samples were shaken (Turbo Mixer; Scientific Industries, Inc., Bohemia) for 3 minutes and microcentrifuged at maximum speed for 3 minutes. The aqueous phase was transferred to a new tube and extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v:v:v; pH 8.0) followed by extraction with an equal volume of chloroform:isoamyl alcohol (24:1, v:v; pH 8.0). The DNA was precipitated with a 0.6 volume of isopropanol at -20°C overnight; the nucleic acid was collected by centrifugation and the remaining solvent was evaporated under vacuum. The nucleic acid was resuspended in 50µl distilled and deionized H_2O with 1µl RNAase solution [0.5 units/µl DNAse-free RNAse A from bovine pancreas (Boehringer Mannheim, Indianapolis), 10mM Tris-Cl (pH 7.5) and 15 mM NaCl] and incubated at 37° C for 1 hour. A Wizard DNA Cleanup kit (Promega, Madison) was used to further purify the DNA prior to PCR. Each filter yielded approximately 0.5 µg of DNA in 50 µl.

PCR and Cloning. The DNA from each sample was subjected to two PCR amplification reactions. The 16S ribosomal DNA was PCR amplified using Ready-To-Go PCR Beads (Pharmacia Biotech, Piscataway, NJ). In the first reaction, the bacterial primer 27f, 5'-AGA GTT TGA TCM TGG CTC AG-3' and the universal primer 1392r, 5'-ACG GGC GGT GTG TRC-3' were used (Lane, 1991). In the second reaction, the archaeal primer 21f, 5'-TTC CGG TTG ATC CYG CCG GA-3' and the universal primer 1392r were used. Template DNA was added to a reaction mixture at a volume of 2µl (0.02 µg), and each primer was added at a concentration of 0.8 µM. The reaction mixtures were heated for 5 minutes at 94 °C. The denaturation, elongation, and annealing conditions were 1 minute at 94 °C, 2 minutes at 72 °C, and 1 minute at 61 °C, respectively. The PCR products were electrophoresed on a 0.8% agarose gel and purified from gel slices using Prep-A-Gene DNA Purification Systems (Biorad, Hercules). The amplified DNA was ligated into vector pCR 2.0 (Invitrogen, Carlsbad) and transformed into *Escherichia coli* Top 10 cells. The PCR inserts in the resulting clones were sequenced at the Molecular Genetics Instrumentation Facility at the University of Georgia (Athens, GA, USA).

Primers 27f and 21f were used to sequence the bacterial and archaeal clones, respectively. Approximately 600 base pairs from each clone were sequenced. The clone SRS62DBA50 was further sequenced with the bacterial primer 327f (5'-ACC GCT TGT GCG GGC CC-3'; (11)) and the universal primer 1392r to yield a sequence with 1,298 nucleotides. The clone SRS62DAR03 was further sequenced with the universal primers 21f, 1392r, 519r (5'-GWA TTA CCG CGG CKG CTG-3'), 907r (5'-CCG TCA ATT CMT TTR AGT TT-3'), and 926f (5'-AAA CTY AAA KGA ATT GAC GG-3'; (26)) to yield a sequence with 1,346 nucleotides.

Phylogenetic Analysis. A FastA search was performed on all sequences to determine which sequences in the GenBank and EMBL databases were most closely related (3; 33). All sequences were compared to each other, and if duplicate sequences occurred (>99.6% sequence similarity), then only one of each type was used for further analysis. The program Check Chimera, from the Ribosomal Database Project was used to determine if any of these sequences were chimeric (28). Phylip 3.5 was used to construct phylogenetic trees on all remaining sequences (15). The Jukes Cantor formula was used to calculate distances, and the Neighbor joining, Parsimony, and Fitch-Margoliash algorithms were used to examine phylogenetic relationships.

Phospholipid Analysis. Two 3-L aliquots of each groundwater sample were vacuum filtered at the same time as the molecular samples. A 3-liter aliquot of groundwater was sequentially passed through 2.7, 1.0, 0.7, and 0.2 μm pore-size cellulose filters (Whatman). All the filters were immediately packed in ethanol-rinsed aluminum foil and frozen at -70° C. The filters were shipped on ice to Microbial Insights, Inc. (Rockford, TN, USA) (30) for PLFA analysis as previously described (37).

BIOLOG Analysis. Aliquots (150 μl) of groundwater samples were directly inoculated into triplicate BIOLOG GN plates (BIOLOG, Inc., Hayward, CA, USA) and incubated at room temperature for up to 6 days. Triplicate control plates were inoculated with autoclaved deionized water. At 0, 1, 2, 3, 4, 5, and 6 days post-inoculation, absorbance (590 nm) was measured using an automated plate reader (Biotek Instruments, Inc., Winooski, VT). Average color well development (AWCD) was determined as described by Garland (17).

RESULTS

Microbiology. Table 1 summarizes the results for total culturable bacteria (1% PTYG plates), total bacteria densities (AODC), methanotrophic bacteria by DFA, and geochemical parameters found in the pristine (LFW43B) and contaminated (LFW62D) groundwater. All microbiological tests revealed higher microbial densities in the contaminated groundwater from LFW62D as compared to LFW 43B. Total cell densities (AODC) and culturable heterotrophs were approximately 2-orders of magnitude greater in contaminated vs. pristine groundwater. The methanotrophic bacteria were also more abundant at LFW 62D.

Geochemistry. The groundwater from contaminated well LFW 62D had higher concentrations for every geochemical parameter measured compared to the non-contaminated groundwater LFW 43B, with the exception of dissolved oxygen (DO) and nitrate (Table 1). It is thought that the lower concentration of DO is a result of greater microbial activity in the contaminated groundwater. Both TCE and PCE were detected as well as daughter biodegradation products cDCE and VC (Table 1). Further, elevated chloride levels (up to 37 ppm) were consistent with the dehalogenation of ppm concentrations of CE know to be in this site in previous years (6). However, the concentration of chloride in the contaminated groundwater well at least an order-of-magnitude greater than the sum of all chlorinated compounds currently present in the same location (ppb range). The elevated chloride concentration could be the result of a separate source of chloride ions, or a residual artifact of previous dechlorination; it is known that historical concentrations of CE were much higher than at present.

Molecular Results. Approximately 130 colonies containing PCR-amplified bacterial 16S rDNA were obtained from the sample from LFW43B. Eight of these clones were sequenced, and they were virtually identical. Four of the sequences had exactly the same sequence, and four contained one or two different substitutions out of 550 positions examined. This level of substitutions is within the range expected from Taq DNA polymerase error (34). Since the sequences were greater than 99.6% similar to each

other, only one was chosen for subsequent analyses (SRS43BBA18). Plasmids representing 18 more clones from LFW 43B were digested with *Eco* R1, and the clones were found to contain a DNA banding pattern identical to that of the sequenced clones. Therefore, all 26 clones examined from LFW 43B examined by either sequencing or restriction endonuclease pattern appeared to be from closely related organisms (Table 2). The sequence of clone SRS43BBA18 was 99.5% similar to that of *Pseudomonas gessardii*. Phylogenetic analysis placed these sequences and *Pseudomonas libaniensis* in the *Pseudomonas* group of the gamma proteobacteria (Figure 2). *P. gessardii* and *P. libaniensis* were first isolated from spring water from France and Lebanon, respectively (36; 10). No PCR products were found after amplification of the sample from LFW 43B with the archaeal primer, even after increasing the template DNA amount to 0.05 µg. Therefore, it was assumed that LFW 43B contained very few archaea.

Approximately 150 colonies containing PCR-amplified bacterial 16S rDNA were obtained from LFW62D, and forty-one of these clones were sequenced. One sequence was found to be chimeric and was eliminated from further analysis. When the remaining sequences were compared to each other, seventeen ribotypes were found (Table 2). Sequences SRS62DBA43, -11, -07, -03, -19, -09, -21, and -05 were related to the beta proteobacteria, and sequences SRS62DBA39, -44, -37, -10, -32, -12, -24, and -01 were related to the gamma proteobacteria. The sequence SRS62DBA50 was of special interest because, while it was not closely related to any cultured organisms, it was related to four uncultured and unclassified eubacterial clones found in a hydrocarbon- and chlorinated-solvent-contaminated aquifer at 87-90% sequence similarity. Sequence SRS62DBA50 had less than 75% sequence similarity to the sequences of any cultured organism in the database.

To determine the most closely related sequences, the clones from the gamma and beta proteobacterial groups and clone SRS62DBA50 were analyzed separately. Within the gamma proteobacteria (Figure 3), SRS62DBA01, -24, -12, -32, -10, and -37 grouped consistently with the Methylococcaceae family. In 93% of the replicates SRS62DBA1, -24 grouped with a cluster of *Methylomonas* species.

SRS62DBA12, -32, -10, and -37 always grouped together with *Methylobacter psychrophilus* and isolate BB5.1, but the branching order within this clade varied (Figure

4). Clones SRS62DBA39 and -44 consistently clustered with the *Pseudomonas* species. Although the branching order within these two clades differed sometimes, SRS62DBA39 was always found clustered with *P. lundensis, P. fluorescens, P. brassicacearum* and *P. syringae*, and SRS62DBA44 was always found grouped with *P. libaniensis* and *P. gessardii*. Very similar results were also obtained by parsimony and the Fitch-Margoliash analyses. Using these algorithms, the SRS clones grouped with the same organisms and clones as found with the neighbor-joining analysis, and differences occurred only within the deepest branches of the trees.

Within the beta proteobacteria (Figure 5), SRS62DBA21 and -05 clustered with the methylotrophs in the beta proteobacteria group 85% of the time; otherwise it grouped with the *Thiobacillus* group. SRS62DBA43, -11, -07 and -03 clustered consistently with *Pseudomonas mephitica* and *Janthinobacterium lividum*. However, the branching order of SRS62DBA07 and -03 varied, which caused some low bootstrap values within this clade. SRS62DBA19 and -09 clustered with *Pseudomonas lemoignei*, but the branching order within this group changed. Similar results were also obtained by parsimony and the Fitch-Margoliash analyses. In these analyses, SRS62DBA09 and –19 remained associated with the clade including *Herbaspirillum*, *Janthinobacterium*, and *Duganella*. However, -09 and –19 did not always associate with *P. lemoignei* and branched deeper within the clade instead. Moreover, within the clade that contained SRS62DBA03 and -07 the branching order varied.

The 500 base pair sequence that resulted from clone SRS62DBA50 possessed only low similarity to other sequences in the Genbank and EMBL databases (3;33), and 798 more positions of this clone were sequenced. A phylogenetic tree was constructed with the almost complete 16S rDNA sequence of SRS62DBA50 and sequences from a diverse collection of cultured prokaryotes (data not shown). SRS63DBA50 did not group consistently with any group of organisms. It showed a very weak association with members of the Gram-positive group when the tree was constructed with the neighbor joining or Fitch-Margoliash criterion, but the bootstrap value for this association was below 50. This association was not observed in the tree constructed by parsimony.

The program Check Chimera indicated that the SRS62DBA50 sequence was not chimeric, but other analyses were done to support this conclusion. FastA analyses were

performed on several different portions of the sequence (300 base pairs) and each portion had relatively the same % sequence similarity to the same sequences in the Genbank and EMBL databases (3, 33). A program called mfold 3.0 (43, 29) was used to predict the secondary structure of the sequence. This secondary structure contained many of the same features as an *E. coli* 16S rRNA secondary structure. Since the sequence folded into a recognizable 16S rRNA molecule and did not possess small regions with high sequence similarity to other sequences, SRS62DBA50 did not appear to be chimeric. Approximately 160 colonies containing PCR-amplified archaeal 16S rDNA were found in the archaeal library from LFW 62D. Fifteen clones were sequenced (Table 2). Sequences of 10 clones had 100% sequence similarity and the remaining 5 each contained 1 independent substitution out of 450 positions. Only one clone (SRS62DAR03) was chosen for further analysis. This clone was fully sequenced (1346 nucleotides) for the phylogenetic analysis. Within the GenBank and EMBL databases (3, 33), the sequence with the highest similarity (89%) to that of SRS62DAR03 was the crenarchaeote clone *Cenarchaeum symbiosum* from marine sponges, (Figure 6). This assignment was robust and detected in phylogenetic trees constructed using neighbor-joining, Fitch-Margoliash, and parsimony analyses. The bootstrap values associated with this grouping were high, indicating a stable relationship in the neighbor-joining tree.

Phospholipid fatty acid analysis. Phospholipids were directly extracted from groundwater microbial biomass captured on membrane filters, without prior plating or culturing on a selective-enrichment medium. As such, no attempt was made to identify individual bacteria comprising the groundwater communities. The total biomass as measured by PLFA was greater in LFW 62D as compared to LFW 43B (Figure 7). This data was in agreement with the measurements of microbial densities and CFUs (Table 1). These contaminants were not at currently toxic levels (Table 1) and could act as potential sources of carbon and energy for the microbial communities in the aquifer and may explain in part why there is a more diverse community in LFW 62D as compared to LFW 43B (Figure 7). In the pristine groundwater (LFW 43B), only four different phospholipids were detected: 18:1w7c, 14:0, 16:0, and 18:0. The distribution was uneven and primarily composed of 18:1w7c (66%), which is common in many pseudomonads.

In contrast to the relatively limited pristine groundwater community, the contaminated groundwater (LFW62D) contained 17 different phospholipids. Monoenoic lipids composed 77% of the PLFA profile and are common in Gram-negative bacteria or proteobacteria (White et al. 1998). The monoenoic PLFA 16:1w8c accounted for 6.5% and was found in *Methylomonas spp*. Other monoenoic PLFAs included 18:1w8c at 5.3%, common in *Methylosinus*; 10me16:0 at 1.1%, common in *Desulfobacter*; and i17:1w7C at 0.4%, common in *Desulfovibrio*. Terminally branched saturated PLFAs at 2.0%, common in Gram-positive or sulfate-reducing bacteria were also detected.
The Gram negative communities in LFW62D contained detectable biomarkers for growth phase (Figure 7) and environmental stress (Figure 8). In contrast LFW43B indicated no detectable turnover rate or signs of environmental stress. The physiological status of Gram-negative communities can be assessed from ratios of different monoenoic biomarkers. Specifically, 16:1ω7c and 18:1ω7c are converted to cyclopropyl fatty acids (cy17:0 and cy19:0) as microbes move from a log to a stationary phase of growth (i.e. slowing of growth). This change is expressed in the two ratios cy17:0 to 16:1ω7c and cy19:0 to 18:1ω7c, which may vary from organism to organism or environment to environment, but usually will fall within the range of 0.1 (log phase) to 5.0 (stationary phase) when summed. This ratio is inversely proportional to the turnover rate (i.e. a lower ratio infers a higher turnover rate). An increase in cyclopropyl formation has also been associated with anaerobic metabolism (37). Gram-negative bacteria also generate trans fatty acids to minimize the permeability of their cellular membranes as adaptation to a more hostile environment. Ratios (16:1ω7t to 16:1ω7c and 18:1ω7t to 18:1ω7c) greater than 0.1 (when summed) have been shown to indicate the adaptation to a toxic environment by lowering the cellular membrane permeability (20).

Although clones related to Methylomonas were common in the library from LFW62D, clones related to *Methylosinus* (a member of the alpha proteobacteria), sulfate-reducing, or Gram-positive bacteria were not found. However, since these PLFA biomarkers represented a low percentage of all the phospholipids present, it is possible these microbes were indeed present in the groundwater samples, but that their 16S rDNA was not detected because of their low abundance. Alternatively, it is possible that these

PLFA markers are not unique, but instead are present in one of the groups of the organisms detected by 16D rDNA sequencing.

BIOLOG. Substrate utilization was defined as a substrate well with an optical density (OD_{590nm}) greater than 0.1 after subtraction of the control well (containing only redox dye groundwater, and no substrate). Inoculum density was not adjusted and was 2-orders of magnitude greater in contaminated vs pristine wells. Microbial communities from pristine groundwater utilized 72 substrates, and from contaminated groundwater used 62 substrates (Figure 9). The rate of color development was estimated by dividing the AWCD during the longest linear increase in color development by corresponding incubation period, as discussed by Garland (17). Microbial communities from both pristine and contaminated groundwater exhibited similar lag phases that occurred at 0-2 days following inoculation. The rate was 0.07 (at 2-5 days) in the pristine groundwater and 0.19 (at 2-5 days) in the contaminated groundwater. Maximum AWCD values (0.6) were reached at 6 days for pristine groundwater and 5 days for contaminated groundwater. In the contaminated groundwater, AWCD decreased after 5 days of incubation; however, it was later determined that the decrease was caused by cellular growth in the control well (containing redox bye and no substrate) at day 5. Because cell densities were not adjusted prior to inoculation in Biolog plates, individual well utilization values were not used to compare metabolic function between the pristine vs. contaminated groundwater samples.

DISCUSSION

Biological and chemical analyses were performed to determine how chlorinated-ethene contamination affects groundwater microbial communities. The study was conducted as part of an environmental restoration program to remediate groundwater contaminated with low-level (<1 ppm) CE-contamination at the NRWF. All microbiological tests revealed higher microbial densities in the contaminated compared to pristine groundwater. Total cell densities (AODC) and culturable heterotrophs were approximately 2-orders of magnitude greater in contaminated vs. pristine wells. This was

expected, since labile carbon sources are thought to move with the CE plume. However, it was of interest that the methanotrophic bacteria densities were elevated in pristine groundwater (2.2×10^2 cells/mL; Table 1) because that area north of the landfill is oligotrophic and historical documents indicate that no known waste had been disposed of there. Additional studies are needed to determine the source and concentration of labile carbon in both the pristine and contaminated groundwater wells although LFW62D is located within a plume of chlorinated hydrocarbon contamination as demonstrated by the geochemical data (Table 1). These contaminants are not at currently toxic levels and act as potential sources of carbon and energy for the microbial communities in the aquifer and may explain in part why there is a more diverse community in LFW62D as compared to LFW43B (Figure 8).

Since the bacterial and archaeal clones SRS62DBA50 and SRS62DAR03 did not cluster with any cultured and/or characterized prokaryote in the Genbank and EMBL databases, little can be said about their presence or functionality in the aquifer. Both seem to be novel organisms. Future attempts to culture these organisms will be helpful to determine the significance of their presence in the groundwater.

The agreement between the molecular and PLFA results on the relative diversity of the two wells is strong. For example, as mentioned the monoenoic biomarker 16:1ω8c accounted for 6.5% of the phospholipids and is found in *Methylomonas*. Similarly, 7.5% of the bacterial clones sequenced from LFW62D grouped with *Methylomonas*. With the exception of SRS62DBA50, all of the bacterial clones sequenced from LFW62D grouped with the proteobacteria, which also concurred with the PLFA results. The higher methane concentration found LFW 62D as compared to LFW 43B is consistent with the methanotrophic bacteria findings (Table 1).

Thirty percent of the bacterial clones sequenced from LFW62D clustered with the *Methylophilus* and *Methylobacillus* members of the β proteobacteria. *Methylophilus* and *Methylobacillus* are methanol oxidizers. Methanol is one of the byproducts of methane oxidation (1), and it is likely that these bacteria are utilizing the methanol produced by the type I methanotrophs. Methanol oxidizers are commonly found in association with methane-oxidizing bacteria for this reason (22).

The pseudomonads and burkholderia groups contain metabolically diverse bacteria. Some pseudomonas and burkholderia, such as *P. putida* and *B. cepacia*, can oxidize TCE (29, 31, 38). Results here demonstrate that 35% and 5% of the bacterial clones sequenced grouped with the burkholderia and pseudomonas groups, respectively. With the exception of a close relative of *P. gessardii* these bacteria were not detected in the uncontaminated well (LFW 43B), and it is likely that the contaminating compounds have enriched for these organisms and act as a source for their carbon and energy. Therefore, these organisms could contribute to the breakdown of TCE and other contaminants found at the site.

The PLFA are components of all cellular membranes, allowing cells to maintain cell fluidity, which enables the transport of nutrients and elimination of metabolic byproducts. In this study all PLFAs were extracted from cells collected on .2 μm filters. PLFAs decompose quickly upon cell death, and are degraded by a variety of enzymes within the microbiota. Different groups of microorganisms (e.g., gram negative; anaerobes) synthesize a variety of specific PLFA through various biochemical pathways. In some cases these associations are so strong that fatty acid biomarkers have been identified for particular organisms including those identified here (35, 37). Knowledge of specific lipid biosynthetic pathways can provide insight into the nutritional status of the groundwater microbial community; the lipid composition of microorganisms is a function of metabolic activity and thus reflects the phenotypic response of the organisms to their environment (13). The general agreement here between the molecular and PLFA data strengthens that point. Further, PLFA analysis provides a quantitative means to directly measure viable microbial biomass, community composition, and nutritional status for quantifying subsurface bioremediation (37).

This study provides significant insight regarding characteristics associated with microbial communities in the CE contaminated NRWDF aquifer. *In situ* biodegradation is the preferred technology for environmental restoration at this and related sites because contaminants are destroyed in place, not simply moved to another location or immobilized (4). Microbial biodegradation of subsurface contaminants, while site specific, is highly dependent on the *in situ* environment (21). The aquifers at the Savannah River Site offer many unique environments, including contaminated and

pristine, that have been proven to host different microbial communities (5). These microbial populations have been significantly altered in some area of SRS to achieve environmental restoration goals (25). Data obtained from 16S rRNA, BIOLOG, and PLFA profiles at this landfill provides evidence that the microbial populations in aquifers can vary based on geochemical parameters. The direct isolation of rRNA and PLFA from groundwater microbial communities eliminates the bias introduced by enrichment and cultivation. Further, the use of BIOLOG to evaluate groundwater physiological potential without processing allows a metabolic "fingerprint" to be taken of a given sample. Moreover, the 16S rRNA, BIOLOG and PLFA data taken from different locations on the same site can demonstrate microbial activity, structure, and dynamics in the presence of chlorinated ethenes. Based on this information, future studies could identify molecular components in the bacteria that provide an enhanced ability to degrade the chlorinated ethenes PCE, TCE, and daughter products cDCE and VC. The data will also provide a significant contribution since the CE natural attenuation rates are being evaluated at this site (16). This information will provide an ability to compare biodegradation rates between groundwater monitoring wells based on different microbial populations. Moreover, using the 16S rRNA, BIOLOG, and PLFA data can potentially validate differences observed in CE biodegradation rates between two distinct groundwater sources. For example, differences in the molecular results (16S rRNA data) and population dynamics (PLFA and BIOLOG) between groundwater sources could be more easily understood if differences are observed simultaneously in the geochemical data. Collectively, data obtained in this study will provide a fundamental understanding of microbial diversity with respect to groundwater contamination. The 16S rRNA and PLFA data provides information on the exact species composition combined with the BIOLOG data as an indication of the metabolic activity allows characterization of the groundwater consortia as it relates to bioremediation potential.

The contaminants found within well LFW 62D included PCE, TCE, cDCE, and VC (Table 1). The methane monooxygenase of methane-oxidizing bacteria (methanotrophs), such as *Methylomonas*, *Methylobacter*, *Methylosinus*, *Methylococcus*, and *Methylomicrobium*, can cometabolize these CE under the right conditions (32). Therefore, an environment with methane and CE may enrich for these methane oxidizers.

The 16S rDNA data showed that 28% of the bacterial clones sequenced clustered with the type I methanotrophs. Since this microbial characterization work was complete, a campaign using biosparging was initiated at the landfill (Figure 1). The results here imply that *in situ* bioremediation utilizing methanotrophs may be effective in the vicinity of LFW 62D. The pumping of methane, air, and other nutrients into this community will potentially enrich the methane-oxidizing members in this community further to co-metabolize the CE. The enrichment of these organisms expediting the degradation of the contaminating plume will be monitored.

Of interest here are the molecular (Table 2 and Figure 2) and PLFA (Figures 7 and 8) results for the groundwater from LFW 43B. This well was selected as a non-contaminated source to compare with the contaminated groundwater LFW 62D as it was upgradient from the CE plume and historically had never demonstrated any groundwater contamination. The phylogenetic analysis revealed a low diversity with all clones closely related to *P. gessardii* and *P. libaniensis* that were previously described in spring water. The PLFA demonstrated similar results with most of the phospholipids common to pseudomonads. Results from BIOLOG demonstrated a wider range of substrate utilization for LFW 43B (Figure 9) potentially due to the greater proportion of pseudomonads.

Results from biological analyses are useful to compare differences in microbial communities from spatially different non contaminated and contaminated groundwater, and develop a baseline for future analyses. Bioremediation is known to be more successful where there is indigenous diverse population of microorganisms capable of mineralizing a wide range of substrates (19). At the SRS, microbial characterization has proven to be of critical importance in determining the successful outcome of field-scale bioremediation deployments (5, 6, 14, 23, and 25). Future microbial analyses at this and other SRS sites will help determine whether and how subsurface microbial communities change as contaminate plumes advance through them. In addition, this information can lead to management practices of these indigenous microbial communities to maintain the diversity and activity needed to achieve long term environmental restoration goals.

Acknowledgements

We are grateful to Marilyn Franck, Topher Berry, and Pam McKinsey of the Savannah River Technology Center for their excellent technical support.

This paper was prepared in connection with work done under Contract No. DE-AC09-96SR18500 with the U. S. Department of Energy. We thank the Oak Ridge Institute for Science and Education (ORISE) for support of Mr. Glucksman and Dr. Furlong.

REFERENCES

1. **Anthony, C.** 1986. Bacterial oxidation of methane and methanol. Advances in Microbial Physiol. **27**:113-210.
2. **Balkwill, D.L., F. R. Leach, J. T. Wilson, J. F. McNabb, and D. C. White.** 1988. Equivalence of microbial biomass measures based on membrane lipid and cell wall components, adenosine triphosphate and direct counts in subsurface sediments. Microbial Ecol. **16**:73-84.
3. **Benson D. A., M. S. Boguski, D. J. Lipman, J. Ostell, B. F. Ouellette, B. A. Rapp, D.L. Wheeler.** 1999. Genbank. Nucleic Acids Res. **27**:12-17.
4. **Bouwer, E., N. Durant, L. Wilson, L. Zhang, and W. A. Cunningham.** 1994. Degradation of xenobiotic compounds in situ: Capabilities and limits. FEMS Microbiology Reviews. **15**:307-315.
5. **Bowman, J.P., L. Jimenez, I. Rosario, T. C. Hazen, G. S. and Sayler.** 1993. Characterization of the methanotrophic bacterial community present in a trichloroethylene-contaminated subsurface groundwater site. Appl. Environ. Microbiol. **59**: 2380-2387.
6. **Brigmon, R.L., D. J. Altman, M. M. Franck, T. C. Hazen, A. W. Bourquin and C. B. Fliermans.** 1999. Evaluation of Methanotrophic Bacteria During Injection of Gaseous Nutrients for *In situ* Trichloroethylene Bioremediation in a Sanitary Landfill. In Engineered Approaches for In Situ Bioremediation of Chlorinated Solvent Contamination. **5**:107-112. Batelle Press Columbus, OH
7. **Brigmon R. L., M. M. Franck, J. S. Bray, S. Lanclos, D. Scott, and C. B. Fliermans.** 1998. Direct immunofluorescence and enzyme-linked immunosorbent assays for evaluating organic contaminant degrading bacteria. *J. Microbiol. Methods.* **32**:1-10.
8. **Brigmon R. L.** 2002. Methanotrophic Bacteria: Use in Bioremediation, p. 1936-1944. *In* G. B. Bitton (ed.), Encyclopedia of Environmental Microbiology. John Wiley & Sons, New York, NY.

9. **Coleman, N. V., T.E. Mattes, J. M. Gossett, and J. C. Spain.** 2002. Biodegradation of *cis*-Dichloroethene as the sole carbon Source by a β-Proteobacterium. Appl. Environ. Microbiol. **68**:2726-2730.
10. **Dabboussi, F., M. Hamze, M. Elomari, S. Verhille, N. Baida, D. Izard, and H. Leclerc.** 1999. *Pseudomonas libanensis* sp. Nov., a new species isolated from Lebanese spring waters. Int. J. Syst. Bacteriol. **49**:1091-1101.
11. **DeLong, E. F., G. J. Olsen, and N. R. Pace.** 1988. Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. J. Bacteriol. **170**:720-726.
12. **Doherty, R. E.** 2000. A History of the Production and Use of Carbon Tetrachloride, Tetrachloroethylene and 1,1,1-Trichloroethane in the United States: Part 1-Historical Background; Carbon Tetrachloride and Tetrachloroethylene. J. Environ. Foren. **1:**69-81.
13. **Dowling, N. J. E., F. Widdel, and D.C. White.** 1986. Phospholipid ester-linked fatty acid biomarkers of acetate-oxidizing sulfate reducers and other sulfide-forming bacteria. J. Gen. Microbiol. **132**:1815-1825.
14. **Enzien, M. V., F. Picardal, T. C. Hazen, R. G. Arnold, and C. B. Fliermans.** 1994. Reductive dechlorination of trichloroethylene and tetrachloroethylene under aerobic conditions in a sediment column. J. Appl. Environ. Microbiol. **60**:2200-2204.
15. **Felsenstein, J.** Phylip ver. 3.5, 1993. University of Washington, Seattle, WA.
16. **Freedman, D. L., J. Cox, L. Baiden, C. Carvalho, C. K. Gunsch, J. Hunt, and R. L. Brigmon.** 2001. Potential for Bioremediation of Groundwater Contaminated with Landfill Leachate, **6**:109-116. *In* V.S. Magar, D.E. Fennell, J.J. Morse, B.C. Alleman, and A. Leeson (ed.), Anaerobic Degradation of Chlorinated Solvents, Proceedings of the Sixth International In Situ and On-Site Bioremediation Symposium, Battelle Press, Columbus, OH.
17. **Garland, J. L.** 1996. Analytical approach to the characterization of samples of microbial communities using pattern of potential C source utilization. Soil Biol. Biochem. **28**:213-221.
18. **Glucksman, A. M., H. D. Skipper, J. Santo Domingo, and R. L. Brigmon.** 2000. Use of fatty acid methyl esters to detect microorganisms from water. J. Appl.

Microbiol. **88**:711-719.

19. **Green, T. G., and K. M. Scow.** 2000. Analysis of phospholipid fatty acids (PLFA) to characterize microbial communities in aquifers. Hydrogeol. J. **8**: 126-141.

20. **Guckert, J.B., Hood, M.A., and D.C. White.** 1986. Phospholipid, ester-linked fatty acid profile changes during nutrient deprivation of *Vibrio cholerae:* increases in the *trans / cis* ratio and proportions of cyclopropyl fatty acids. Appl. Environ. Microbiol. **52**:794-801.

21. **Haack, S. K., and B. A. Bekins.** 2000. Microbial populations in contaminant plumes. Hydrogeol. J. **8**: 63-76.

22. **Hanson R.S.** 1980. Ecology and diversity of methylotrophic organisms. Adv. Appl. Microbiol. **26**:2-39.

23. **Hazen, T. C., B. B. Looney, M. Enzien, J. M. Dougherty, J. Wear, C. B. Fliermans, and C. A. Eddy.** 1996. In Situ Bioremediation Via Horizontal Wells, p. 79-86 *In* R. F. Hickey and G. Smith (eds.) Biotechnology in Industrial Waste Treatment and Bioremediation, Batelle Press, Columbus, OH.

24. **Hendrickson, E. R., J. A. Payne, R. M. Young, M. G. Starr, M. P. Perry, S. Fahnestock, D. E. Ellis, and R. C. Ebersole**. 2002. Molecular Analysis of *Dehalococcoides* 16S Ribosomal DNA from Chloroethene-Contaminated Sites throughout North America and Europe. Appl. Environ. Microbiol. **68:**485-495.

25. **Kastner J.R., J. Santo Domingo, M. Denham, M. Molina, R. Brigmon.** 2000. Effect of Chemical Oxidation on Subsurface Microbiology and Trichloroethylene (TCE). Biodegradation. Bioremediation J. **4:** 219-236

26. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115-175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic Acid Technology in Bacterial Systematics. John Wiley and Sons, New York.

27. **Li, S., and L. P. Wackett,** 1992. Trichloroethylene oxidation by toluene dioxygenase. Biochem. Biophys. Res. Commun. **185**:443-451.

28. **Maidak, B. L., J. R. Cole, C. T. Parker Jr., G. M. Garrity, N. Larsen, B. Li, T. G. Lilburn, M. J. McCaughey, G. J. Olsen, R. Overbeek, S. Pramanik, T. M. Schmidt, J. M. Tiedje, and C. R. Woese.** 1999. A new version of the RDP (Ribosomal Database Project). Nucleic Acids Res. **27**:171-173.

29. **Mathews, D.H., J. Sabina, M. Zuker and D. H. Turner.** 1999. Expanded sequence dependence of thermodynamic parameters provides robust prediction of RNA secondary structure. J. Mol. Biol. **288**: 911-940.

30. **Microbial Insights, Inc.** 1999. Rockford, TN.

31. **Newman, L. M. and L. P. Wackett**. 1995. Purification and characterization of toluene 2-monoxygenase from *Burkholderia cepacia*. Biochemistry **34**:14066-14076.

32. **Rosenzweig, A. C., C. A. Frederick, S. J. Lippard, and P. Nordlund. 1993.** Crystal structure of a bacterial non-haem iron hydroxylase that catalyses the biological oxidation of methane. Nature **366**:537-543.

33. **Stoesser, G., W. Baker, A. E. van den Broek, E. Camon, P. Hingamp, P. Sterk, and M. A. Tuli.** 2000. The EMBL nucleotide sequence database. Nucleic Acids Res. **28**: 19-23.

34. **Takai, K. and K. Horikoshi.** 1999. Genetic diversity of archaea in deep-sea hydrothermal vent environments. Genetics **152**:1285-1297.

35. **Tunlid, A., and D. C. White.** 1991. Biochemical analysis of biomass, community structure, nutritional status and metabolic activity of the microbial communities in soil, in Soil Biochemistry, Vol. 7, (Bollag, J.M., Stotzky, G. eds.), pp. 229-262.

36. **Verhille, S., N. Baida, F. Daboussi, M. Hamze, D. Izard, and H. Leclerc.** 1999. *Pseudomonas gessardii* sp. Nov. and *Pseudomonas migulae* sp. Nov., two new species isolated from natural mineral waters. Int. J. Syst. Bacteriol. **49**: 1559-1572.

37. **White, D.C., C.A. Flemming, K. T. Leung, and S. J. Macnaughton.** 1998. In situ microbial ecology for quantitative appraisal, monitoring, and risk assessment of pollution remediation in soils, the subsurface, the rhizosphere and in biofilms. J. Microbiol. Methods. **32:**93-106.

38. **Whittaker, M., D. Monroe, and D. J. Oh.** 1999. University of Minnesota. http://www.labmed.umn.edu/umbbd/tce/tce_map.html

39. **Wiedemeir, T.H., M.A. Swanson, J.T. Wilson, D.H. Kampbell, R.N. Miller, and J.E. Hansen.** 1995. Patterns of Intrinsic Bioremediation at Two U.S. Air Force Bases. In: Intrinsic Bioremediation, R.E. Hinchee, John T. Wilson, Douglas C. Downey (eds), Battelle Press, Columbus, OH, pp. 31-52.

40. **Wise, M.G., J. Vaun McArthur, and L.J. Shimkets.** 1999. Methanotroph Diversity in Landfill Soil: Isolation of Novel Type I and Type II Methanotrophs Whose Presence Was Suggested by Culture Independent 16S Ribosomal DNA Analysis. Appl. Environ. Microbiol. **65**:4887-4897.

41. **WSRC**. 1996, *Sanitary Landfill In situ Bioremediation Optimization Test Final Report*, Westinghouse Savannah River Company, Aiken, SC, 29808, WSRC-TR-96-0065.

42. **Zhou, J., B. Xia, D. S. Treves, L.-Y. Wu, T. L. Marsh, R.V. O'Neill, A.V. Palumbo, and J.M. Tiedje.** 2002. Spatial and Resource factors influencing high microbial diversity in soil. Appl. Environ. Microbiol. **68**:326-334.

43. **Zuker, M., D.H. Mathews, and D. H. Turner.** 1999. Algorithms and thermodynamics for RNA secondary structure prediction: a practical guide. *In* RNA Biochemistry and Biotechnology. J. Barciszewski & B.F.C. Clark, eds. NATO ASI Series, Kluwer Academic Publishers.

List of Figures

FIG 7. Groundwater biomass content and diversity as measured by percentage of total groundwater PLFA for LFW 43B and LFW62D.

FIG 8. Growth phase and comparative membrane permeability in the LFW Gram negative communities.

FIG 9. Average BIOLOG well color development in LFW 43B; OCRD = 0.131 and in LFW 62D; OCRD = 0.220 and number of individual BIOLOG substrates utilized (>0.1 OD_{590nm}) over time in LFW 62Dand LFW 43B.

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LIST OF TABLES

Figures

FIG. 1. Location of groundwater monitoring wells LFW 43B and LFW 62D relative to the Vinyl Chloride plume at the SRS Sanitary Landfill.

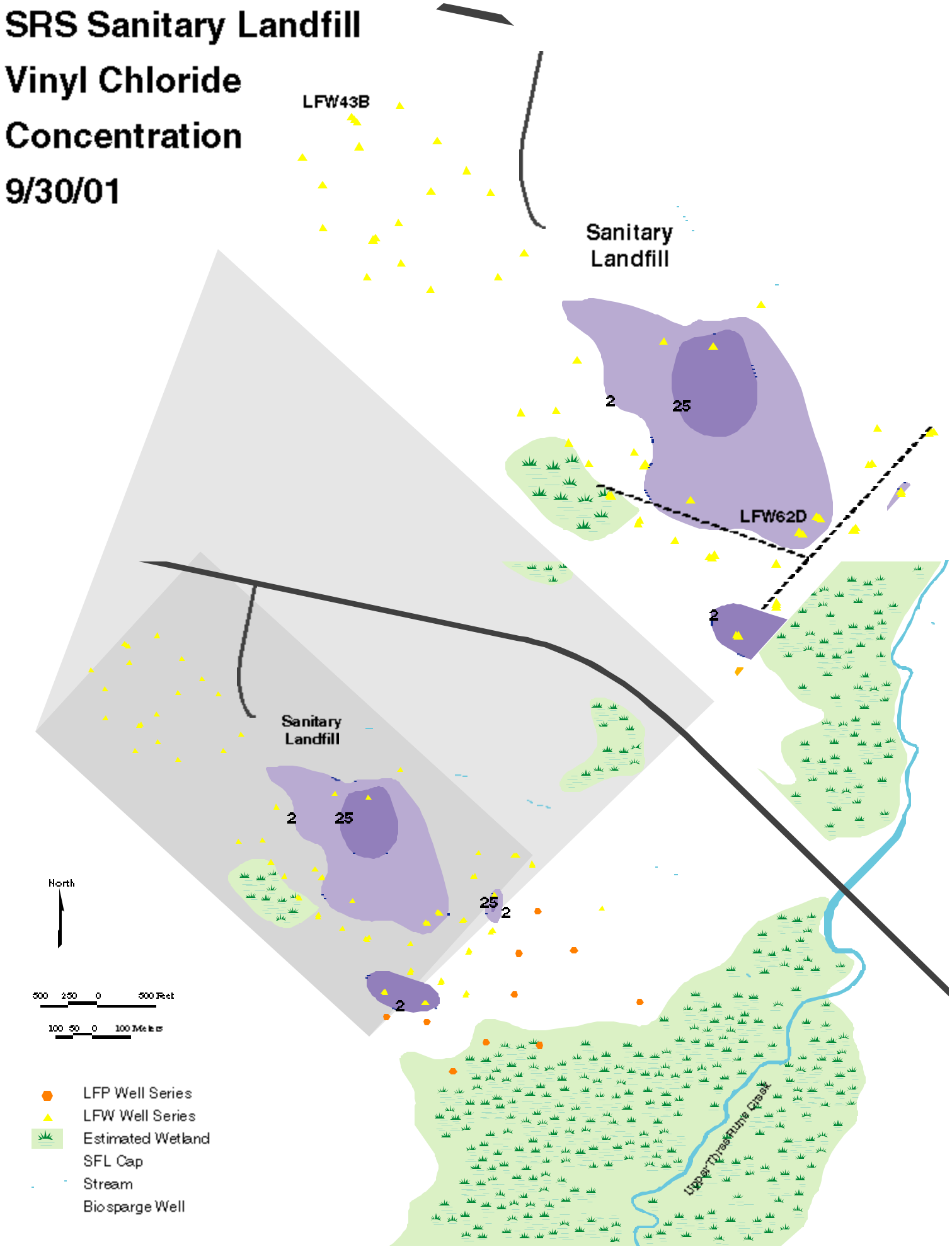
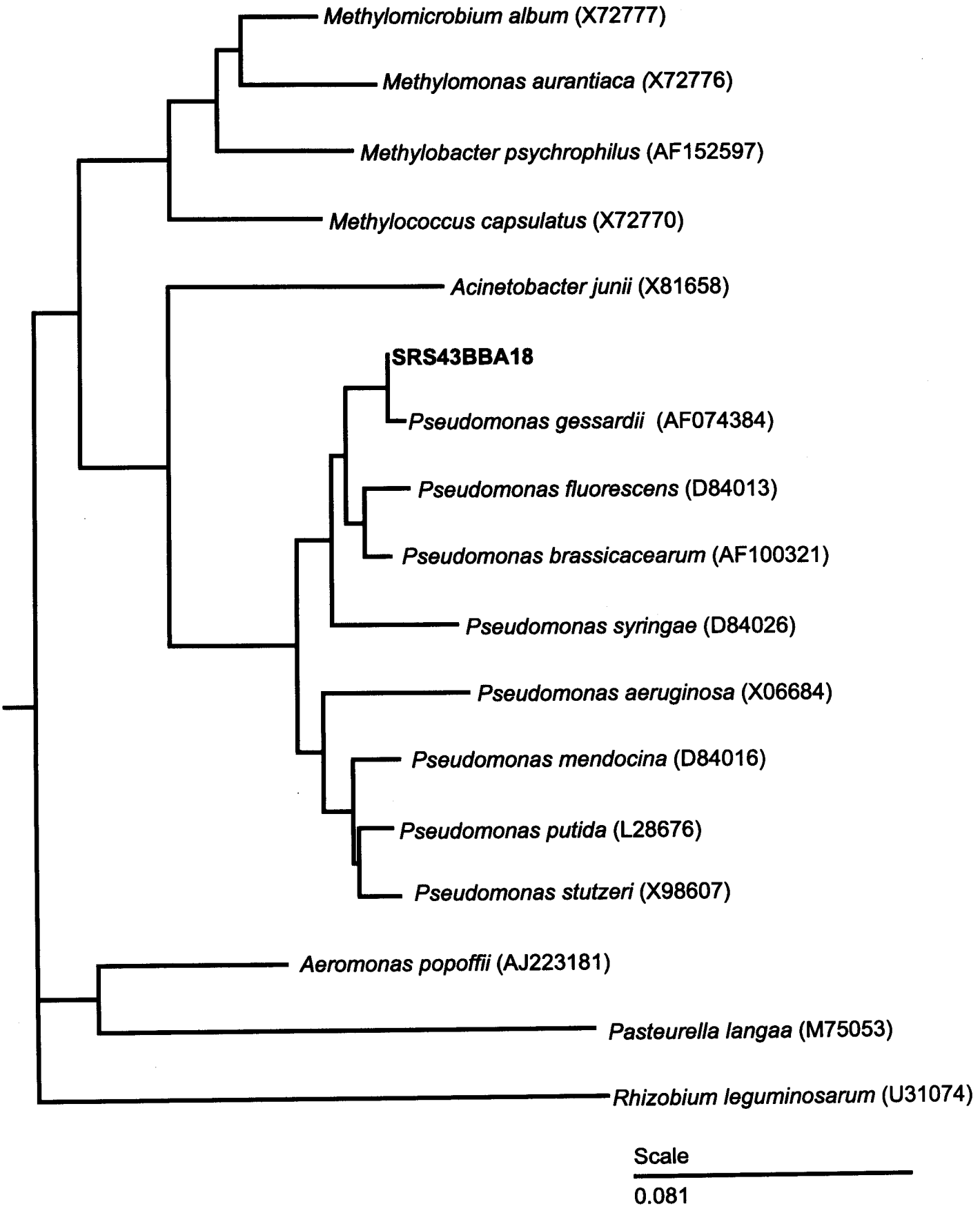

SRS Sanitary Landfill
Vinyl Chloride
Concentration
9/30/01
LFW43B
Sanitary
Landfill
2
25
LFW62D
2
Sanitary
Landfill
2
25
25
2
2
North
500 250 0 500 Feet
100 50 0 100 Meters
LFP Well Series
LFW Well Series
Estimated Wetland
SFL Cap
Stream
Biosparge Well
Upper Three Runs Creek

FIG. 2. Phylogenetic tree generated by the neighbor-joining method from an alignment of 559 nucleotide positions. The clones from LFW 43B were related to the γ-Proteobacteria. The scale bar indicates the Jukes Cantor distance. The number in parentheses indicates the accession number for the sequence.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Methylomonas aurantiaca (X72776)
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Methylococcus capsulatus (X72770)
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SRS43BBA18
Pseudomonas gessardii (AF074384)
Pseudomonas fluorescens (D84013)
Pseudomonas brassicacearum (AF100321)
Pseudomonas syringae (D84026)
Pseudomonas aeruginosa (X06684)
Pseudomonas mendocina (D84016)
Pseudomonas putida (L28676)
Pseudomonas stutzeri (X98607)
Aeromonas popoffii (AJ223181)
Pasteurella langaa (M75053)
Rhizobium leguminosarum (U31074)
Scale
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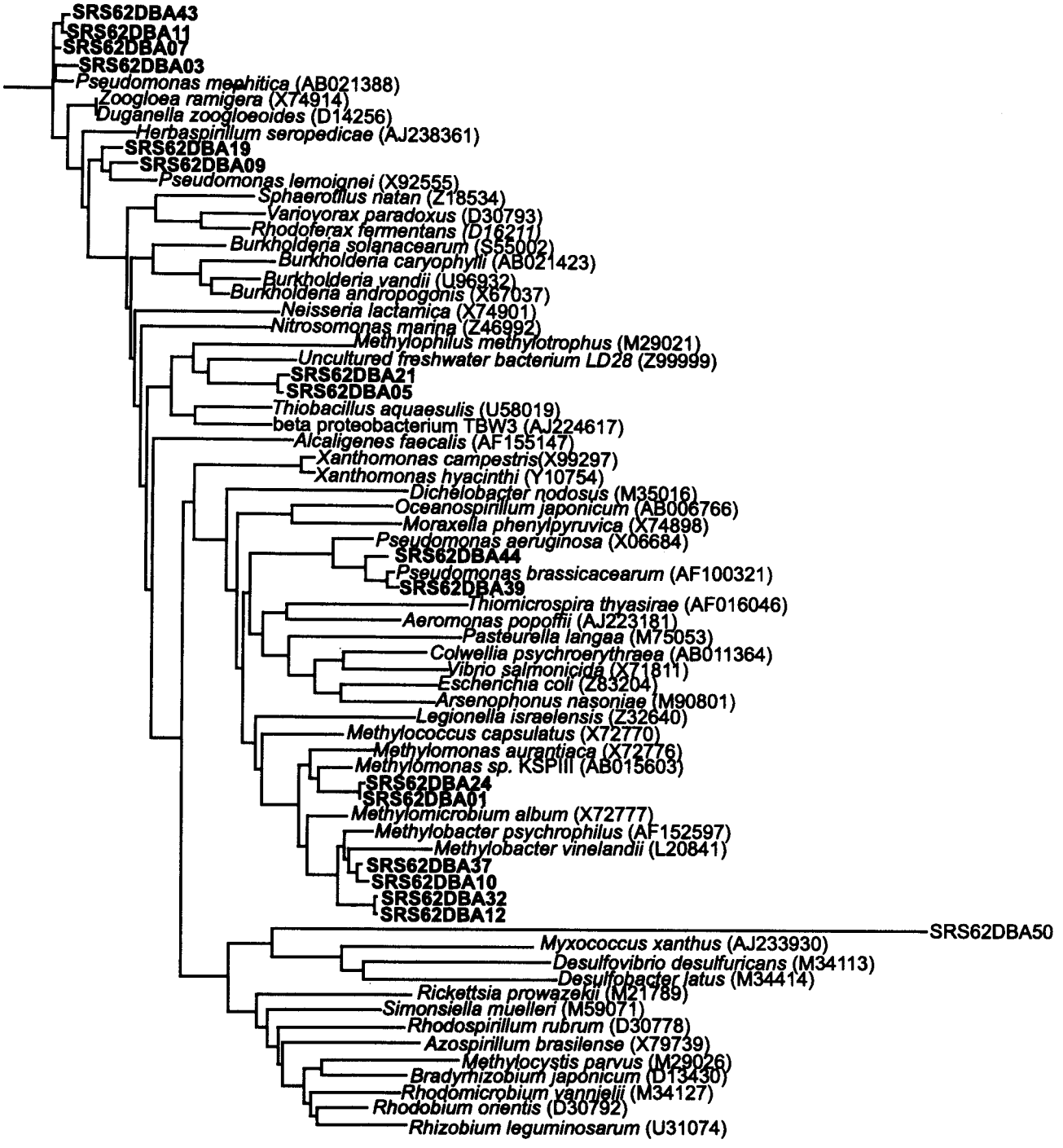
FIG. 3. Phylogenetic tree generated by the neighbor-joining method from an alignment of 490 nucleotide positions, showing the relationships between LFW 62D clones and clones related to γ--Proteobacteria. The scale bar indicates the Jukes Cantor distance. The number in parentheses indicates the accession number for the sequence.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SRS62DBA07
SRS62DBA03
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Zoogloea ramigera (X74914)
Duganella zoogloeoides (D14256)
Herbaspirillum seropedicae (AJ238361)
SRS62DBA19
SRS62DBA09
Pseudomonas lemoignei (X92555)
Sphaerotilus natan (Z18534)
Variovorax paradoxus (D30793)
Rhodoferax fermentans (D16211)
Burkholderia solanacearum (S55002)
Burkholderia caryophylli (AB021423)
Burkholderia vandii (U96932)
Burkholderia andropogonis (X67037)
Neisseria lactamica (X74901)
Nitrosomonas marina (Z46992)
Methylophilus methylotrophus (M29021)
Uncultured freshwater bacterium LD28 (Z99999)
SRS62DBA21
SRS62DBA05
Thiobacillus aquaesulis (U58019)
beta proteobacterium TBW3 (AJ224617)
Alcaligenes faecalis (AF155147)
Xanthomonas campestris(X99297)
Xanthomonas hyacinthi (Y10754)
Dichelobacter nodosus (M35016)
Oceanospirillum japonicum (AB006766)
Moraxella phenylpyruvica (X74898)
Pseudomonas aeruginosa (X06684)
SRS62DBA44
Pseudomonas brassicacearum (AF100321)
SRS62DBA39
Thiomicrospira thyasirae (AF016046)
Aeromonas popoffii (AJ223181)
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Colwellia psychroerythraea (AB011364)
Vibrio salmonicida (X71811)
Escherichia coli (Z83204)
Arsenophonus nasoniae (M90801)
Legionella israelensis (Z32640)
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SRS62DBA01
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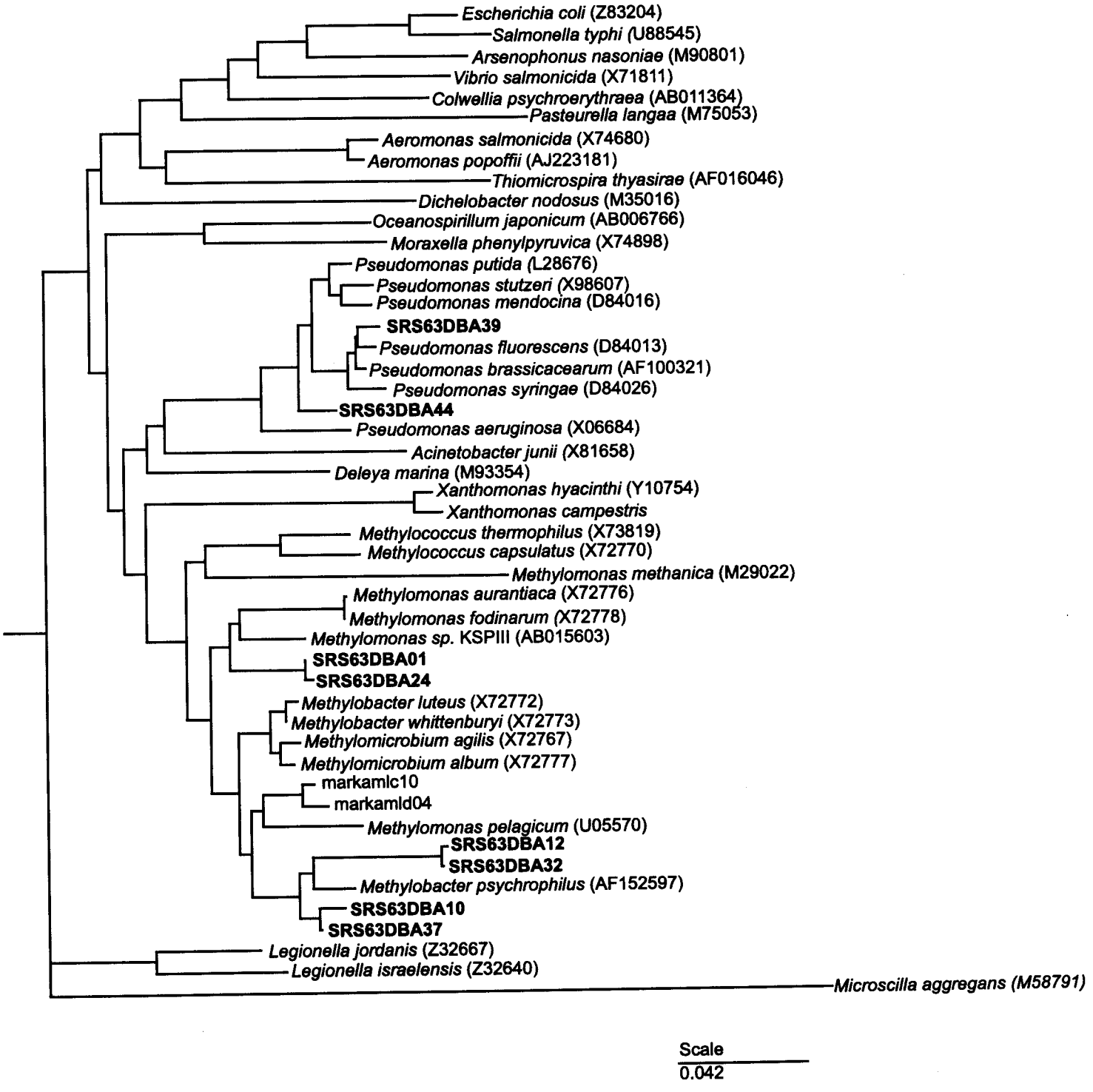
Methylobacter psychrophilus (AF152597)
Methylobacter vinelandii (L20841)
SRS62DBA37
SRS62DBA10
SRS62DBA32
SRS62DBA12
SRS62DBA50
Myxococcus xanthus (AJ233930)
Desulfovibrio desulfuricans (M34113)
Desulfobacter latus (M34414)
Rickettsia prowazekii (M21789)
Simonsiella muelleri (M59071)
Rhodospirillum rubrum (D30778)
Azospirillum brasilense (X79739)
Methylocystis parvus (M29026)
Bradyrhizobium japonicum (D13430)
Rhodomicrobium vannielii (M34127)
Rhodobium orientis (D30792)
Rhizobium leguminosarum (U31074)
Distance Scale
0.114

FIG. 4. Phylogenetic tree generated by the neighbor-joining method from an alignment of 502 nucleotide positions, showing the relationships between LFW 62D clones and clones related to the γ--Proteobacteria. The scale bar indicates the Jukes Cantor distance. The number in parentheses indicates the accession number for the sequence. markamlc10 and markamld04 refer to two novel type I methanotrophic isolates obtained from a landfill by Wise et al 1998.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Aeromonas salmonicida (X74680)
Aeromonas popoffii (AJ223181)
Thiomicrospira thyasirae (AF016046)
Dichelobacter nodosus (M35016)
Oceanospirillum japonicum (AB006766)
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Pseudomonas stutzeri (X98607)
Pseudomonas mendocina (D84016)
SRS63DBA39
Pseudomonas fluorescens (D84013)
Pseudomonas brassicacearum (AF100321)
Pseudomonas syringae (D84026)
SRS63DBA44
Pseudomonas aeruginosa (X06684)
Acinetobacter junii (X81658)
Deleya marina (M93354)
Xanthomonas hyacinthi (Y10754)
Xanthomonas campestris
Methylococcus thermophilus (X73819)
Methylococcus capsulatus (X72770)
Methylomonas methanica (M29022)
Methylomonas aurantiaca (X72776)
Methylomonas fodinarum (X72778)
Methylomonas sp. KSPIII (AB015603)
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SRS63DBA24
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Methylobacter whittenburyi (X72773)
Methylomicrobium agilis (X72767)
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markamld04
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SRS63DBA32
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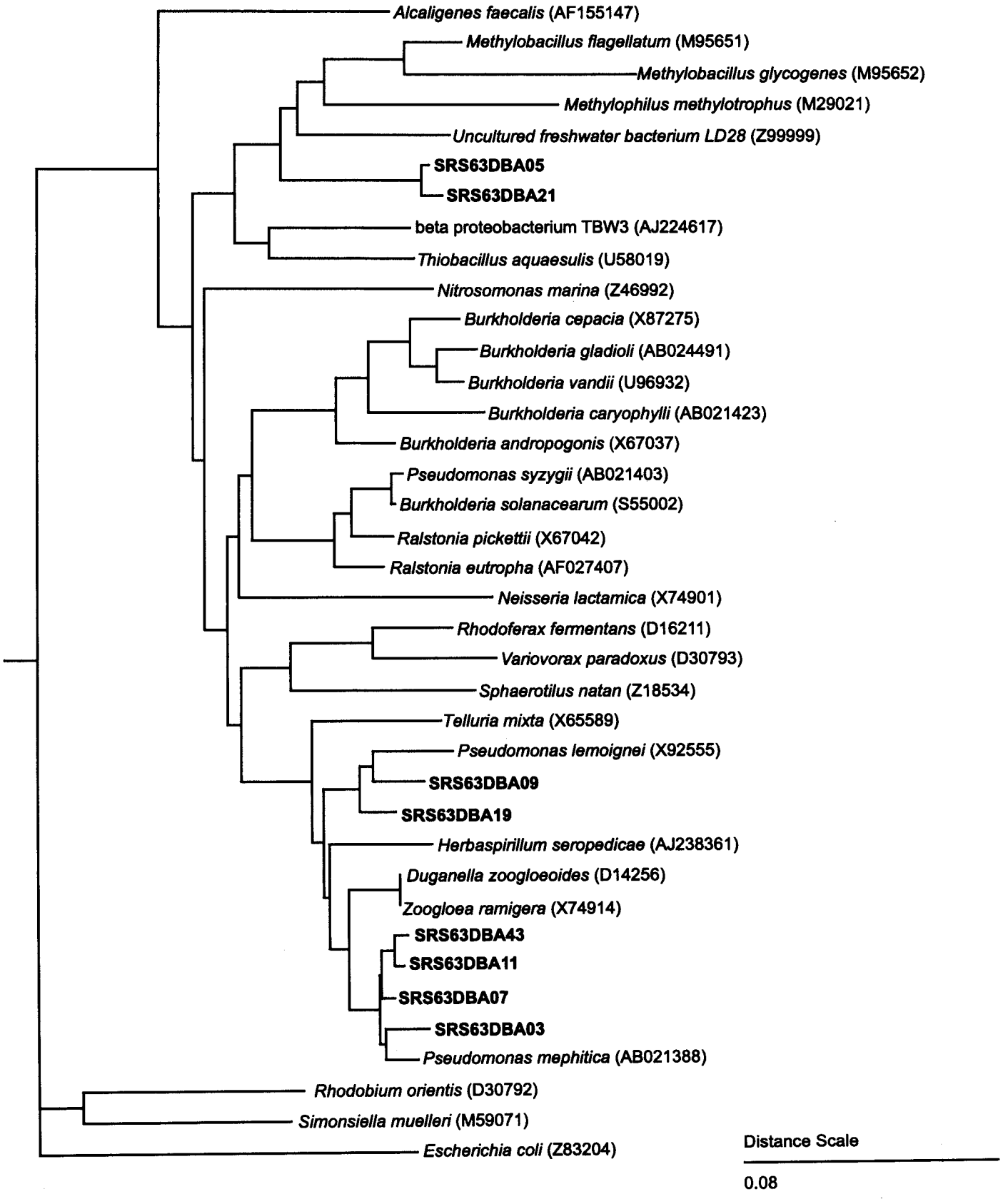
SRS63DBA37
Legionella jordanis (Z32667)
Legionella israelensis (Z32640)
Microscilla aggregans (M58791)
Scale
0.042

FIG. 5. Phylogenetic tree generated by the neighbor-joining method from an alignment of 469 nucleotide positions, showing the relationships between LFW 62D clones and clones related to the β-Proteobacteria. The scale bar indicates the Jukes Cantor distance. The number in parentheses indicates the accession number for the sequence.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SRS63DBA21
beta proteobacterium TBW3 (AJ224617)
Thiobacillus aquaesulis (U58019)
Nitrosomonas marina (Z46992)
Burkholderia cepacia (X87275)
Burkholderia gladioli (AB024491)
Burkholderia vandii (U96932)
Burkholderia caryophylli (AB021423)
Burkholderia andropogonis (X67037)
Pseudomonas syzygii (AB021403)
Burkholderia solanacearum (S55002)
Ralstonia pickettii (X67042)
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Neisseria lactamica (X74901)
Rhodoferax fermentans (D16211)
Variovorax paradoxus (D30793)
Sphaerotilus natan (Z18534)
Telluria mixta (X65589)
Pseudomonas lemoignei (X92555)
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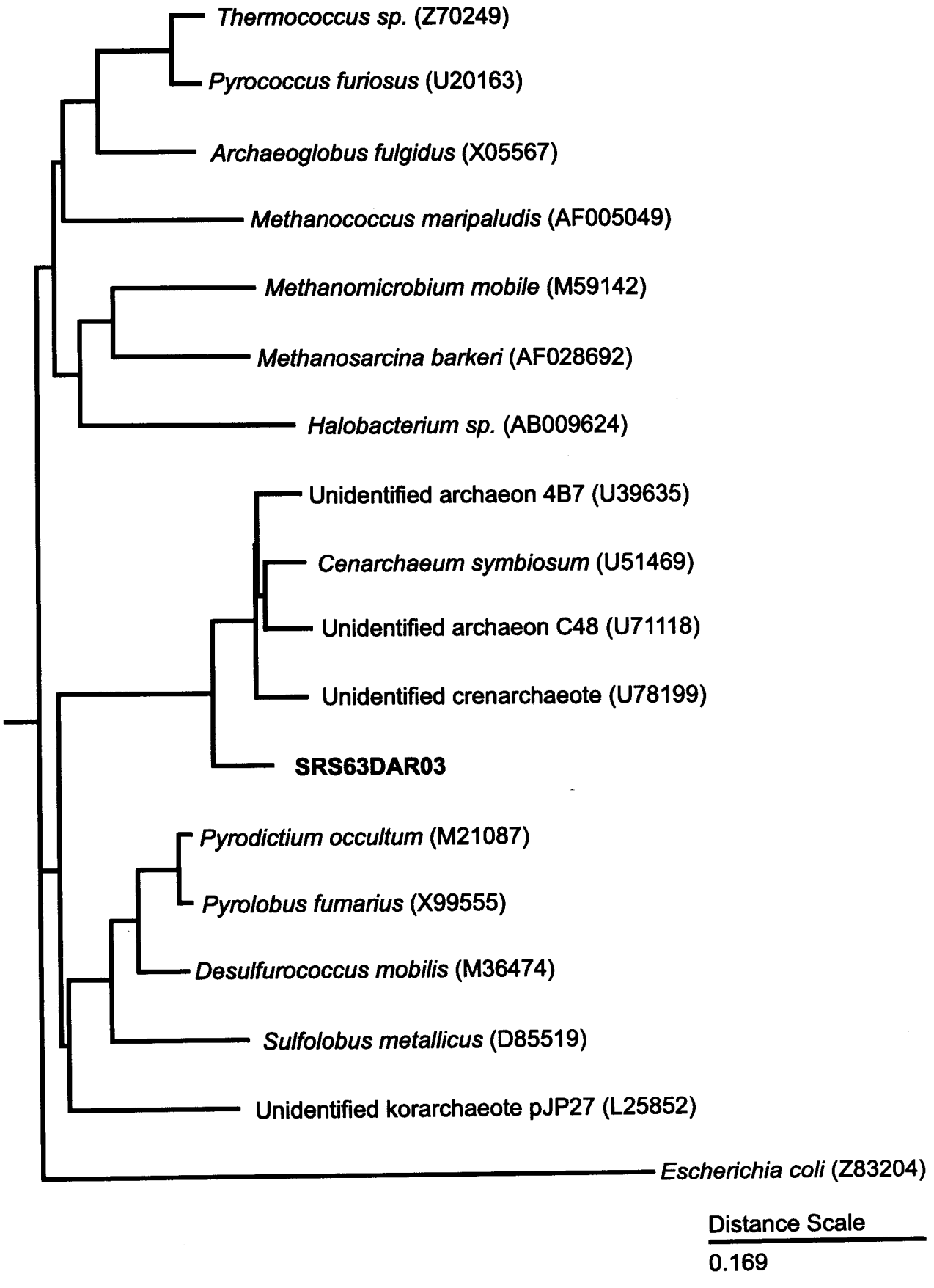
SRS63DBA19
Herbaspirillum seropedicae (AJ238361)
Duganella zoogloeoides (D14256)
Zoogloea ramigera (X74914)
SRS63DBA43
SRS63DBA11
SRS63DBA07
SRS63DBA03
Pseudomonas mephitica (AB021388)
Rhodobium orientis (D30792)
Simonsiella muelleri (M59071)
Escherichia coli (Z83204)
Distance Scale
0.08

FIG. 6. Phylogenetic tree generated by the neighbor-joining method from an alignment of 1,339 nucleotide positions, showing the relationships between LFW 62D archaeal clones and clones related to the organisms in the Archaea domain. The scale bar indicates the Jukes Cantor distance. The number in parentheses indicates the accession number for the sequence.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Methanosarcina barkeri (AF028692)
Halobacterium sp. (AB009624)
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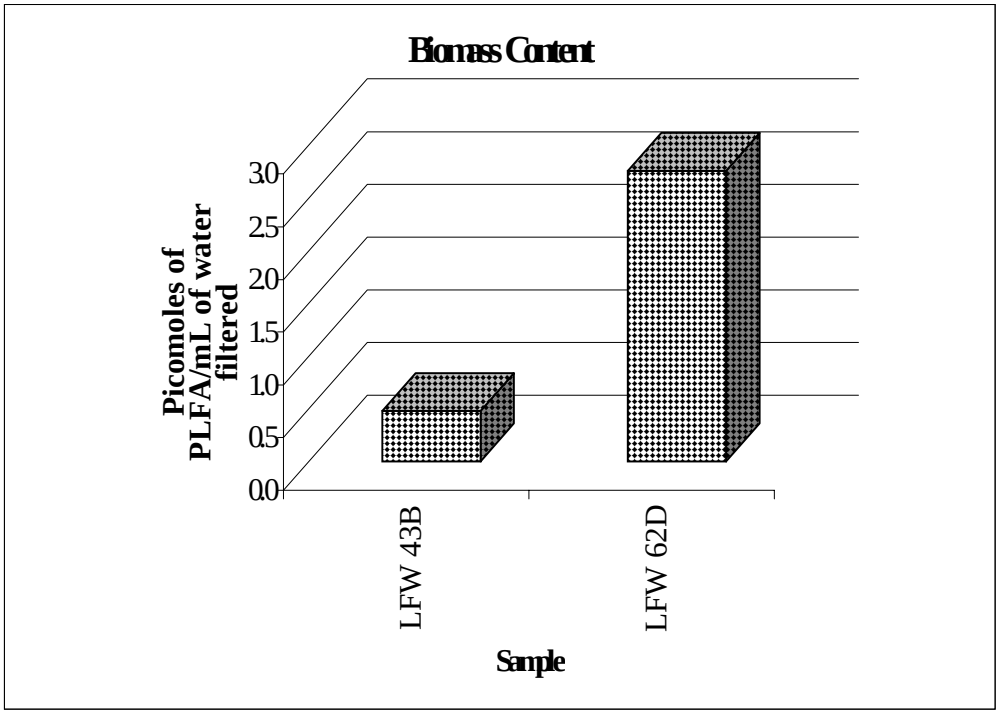
Cenarchaeum symbiosum (U51469)
Unidentified archaeon C48 (U71118)
Unidentified crenarchaeote (U78199)
SRS63DAR03
Pyrodictium occultum (M21087)
Pyrolobus fumarius (X99555)
Desulfurococcus mobilis (M36474)
Sulfolobus metallicus (D85519)
Unidentified korarchaeote pJP27 (L25852)
Escherichia coli (Z83204)
Distance Scale
0.169

FIG 7. Groundwater biomass content and diversity as measured by percentage of total groundwater PLFA for LFW 43B and LFW62D.


Biomass Content
Picomoles of PLFA/mL of water filtered
3.0
2.5
2.0
1.5
1.0
0.5
0.0
LFW 43B
LFW 62D
Sample

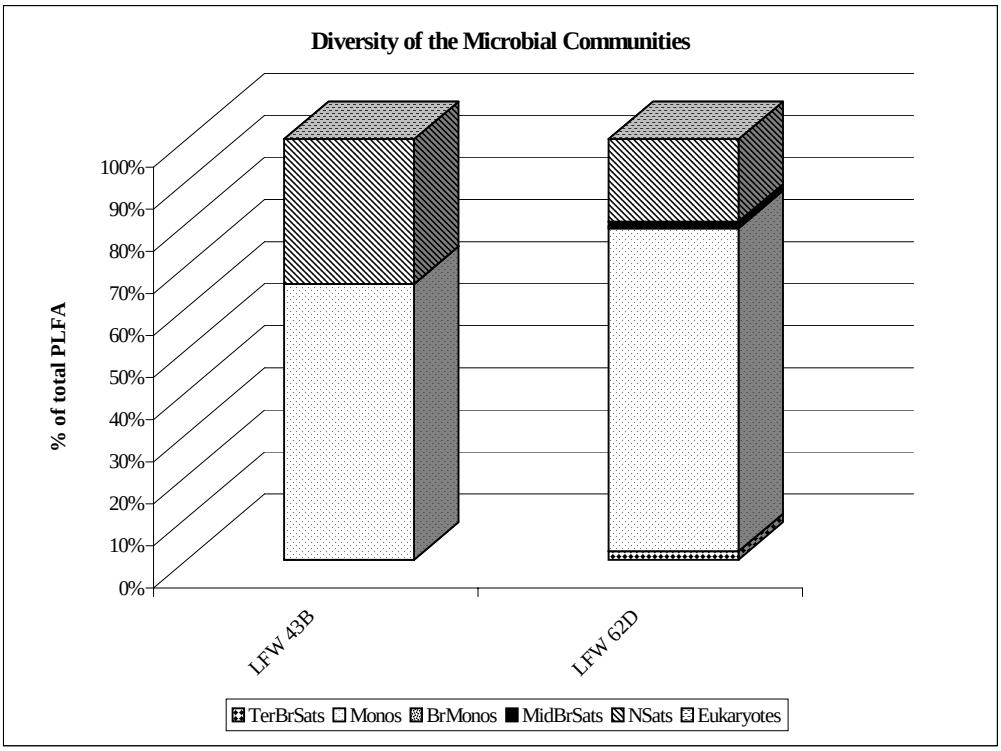
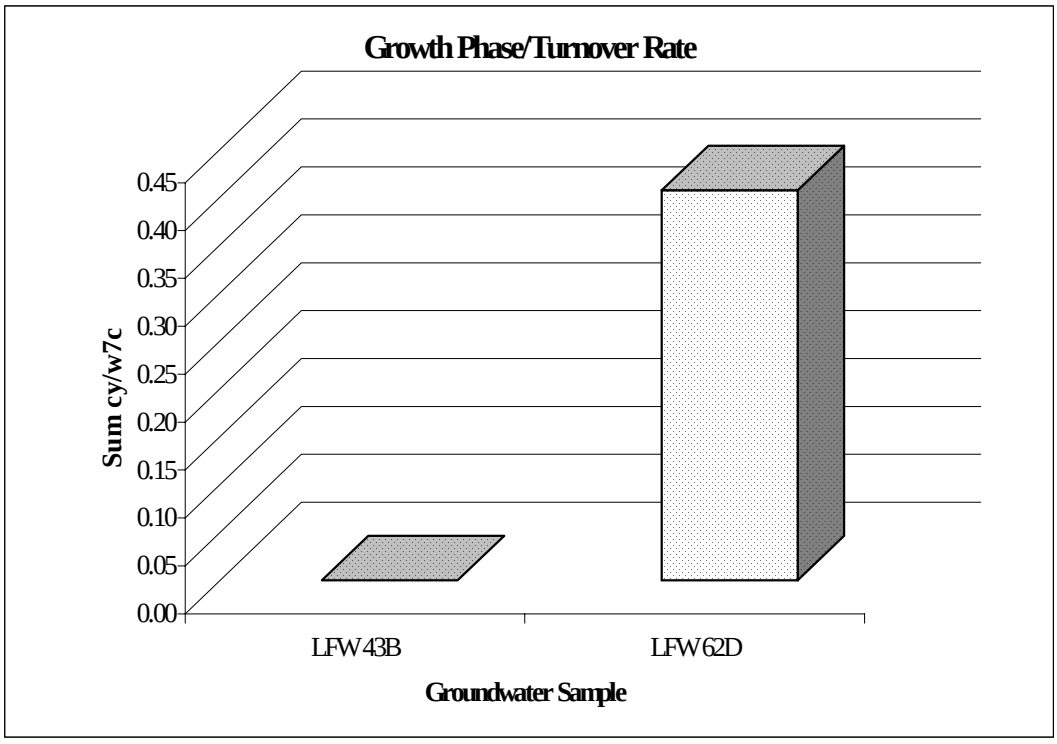

Diversity of the Microbial Communities
% of total PLFA
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
LFW 43B
LFW 62D
TerBrSats
Monos
BrMonos
MidBrSats
NSats
Eukaryotes

FIG 8. Growth phase and comparative membrane permeability in the LFW Gram negative communities.


Growth Phase/Turnover Rate
Sum cy/w7c
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
LFW43B
LFW62D
Groundwater Sample

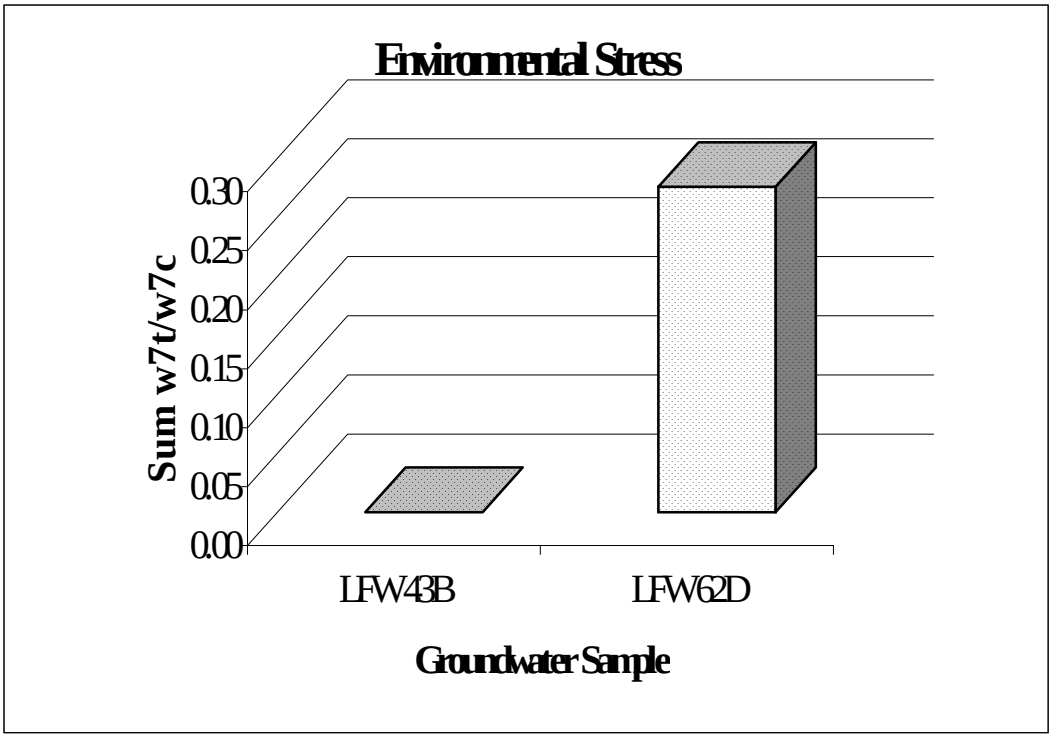
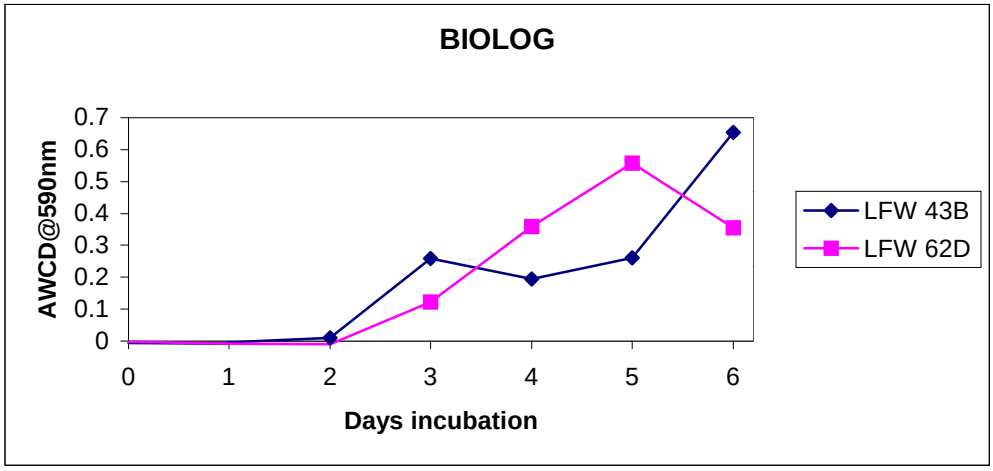

Environmental Stress
Sum w7t/w7c
0.30
0.25
0.20
0.15
0.10
0.05
0.00
LFW43B
LFW62D
Groundwater Sample

FIG 9. Average BIOLOG well color development in LFW 43B; OCRD = 0.131 and in LFW 62D; OCRD = 0.220 and number of individual BIOLOG substrates utilized (>0.1 OD_{590nm}) over time in LFW 62Dand LFW 43B.


BIOLOG
AWCD@590nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
5
6
Days incubation
LFW 43B
LFW 62D

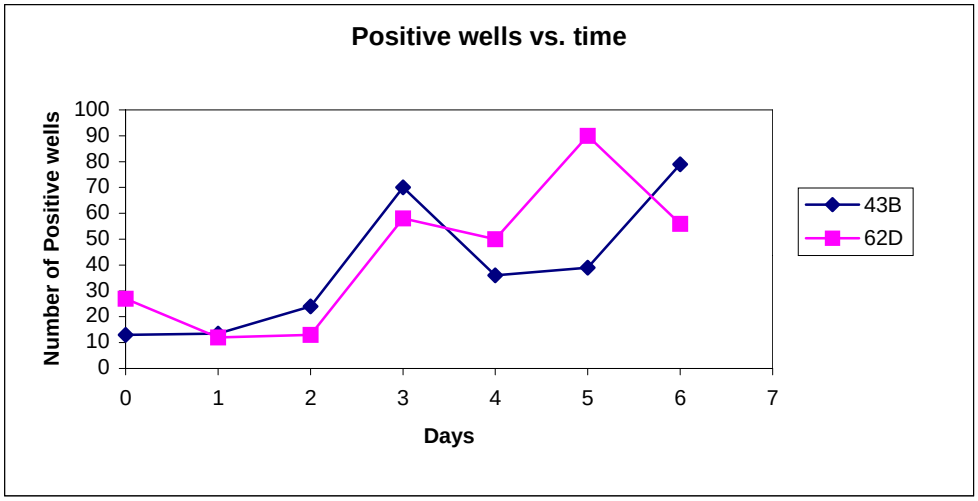

Positive wells vs. time
Number of Positive wells
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
Days
43B
62D

TABLE 1. Summary of microbiological and geochemical analysis on groundwater samples from wells LFW 43B and LFW 62D.

Parameter	Unit	LFW 43B Pristine	LFW 62D Contaminated
Plate Counts	cfu/ml	1.05E+02	8.25E+03
AODC	Cells/ml	1.32E+04	2.27E+06
DFA's	Cells/ml	2.24E+01	1.63E+02
TCE	(ug/l)	ND*	10
c DCE	(ug/l)	ND	70
PCE	(ug/l)	ND	6
VC	(ug/l)	ND	26
CB	(ug/l)	ND	22
Methane	(ppmv in 10 ml sample)	15	11085
Total Iron	UGL	27.4	1780
Chloride	(mg/l)	1.9	37
Nitrate	(mg/l)	4	<1.0
Sulfate	(mg/l)	1.6	5.8
Temperature	C	18.8	21.7
Conductivity	μS/cm	21	170
Alkalinity	mg/l	1	7
DO	Mg/L	9.82	3.57
Turbidity	NTU	0.3	14.7
Depth	Ft	37.2	21.9

*ND=Non Detected

TABLE 2. 16S rDNA sequences from LFW 62D and 43B amplified with bacterial primers and archaeal primers.

Representative Clones	Number of clones found with this ribotype[a]	Closest Relative[b]	Percent similarity[c]
SRS43BBA18	26	*Pseudomonas gessardii*	99.5
SRS62DBA05	7	beta proteobacterium TBW3	90.8
SRS62DBA21	5	beta proteobacterium TBW3	90.3
SRS62DBA03	1	Zoogloea ramigera	96.8
SRS62DBA07	1	*Zoogloea ramigera*	97.8
SRS62DBA11	1	*Zoogloea ramigera*	97.2
SRS62DBA19	1	*Zoogloea ramigera*	96.2
SRS62DBA43	1	*Pseudomonas mephitica*	98.0
SRS62DBA09	9	*Pseudomonas lemoignei*	94.2
SRS62DBA12	3	*Methylobacter psychrophilus*	97.9
SRS62DBA32	1	*Methylobacter psychrophilus*	97.5
SRS62DBA10	1	*Methylomonas sp.* T20	97.4
SRS62DBA37	3	*Methylomonas sp.* T20	98.2
SRS62DBA01	2	*Methylomonas sp.* KSPIII	96.2
SRS62DBA24	1	*Methylomonas sp.* KSPIII	95.9
SRS62DBA39	1	*Pseudomonas brassicacearum*	98.9
SRS62DBA44	1	*Pseudomonas gessardii*	99.8
SRS62DBA50	1	*Acetobacterium wieringae*	71.6
SRS62DAR03	15	*Cenarchaeum symbiosum*	89.3

[a]Ribotype includes all sequences with ≥99.6% sequence similarity.
[b]Refers to the sequence in the Genbank or EMBL databases (Benson *et al*., 1999; Stoesser *et al*., 2000) that possessed the highest percent similarity.
[c]Percent similarity = 100 X (number of identical bases) / (total number of positions compared)