PHYLOGENY OF ARCHAEA AND BACTERIA ISOLATED FROM DEEP SOUTH AFRICAN GOLD AND DIAMOND MINES

by

Sean M. McCuddy

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New Mexico Institute of Mining and Technology

Department of Earth and Environmental Science

Socorro, New Mexico

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To my parents, Steven and Vickie McCuddy

ABSTRACT

The retrieval of subsurface samples for microbial analysis has typically occurred via drilling from the surface, an expensive and contamination-prone process. Deep mines located in and around the Witwatersrand basin in South Africa offer comparatively easy access to the deep subsurface. Here the hypothesis that an indigenous population of microorganisms, not introduced by mining activities, exists in the deep subsurface and varies in composition according to local physical and geochemical characteristics of the host waters was examined. Borehole and fissure water samples collected from the subsurface were used to inoculate various growth media. DNA was extracted from 22 positive enrichments, and 16S rRNA genes were amplified, cloned, screened by RFLP analysis, and sequenced. Phylogenetic analysis of the resulting three archaeal sequences and thirty-eight bacterial sequences indicated a mix of indigenous and contaminating organisms. A deeply-branching, possibly hyperthermophilic crenarchaeote, unrelated to any cultured archaea, was identified. Two apparently novel thermophilic crenarchaeotes from the non-thermophilic Crenarchaeota group were cultured. Most of the thermophilic bacteria were spore formers related to *Bacillus* or *Clostridium* species. Two clostridia probably represent new species. The majority of organisms from room-temperature enrichments were from the *Pseudomonas* and *Aeromonas* genera. Bacteria that may be participating in the anaerobic oxidation of methane were identified in two enrichments with CH_4 and SO_4^{2-} provided as the only intended electron donor and acceptor,

respectively. The novel archaea and thermophilic spore-forming bacteria were associated with old, warm saline waters and are taken to be members of an indigenous deep subsurface community not associated with mining contamination. Mesophilic bacteria closely related to widespread surface organisms and associated with cooler, less saline waters likely represent contaminants introduced into the subsurface by mining. Some mesophilic bacteria with novel 16S rRNA sequences and/or novel metabolic capabilities may be native to the subsurface.

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INTRODUCTION

Life in the deep subsurface

In recent decades it has become apparent that life exists in many places previously thought to be uninhabitable. Microbial life has been discovered nearly everywhere liquid water is present and temperatures are less than ~120°C (Rothschild and Mancinelli, 2001). Life has been shown to exist in many so-called “extreme environments,” from boiling hot springs in Yellowstone National Park (Barns et al., 1994) to sea ice in Antarctica (Thomas and Dieckmann, 2002) to volcanic rocks buried hundreds of meters below the sea floor (Furnes and Staudigel, 1999). Microorganisms isolated from such environments may have important biotechnological applications in fields ranging from medicine to bioremediation (Deming, 1998; Franco and McClure, 1998; Fredrickson and Onstott, 1996; Niehaus et al., 1999; Omura, 1992; Ralebitso et al., 2002; Vandamme, 1994).

The deep terrestrial subsurface represents another extreme environment where microbes have been found and studied in recent years (Chapelle et al., 2002; Colwell et al., 1997; Krumholz et al., 1997; L'haridon et al., 1995; Onstott et al., 1998; Pedersen, 1997; Slobodkin et al., 1999; Stetter et al., 1993; Stevens and McKinley, 1995). Extreme conditions that can be found in the subsurface include high temperature and pressure, a paucity of available energy sources and/or electron acceptors, high salinity, and high levels of ambient radiation (Onstott et al., 1997). Due to limited and difficult access to

the subsurface, comparatively little is known about the organisms inhabiting this environment. Subsurface life, however, may be widespread and comprise a significant component of the earth's biosphere (Gold, 1992).

In addition to yielding novel microbes with potential applications in areas such as bioremediation (Fredrickson et al., 1991), the study of subsurface microbial life may offer insights into the origin and early evolution of life on Earth, as well as the possibility of life on other planets such as Mars. Few, if any, problems in science have been studied as intensely as the origin of life. Yet relatively little is known about how it may have actually occurred. It is difficult enough to find clues about life's earliest existence due to an absent or intensely deformed early geological record. As the oldest known rocks are 4.03 Gyr old (Bowring and Williams, 1999), a record of the first 500 million years of Earth's history does not exist apart from few ancient zircons (Mojzsis et al., 2001; Wilde et al., 2001). The first reported evidence of cellular fossils in the geological record occurs in the 3.465-Gyr-old Apex chert in Australia (Schopf, 1993; Schopf et al., 2002). However, this claim has recently been disputed (Brasier et al., 2002). Likewise, claims to the earliest isotopic evidence of life at Akilia, Greenland, reported at ~3.85 Ga (Mojzsis et al., 1996), have also been questioned (Fedo and Whitehouse, 2002). The best documented evidence for the earliest life on Earth, therefore, comes from ^{13}C-depleted graphite particles in >3.7 Ga turbiditic and pelagic sedimentary rocks from west Greenland (Rosing, 1999). Life may not have been able to exist much before this time, at least not continuously (Maher and Stevenson, 1988), due to the high incidence of impact events during the 'late heavy bombardment' (Kasting, 1993; Nisbet and Sleep, 2001), which ended approximately 3.8 Ga. Alternatively, life may have survived the harsh

surface conditions created by the bombardment if it had evolved to fill protected niches in the subsurface (Chyba, 1993; Sleep and Zahnle, 1998). The deep subsurface has even been suggested as a possible location for the origin of life (Pedersen, 1997). Continuing studies of microbial life in the deep subsurface of Earth may shed light on the origin and early evolution of life.

Today the surface of Mars is a cold, dry, inhospitable desert. It has become clear, however, that early in its history Mars was much more dynamic and had an active surface hydrological cycle (Farmer and Des Marais, 1999; Squyres and Kasting, 1994). If life ever evolved on the surface of Mars, it would have had to migrate into the subsurface as conditions at the surface became more and more inhospitable. If it is assumed that liquid water currently exists below the surface of Mars (due to internal heat), then it is possible that life, if it ever evolved on Mars, could still exist today in the subsurface (Boston et al., 1992; Clifford, 1993; Farmer and Des Marais, 1999; Fisk and Giovannoni, 1999; Jakosky, 1998; Max and Clifford, 2000; Nealson, 1997). Such hypothetical ecosystems would likely be similar to H_2-based communities found in the subsurface of the earth (Chapelle et al., 2002; Pedersen, 1997; Stevens and McKinley, 1995). Insights gained from the examination of subsurface life on Earth will continue to be helpful in assessing the possibility of past or present life on Mars, as well as how to best to focus the search for such life.

Deep subsurface life in South African gold mines – project background

Although interest in deep subsurface microbiology has increased rapidly in recent years, easy access to the subsurface has not. Most subsurface samples have been obtained by drilling from the surface. Thus, contamination of the sample, whether rock,

sediment, or ground water, by drilling fluid and unsterile equipment is a major source of concern and frustration. Various tracers can be employed to quantify the degree of sample contamination (Fredrickson et al., 1997; Pedersen, 1993), but retrieving truly pristine subsurface samples is not possible. Moreover, drilling boreholes from the surface is expensive and does not provide a good representation of the microbial processes occurring laterally at depth.

Accessing the subsurface via existing mine shafts and tunnels offers an attractive alternative to drilling from the surface. While subsurface access through mines is less expensive and negates the need to drill through hundreds or thousands of meters of overlying rock, it is not without its own set of contamination problems (Onstott et al., 2002). Nonetheless, mines provide a good opportunity to investigate the biogeochemistry of the subsurface. The deep gold mines in the Witwatersrand basin, South Africa, with depths reaching >3 km below the surface (kmbls), are particularly well suited to the investigation of microbial life in the deep subsurface. Over a hundred years of mining in the region has resulted in one of the most extensive mining infrastructures in the world. Such an infrastructure is conducive to collecting samples over a wide lateral and vertical range. Currently, a team of scientists led by geologist Tullis Onstott at Princeton University is exploring subsurface life in these gold mines (Kerr, 2002), as well as in both a platinum mine and a diamond mine located north of the basin.

The group has already produced some interesting results. Initial studies (Onstott et al., 1997) in the deep gold mines focused on the feasibility of collecting high quality, asceptic microbial samples, the presence or absence of indigenous microbes in these

samples, and the possible role played by microorganisms in the mineralization of the Carbon Leader, the most productive gold reef mined. The Carbon Leader is a thin (maximum thickness of 13 mm), organic-rich layer with concentrated deposits of gold and uranium. It occurs at the contact between the Jeppestown shale and Johannesburg quartzite. Results from samples collected at Western Deep Levels, No. 1, level 109 (3.231 kmbls), indicated the presence of possibly indigenous sulfate-reducing and iron-reducing microorganisms from the quartzite immediately above the Carbon Leader. A metal-reducing *Thermus* species was isolated from water emanating from a borehole drilled near the Carbon Leader. A variety of contaminating bacteria and some fungi, both introduced into the subsurface by mining, were also recovered. Radiolysis of water (e.g., around the U-enriched Carbon Leader) was hypothesized as a possible source of H_2 for subsurface lithoautotrophy (Lin et al., 2002).

Characterization of the metal-reducing *Thermus* species, designated *Thermus* strain SA-01, was carried out by Kieft et al. (1999). The authors found the thermophilic bacterium capable of using O_2, NO_3^-, Fe(III), and S^0 as terminal electron acceptors for growth. *Thermus* strain SA-01 was also capable of reducing Mn(IV), Co(III)-EDTA, Cr(VI), and U(VI). Growth did not occur fermentatively. The optimal temperature and pH for growth were 65°C and 6.5-7.0, respectively. Although the genus *Thermus* has been studied extensively, the metabolic diversity exhibited by strain SA-01 had not previously been shown. Strain SA-01 was also shown to be phylogenetically related to *Thermus* strains NMX2 A.1 (Morgan, unpublished data) and VI-7 (Williams and Sharp, 1995), from New Mexico and Portugal, respectively. Environmental 16S rDNA with >99% homology to strain SA-01 was extracted from rock samples near the borehole from

which strain SA-01 was isolated (Fredrickson and Bailey, unpublished results). It is possible that metal-reducing microorganisms such as *Thermus* strain SA-01 played a role in the mineralization of both the Carbon Leader and similar ore deposits.

A novel, extremely alkaliphilic bacterium was isolated from a containment dam located 3.2 kmbls in a Driefontein gold mine (Takai et al., 2001b). The isolate, designated SAGM1, with a proposed name of *Alkaliphilus transvaalensis*, was shown to grow over a temperature range of 20-50°C, with an optimum at 40°C and an associated doubling time of 45 minutes. A pH range of 8.5-12.5 was required for growth, with the optimum pH being 10.0. The organism is a member of cluster XI in the low G+C Gram-positive bacteria, but is only distantly related to previously described members (87.8-91.8% homology). It is a strictly anaerobic chemoorganotroph capable of using proteinaceous substrates such as yeast extract, peptone, and tryptone for growth. SAGM1 is one of the most alkaliphilic microorganisms described to date and is proposed to be a consumer of dissolved and particulate organic matter in alkaline deep subsurface waters.

Bacteria and fungi are not the only microorganisms to have been found in the deep subsurface. A phylogenetically diverse group of archaea, including both crenarchaeotes and euryarchaeotes, also inhabits waters in the deep subsurface (Takai et al., 2001a). Using culture-independent methods, Takai et al. (2001a) assessed the archaeal communities in deep fissure water, in mine service water, and in water from an overlying dolomite aquifer. Samples were collected from the Driefontein, Kloof, and Beatrix mines in the Witwatersrand basin. Distribution of the archaea varied with the source of the waters (e.g., fissure vs. service). The authors found that, in general, the

archaea present in the gold mines are distantly associated with archaea from other environments. Several novel archaeal lineages were also discovered. In a fissure water sample taken at 3.08 kmbls from Kloof Mine, 16S rDNA with 99.4% homology to that of *Pyrococcus abyssi* was recovered. *Pyrococcus* species are hyperthermophilic archaea that inhabit marine hydrothermal vent systems. The presence of *Pyrococcus*-like species in the Kloof sample suggests that hot, saline, anaerobic water containing hyperthermophiles may have migrated upward from a depth of 5-6 kmbls, where ambient temperatures are in the range of hyperthermophiles, and mixed with meteoric water.

Baker et al. (2002) examined sulfate-reducing bacteria in gold mine borehole environments. The authors installed sterile cartridges filled with crushed country rock on two chemically distinct, slowly draining boreholes at depths of 3.21 and 2.7 kmbls. The cartridges remained in place for 47 and 61 days, respectively, to allow biofilm accumulation on the crushed rock. Genes for dissimilatory sulfite reductase (DSR) and 16S rRNA were sequenced from clones derived from DNA extracts from the cartridge material. Phylogenetic analysis of the 16S rRNA genes revealed a cluster of organisms most closely related to *Desulfotomaculum thermosapovorans* and *Desulfotomaculum geothermicum*. However, the DSR sequences from the two boreholes were found to be phylogenetically distinct. A novel, deeply branching cluster related to *Thermodesulfovibrio yellowstonii* may represent a new group of sulfate-reducing bacteria.

Using the dissolved noble gas isotopes ^{4}He, ^{40}Ar, ^{134}Xe, and ^{136}Xe, as well as ^{36}Cl data, Lippmann et al. (2002) dated waters ranging in depth from 0.718 to 3.3 kmbls in the South African gold mines. Low $^{36}Cl/Cl$ ratios indicated that the fissure waters were all

older than 1.5 Myr, or 5 times the half-life of ^{36}Cl. Noble gas model ages, based on a range of assumptions, yielded subsurface residence times between 3 Myr and 418 Myr for the waters. The possibility of mixing between small quantities of younger water and the old water could not be ruled out.

Omar and Onstott (2002) have recently described the thermal history of the northern margin of the Witwatersrand basin. The authors performed fission track analyses of detrital apatite grains from the subsurface in order to constrain the maximum possible age of any indigenous microbial communities found there. The region examined has produced evidence of thermophilic and hyperthermophilic microorganisms at depth (Kieft et al., 1999; Takai et al., 2001a). Forty-seven samples from four exploratory cores were analyzed. The data suggest that the rocks from the cores had cooled to ~120°C between 422 to 21 Ma. Modeling of one sample collected at 3.7 kmbls indicates the sample area reached present day temperatures at 30 Ma from 120°C at 80 Ma. The fission track results suggest that non-hyperthermophilic organisms living at or below the present depth of 1.7 kmbls must have been transported to their current location after 70 Ma. Additionally, the authors suggest that hyperthermophiles found at present depths of 1.2-3.7 kmbls could be descended from hyperthermophilic microorganisms that entered the subsurface in the early Paleozoic era.

In addition to water and rock samples, gas samples from the deep subsurface in South Africa have also been analyzed. High-pressure pockets of water and gas coexist in the deep subsurface and are often intersected during normal drilling operations. The gases are a mix of hydrogen gas and hydrocarbon gas. Most hydrocarbon gases are formed by thermal decomposition of organic matter or by microbial processes, but an

abiogenic source of hydrocarbons has also been shown to exist (Lollar et al., 2002a). Lollar et al. (2002a) provided evidence of abiogenic hydrocarbon formation in the form of unusual patterns of $\delta^{13}C$ values, as well as the relationship between $\delta^{13}C$ and $\delta^{2}H$, in C_1-C_4 alkanes from Kidd Creek Mine in Timmins, Ontario, Canada. The same isotope enrichment and depletion trends used to identify the abiogenic gases at Kidd Creek Mine are presently being applied to gases from the deep mines in the Witwatersrand basin (Lollar et al., 2002b) to distinguish abiogenic gases from more typical sources, such as microbial methanogenesis. The extent to which hydrocarbon and hydrogen gases support deep subsurface microbial communities is also being examined. Recent results appear to indicate that methane and other hydrocarbons from depths greater than 2 kmbls originate by abiogenic reduction of CO_2 and oxidation of H_2 (Onstott et al., 2003). In contrast, gases from depths less than 2 kmbls contain a significant microbial methane component (Onstott et al., 2003).

H_2 is thought to be an important source of energy in the deep subsurface, where organic compounds are typically scarce, and forms the basis of lithoautotrophic communities that exist independent of photosynthesis (Chapelle et al., 2002; Stevens and McKinley, 1995). Analyses of dissolved H_2 and He from deep waters in South African gold mines suggest that a substantial amount of H_2 is generated in the subsurface by radiolysis of water on a time scale of millions to tens of millions of years (Lin et al., 2002). The H_2 and He concentrations in one analyzed sample agreed exactly with the concentrations predicted by radioactive decay in the host rock and indicate an annual H_2 production rate of 0.1 to 1 nanomoles/liter/year. Other samples had concentrations less

than predicted and indicate the presence of one or more H_2 sinks, which may include microbial H_2 consumption or abiogenic formation of hydrocarbon gases, or both.

Finally, the problem of mining-induced contamination of the subsurface environment in the South African gold mines is addressed in Onstott et al. (2002). Hand samples, block samples, and core samples, as well as associated service water used during mining operations, were collected from an actively mined region of the Carbon Leader. Rhodamine WT dye and fluorescent microspheres were used to quantify the degree of contamination of the rock samples by the service water. Despite contamination levels $<0.01\%$ in the pared rock samples, most of the microorganisms present were found to be closely related to organisms in the service water. Mining-induced fractures in the low permeability rocks are thought to enable the contaminants to infiltrate the rocks via service water and mine air before sampling. Concentrations of indigenous microorganisms in the rocks were found to be less than 100 cells per gram. The authors conclude that the impact of fracturing on indigenous microbial communities in the rock should not be underestimated and stress that samples for microbial analysis should be obtained through exploration coring (past the zone of fracturing) when possible.

Ongoing research by the South Africa group is continuing to shed light on the biogeochemistry of the deep subsurface. In this paper, the cultivation, identification, and phylogeny of microorganisms collected from mines in the Witwatersrand basin and from the Premier Mine kimberlite north of the basin are described. The hypothesis that an indigenous population of microorganisms, not introduced by mining activities, exists in the deep subsurface and varies in composition according to local physical and geochemical characteristics of the host waters is examined. The results indicate a

probable mix of indigenous microbes that may have been present in the deep subsurface for millions of years and contaminants from the surface, introduced via mining activity. Phylogenetic analyses indicate some of the cultivated microorganisms may be previously undescribed. Evidence bearing on the previous suggestion that hot water containing hyperthermophiles may be migrating upward from greater depths in the basin (Takai et al., 2001a) is also discussed.

Geology of the Witwatersrand basin and Premier kimberlite, South Africa

Witwatersrand basin. The Witwatersrand basin is an integral part of the Kaapvaal craton, one of the oldest Archean cratons known (de Wit et al., 1992). The basin forms an elongate saucer-shaped structure (Coward et al., 1995) approximately 360 km by 200 km and has a long axis trending NE-SW. The basin consists of approximately 7 km of mainly arenaceous and argillaceous rocks, with minor rudaceous components, deposited episodically in a fluvio-deltaic environment over a period of 360 Myr (Robb and Meyer, 1995). The basin is undoubtedly the world's most important gold province. Nearly 40% of all the gold mined during the course of recorded history has come from the Witwatersrand basin over the past 120 years (Frimmel, 2002). The Witwatersrand Supergroup is the central component of the basin and is subdivided into the lower West Rand Group and the upper Central Rand Group. Underlying the Witwatersrand Supergroup are the volcano-sedimentary rocks of the Dominion Group, and covering the Witwatersrand Supergroup are the mainly volcanic rocks of the Ventersdorp Supergroup. Together, the Dominion, Witwatersrand, and Ventersdorp are commonly known as the Witwatersrand Triad (Coward et al., 1995). Throughout much of the basin the

Witwatersrand Triad is overlain by the Transvaal Supergroup or by a thin cover of Karoo sediments.

U-Pb isotope geochronology has been used to constrain the ages of deposition for the various units making up the volcano-sedimentary sequence in the Witwatersrand basin and is summarized in Robb and Meyer (1995). Sediments comprising the lower portion of the Dominion Group were deposited over a fairly short period, between 3086 ± 3 Ma, which is the age of the Westerdam granite, on which the Dominion sediments were deposited, and 3074 ± 6 Ma, the age of the lavas at the top of the Dominion Group. The Witwatersrand sediments overlie either the Archean granite-greenstone basement complex or the Dominion Group. Hence, the maximum age of the onset of sedimentation within the Witwatersrand Supergroup is 3074 ± 6 Ma. Lavas that immediately overlie the Witwatersrand Supergroup over wide areas form the base of the Ventersdorp Supergroup and have been dated to 2714 ± 8 Ma. Thus, the Witwatersrand sediments were deposited over a period of 360 Myr. Deciphering the ages of the various pulses of sedimentation within the sequence, however, has proven difficult. Detrital zircons dated at 2970 ± 3 Ma have been found in the Promise reef of the West Rand Group. These zircons set a maximum constraint on the age of the West Rand Group and indicate that a significant gap (~100 million years) existed between the end of Dominion deposition and the onset of Witwatersrand sedimentation. The Crown lava, occurring just below the West Rand Group – Central Rand Group transition has been dated to 2914 ± 8 Ma and thus provides approximate minimum and maximum age constraints on West Rand and Central Rand deposition, respectively. Central Rand Group deposition had terminated by the onset of Ventersdorp volcanism at ~2714 Ma. A maximum age of 2890 Ma for the

Central Rand Group has recently been obtained from detrital zircons in the lower part of the sequence (Poujol et al., 1999). A minimum age of 2760 Ma for the group has recently been determined based on the oldest authigenic xenotime Pb ages in the sequence (England et al., 2001).

Evidence for greenschist facies metamorphism in the Witwatersrand basin is widespread (Phillips and Law, 1994). Silicate mineral assemblages from all the major gold fields in the basin indicate peak metamorphic conditions equivalent to the chlorite zone, with peak conditions having reached 350 ± 50°C and up to 3 kb. In three isolated areas of the basin, however, higher metamorphic grades are evident. These areas include the northwest margin of the basin, an area northeast of the Evander gold field, and the collar of the Vredefort Dome. Three principle episodes of metamorphism in the basin are recognized (Robb and Meyer, 1995). The first two, at about 2500 Ma and 2300 Ma, are probably related to progressive burial metamorphism caused by the deposition of the lower and upper portions of the Transvaal Supergroup. The third episode, at ~2000 Ma, is likely related to either the Vredefort impact event or the intrusion of the Bushveld Igneous Complex, or both.

Most of the economically important gold deposits in the Witwatersrand basin are found in the conglomerates of the Central Rand Group (Robb and Meyer, 1995). There has been a long-standing debate over the origin of the gold deposits, with some arguing for a sedimentary placer origin (Minter, 1999) and others for a hydrothermal origin (Barnicoat et al., 1997). A recent study (Kirk et al., 2002) seems likely to put an end to the debate. Kirk et al. (2002), using the Re-Os dating method, found gold and rounded pyrites from conglomerates in the Central Rand Group to be 3.03 ± 0.02 Gyr. This age is

older than the 2.89- to 2.76-Gyr host conglomerates of the Central Rand Group and indicates that the gold was detrital in origin.

Premier kimberlite. The Premier kimberlite is also found in the Kaapvaal craton in South Africa. It is located about twenty miles east of Pretoria, South Africa, at coordinates of 25°49'S, 28°30'E (Janse and Sheahan, 1995). The kimberlite had been thought to be Cretaceous in age, similar to other South African kimberlites, but was later shown to be Proterozoic in age (Allsopp et al., 1967). The diatreme has yielded the world's largest gem diamonds, as well as an array of inclusion-bearing diamonds that have been studied extensively (Burgess et al., 1989; Phillips et al., 1989; Richardson, 1986; Richardson et al., 1993; Richardson et al., 1990).

The Premier diatreme itself is a composite body, consisting of three major intrusions of kimberlite, which was subsequently cut by an 80-m thick gabbro sill and magnetite-serpentine-calcite dykes (Robinson, 1975). The three main kimberlite intrusions are known as the Brown Kimberlite, Grey Kimberlite, and Black Kimberlite. The preferred emplacement age of the Premier diatreme is 1,180 ± 30 Ma (Richardson et al., 1993), and the sill has been dated to 1115 ± 15 Ma (Allsopp et al., 1967). The kimberlite pipe intrudes the Transvaal Supergroup and the Waterburg Group fluvial sediments, as well as a norite phase of the Bushveld Complex (Richardson et al., 1993). The 2060 Ma Bushveld Complex is one of the largest layered intrusions known, containing 66,000 km^2 of mafic rocks alone, and has a thickness of 7-9 km (Eales et al., 1993).

MATERIALS AND METHODS

Sample collection

Between June and September 2001, subsurface borehole and fissure water samples were collected from the Evander, Kloof, and Driefontein mines in the Witwatersrand basin and from the Premier Mine kimberlite near Pretoria. At Evander, samples were taken from 8 shaft, 18 level (hereafter designated EV818); 2 shaft, 19 level (EV219); and 8 shaft, 21 level (EV821). At Kloof, samples were collected from 7 shaft, 39 level (KL739); 4 shaft, 43 level (KL443); and 7 shaft, 37 level (KL737). Driefontein samples were taken from two sites, designated hole 1 and hole 2, at 9 shaft, 38 level (DR938H1 and DR938H2, respectively) and from 5 shaft, 48 level (DR548). One sample was collected from 763 level of the Premier diamond mine (PR763). Sample depths in the three gold mines ranged from 1.474 – 3.4 kmbls; the Premier sample was collected at 0.763 kmbls.

At each site, a suite of samples was collected for chemical, gas, and biological analyses. However, a complete set of samples could often not be collected due to uncontrollable factors such as borehole/fissure quality and location, safety precautions, and mine-imposed time constraints. Moreover, not all samples collected were of the highest quality. Samples were collected for the following: anions, cations, dissolved ammonia ^{15}N, dissolved iron, phospholipid fatty acids, environmental DNA, fluorescent in situ hybridization, microbial enrichments, dissolved inorganic carbon (DIC), dissolved

O_2, total ammonia ^{15}N, phosphorus, formate, acetate, dissolved organic carbon (DOC), total organic carbon, volatile organic carbon, sulfide, sulfite, thiosulfate, polysulfide, $\delta^{34}S$, δD, $\delta^{18}O$, $\delta^{13}C$ DIC, ^{14}C, ^{36}Cl, ^{3}H, $\delta^{13}C$ DOC, dissolved gases, and dissolved noble gases. The present study focuses on microbial enrichments, with some reference to the other data sets. Details of the geochemical, gas, and other biological analyses will be reported elsewhere.

Before sampling, several 140 ml serum vials were sealed with butyl rubber stoppers and metal rings, flushed with nitrogen, and then autoclaved with the other equipment to be used for subsurface sampling. All equipment used in sample collection was wrapped, autoclaved, and kept sterile until used in the field. Underground, an adjustable packing system designed to enable asceptic water sampling (Moser et al., 2002) was inserted into the borehole. A manifold was connected to the end of the packing system. This manifold consisted of a primary valve to control the water flow rate and six secondary valves for connecting tubes and taking samples. To collect the samples for microbial enrichments, a sterile tube with a syringe tip at one end was connected to the manifold. A needle was attached to the syringe tip and inserted into the 140 ml serum vials. As they filled, the vials were periodically vented using a smaller gauge needle.

Samples for microbial enrichments could not always be taken in this manner, in light of the uncontrollable factors listed above. When controlled sampling was not possible, samples were collected by alternative means, with the highest regard to asceptic sampling technique. For the DR548 sample, the metal ring and butyl stopper were removed from a 140 ml vial underground. The vial was filled with water trickling out of

a pipe connected to the borehole and then re-stoppered and sealed. For the DR938H2 sample, a 10-ml syringe was used to withdraw water from a high-pressure stream of water emerging from a previously sealed borehole, which was opened immediately prior to sampling. After each fill, a new sterile needle was attached to the syringe and the water injected into the 140 ml collection vial. For the EV821 sample, an unsterile serum vial was used to collect water trickling out of a pipe connected to the drill, which was still inserted in the borehole. The sample was intended for chemical analysis only, as it was obviously contaminated, but it was later used for high temperature enrichments as well when it was decided that the intersected water could be quite ancient and saline. For the KL443 sample, a 10-ml syringe with needle was inserted into a fractured borehole. Water was withdrawn and subsequently injected into the 140 ml vial. For the KL737 sample, the stopper and seal were removed from a 140-ml vial, and water was collected as it seeped from a fault contact. For the PR763 sample, a 10-ml syringe with needle was used to withdraw water from a large, flowing borehole and inject it into a 140 ml vial.

Subsurface samples were transported to a field lab in Glenharvie in the Witwatersrand basin and processed or preserved for transport to the United States. The samples collected for microbial enrichments were used to inoculate various growth media, usually within one day of sample collection, in an attempt to cultivate microorganisms inhabiting the water.

Microbial enrichments

Two types of media were used. The first, designated TYG/NO_3^-, was a heterotrophic medium with nitrate (Kieft et al., 1999) (Appendix 1). The medium was dispensed into Balch tubes (Bellco, Vineland, NJ) (10 ml per tube), gassed with a mixture

of 80% N_2 and 20% CO_2 for 10 minutes, sealed with butyl rubber stoppers and metal rings, and autoclaved.

The second type of enrichment medium used was a basal salts medium (Kieft et al., 1999) (Appendix 1) amended with various combinations of electron donors and acceptors. Eight ml of the basal medium was dispensed into each Balch tube. The tubes were gassed with 80% N_2 and 20% CO_2 for 10 minutes, sealed with butyl rubber stoppers and metal rings, and autoclaved. After autoclaving, 0.15 ml of Wolfe's vitamin solution (Atlas, 1993) (Appendix 1) and 0.1 ml of Wolfe's mineral solution (Atlas, 1993) (Appendix 1), both anaerobic, were added to each tube. Each tube was then amended with ~1 ml each of 100 mM sodium acetate, sodium lactate, and sodium pyruvate, all anaerobic, and the headspace was filled with 10 ml of hydrogen. One ml of an electron acceptor (100mM), made anaerobic, was also added to each tube. Electron acceptors used included hydrous ferric oxide (HFO) (Lovley and Phillips, 1986), iron citrate, iron chelated with nitrilotriacetic acid (NTA), sulfate, thiosulfate, and manganese oxide (MnO_2). In some cases, only H_2 or only H_2 and acetate were provided as electron donors in an attempt to cultivate autotrophic or methanogenic organisms, respectively.

In the field lab in South Africa, the Balch tubes containing growth media were inoculated with 1 ml of water collected in the mines and incubated at room temperature, 60°C, and 80°C. Enrichments were checked often for growth by visual turbidity and by phase contrast microscopy. Transfers of positive enrichments into fresh media were performed as necessary. Enrichments showing growth were transported to the United States for further study. Upon arrival, they were incubated at the same temperatures at which they had been growing in South Africa. Transfers continued as needed.

DNA extraction and PCR amplification of 16S rDNA

Organisms in positive enrichments were identified by 16S ribosomal DNA (16S rDNA) sequencing. DNA from each sample was extracted using the UltraClean Soil DNA Kit (MoBio Laboratories, Inc., Solana Beach, CA), following the manufacturer's protocol but substituting 500 μl of enrichment culture for the soil. In some cases involving cultures with low microbial density, it was necessary to use more than 500 μl in order to obtain enough amplifiable DNA. In such cases, cells from up to ~30 ml of culture were concentrated by centrifugation and then resuspended before beginning the extraction protocol. In the final step, DNA was eluted from the spin filter with 35-50 μl of molecular grade water and stored at –20°C.

The extracted DNA was amplified by the polymerase chain reaction (PCR). Universal bacterial primers 7F (5′-AGA GTT TGA TCN TGG CTC AG-3′) and 1392R (5′-ACG GGC GGT GTG TRC-3′) and universal archaeal primers Arch21F (5′-TTC CGG TTG ATC CYG CCG GA-3′) and Arch915 (5′-GTG CTC CCC CGC CAA TTC CT-3′) were used to amplify the 16S rRNA gene (Chandler et al., 1998). All primers were from Integrated DNA Technologies (IDT), Inc. (Coralville, IA). Separate reactions were run for each primer set. Reactions were run under mineral oil with 1-10 μl of DNA template from the DNA extractions, 0.4 μM each primer, 0.07 mM each dNTP, 2 mM $MgCl_2$, 1X Buffer II (Perkin Elmer, Wellesley, MA), and 1 unit AmpliTaq DNA Polymerase LD (Perkin Elmer). PCR was performed with a Perkin Elmer DNA Thermal Cycler 480. Template DNA and primers were heated to 85°C for 5 minutes before the other reaction components were added. 16S rDNA was amplified by 5 cycles of 94°C for 40 seconds, 55°C for 15 seconds, and 72°C for 1 minute, followed by 35 cycles of 94°C

for 15 seconds, 65°C for 15 seconds, and 72°C for 1 minute. Reactions were cooled to and held at 4°C following a final extension step lasting 10 minutes at 72°C.

After the PCR, the amplified products were separated by gel electrophoresis using a 0.7-1.0% low melt agarose gel. Bands of appropriate size (~1400 bases for bacteria; ~900 bases for archaea) were excised and cleaned using the Wizard PCR Preps DNA Purification System (Promega Corporation, Madison, WI) and following the manufacturer's protocol. The cleaned DNA products were stored at -20°C.

Cloning of PCR products

The amplified 16S rDNA fragments were cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. After the SOC medium incubation step, the transformed cells were spread evenly onto 1 to 3 LB/ampicillin agar plates, with 75-150 μl of the cell-containing SOC medium used on each plate, and incubated at 37°C for approximately 15 hours. In most cases, 15 to 20 clones for each sample were picked from the plates and grown in liquid LB/ampicillin medium (99 mg ampicillin per liter LB medium) for approximately 15 hours. Frozen stock cultures of each clone were saved in 16% glycerol at –80°C to create clone libraries for later access.

DNA from each clone was extracted using the freeze-thaw method. Cells from 600 μl of the LB/ampicillin culture were concentrated by centrifugation and then resuspended in 45-50 μl of molecular grade water by vortexing. Next, the cells were frozen at –80°C for at least 20 minutes and then quickly thawed at 99°C for 15 minutes to lyse the cells. Cellular debris from the thawing step was concentrated by centrifugation.

The DNA-containing supernatants were transferred to clean microfuge tubes and stored at –20°C or processed immediately.

The 16S rDNA inserts were amplified by PCR with the vector-specific T3 (5′-ATT AAC CCT CAC TAA AGG GA-3′) and T7 (5′-TAA TAC GAC TCA CTA TAG GG-3′) primers. The final 25-μl reaction volume contained 1 μl of the freeze-thaw DNA template, 0.8 μM each primer, 0.07 mM each dNTP, 2 mM $MgCl_2$, 1X Buffer II (Perkin Elmer), and 1 unit KlenTaq LA DNA Polymerase (Sigma, St. Louis, MO). PCR was again performed under mineral oil with a Perkin Elmer DNA Thermal Cycler 480. The 16S rDNA inserts were amplified by 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute. Reactions were cooled to and held at 4°C following a final extension step lasting 10 minutes at 72°C.

RFLP analysis and 16S rDNA sequencing

After amplification, the PCR products were cut with the *Hha*1 restriction endonuclease (Invitrogen). A mixture containing 10 μl of PCR product, 5 units of *Hha*1, and 1X React 2 buffer (Invitrogen) was incubated under mineral oil at 37°C for approximately 15 hours. The restriction digests were separated by gel electrophoresis using a 3% standard agarose gel. The gel was viewed on a UV light transillumínator and a digital photograph was taken. Clones were assigned to groups based on the restriction fragment length polymorphism (RFLP) patterns of their 16S rDNA inserts, with clones having the same RFLP pattern being placed in the same group.

A representative clone from each group was selected, and the frozen stock cultures of these clones were cultivated in 6-7 ml of LB/ampicillin broth in preparation for DNA sequencing. For each clone, a replacement stock culture was made, and the

remainder of the culture was centrifuged to concentrate the cells. The QIAprep Spin Miniprep Kit (Qiagen, Inc., Valencia, CA) was used to extract DNA from the cells in preparation for DNA sequencing. The manufacturer's protocol was followed. DNA from each clone was quantified by UV spectroscopy and sent to the University of Maine DNA sequencing facility (Orono, ME) for sequencing on an ABI Prism 377. In most cases, the 16S rDNA inserts were sequenced from the T3 priming site. In some cases, however, sequences from both the T3 and T7 priming sites were obtained.

Results from the University of Maine were received as chromatogram files. Sequences were evaluated and edited using the ABIview (David Klatte, University of Chicago, http://bioinformatics.weizmann.ac.il/software/abiview/abiview.html) and BioEdit (Tom Hall, North Carolina State University, http://www.mbio.ncsu.edu/BioEdit/bioedit.html) programs. Base changes were made where appropriate by examining the color peak data in the chromatograms. Sequences were truncated when the automated base calls could no longer be confirmed or changed with confidence.

Phylogenetic analysis

BLAST and RDP searches. The edited sequences were then used for similarity searches in both the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/) and Ribosomal Database Project II (RDP) (http://rdp.cme.msu.edu/html/) databases. The standard nucleotide-nucleotide search was used in BLAST. The Sequence Match program was used in RDP. Organisms with the highest similarity rankings in both BLAST and RDP were later used in constructing phylogenetic trees and also aided in selecting template organisms to use in aligning the

16S rDNA sequences to 16S rRNA secondary structures. In addition, sequences were checked for chimeric artifacts using the Chimera Check program at the RDP website.

Secondary structures. To ensure the accuracy of the edited 16S rDNA sequences and to help facilitate sequence alignment, the sequences were aligned manually to the 16S rRNA secondary structure of the closest relative with an available secondary structure diagram. The secondary structures were obtained from the Comparative RNA Web Site (http://www.rna.icmb.utexas.edu/). Base changes were noted on the diagram and then later checked for correct base pairings. Base pair discrepancies (e.g., a cytosine-uracil pair) were checked with the chromatograms to make sure the correct bases had been called. Corrected sequences were resubmitted to the BLAST and RDP databases.

Sequence alignment and tree generation. The final edited sequences were used in the construction of sequence alignments. For each sequence or group of similar sequences, an alignment was constructed using the top sequence matches from both the BLAST and RDP databases. Sequences obtained from the University of Maine, as well as those imported from RDP and BLAST, were manually aligned to the pre-aligned RDP sequences. The BioEdit program (Tom Hall, North Carolina State University, http://www.mbio.ncsu.edu/BioEdit/bioedit.html) was used to align all sequences. Individual and group alignments were merged into four final sequence alignments to be used for phylogenetic analysis. The Phylogenetic Analysis Using Parsimony (PAUP) program (version 4.0b 10, David Swofford, Sinauer Associates, Inc., Sunderland, MA) was used to generate phylogenetic trees. Trees were constructed primarily by the distance method with the Jukes-Cantor substitution model (Jukes and Cantor, 1969). One

hundred bootstrap replicates were run at the >50% confidence limit. Some parsimony and maximum likelihood trees were generated as well. Distance matrices were used to compare the sequence similarities of select organisms.

Nucleotide sequence accession numbers. The 16S rDNA sequences determined in this study have been deposited in the GenBank database under accession numbers AY187879 – AY187919 (Appendix 2).

RESULTS

Enrichments

Thirty of the microbial enrichments performed in South Africa yielded growth. Samples producing the positive enrichments were obtained from a total of six locations in the Evander, Kloof, and Premier mines (Table 1). Enrichments using samples from KL737, DR938H1, DR938H2, and DR548 showed no growth. The majority of the positive enrichments were at room temperature, with the remainder at 60°C (Table 1). No growth was observed in any of the 80°C enrichments. Although initially showing strong growth (high turbidity) through the first couple of transfers, all three EV821 cultures showed diminished growth in successive subcultures. The lack of continued growth likely indicated that the organisms in the cultures required one or more nutrients present in the EV821 fissure water, and those nutrients were diluted out with successive transfers. Attempts to revive the cultures have been unsuccessful. Growth in all of the other 60°C cultures was notably sparse, and phase contrast microscopy was necessary to confirm growth. Enrichments with Fe-NTA, Fe-citrate, HFO (hydrous ferric oxide), MnO_2, or TYG/NO_3^- showed growth only at room temperature (Table 1). The KL443 enrichment with H_2 and CO_2 was first inoculated with 1 ml of medium from the second subculture of KL443 with H_2, acetate, and CO_2 to determine whether or not autotrophic growth could occur on H_2. Transfers for both enrichments continued. Two enrichments, both at room temperature, showed apparent growth on CH_4 coupled with sulfate. Five

positive enrichments were obtained on an amended basal medium designed to select for methanogens.

16S rDNA sequencing and BLAST and RDP results

16S rRNA genes were amplified from total-DNA extracts from twenty-two positive enrichments. No 16S rDNA could be amplified from DNA extracted from the 60°C KL739 thiosulfate enrichment. No attempt to extract DNA was made for seven of the enrichments. Included in this group were the three 60°C EV821 enrichments, all three of the room temperature thiosulfate cultures, and the room temperature H_2/sulfate enrichment from EV818. In the case of the EV821 samples, DNA extraction was not attempted because the enrichments were thought to contain an inadequate number of cells, if any. DNA was not extracted from the remaining four enrichments because it was thought that the results would be similar to those from most of the other room temperature enrichments. The 60°C enrichments yielded low quantities of 16S rDNA, as indicated by the faint bands obtained during gel electrophoresis.

From the twenty-two cultures characterized by 16S rDNA, 501 clones were screened by RFLP analysis and 43 clones were sequenced (Table 2). Two clones, I3 and R6, were found to be chimeric sequences and were excluded from the rest of the analysis. The remaining forty-one clones included three archaeal sequences and thirty-eight bacterial sequences. For four of the clones (two archaea and two bacteria) the entire 16S rDNA insert was sequenced. For the other thirty-seven clones, only one end of the molecule was sequenced. Sequencing revealed that some clones showing different RFLP patterns had the same or very similar sequences. For all clones except one, the top BLAST matches had E values of 0.0 (Table 3). The top match for clone H9 had had an E

value of e^{-159}. RDP similarity scores ranged from 0.406 for clone H9 to 1.000 for clones C37, L4, and I15 (Table 3). Agreement between the BLAST and RDP results was generally good, with the same or similar organisms being the top matches in both databases. Some variability between the results of the two databases was expected due to the much larger size of the BLAST database and the different algorithms used by the BLAST and RDP sequence match programs.

As revealed by 16S rDNA sequencing, the groups of microorganisms isolated from subsurface water at the five locations (EV821 excluded) were generally different from each other (Table 3). In addition, the microorganisms that grew at 60°C were different from those that grew at room temperature. The exception to these two general trends was the group of clones represented by the BLAST sequence of uncultured bacterium clone Ebpr3, which was the top BLAST match to clones L7, CC14, CC4, X4, Y10, and GG8. These clones represent four of the five sampling locations and both the 60°C and room temperature enrichments. The room-temperature enrichments were dominated by species of both *Aeromonas* and *Pseudomonas*. No archaea were found in the room temperature cultures, but three archaea, all crenarchaeotes, were present in the 60°C enrichments. Both *Clostridium* and *Bacillus* species were also found in the 60°C enrichments.

Borehole and fissure water geochemistry

Available field and geochemical data for the sample locations that yielded positive enrichments are presented in Appendices 3a-e (data provided by T.C. Onstott, Princeton University). Samples were collected from 0.763 to 3.4 kmbls. Measured water

temperatures at the time of sampling ranged from 25°C to 54°C, and pH values ranged from about 5.8 to 10.3. EV818 and KL739 were somewhat reducing, with Eh values of -240 and -220 mV, respectively. Samples had little to no dissolved iron or oxygen. Ammonia ranged from 3 to 7 ppm. EV219 had comparatively high levels of dissolved inorganic carbon (>143 ppm). Sulfide was detected in all samples (0.32 to >10 ppm), with EV818 having the highest concentration (>10 ppm). For samples EV818 and KL739, both $\delta^{34}S$ of SO_4^{2-} and $\delta^{34}S$ of S^{2-} were measured. Fractionation between the sulfur species in both EV818 and KL739 (22.6‰ and 18.9 ‰, respectively) is consistent with bacterial sulfate reduction (Hoefs, 1997). The isotopic data alone, however, do not indicate the presence of sulfate-reducing bacteria in the two waters (Pedersen, 1997).

The EV818, KL443, and KL739 samples were relatively saline (Fig. 1) and may indicate a long residence time in the subsurface. The Br and Cl concentrations in the three samples were closer to those of seawater than to those of many of the other South African mine waters. The EV818, KL443, and KL739 waters were much more saline than the mine service waters and the groundwater from the dolomite aquifer in the lower portion of the Transvaal Supergroup. Samples EV219 and PR763 were typical of the less saline waters encountered in the mines.

Relatively high calcium and sodium concentrations also indicated the saline nature of EV818 and KL739 (Fig. 2). Ca and Na data were not available for KL443. EV818 and KL739 are more Ca-rich than seawater but less Na-rich. EV818 and KL739 are also more saline than water from thermal springs across South Africa. PR763 and EV219 had Ca and Na concentrations typical of both the less saline mine waters and the most saline thermal spring waters.

δD and $\delta^{18}O$ values were measured for EV818, KL739, and PR763 and plotted against the values measured for waters at other surface and subsurface locations in South Africa (Fig. 3). PR763 fell near both the global and local meteoric water lines, along with most of the other water samples, indicating a relatively recent meteoric origin for the waters. Most of these waters, however, including PR763, were depleted in D and ^{18}O with respect to Pretoria mean meteoric water (IAEA/WMO, 2001). Thus, these samples may represent precipitation falling at higher altitudes than in Pretoria, or meteoric water precipitated during colder climates (e.g. during the Pleistocene), or a combination of the two (Duane et al., 1997).

In contrast to PR763, samples KL739 and EV818 were shifted left of the meteoric water lines (Fig 3). Also shifted left of the meteoric water lines were a sample from Western Deep Levels Mine (Duane et al., 1997), a sample from Driefontein Mine (Lippmann et al., 2002b), and some Kloof and Evander samples (Onstott, unpublished data). An isotopic shift left of the global meteoric water line indicates a depletion of ^{18}O in the waters. Such a depletion may be the result of hydration of the host rocks (Duane et al., 1997), which would preferentially remove ^{18}O from the waters. Hydration would also preferentially remove deuterium from the waters.

Phylogenetic analysis

For phylogenetic analysis, the forty-one sequences were separated into the following four groups: 1) Archaea, 2) Gram positive bacteria, 3) *Cytophagales/Flavobacteria/Bacteroides* (CFB) group bacteria, and 4) Proteobacteria (including alpha, beta, and gamma subdivisions). The affiliation of clone H9 was uncertain, but it was thought to be most closely related to the CFB group and was thus

included with that group for sequence alignment and tree generation. All clones were sequenced from the T3 primer site in the cloning vector. Four of the clones were also sequenced from the T7 primer site. For the T3-only clones, the portion of the 16S rDNA insert that was sequenced depended on which way the molecule inserted into the cloning vector during the annealing reaction (Appendix 4). Because each group of sequences had some clones with the 5′ end of the 16S rDNA molecule sequenced and some clones with the 3′ end of the molecule sequenced, two sets of sequence alignments were constructed for each group. One set included the 5′ ('forward') inserts and the other included the 3′ ('reverse') inserts. Subsequently, two phylogenetic trees were generated for each group of sequences. Because clones J6 and BB7 were sequenced in their entirety and because most of the AA2 sequence was obtained from sequencing from the T3 site alone, only one sequence alignment was needed for the archaeal clones.

The three archaeal sequences fell within the Crenarchaeota (Fig 4). Clones AA2 and J6 were closely related to non-thermophilic archaea that have been found, but rarely cultured, in various soil and water environments at the surface. Clone AA2 was 99.3% homologous to uncultured archaeon clone HTA-C5, and clone J6 was 99.3% homologous to uncultured Front Range soil crenarchaeote FRD9. Clones AA2 and J6 shared a sequence similarity of 95.9%, indicating that they are different species of archaea. Clone BB7 represents a deep-branching crenarchaeote and is phylogenetically related to a variety of hyperthermophilic archaea. It is 99.8% homologous to uncultured thermal soil archaeon clone YNPFFA4, detected in a thermal soil in Yellowstone National Park. Interestingly, clone BB7 is closely related to three clones obtained from DNA previously extracted (McCuddy and Kieft, unpublished data) from the W6-38-Bh1 (Fig. 1) borehole

cartridge experiment discussed in Baker et al. (2002). The clones, designated W6Arch8, W6Arch34, and W6Arch39 (Fig. 4), are 99.2 to 99.7% homologous to clone BB7.

Only one of the Gram positive bacteria group clones, X13, had a forward 16S rDNA insert. Clone X13 was found to be a *Bacillus* species (Fig. 5). Its sequence was 99.2% similar to that of *Bacillus* sp. IZ5.

Clones CC9, GG13, and GG15 branched with various species of *Clostridium*, while clone M3 grouped with several strains of *Propionibacterium acnes* and similar uncultured clones (Fig 6). Clone CC9 was only 93.4% homologous to its nearest phylogenetic relative, *Clostridium subatlanticum*, indicating that clone CC9 is a new species of *Clostridium*. Clones GG13 and GG15 likely represent the same organism but showed different RFLP patterns. They were ~98.2% homologous to *Clostridium aerotolerans*.

Clones CC4, Y10, CC14, and X4 formed a coherent cluster within the *Cytophaga*, *Flavobacterium*, *Bacteroides* (CFB) group of bacteria (Fig. 7). They appeared to represent a new species, as they all had <97% sequence homology with uncultured bacterium clone Ebpr3, the most closely related of the BLAST and RDP matches used in the tree construction. However, some sequences had to be excluded from the analysis presented in Fig. 7 because they were short sequences that did not have good overlap with the other sequences in the alignment. Upon further examination it was discovered that clones CC4, Y10, CC14, and X4, as well as clones GG8 and L7 (Fig. 8), were identical or nearly identical to one of the excluded sequences. The excluded sequence was uncultured eubacterium clone MT10, which has been identified as a contaminant in one or more of the reagents used in DNA extraction or amplification (Tanner et al.,

1998). All six clones were 99.6-100% homologous to clone MT10. It was therefore concluded that clones CC4, Y10, CC14, X4, GG8, and L7 represent contaminating DNA in the MoBio UltraClean Soil DNA Kit or in one or more of the PCR reagents. This low level of contamination is likely most evident when extracting and amplifying DNA from enrichments containing very little biomass. Ultimately, not enough sample DNA was recovered to dilute the contaminating DNA (Tanner et al., 1998).

Clones H9 and M2 remained as the only genuine subsurface clones in the two CFB group alignments. Clone M2 (Fig. 8) was most closely related to an unidentified CFB group bacterium and a *Bacteroides* termite symbiont. Clone H9 (Fig. 7) was not closely related to any cultured or uncultured bacteria. It was only 78% homologous to uncultured soil bacterium PBS-II-37. Although there were a few BLAST matches higher than this sequence, they all had very low E values as well. They could not be used in the sequence alignment because they did not overlap sufficiently with the other sequences in the alignment. Because of the low E values, however, it seems unlikely that clone H9 is much more than 80% homologous to any cultured or uncultured bacterium known.

The majority of the forty-one clones examined fell within the Proteobacteria (Figs. 9 and 10) and included organisms from the alpha, beta, and gamma subdivisions. Clones I19, G5, W9, W12, L4, Q2, T8, and G7 clustered with various *Aeromonas* species. Clones H3, M1, C23, P5, K7, N3, U9 and C37 were found to be species of *Pseudomonas*. Clones C23, K7, N3, U9, and C37 were ≥99.6% homologous to their closest phylogenetic matches while clones P5 and M1 were 98.9% and 98.4% homologous, respectively, to their closest relatives. Clone H3 was the least similar (97.1%) to its closest phylogenetic matches, which included both *Pseudomonas*

pseudoalcaligenes and *P. alcalophila*. Clones R10 and O4 were 99.9% homologous and were closely related to *Hydrogenophaga flava*. Clone S2 was 97.7% homologous to *Thiomonas* sp. Ynys3 (Fig. 10), and clone I12 was 99.1% homologous to isolate Boom-7m-04 (Fig. 9). Clone I13 was very similar to petroleum-degrading bacterium HD-1 and showed a sequence similarity of 99.7%. Clone V3 was found to be a soil bacterium 99.4% homologous to *Agrobacterium tumefaciens* isolate C4 and two uncultured relatives. Clone I15 was identical to four strains of *Caulobacter*, with 654 positions of sequence examined. Clone M9 was 99.7% homologous to *Phaeospirillum fulvum* isolate S3, and clone CC8 was 99.7% similar to two strains of *Bosea thiooxidans*.

The environment of origin for some of the sequences that are phylogenetically related to the clones described in this study are listed in Table 4. Accession numbers are also provided.

DISCUSSION

Samples KL739 and EV818 are particularly interesting because they are relatively saline (Figs. 1 and 2) and are shifted left of the global meteoric water line (Fig. 3). Most processes, such as evaporation or high temperature water-rock isotope exchange, tend to shift the compositions of meteoric waters to the right of the meteoric water line. Waters showing an isotopic shift left of the meteoric water line were long thought to be rare. Such waters have recently been suggested to occur commonly in deep crystalline basements with slow water movement and relatively low temperatures (Kloppmann et al., 2002). The supposed rareness of these waters may thus result from sampling bias. The shift to the left of the meteoric water line may result from low temperature exchange with silicate or carbonate rocks or from the hydration of host rocks and the formation of clay minerals (Duane et al., 1997; Frape and Fritz, 1982). Regarding waters encountered in the gold mines of South Africa, Duane et al. (1997) considered isotopic exchange with surrounding rocks unlikely because of the low susceptibility of the host quartzites to isotopic exchange at temperatures $<100°C$. Instead, they considered hydration of the Ventersdorp lavas or similar formations to be responsible for isotopic shifts to the left of the meteoric water line. By this process, ^{18}O and D would have been preferentially taken up by the lavas during hydration and would have led to a depletion of ^{18}O and D in the remaining water. Low water/rock ratios would have been required for large shifts in $\delta^{18}O$ and δD. It is likely that KL739 and EV818 became depleted in ^{18}O and D by the

hydration of host rocks in the subsurface. It is worth noting that the KL739 sample came from a water pocket intersected during drilling into the Ventersdorp lavas (Appendix 3a). The saline and ^{18}O-depleted nature of the KL739 and EV818 waters suggests that they had resided in the subsurface for an extended period of time and were not recently introduced meteoric waters.

In this study, strict anaerobic techniques were employed during media preparation. However, as no reducing agent was used in any of the media, the possibility that some of the microorganisms grew on trace quantities of oxygen in the media cannot be excluded. It is possible that the presence of some oxygen in the media could have enabled limited growth of obligate aerobes, facultative aerobes, or aerotolerant organisms. The metabolic activities of the microorganisms growing in the enrichments cannot therefore be assumed to be anaerobic. The same media and media preparation techniques used in this study have previously been used to cultivate known anaerobes (e.g., sulfate-reducing bacteria) (Kieft et al., 1999; McCuddy and Kieft, unpublished data). The detection of large numbers of predominantly aerobic organisms such as *Pseudomonas* and *Aeromonas*, as well as aerotolerant microorganisms (e.g., *Clostridium aerotolerans*), and the fact that no sulfide was ever detected (by smell) in the sulfate and thiosulfate enrichments during transfers, underscore the possibility that the ability either to use or to tolerate oxygen was a selective factor.

The presence of facultative aerobes or aerotolerant microorganisms in the deep subsurface does not necessarily indicate that the organisms are contaminants from the surface. The ability to utilize a variety of electron donors and acceptors for growth would be advantageous for organisms inhabiting the subsurface, where nutrients can be scarce

and water flow can transport microorganisms into and out of different geochemical environments. Microorganisms transported into the deep subsurface from oxygenated surface environments even millions of years ago may not have lost the ability to metabolize oxygen. Microorganisms from the deep subsurface that can reduce oxygen and also a variety of metals have previously been described (Kieft et al., 1999). Further work is needed to determine the metabolic capabilities of the organisms cultured in this study.

This study is the first to culture thermophilic archaea from the non-thermophilic Crenarchaeota group. Clones AA2 and J6 are >99% homologous to archaea from low-temperature surface environments but grew in enrichments at 60°C. Archaea from the non-thermophilic Crenarchaeota group have been found in oceans (Delong, 1992; Delong et al., 1994; Fuhrman et al., 1992), freshwater reservoirs (Stein et al., 2002), soils (Bintrim et al., 1997), and more recently in the deep subsurface of South Africa (Takai et al., 2001a). The presence of these archaea in enrichment cultures at 60°C suggests either that some of these non-thermophilic crenarchaeotes are capable of growth over large temperatures ranges, including thermophilic temperatures, or that they represent a previously unknown group of thermophilic crenarchaeotes. It is possible that the microorganisms represented by clones AA2 and J6 are descendents of non-thermophilic crenarchaeotes that were transported into the deep subsurface at some time in the past and have since adapted to life at higher temperatures. Clone AA2 was isolated from the 45°C EV818 sample, and clone J6 was isolated from the 54°C KL739 sample. Based on salinity (Figs. 1 and 2), δD, and $\delta^{18}O$ values (Fig. 3), EV818 and KL739 may have had long residence times in the subsurface. Further support for the old age of these two

waters has been offered by Lippmann et al. (2002b). Using the ^{36}Cl dating technique, the authors found EV818 and KL739 to be at least 1.5 Myr old. Due to the relatively short half-life of ^{36}Cl (~301 Kyr), this date is an absolute minimum based on about five half-lives of ^{36}Cl, which is the detection limit using conventional accelerated mass spectrometry. Model ages based on the concentrations of dissolved noble gases indicate that the two waters could be significantly older. Calculated model ages for ^{136}Xe concentrations suggest that EV818 is 88 ± 44 Myr old and KL739 is 418 ± 209 Myr old (Lippmann et al., 2002b). Unless the J6 and AA2 organisms are actually hyperthermophiles, which seems unlikely, they must have migrated to the locations from which they were sampled at some time after 70 Ma (Omar and Onstott, 2002). Before this time, temperatures at these depths would have been too high to support non-hyperthermophilic life. This time constraint, based on fission-track analyses of subsurface apatites, means that the KL739 water must have come into contact with younger water(s) some time after 70 Ma in order for the J6 organism to be introduced into the KL739 water. The amount and age of the younger water(s) is unknown but must be ≤70 Myr old. In contrast, it is possible that the EV818 water entered the subsurface ~40-70 Ma, consistent with both the ^{136}Xe and fission-track data, and has not been influenced by other water sources since that time. The AA2 organism could thus be the descendent of a non thermophilic crenarchaeote that entered the subsurface in the late Cretaceous or early Tertiary period.

The archaea represented by clones AA2 and J6 both grew in a medium containing sulfate as the only electron acceptor, which suggests that they could be involved in the reduction of sulfate in the subsurface. Many members of the Crenarchaeota require

elemental sulfur or sulfur-containing anions such as thiosulfate or sulfite for growth (Pace, 1997; Schönheit and Schäfer, 1995). Curiously, however, no sulfate-reducing crenarchaeotes are known. Species of *Archaeoglobus* (Euryarchaeota) are the only archaea known to reduce sulfate (Madigan et al., 2000; Schönheit and Schäfer, 1995). Sulfate and sulfide were both detected in EV818 and KL739. EV818 had the most sulfide of any of the samples measured. The sulfur isotope data for EV818 and KL739 are consistent with, but do not confirm, the presence of sulfate-reducing microorganisms in the two waters. Acetate was one of three organic electron donors in the sulfate-containing medium and was also detected in both EV818 and KL739. The archaea may be utilizing this compound as a source of carbon and electrons in the subsurface. Hydrogen was also used in the medium as a possible electron donor and was detected in sample KL739. Hydrogen would thus have been available as an energy source to support lithoautotrophic growth in the KL739 water. Hydrogen is commonly used by archaea as a source of energy (Pace, 1997).

Clone BB7, from the EV818 sample, represents a deep-branching crenarchaeote that is not closely related to any cultured archaeon (Fig. 4). It is, however, very similar to a group of uncultured thermal soil archaea from Yellowstone National Park. Clone BB7 also shares a high sequence homology with the three W6Arch clones (McCuddy and Kieft, unpublished data). The W6Arch sequences were amplified from DNA extracted from the W6-38-Bh1 borehole cartridge material discussed in Baker et al. (2002). The cartridge was attached to a borehole located 2.7 kmbls in Driefontein Mine and allowed to incubate for 61 days. The temperature of the borehole water was 37°C. The samples yielding the BB7 and W6Arch sequences were collected approximately 160 km apart in

the Witwatersrand basin. Phylogenetically, clone BB7 and the W6Arch clones lie in a region of the 16S rRNA tree of life that is characterized by hyperthermophilic organisms (Pace, 1997; Stetter, 1996). Based on their phylogenetic positions, it is possible that clone BB7 and the W6Arch clones represent hyperthermophilic microorganisms. It is interesting, therefore, that these organisms were found in waters having temperatures of 45°C and 37°C, respectively. Hyperthermophilic microorganisms would not be expected to grow under these conditions. Thus, it is possible that the W6Arch and BB7 clones are not from hyperthermophilic organisms but from thermophilic or mesophilic organisms instead. In this case, the clones may represent a new group of lower temperature, deep-branching crenarchaeotes. The fact that the clones are so closely related to those from thermal soil archaea in Yellowstone National Park, however, suggests that the BB7 and W6Arch clones are from organisms growing in at least a thermophilic temperature range. Moreover, as clone BB7 grew in a 60°C enrichment, it is not likely to be mesophilic. Hyperthermophiles may be capable of surviving for long periods of time at lower temperatures, an ability which may aid in their dispersion between hyperthermophilic environments (Stetter, 1996).

If the BB7 and W6Arch clones do indeed represent hyperthermophilic archaea, their discovery in 45°C and 37°C waters may provide further support for the suggestion that hot, hyperthermophile-containing water may be migrating upward from greater depths in the Witwatersrand basin and mixing with cooler water (Takai et al., 2001a). The growth of the BB7 organism in a 60°C enrichment does not preclude the organism from being a hyperthermophile. There are several known examples of hyperthermophilic archaea that are capable of growth at or below 60°C (Blöchl et al., 1995; Stetter, 1996).

The archaeon represented by clone BB7 may not have grown well in the 60°C culture because the temperature was not high enough. However, it is perhaps more likely that the medium lacked enough of one or more essential nutrients needed to sustain robust growth. Further work is needed to determine whether or not the BB7 and W6Arch clones represent hyperthermophilic archaea. If these archaea can be shown to have temperature optima characteristic of hyperthermophiles, the results will strongly support the hypothesis advanced by Takai et al. (2001) that life may exist at greater depths in the crust than presently known. According to Omar and Onstott (2002), hyperthermophiles found at present-day depths of 1.2-3.7 kmbls in the Witwatersrand basin could be descendents of hyperthermophiles that were transported into the deep subsurface during the early Paleozoic Era.

The crenarchaeote represented by clone BB7 grew in a medium containing thiosulfate as the only intended electron acceptor. Many deep-branching crenarchaeotes couple the oxidation of H_2 with the reduction of a sulfur compound to derive energy for growth (Pace, 1997; Schönheit and Schäfer, 1995). It seems likely, therefore, that the BB7 microorganism utilized H_2 and thiosulfate for growth. Thiosulfate was not measured for sample EV818, but sulfide was detected in relatively high quantities. The presence of sulfide is consistent with the reduction of thiosulfate to sulfide in the EV818 water.

In addition to the three thermophilic archaea, four thermophilic bacteria were isolated from the subsurface. The thermophilic nature of these archaea and bacteria is consistent with their being members of an indigenous subsurface community, which would be expected to be adapted to the higher ambient temperatures of the deep

subsurface. These thermophilic organisms, which were isolated from waters having temperatures in the thermophilic range, are less likely to have been introduced into the subsurface by mining activity. Mining operations make use of chilled service water and chilled air for drilling and temperature control in the active areas of the mines (Onstott et al., 2002). Although possible, it does not seem likely that this water and air would harbor significant numbers of thermophilic organisms. Contaminants from the surface are predominantly mesophilic and very similar to microorganisms found in surface environments.

The thermophilic bacteria cultured in this study, with one exception (clone CC8), are related to spore-forming organisms of the genera *Bacillus* and *Clostridium*. The presence of spore formers in the deep subsurface is consistent with the existence of an indigenous microbial community there. The ability to form spores would be advantageous to bacteria inhabiting the deep subsurface, where conditions can be inimical to life. Spores would offer some degree of protection from harsh elements, such as high temperatures and lack of essential nutrients, often encountered in the subsurface. Thus, spores would be expected to survive in the subsurface longer than vegetative cells. In addition to several durable outer layers, spores have internal mechanisms to protect their DNA from heat and radiation damage (Nicholson et al., 2002; Setlow, 1995). These protective mechanisms allow them to survive in a dormant state for extended periods of time and then successfully convert back into vegetative cells when conditions become more favorable. Spore-forming species are well represented among microorganisms that may have been preserved for long periods of time (up to millions of years) and subsequently revived (Kennedy et al., 1994). Spores of a *Bacillus* species have been

successfully revived from the gut of an extinct bee that was preserved for 25-40 Myr in buried Dominican amber (Cano and Borucki, 1995). In a more controversial claim, Vreeland et al. (2000) purportedly isolated and grew a *Bacillus* species from a brine inclusion in a 250-Ma halite crystal. In addition to surviving for millions of years trapped in geological materials, bacterial spores have been shown to survive in the harsh environment of space (Horneck et al., 1994; Horneck et al., 2001b). Consequently, they have also been implicated in the possible interplanetary transfer of life (Horneck et al., 2001a; Mastrapa et al., 2001; Nicholson et al., 2000) and in the possible existence of life on Mars (Horneck, 2000; Koike et al., 1994). Because spores are so robust and confer survival advantages on microorganisms capable of producing them, it is not surprising to find them in the deep subsurface, nor is it likely a coincidence. The ability to form spores is probably a selective advantage for life inhabiting the subsurface. Indeed, spores have previously been found in various subsurface environments (Cayol et al., 1995; Colwell et al., 1997; Nakagawa et al., 2002; Nazina et al., 2001). Although they are in a dormant state, spores may not be entirely passive in their environment and may continue to interact chemically with their surroundings (Francis and Tebo, 2002; Mandernack et al., 1995).

The spore-forming bacteria cultured in this study include two species of *Clostridium* (clones CC9 and GG13/GG15) and one species of the genus *Bacillus* (clone X13). Clone CC9 represents a new *Clostridium* species, as its 16S rDNA sequence is only 93.4% homologous (over 746 bases) to its closest relative, *Clostridium subatlanticum* (Brisbarre et al., 2003). Clone CC9 came from the same culture as clone BB7, the 60°C thiosulfate enrichment from EV818 (Table 2). It is not immediately clear

how the new *Clostridium* species obtained energy for growth. Most *Clostridium* strains are strictly anaerobic, fermentative organisms (Madigan et al., 2000). Although the medium was not designed to culture fermentative organisms, it is possible that the new species grew by fermenting lactate, similar to *Clostridium propionicum*. The new *Clostridium* species could also be acetogenic, producing acetate from H_2-CO_2, lactate, or pyruvate (Kusel et al., 2000). Because the new *Clostridium* species is not closely related to any described *Clostridium* species, it is possible that it utilizes a metabolic pathway that is novel within the genus *Clostridium*. This pathway may involve the respiration of thiosulfate, as indicated by its growth in a thiosulfate medium.

The third organism that grew in the EV818 60°C thiosulfate culture, represented by clone CC8, appears to be a strain of *Bosea thiooxidans*, a Gram-negative bacterium capable of oxidizing reduced sulfur compounds (Das et al., 1996; Stubner et al., 1998). This study appears to be the first to report a thermophilic *Bosea thiooxidans* strain from the deep subsurface. In the culture with the new *Clostridium* species and the deep-branching crenarchaeote (BB7), *B. thiooxidans* may have oxidized thiosulfate with trace quantities of oxygen present in the medium, perhaps creating strict anaerobic conditions necessary for the growth of the aforementioned organisms. It is not known what role *B. thiooxidans* might play in the deep subsurface, where the waters are presumably anoxic, unless it can oxidize thiosulfate with a compound other than oxygen. Alternatively, it may have other metabolic capabilities involving thiosulfate or possibly unrelated to thiosulfate.

The other *Clostridium* species (clone GG13/GG15) cultured in this study was ~98.2% homologous to *C. aerotolerans* (Collins et al., 1994). Although the 16S rDNA

sequences of *C. aerotolerans* and the *Clostridium* species represented by clones GG13 and GG15 are somewhat divergent, the difference is not enough to distinguish a new species based on 16S rDNA alone. Like the CC9 *Clostridium*, it is not clear how the GG13/GG15 *Clostridium* grew in the KL739 sulfate medium (Table 2) or what role it plays in the subsurface. It is interesting to note that when oxygen (sterile atmosphere) was injected into the headspace of cultures containing the GG13/GG15 organism, the cultures became much more turbid, indicating greater concentrations of microorganisms. Additionally, the GG13/GG15 organism grew rapidly when the medium containing it was streaked onto R2A plates and incubated aerobically at 60°C. Colonies were easily visible after overnight incubation and spores were abundant, as indicated by phase contrast microscopy. These observations suggest that the GG13/GG15 *Clostridium* is a facultative aerobe. To the author's knowledge, no such *Clostridium* spp. have previously been cultured. Although the known *Clostridium* spp. cannot use oxygen for growth, some of them are aerotolerant. When oxygen is present in low concentrations, some *Clostridium* spp. appear to consume the oxygen but do not resume growth until the oxygen is gone (Kawasaki et al., 1998), do not grow as well in the presence of oxygen (Kusel et al., 2001), or undergo a lag phase in growth (Karnholz et al., 2002). In contrast, the *Clostridium* represented by clones GG13 and GG15 showed robust growth even under atmospheric oxygen levels, and the addition of oxygen stimulated growth rather than stifled it.

The *Bacillus* species cultured here, represented by clone X13, clustered with a group of thermophilic *Bacillus* strains in the *B. stearothermophilus* subgroup (Fig. 5). The X13 *Bacillus* grew in an enrichment with only acetate, H_2, and CO_2 added to the

basal salts medium (Table 2). It was intended to culture acetogens or methanogens, but it is unlikely that the X13 *Bacillus* is either one of these. How it derived energy for growth in this culture is unknown. Because most *Bacillus* strains are known to be aerobic or facultatively aerobic (Madigan et al., 2000), it is possible that the X13 organism oxidized the acetate with trace quantities of oxygen in the medium. Its role in the subsurface is also uncertain but could involve fermentation or the reduction of metals such as Fe(III) or Mn(IV) (Boone et al., 1995).

The organisms that grew in the room-temperature enrichments are more likely than the thermophilic microorganisms to be contaminants from the surface. Even so, some of the room-temperature organisms may be indigenous to the subsurface. The most likely candidates for members of an indigenous subsurface microbial community are those organisms that are not closely related to microorganisms widespread at the surface (Lovley and Chapelle, 1995). However, some common surface organisms may have also moved into the subsurface in the past. Underground, they may carry out metabolic activities different from those that they would at the surface. On the first consideration, it is tempting to assign all the *Pseudomonas* and *Aeromonas* species to the surface contaminants group. These organisms are widespread in a variety of environments at the surface and are predominantly aerobic. It is possible, however, that some of these organisms, especially if they are facultative anaerobes, are indigenous to the subsurface and were not introduced by mining. In addition to oxygen, some *Aeromonas* species can use other electron acceptors such as Fe(III), NO_3^-, and Co(III) for growth (Cunliffe and Adcock, 1989; Kelso et al., 1997; Knight and Blakemore, 1998; Srinath et al., 2001). Some species of *Pseudomonas* are similarly diverse in their metabolic capabilities

(Altenschmidt and Fuchs, 1991; Balashova et al., 1991; Chayabutra and Ju, 2000; Macy et al., 1989; Samuelsson et al., 1988; Sorokin et al., 1999; Wang et al., 2002). Thus, it appears possible that some of the *Aeromonas* and *Pseudomonas* species cultured in this study did not grow using trace quantities of oxygen in the media. It follows that some of them may be part of an indigenous subsurface community, gaining energy by reducing compounds such as Fe(III) or NO_3^-.

All of the room-temperature clones except five had 16S rDNA similarities >99% with their closest phylogenetic match. The five that did not are considered most likely to be indigenous to the subsurface and warrant further study. Clones in this group include P5, H3, M1, S2, and H9 (Tables 2 and 3). Three of these, P5, H3, and M1 represent *Pseudomonas* spp. from the PR763 sample. Clones H3 and M1 are only 98.6% homologous and thus are not from the same organism. The *Pseudomonas* sp. represented by clone H3 is possibly a new species based on its 16S rDNA sequence alone, which is ~97.1% homologous to the two most similar sequences from the BLAST and RDP databases. Because it is from an enrichment designed to cultivate anaerobic methanotrophs, the H3 organism is of particular interest.

The anaerobic oxidation of methane (AOM) is a globally important process that prevents CH_4, a potent greenhouse gas, from accumulating in the atmosphere. Although the organisms involved have yet to be cultured, AOM is thought to be mediated by a consortium of methane-oxidizing archaea and sulfate-reducing bacteria (Hinrichs et al., 1999; Orphan et al., 2001; Valentine, 2002; Valentine and Reeburgh, 2000). Geochemical evidence of AOM is well documented and comes from a variety of environments including seafloor cold seeps (Tsunogai et al., 2002), gas hydrates (Zhang

et al., 2002), euxinic waters (Michaelis et al., 2002; Schouten et al., 2001), marine sediments (Pancost et al., 2000; Thomsen et al., 2001), and hydrothermal vent sediments (Teske et al., 2002). AOM may also be an important process in the deep subsurface (Kotelnikova, 2002).

In this study, two anaerobic methanotroph cultures were obtained, one from EV219 and one from PR763 (Table 2). Currently, it is not known whether or not these two cultures were indeed completely anaerobic. As mentioned before, the presence of trace quantities of oxygen in the medium cannot be excluded. Additionally, if these cultures do contain anaerobic methane oxidizers, they are different from the archaea-bacteria consortia that syntrophically oxidize methane and reduce sulfate, because no archaea were detected in either culture. Many examples of aerobic methanotrophs are known. It seems likely that electron acceptors other than oxygen, such as Fe(III) and SO_4^{2-}, could be used in the oxidation of methane, as such couples are energetically favorable. There may be yet undiscovered examples of methanotrophic bacteria that can use these and other electron acceptors to oxidize methane anaerobically.

The EV219 CH_4/SO_4^{2-} culture yielded sequences from four microorganisms (Tables 2 and 3). Clone I3, a fifth clone from the culture, was a chimera. Two of these organisms, represented by clones I15 and I19, belong to the genera *Caulobacter* and *Aeromonas*, respectively. Because they are identical or nearly identical to known species, it is not clear how they grew in the enrichment. It seems unlikely that they participated in the oxidation of methane, but they may have utilized metabolic waste products from other microorganisms in the culture. Clone I12 represents a bacterium similar to a clone from a 35-Myr-old subsurface clay formation (BoivinJahns et al.,

1996), but nothing is known about its metabolism. The I13 organism, similar to petroleum-degrading bacterium HD-1 (Figs. 9 and 10) (Morikawa and Imanaka, 1993), seems to be the most likely candidate for an anaerobic methanotroph in the culture. Strain HD-1 can grow autotrophically and is capable of both anaerobically using alkanes as energy sources (Morikawa et al., 1996) and anaerobically producing alkanes and alkenes (Morikawa et al., 1998). Methane is the simplest alkane and was the only energy source available in the EV219 CH_4/SO_4^{2-} enrichment. In light of the previous research performed on strain HD-1, it seems likely that the very similar I13 organism could have grown anaerobically on methane in the EV219 culture. It is not known whether the sulfate in the culture medium was reduced by microbial metabolism. When the EV219 borehole was sampled in August 2001, no gas flow was detected, and hence, no gas sample was collected. Methane was detected in samples collected in March 2001 from two other boreholes at the same location in the mine (Onstott, unpublished data). All boreholes were drilled into the same rock face and clustered within an area of a few m^2. Although no gas flow was detected from the EV219 borehole, gases, including CH_4, could have been dissolved in the water. Furthermore, the gas flow from the borehole could simply have been too low to detect. The possibility that the I13 organism was using methane anaerobically in the subsurface cannot be ruled out.

The positive methanotrophic culture from PR763 yielded two organisms (Tables 2 and 3). The H3 *Pseudomonas* may be a new species. As discussed above, *Pseudomonas* sp. can be facultatively anaerobic. Some *Pseudomonas* sp. can be methylotrophic, growing on one-carbon compounds such as methanol (Madigan et al., 2000; Narbad et al., 1989; Olechnovich et al., 1987). As CH_4 is the only known electron donor in the

PR763 CH_4/SO_4^{2-} enrichment, it is possible, although only speculative at this point, that the H3 *Pseudomonas* is not only methylotrophic, but also methanotrophic and actually used the methane in the culture for growth. Because the H9 organism is not closely related to any bacterium, cultured or uncultured, it is difficult even to speculate about how it grew in the methanotrophic culture or how it lives in the subsurface. The discovery of a novel metabolic pathway in this organism, such as methane oxidation coupled to sulfate reduction, is possible given its phylogenetic distance from other bacteria. Unfortunately, the large borehole size at the PR763 site prevented a gas sample from being collected, so it is not known whether the water contained methane.

If none of the microorganisms in the methanotroph cultures used methane, then it is unclear how grow of the organisms was sustained. Any other source of energy in the medium would have been extremely dilute and not likely to permit growth. Even if some or all the organisms in the two cultures used trace quantities of oxygen in the medium, the energy source, if it was not methane, remains problematic. None of the microorganisms in the enrichments are known to be aerobic methanotrophs. Clearly, further work on both of the apparently anaerobic methanotroph cultures is needed.

Many of the organisms isolated from deep-subsurface waters in this study have phylogenetic relatives that have been found in other extreme and/or interesting environments (Table 4). Sequences similar to the clones discussed in this study have been discovered in environments such as thermal soils, deep-sea sediments, the terrestrial subsurface, hypersaline lakes, deep-sea hydrothermal vents, gas hydrates, and uranium mining waste piles (Table 4). This observation supports the idea that many of the microorganisms from South African mine waters are indigenous to the subsurface and are

not simply surface contaminants introduced into the deep subsurface by mining in recent times.

CONCLUSIONS

Microorganisms isolated in culture from gold and diamond mines in South Africa were identified by 16S rDNA sequencing. Three archaeal sequences and thirty-eight bacterial sequences were obtained. Phylogenetic analyses of these sequences yielded evidence for the existence of a native deep subsurface microbial community not associated with mining contamination.

Geochemical data revealed that two of the water samples were relatively saline and had δD and $\delta^{18}O$ values that were shifted left of the meteoric water line. These samples had likely resided in the subsurface for an extended period of time and had not been influenced by recent meteoric water or by mine service water. Most of the mine waters plotted along the meteoric water line, indicating a more recent meteoric origin. These waters are more likely to have been influenced by mine service water. In general, the organisms identified as being the best possibilities for indigenous deep subsurface inhabitants were isolated from warmer, more saline waters. Cooler, less saline waters yielded many organisms that are probably mesophilic contaminants from the surface. Some of the mesophilic organisms, however, may be native to the deep subsurface.

Several of the organisms cultured in this study are probably novel species or new strains of previously described species. Two of the three archaeal sequences represent thermophilic archaea from the non-thermophilic Crenarchaeota group. To the author's knowledge, these are the first species from this group to be cultured. In addition, their

thermophilic nature indicates that some of the so-called non-thermophilic crenarchaeotes grow at high temperatures.

The third archaeal sequence represents a deeply-branching crenarchaeote related to a group of uncultured archaea from an extreme thermal soil in Yellowstone National Park. The phylogenetic position of this archaeon is interesting because it suggests that it may be hyperthermophilic. If it can be observed to grow optimally at 80°C or higher, then it may provide support for the previously proposed idea that hot, hyperthermophile-containing water is migrating upward from deeper in the Witwatersrand basin. This support would come from the fact that the archaeon was associated with borehole water that was well below hyperthermophilic temperatures.

Three thermophilic, spore-forming bacteria from the *Bacillus* and *Clostridium* genera were cultured. Their thermophilic nature and spore-forming ability are consistent with their being indigenous to the deep subsurface. The ability to form spores probably offers a survival advantage to microorganisms inhabiting the deep subsurface. The *Clostridium* spp. included a new species <94% similar to its closest phylogenetic relative, *Clostridium subatlanticum*, an isolate from a deep-sea hydrothermal chimney. A new strain of *Clostridium aerotolerans* was also cultured. The new strain is a facultative aerobe whose growth is enhanced, not inhibited, by the presence of oxygen, a previously undescribed trait for any strain of *C. aerotolerans*.

Two enrichments with methane as the only intended energy source and sulfate as the only intended electron acceptor showed microbial growth. Whether these two cultures indeed contain organisms participating in the anaerobic oxidation of methane (AOM) is uncertain. Because no archaea were identified in either of the anaerobic

methanotroph cultures, the microorganisms in these cultures, if they are truly participating in AOM, are doing so by an undescribed metabolic pathway.

This culture-dependent study of microorganisms isolated from waters from South African gold and diamond mines has expanded knowledge of microbial life in the deep subsurface. The presence of thermophiles, possible hyperthermophiles, spore formers, new species and strains of microorganisms, and potentially novel metabolic pathways strongly suggest that an indigenous population of subsurface microorganisms, not introduced via mining in recent times, exists at depths to at least 3.4 km below the surface in the Witwatersrand basin. Further research on the physiology of the cultured organisms will likely yield essential insights into the metabolic capabilities of these deep subsurface inhabitants and their relationship to local geochemical environments.

Table 1. Positive microbial enrichments.

Sample	Enrichment Temperature (°C)	Electron Donor(s)	Electron Acceptor(s)
Evander 8-18	RT	APL, H_2	sulfate
Evander 8-18	RT	APL, H_2	thiosulfate
Evander 8-18	RT	H_2/acetate	CO_2/acetate
Evander 8-18	RT	H_2	sulfate
Evander 8-18	60	APL, H_2	sulfate
Evander 8-18	60	APL, H_2	thiosulfate
Evander 8-18	RT	TYG	NO_3^-
Evander 8-18	RT	APL, H_2	HFO
Evander 8-18	RT	APL, H_2	MnO_2
Kloof 4-43	60	H_2/acetate	CO_2/acetate
Kloof 4-43	60	H_2	CO_2
Kloof 7-39	60	APL, H_2	sulfate
Kloof 7-39	60	APL, H_2	thiosulfate
Premier 763	RT	APL, H_2	Fe-NTA
Premier 763	RT	APL, H_2	sulfate
Premier 763	RT	APL, H_2	thiosulfate
Premier 763	RT	H_2/acetate	CO_2/acetate
Premier 763	RT	CH_4	sulfate
Premier 763	RT	APL, H_2	MnO_2
Evander 8-21	60	APL, H_2	sulfate
Evander 8-21	60	APL, H_2	thiosulfate
Evander 8-21	60	H_2/acetate	CO_2/acetate
Evander 2-19	RT	APL, H_2	sulfate
Evander 2-19	RT	APL, H_2	thiosulfate
Evander 2-19	RT	APL, H_2	MnO_2
Evander 2-19	RT	H_2/acetate	CO_2/acetate
Evander 2-19	RT	CH_4	sulfate
Evander 2-19	RT	APL, H_2	Fe-citrate
Evander 2-19	RT	APL, H_2	HFO
Evander 2-19	RT	TYG	NO_3^-

RT = room temperature; APL = acetate, pyruvate, lactate
TYG = tryptone, yeast, glucose; HFO = hydrous ferric oxide

Table 2. Positive microbial enrichments with associated clones.

Sample	Enrichment Temperature (°C)	Electron Donor(s)	Electron Acceptor(s)	Clones sequenced
Evander 8-18	RT	APL, H_2	sulfate	C23, C37
Evander 8-18	RT	H_2/acetate	CO_2/acetate	N3
Evander 8-18	60	APL, H_2	sulfate	AA2
Evander 8-18	60	APL, H_2	thiosulfate	BB7, CC4, CC8, CC9, CC14
Evander 8-18	RT	TYG	NO_3^-	K7
Evander 8-18	RT	APL, H_2	HFO	U9
Evander 8-18	RT	APL, H_2	MnO_2	V3
Kloof 4-43	60	H_2/acetate	CO_2/acetate	X4, X13
Kloof 4-43	60	H_2	CO_2	Y10
Kloof 7-39	60	APL, H_2	sulfate	J6, GG8, GG13, GG15
Premier 763	RT	APL, H_2	Fe-NTA	P5
Premier 763	RT	APL, H_2	sulfate	M1, M2, M3, M9
Premier 763	RT	H_2/acetate	CO_2/acetate	O4
Premier 763	RT	CH_4	sulfate	H3, H9
Premier 763	RT	APL, H_2	MnO_2	R6, R10
Evander 2-19	RT	APL, H_2	sulfate	L4, L7
Evander 2-19	RT	APL, H_2	MnO_2	G5, G7
Evander 2-19	RT	H_2/acetate	CO_2/acetate	W9, W12
Evander 2-19	RT	CH_4	sulfate	I3, I12, I13, I15, I19
Evander 2-19	RT	APL, H_2	Fe-citrate	T8
Evander 2-19	RT	APL, H_2	HFO	Q2
Evander 2-19	RT	TYG	NO_3^-	S2

RT = room temperature; APL = acetate, pyruvate, lactate
HFO = hydrous ferric oxide; TYG = tryptone, yeast, glucose

Table 3. BLAST and RDP results.

Sample location	Enrichment temp. (°C)[1]	Clone	Top BLAST hit	E value	Top RDP hit[2]	Similarity score
Evander 2-19	RT	G5	*Aeromonas punctata*	0.0	*Aeromonas caviae*	0.973
Evander 2-19	RT	G7	*Aeromonas caviae*	0.0	*Aeromonas caviae*	0.957
Evander 2-19	RT	I12	Uncultured beta proteobacterium clone ccspost2132	0.0	clone CL03[3] (*Burkholderia glathei* subgroup)	0.983
Evander 2-19	RT	I13	Petroleum-degrading bacterium HD-1	0.0	str. HD-1 (*Azospirillum lipoferum* subgroup)	0.969
Evander 2-19	RT	I15	*Caulobacter vibrioides*	0.0	*Caulobacter vibrioides*	1.000
Evander 2-19	RT	I19	*Aeromonas punctata*	0.0	*Aeromonas caviae*	0.981
Evander 2-19	RT	L4	*Aeromonas caviae*	0.0	*Aeromonas caviae*	1.000
Evander 2-19	RT	L7	Uncultured bacterium clone Ebpr3	0.0	*Flavobacterium ferrugineum*	0.682
Evander 2-19	RT	Q2	*Aeromonas caviae*	0.0	*Aeromonas caviae*	0.994
Evander 2-19	RT	S2	*Thiomonas* sp. Ynys3	0.0	*Leptothrix discophora* str. SS-1	0.782
Evander 2-19	RT	T8	*Aeromonas hydrophila* subsp. dhakensis	0.0	*Aeromonas caviae*	0.988
Evander 2-19	RT	W12	*Aeromonas caviae*	0.0	*Aeromonas caviae*	0.991
Evander 2-19	RT	W9	*Aeromonas punctata*	0.0	*Aeromonas hydrophila*	0.975
Evander 8-18	60	AA2	Uncultured archaeon clone HTA-C5	0.0	clone SCA1145 (marine crenarchaeota group)	0.966
Evander 8-18	60	BB7	Uncultured thermal soil archaeon clone YNPFFA108	0.0	clone pOWA114 (*Aeropyrum pernix* subgroup) and *Staphylothermus achaiicus* str. P8	0.456

Table 3 (continued).

Sample location	Enrichment temp. (°C)[1]	Clone	Top BLAST hit	E value	Top RDP hit[2]	Similarity score
Evander 8-18	60	CC14	Uncultured bacterium clone Ebpr3	0.0	*Flavobacterium ferrugineum*	0.611
Evander 8-18	60	CC4	Uncultured bacterium clone Ebpr3	0.0	*Flavobacterium ferrugineum*	0.600
Evander 8-18	60	CC8	Alpha proteobacterium 63286	0.0	*Bosea thiooxidans* str. RpPl3-VS	0.960
Evander 8-18	60	CC9	*Clostridium subatlanticum*	0.0	*Clostridium felsineum*	0.714
Evander 8-18	RT	C23	*Pseudomonas stutzeri* strain JJ	0.0	*Pseudomonas stutzeri*	0.957
Evander 8-18	RT	C37	*Pseudomonas chloritidismutans*	0.0	*Pseudomonas stutzeri*	1.000
Evander 8-18	RT	K7	*Pseudomonas stutzeri* strain LS401	0.0	*Pseudomonas stutzeri* str. LS401	0.972
Evander 8-18	RT	N3	*Pseudomonas stutzeri* strain LS401	0.0	*Pseudomonas stutzeri* str. LS401	0.973
Evander 8-18	RT	U9	*Pseudomonas stutzeri* strain LS401	0.0	*Pseudomonas stutzeri* str. LS401	0.983
Evander 8-18	RT	V3	Uncultured alpha proteobacterium clone ccspost2147	0.0	*Rhizobium* sp. str. Esparseta 3	0.911
Kloof 4-43	60	X13	*Bacillus* sp.	0.0	*Bacillus* sp. (*B. stearothermophilus* subgroup)	0.948
Kloof 4-43	60	X4	Uncultured bacterium clone Ebpr3	0.0	*Flavobacterium ferrugineum*	0.618
Kloof 4-43	60	Y10	Uncultured bacterium clone Ebpr3	0.0	*Flavobacterium ferrugineum*	0.610
Kloof 7-39	60	GG13	*Clostridium xylanolyticum*	0.0	*Clostridium aerotolerans*	0.898
Kloof 7-39	60	GG15	*Clostridium xylanolyticum*	0.0	*Clostridium aerotolerans*	0.898
Kloof 7-39	60	GG8	Uncultured bacterium clone Ebpr3	0.0	clone P48[3] (*Flexibacter sancti* subgroup)	0.704
Kloof 7-39	60	J6	Uncultured archaeon clone HTA-H8	0.0	clone KBSCul1 and clone SCA1175 (both marine crenarchaeota group)	0.904

Table 3 (continued).

Sample location	Enrichment temp. (°C)[1]	Clone	Top BLAST hit	E value	Top RDP hit[2]	Similarity score
Premier 763	RT	H3	*Pseudomonas nitroreducens* strain 0802	0.0	*Pseudomonas pseudoalcaligenes* subsp. pseudoalcaligenes	0.847
Premier 763	RT	H9	Unidentified bacterium wb1_D18	e-159	str. KOLL2a (*Nitrospina* subdivision)	0.406
Premier 763	RT	M1	*Pseudomonas nitroreducens* strain 0802	0.0	str. LT1 (*Pseudomonas mendocina* subgroup)	0.888
Premier 763	RT	M2	Unidentified *Bacteroides/Flavobacteria/Cytophaga* clone UN85	0.0	clone UN85[3] (*Porphyromonas macacae* subgroup)	0.860
Premier 763	RT	M3	Uncultured hydrocarbon seep bacterium BPC009	0.0	clone BPC009 (*Propionibacterium acnes* subgroup)	0.985
Premier 763	RT	M9	*Phaeospirillum fulvum* isolate S3	0.0	*Acidiphilium acidophilum*[3]	0.718
Premier 763	RT	O4	*Hydrogenophaga flava*	0.0	*Hydrogenophaga flava*	0.983
Premier 763	RT	P5	*Pseudomonas* sp. SMCC D0715	0.0	*Pseudomonas pseudoalcaligenes* subsp. pseudoalcaligenes	0.951
Premier 763	RT	R10	*Hydrogenophaga flava*	0.0	*Hydrogenophaga flava*	0.991

[1]RT designates room temperature

[2]Does not include unclassified/unaligned organisms

[3]Sequence is less than 500 bases

Table 4. Environment of origin for sequences related to clones from this study.

Clones (This Study) and Similar Sequences	Environment	Accession Number	Reference
AA2	**deep South African gold mine water**	**AY187879**	**This study**
Uncultured archaeon clone HTA-C5	associated with metal-rich particles from a freshwater reservoir	AF418930	(Stein et al., 2002)
Uncultured Front Range soil crenarchaeote FRB15	coniferous forest soil	AY016478	(Oline et al., unpublished data)
clone SCA1145	soil	U62811	(Bintrim et al., 1997)
Uncultured crenarchaeote TRC132-7	terrestrial plant roots	AF227637	(Simon et al., unpublished data)
Uncultured archaeon SAGMA-P	deep South African gold mine water	AB050221	(Takai et al., 2001a)
BB7	**deep South African gold mine water**	**AY187880**	**This study**
Uncultured thermal soil archaeon clone YNPFFA108	extreme thermal soil	AF391993	(Botero et al., unpublished data)
Uncultured thermal soil archaeon clone YNPFFA4	extreme thermal soil	AF391990	(Botero et al., unpublished data)
CC4	**deep South African gold mine water**	**AY187881**	**This study**
Uncultured eubacterium clone MT10	reagents used to prepare genomic DNA	AF058376	(Tanner et al., 1998)
Uncultured Cytophagales bacterium clone 09	mesoeutrophic reservoir	AF361195	(Simek et al., 2001)
Uncultured Bacteroidetes bacterium clone CLi112	PCE-contaminated site	AF529321	(Carroll and Zinder, unpublished data)
Uncultured CFB group bacterium clone TAF-B2	epilithic biofilm in river (River Taff epilithon in South Wales, UK)	AY038772	(O'Sullivan et al., 2002)
Uncultured bacterium clone Ebpr3	sewage	AF255636	(Liu et al., 2001)
CC14	**deep South African gold mine water**	**AY187882**	**This study**

Table 4 (continued).

Clones (This Study) and Similar Sequences	Environment	Accession Number	Reference
Uncultured eubacterium clone MT10	reagents used to prepare genomic DNA	AF058376	(Tanner et al., 1998)
Uncultured Cytophagales bacterium clone 09	mesoeutrophic reservoir	AF361195	(Simek et al., 2001)
Uncultured Bacteroidetes bacterium clone CLi112	PCE-contaminated site	AF529321	(Carroll and Zinder, unpublished data)
Uncultured CFB group bacterium clone TAF-B2	epilithic biofilm in river (River Taff epilithon in South Wales, UK)	AY038772	(O'Sullivan et al., 2002)
Uncultured bacterium clone Ebpr3	sewage	AF255636	(Liu et al., 2001)
CC8	**deep South African gold mine water**	**AY187883**	**This study**
str. 5Z2111	rice field soil	AJ224610	(Stubner et al., 1998)
Bosea thiooxidans str. KB13-VS	rhizoplane of *Medicago sativa*	AJ250800	(Goetz et al., unpublished data)
Alpha proteobacterium 63286	hospital water supplies	AF288308	(La Scola et al., 2000)
Bosea thiooxidans str. RpPl5-VS	rhizoplane of *Medicago sativa*	AJ250797	(Goetz et al., unpublished data)
CC9	**deep South African gold mine water**	**AY187884**	**This study**
Clostridium subatlanticum	Atlantic deep-sea hydrothermal chimney	AF458779	(Brisbarre et al., 2003 (in press))
Alkaliphilus transvaalensis	deep South African gold mine	AB037677	(Takai et al., 2001b)
str. BD2-4 (unidentified proteobacterium strain BD2 4)	deep-sea sediment	AB015534	(Li et al., 1999)
str. 48.h (unidentified bacterium clone NB1-o)	Japan Trench sediment (depth of 6292 m)	AB013836	(Yanagibayashi et al., 1999)
Y10	**deep South African gold mine water**	**AY187885**	**This study**
Uncultured eubacterium clone MT10	reagents used to prepare genomic DNA	AF058376	(Tanner et al., 1998)

Table 4 (continued).

Clones (This Study) and Similar Sequences	Environment	Accession Number	Reference
Uncultured Cytophagales bacterium clone 09	mesoeutrophic reservoir	AF361195	(Simek et al., 2001)
Uncultured Bacteroidetes bacterium clone CLi112	PCE-contaminated site	AF529321	(Carroll and Zinder, unpublished data)
Uncultured CFB group bacterium clone TAF-B2	epilithic biofilm in river (River Taff epilithon in South Wales, UK)	AY038772	(O'Sullivan et al., 2002)
Uncultured bacterium clone Ebpr3	sewage	AF255636	(Liu et al., 2001)
C23	**deep South African gold mine water**	**AY187886**	**This study**
Pseudomonas stutzeri	marine, waste water, clinical, and soil samples	AJ006107	(Sikorski et al., 1999)
C37	**deep South African gold mine water**	**AY187887**	**This study**
Pseudomonas stutzeri	marine, waste water, clinical, and soil samples	AJ006107	(Sikorski et al., 1999)
Pseudomonas chloritidismutans	anaerobic chlorate-reducing bioreactor	AY017341	(Wolterink et al., 2002)
Pseudomonas mosselii CIP 105259	clinical specimans	AF072688	(Elomari, unpublished data)
Pseudomonas sp. SMCC D0715	deep subsurface	AF336311	(Vepritskiy et al., 2002)
Pseudomonas mendocina	river continuously polluted with phenolic compounds	AF232713	(Heinaru et al., 2000)
str. LE1	compost biofilter	AJ007004	(Juteau et al., 1999)
str. HTB147 (unidentified gamma proteobacterium strain HTB147)	deep-sea mud samples (depths of 1,050-10,897 m) near southern Japan	AB010850	(Takami et al., 1999)
Pseudomonas pseudoalcaligenes	gold mine tailings dams	AF238494	(Brinne, unpublished data)

Table 4 (continued).

Clones (This Study) and Similar Sequences	Environment	Accession Number	Reference
G5	**deep South African gold mine water**	**AY187888**	**This study**
Aeromonas sp. VKM B-2261	estuarine environment	AF430120	(Gonzalez et al., unpublished data)
Aeromonas sp. strain T8	fish with epizootic ulcerative syndrome (EUS) in Southeast Asian countries	AF099027	(Iqbal et al., unpublished data)
Aeromonas hydrophila subsp. dhakensis	children with diarrhea in Bangladesh	AJ508765	(Huys et al., 2002)
G7	**deep South African gold mine water**	**AY187889**	**This study**
Aeromonas sp. VKM B-2261	estuarine environment	AF430120	(Gonzalez et al., unpublished data)
Aeromonas hydrophila subsp. dhakensis	children with diarrhea in Bangladesh	AJ508765	(Huys et al., 2002)
Aeromonas hydrophila str. T20	fish with epizootic ulcerative syndrome (EUS) in Southeast Asian countries	AF099022	(Iqbal et al., unpublished data)
Aeromonas culicicola strain MTCC 3249 substrain SLH	midgut of *Culex quinquefasciatus*	AY130992	(Jangid et al., unpublished data)
GG13	**deep South African gold mine water**	**AY187890**	**This study**
Clostridium aerotolerans DSM 5434 (T)	corn stover and rumina of sheep fed corn stover	X76163	(Collins et al., 1994)
Clostridium xylanolyticum DSM 6555 (T)	decayed *Pinus patula* wood chips	X76739	(Collins et al., 1994)
Clostridium sp. str. DR7	rumen of red deer	Y10030	(Jarvis et al., unpublished data)
Clostridium saccharolyticum strain DSM 2544	sewage sludge	Y18185	(Stackebrandt et al., 1999)

Table 4 (continued).

Clones (This Study) and Similar Sequences	Environment	Accession Number	Reference
Clostridium methoxybenzovorans str. SR3 DSM 12182	olive mill wastewater treatment digester	AF067965	(Mechichi et al., 1999)
GG15	**deep South African gold mine water**	**AY187891**	**This study**
Clostridium aerotolerans DSM 5434 (T)	corn stover and rumina of sheep fed corn stover	X76163	(Collins et al., 1994)
Clostridium xylanolyticum DSM 6555 (T)	decayed *Pinus patula* wood chips	X76739	(Collins et al., 1994)
Clostridium sp. str. DR7	rumen of red deer	Y10030	(Jarvis et al., unpublished data)
Clostridium saccharolyticum strain DSM 2544	sewage sludge	Y18185	(Stackebrandt et al., 1999)
Clostridium methoxybenzovorans str. SR3 DSM 12182	olive mill wastewater treatment digester	AF067965	(Mechichi et al., 1999)
GG8	**deep South African gold mine water**	**AY187892**	**This study**
Uncultured eubacterium clone MT10	reagents used to prepare genomic DNA	AF058376	(Tanner et al., 1998)
Uncultured Cytophagales bacterium clone 09	mesoeutrophic reservoir	AF361195	(Simek et al., 2001)
Uncultured Bacteroidetes bacterium clone CLi112	PCE-contaminated site	AF529321	(Carroll and Zinder, unpublished data)
Uncultured bacterium FukuN24	lake (bacterioplankton)	AJ289995	(Glockner et al., 2000)
Uncultured bacterium clone Ebpr3	sewage	AF255636	(Liu et al., 2001)
Uncultured CFB group bacterium clone TAF-B2	epilithic biofilm in river (River Taff epilithon in South Wales, UK)	AY038772	(O'Sullivan et al., 2002)

Table 4 (continued).

Clones (This Study) and Similar Sequences	Environment	Accession Number	Reference
H3	**South African diamond mine water**	**AY187893**	**This study**
Pseudomonas alcalophila	seawater off the coast of Rumoi, Hokkaido, Japan	AB030583	(Yumoto et al., 2001)
Pseudomonas pseudoalcaligenes	gold mine tailings dams	AF238494	(Brinne, unpublished data)
str. LE1	compost biofilter	AJ007004	(Juteau et al., 1999)
Pseudomonas chloritidismutans	anaerobic chlorate-reducing bioreactor	AY017341	(Wolterink et al., 2002)
str. HTB110 (unidentified gamma proteobacterium strain HTB110)	deep-sea mud samples (depths of 1,050-10,897 m) near southern Japan	AB010851	(Takami et al., 1999)
Pseudomonas pseudoalcaligenes str. ML-052	lake sediment	AF139995	(Venkateswaran and Nealson, unpublished data)
Pseudomonas sp. SMCC B0310	deep subsurface	AF500620	(Vepritskiy et al., 2002)
H9	**South African diamond mine water**	**AY187894**	**This study**
str. KOLL2a (anaerobic ammonium-oxidizing planctomycete KOLL2a)	rotating biological contactor treating ammonium-rich leachate (near Kolliken, Switzerland)	AJ250882	(Egli et al., 2001)
str. BD7-11	deep-sea sediments	AB015586	(Li et al., 1999)
Uncultured soil bacterium PBS-II-37	bulk soil and rice roots of flooded rice microcosms	AJ390447	(Derakshani et al., 2001)
Unidentified planctomycete Om190	marine coastal picoplankton from continental shelf off Cape Hatteras, NC	U70712	(Rappe et al., 1997)
Marine eubacterial sp. (aggregate agg27)	aggregate-attached marine bacterial assemblages	L10943	(DeLong et al., 1993)

Table 4 (continued).

Clones (This Study) and Similar Sequences	Environment	Accession Number	Reference
Uncultured planctomycete clone KSU-1	anoxic sludge	AB057453	(Sugino, unpublished data)
I12	**deep South African gold mine water**	**AY187895**	**This study**
isolate Boom-7m-04	deep-subsurface clay	Z73450	(Boivin-Jahns et al., 1996)
Uncultured bacterium H20	activated sludge from an industrial sewage treatment plant	AF072920	(Juretschko et al., 2002)
Uncultured beta proteobacterium SBR1001	wastewater/sewage	AF204252	(Crocetti et al., 2000)
I13	**deep South African gold mine water**	**AY187896**	**This study**
str. HD-1 (Petroleum-degrading bacterium HD-1)	oil field in Shizuoka, Japan	D45202	(Morikawa and Imanaka, 1993; Morikawa et al., 1996; Morikawa et al., 1998)
Uncultured bacterium clone d064	trichloroethene-contaminated Superfund site undergoing intrinsic *in situ* reductive dechlorination	AF422655	(Lowe et al., 2002)
I15	**deep South African gold mine water**	**AY187897**	**This study**
Caulobacter segnis str. MBIC2835	oligotrophic environments	AB023427	(Hamada and Suzuki, unpublished data)
I19	**deep South African gold mine water**	**AY187898**	**This study**
Aeromonas sp. VKM B-2261	estuarine environment	AF430120	(Gonzalez et al., unpublished data)

Table 4 (continued).

Clones (This Study) and Similar Sequences	Environment	Accession Number	Reference
Aeromonas sp. strain T8	fish with epizootic ulcerative syndrome (EUS) in Southeast Asian countries	AF099027	(Iqbal et al., unpublished data)
Aeromonas hydrophila subsp. dhakensis	children with diarrhea in Bangladesh	AJ508765	(Huys et al., 2002)
J6	**deep South African gold mine water**	**AY187899**	**This study**
Uncultured Front Range soil crenarchaeote FRD9	coniferous forest soil	AY016505	(Oline et al., unpublished data)
clone KBSCul1	soil	AF058719	(Buckley et al., 1998)
clone SCA1175	soil	U62819	(Bintrim et al., 1997)
clone ST1-8	anoxic rice field soil	AJ236459	(Chin et al., 1999a)
Uncultured archaeon clone HTA-H8	associated with metal-rich particles from a freshwater reservoir	AF418939	(Stein et al., 2002)
K7	**deep South African gold mine water**	**AY187900**	**This study**
Pseudomonas stutzeri	marine, waste water, clinical, and soil samples	AJ006107	(Sikorski et al., 1999)
Pseudomonas chloritidismutans	anaerobic chlorate-reducing bioreactor	AY017341	(Wolterink et al., 2002)
Pseudomonas mosselii CIP 105259	clinical specimans	AF072688	(Elomari, unpublished data)
Pseudomonas sp. SMCC D0715	deep subsurface	AF336311	(Vepritskiy et al., 2002)
Pseudomonas mendocina	river continuously polluted with phenolic compounds	AF232713	(Heinaru et al., 2000)
str. LE1	compost biofilter	AJ007004	(Juteau et al., 1999)
str. HTB147 (unidentified gamma proteobacterium strain HTB147)	deep-sea mud samples (depths of 1,050-10,897 m) near southern Japan	AB010850	(Takami et al., 1999)
Pseudomonas pseudoalcaligenes	gold mine tailings dams	AF238494	(Brinne, unpublished data)

Table 4 (continued).

Clones (This Study) and Similar Sequences	Environment	Accession Number	Reference
L4	**deep South African gold mine water**	**AY187901**	**This study**
Aeromonas sp. VKM B-2261	estuarine environment	AF430120	(Gonzalez et al., unpublished data)
Aeromonas hydrophila subsp. dhakensis	children with diarrhea in Bangladesh	AJ508765	(Huys et al., 2002)
Aeromonas hydrophila str. T20	fish with epizootic ulcerative syndrome (EUS) in Southeast Asian countries	AF099022	(Iqbal et al., unpublished data)
Aeromonas culicicola strain MTCC 3249 substrain SLH	midgut of *Culex quinquefasciatus*	AY130992	(Jangid et al., unpublished data)
L7	**deep South African gold mine water**	**AY187902**	**This study**
Uncultured eubacterium clone MT10	reagents used to prepare genomic DNA	AF058376	(Tanner et al., 1998)
Uncultured Cytophagales bacterium clone 09	mesoeutrophic reservoir	AF361195	(Simek et al., 2001)
Uncultured Bacteroidetes bacterium clone CLi112	PCE-contaminated site	AF529321	(Carroll and Zinder, unpublished data)
Uncultured bacterium FukuN24	lake (bacterioplankton)	AJ289995	(Glockner et al., 2000)
Uncultured bacterium clone Ebpr3	sewage	AF255636	(Liu et al., 2001)
Uncultured CFB group bacterium clone TAF-B2	epilithic biofilm in river (River Taff epilithon in South Wales, UK)	AY038772	(O'Sullivan et al., 2002)
M1	**South African diamond mine water**	**AY187903**	**This study**
Pseudomonas alcalophila	seawater off the coast of Rumoi, Hokkaido, Japan	AB030583	(Yumoto et al., 2001)
Pseudomonas pseudoalcaligenes	gold mine tailings dams	AF238494	(Brinne, unpublished data)
str. LE1	compost biofilter	AJ007004	(Juteau et al., 1999)

Table 4 (continued).

Clones (This Study) and Similar Sequences	Environment	Accession Number	Reference
Uncultured Green Bay ferromanganous micronodule bacterium MNG3	freshwater ferromanganous micronodules and sediments	AF293000	(Stein et al., 2001)
N3	**deep South African gold mine water**	**AY187907**	**This study**
Pseudomonas stutzeri	marine, waste water, clinical, and soil samples	AJ006107	(Sikorski et al., 1999)
Pseudomonas chloritidismutans	anaerobic chlorate-reducing bioreactor	AY017341	(Wolterink et al., 2002)
Pseudomonas mosselii CIP 105259	clinical specimans	AF072688	(Elomari, unpublished data)
Pseudomonas sp. SMCC D0715	deep subsurface	AF336311	(Vepritskiy et al., 2002)
Pseudomonas mendocina	river water continuously polluted with phenolic compounds	AF232713	(Heinaru et al., 2000)
str. LE1	compost biofilter	AJ007004	(Juteau et al., 1999)
str. HTB147 (unidentified gamma proteobacterium strain HTB147)	deep-sea mud samples (depths of 1,050-10,897 m) near southern Japan	AB010850	(Takami et al., 1999)
Pseudomonas pseudoalcaligenes	gold mine tailings dams	AF238494	(Brinne, unpublished data)
O4	**South African diamond mine water**	**AY187908**	**This study**
Uncultured sludge bacterium A6b	nitrifying-denitrifying activated sludge from an industrial sewage treatment plant	AF234720	(Juretschko et al., 2002)
str. mz1L (beta proteobacterium mz1L)	industrial activated-sludge wastewater treatment plant	AF110006	(Lajoie et al., 2000)

Table 4 (continued).

Clones (This Study) and Similar Sequences	Environment	Accession Number	Reference
Unidentified bacterium clone 19523	activated sludge from an industrial wastewater treatment system	AF097797	(Layton et al., 2000)
P5	**South African diamond mine water**	**AY187909**	**This study**
Pseudomonas stutzeri	marine, waste water, clinical, and soil samples	AJ006107	(Sikorski et al., 1999)
Pseudomonas chloritidismutans	anaerobic chlorate-reducing bioreactor	AY017341	(Wolterink et al., 2002)
Pseudomonas mosselii CIP 105259	clinical specimans	AF072688	(Elomari, unpublished data)
Pseudomonas sp. SMCC D0715	deep subsurface	AF336311	(Vepritskiy et al., 2002)
Pseudomonas mendocina	river water continuously polluted with phenolic compounds	AF232713	(Heinaru et al., 2000)
str. LE1	compost biofilter	AJ007004	(Juteau et al., 1999)
str. HTB147 (unidentified gamma proteobacterium strain HTB147)	deep-sea mud samples (depths of 1,050-10,897 m) near southern Japan	AB010850	(Takami et al., 1999)
Pseudomonas pseudoalcaligenes	gold mine tailings dams	AF238494	(Brinne, unpublished data)
Q2	**deep South African gold mine water**	**AY187910**	**This study**
Aeromonas sp. VKM B-2261	estuarine environment	AF430120	(Gonzalez et al., unpublished data)
Aeromonas hydrophila subsp. dhakensis	children with diarrhea in Bangladesh	AJ508765	(Huys et al., 2002)
Aeromonas hydrophila str. T20	fish with epizootic ulcerative syndrome (EUS) in Southeast Asian countries	AF099022	(Iqbal et al., unpublished data)

Table 4 (continued).

Clones (This Study) and Similar Sequences	Environment	Accession Number	Reference
Aeromonas culicicola strain MTCC 3249 substrain SLH	midgut of *Culex quinquefasciatus*	AY130992	(Jangid et al., unpublished data)
R10	**South African diamond mine water**	**AY187911**	**This study**
Uncultured sludge bacterium A6b	nitrifying-denitrifying activated sludge from an industrial sewage treatment plant	AF234720	(Juretschko et al., 2002)
str. mz1L (beta proteobacterium mz1L)	industrial activated-sludge wastewater treatment plant	AF110006	(Lajoie et al., 2000)
Unidentified bacterium clone 19523	activated sludge from an industrial wastewater treatment system	AF097797	(Layton et al., 2000)
S2	**deep South African gold mine water**	**AY187912**	**This study**
Thiomonas sp. Ynys3	acid mine drainage waters	AF387303	(Dennison et al., 2001)
T8	**deep South African gold mine water**	**AY187913**	**This study**
Aeromonas sp. VKM B-2261	estuarine environment	AF430120	(Gonzalez et al., unpublished data)
Aeromonas hydrophila subsp. dhakensis	children with diarrhea in Bangladesh	AJ508765	(Huys et al., 2002)
Aeromonas hydrophila str. T20	fish with epizootic ulcerative syndrome (EUS) in Southeast Asian countries	AF099022	(Iqbal et al., unpublished data)
Aeromonas culicicola strain MTCC 3249 substrain SLH	midgut of *Culex quinquefasciatus*	AY130992	(Jangid et al., unpublished data)

Table 4 (continued).

Clones (This Study) and Similar Sequences	Environment	Accession Number	Reference
U9	**deep South African gold mine water**	**AY187914**	**This study**
Pseudomonas stutzeri	marine, waste water, clinical, and soil samples	AJ006107	(Sikorski et al., 1999)
Pseudomonas chloritidismutans	anaerobic chlorate-reducing bioreactor	AY017341	(Wolterink et al., 2002)
Pseudomonas mosselii CIP 105259	clinical specimans	AF072688	(Elomari, unpublished data)
Pseudomonas sp. SMCC D0715	deep subsurface	AF336311	(Vepritskiy et al., 2002)
Pseudomonas mendocina	river water continuously polluted with phenolic compounds	AF232713	(Heinaru et al., 2000)
str. LE1	compost biofilter	AJ007004	(Juteau et al., 1999)
str. HTB147 (unidentified gamma proteobacterium strain HTB147)	deep-sea mud samples (depths of 1,050-10,897 m) near southern Japan	AB010850	(Takami et al., 1999)
Pseudomonas pseudoalcaligenes	gold mine tailings dams	AF238494	(Brinne, unpublished data)
V3	**deep South African gold mine water**	**AY187915**	**This study**
Uncultured alpha proteobacterium clone ccspost2147	TCE-contaminated site	AY133103	(Carroll and Zinder, unpublished data)
Uncultured alpha proteobacterium clone FTLM218	trichloroethene-contaminated site undergoing in situ bioremediation treatment	AF529121	(Carroll and Zinder, unpublished data)

Table 4 (continued).

Clones (This Study) and Similar Sequences	Environment	Accession Number	Reference
Uncultured bacterium clone d041	trichloroethene-contaminated Superfund site undergoing intrinsic *in situ* reductive dechlorination	AF422650	(Lowe et al., 2002)
Agrobacterium tumefaciens strain IrT-JG-6	uranium mining waste piles	AJ295683	(Selenska-Pobell, unpublished data)
Alpha proteobacterium RSHD3S8	bacterial community associated with laboratory cultures of four '*Pfiesteria* -like' dinoflagellates isolated from 1997 fish killing events in Chesapeake Bay	AF190213	(Alavi et al., 2001)
Agrobacterium sp. LMG 11915	nodules of tropical legumes	AJ130720	(de Lajudie et al., unpublished data)
W12	**deep South African gold mine water**	**AY187916**	**This study**
Aeromonas sp. VKM B-2261	estuarine environment	AF430120	(Gonzalez et al., unpublished data)
Aeromonas hydrophila subsp. dhakensis	children with diarrhea in Bangladesh	AJ508765	(Huys et al., 2002)
Aeromonas hydrophila str. T20	fish with epizootic ulcerative syndrome (EUS) in Southeast Asian countries	AF099022	(Iqbal et al., unpublished data)
Aeromonas culicicola strain MTCC 3249 substrain SLH	midgut of *Culex quinquefasciatus*	AY130992	(Jangid et al., unpublished data)
W9	**deep South African gold mine water**	**AY187917**	**This study**

Table 4 (continued).

Clones (This Study) and Similar Sequences	Environment	Accession Number	Reference
Aeromonas sp. VKM B-2261	estuarine environment	AF430120	(Gonzalez et al., unpublished data)
Aeromonas sp. strain T8	fish with epizootic ulcerative syndrome (EUS) in Southeast Asian countries	AF099027	(Iqbal et al., unpublished data)
Aeromonas hydrophila subsp. dhakensis	children with diarrhea in Bangladesh	AJ508765	(Huys et al., 2002)
X13	**deep South African gold mine water**	**AY187918**	**This study**
Bacillus sp. B-3	compost	AB040122	(Karita et al., 2001)
Bacillus pallidus DSM 3670 (T)	sewage	Z26930	(Rainey et al., 1994)
Saccharococcus thermophilus str. 657 ATCC 43125 (T)	beet sugar extraction	L09227	(Rainey and Stackebrandt, 1993)
str. BD5-12 (unidentified proteobacterium strain BD5 12)	deep-sea sediments	AB015568	(Li et al., 1999)
clone BPC060	hydrocarbon seep sediment	AF154081	(O'Neill et al., unpublished data)
Bacillus SB45 str. SB45	anoxic bulk soil of rice paddy microcosms	AJ229238	(Chin et al., 1999b)
Uncultured bacterium clone DA036	grassland soils	AJ000981	(Felske et al., 1998)
X4	**deep South African gold mine water**	**AY187919**	**This study**
Uncultured eubacterium clone MT10	reagents used to prepare genomic DNA	AF058376	(Tanner et al., 1998)
Uncultured Cytophagales bacterium clone 09	mesoeutrophic reservoir	AF361195	(Simek et al., 2001)
Uncultured Bacteroidetes bacterium clone CLi112	PCE-contaminated site	AF529321	(Carroll and Zinder, unpublished data)
Uncultured CFB group bacterium clone TAF-B2	epilithic biofilm in river (River Taff epilithon in South Wales, UK)	AY038772	(O'Sullivan et al., 2002)

Table 4 (continued).

Clones (This Study) and Similar Sequences	Environment	Accession Number	Reference
Uncultured bacterium clone Ebpr3	sewage	AF255636	(Liu et al., 2001)

Figure 1. Br versus Cl for South African mine waters. Seawater included as reference.

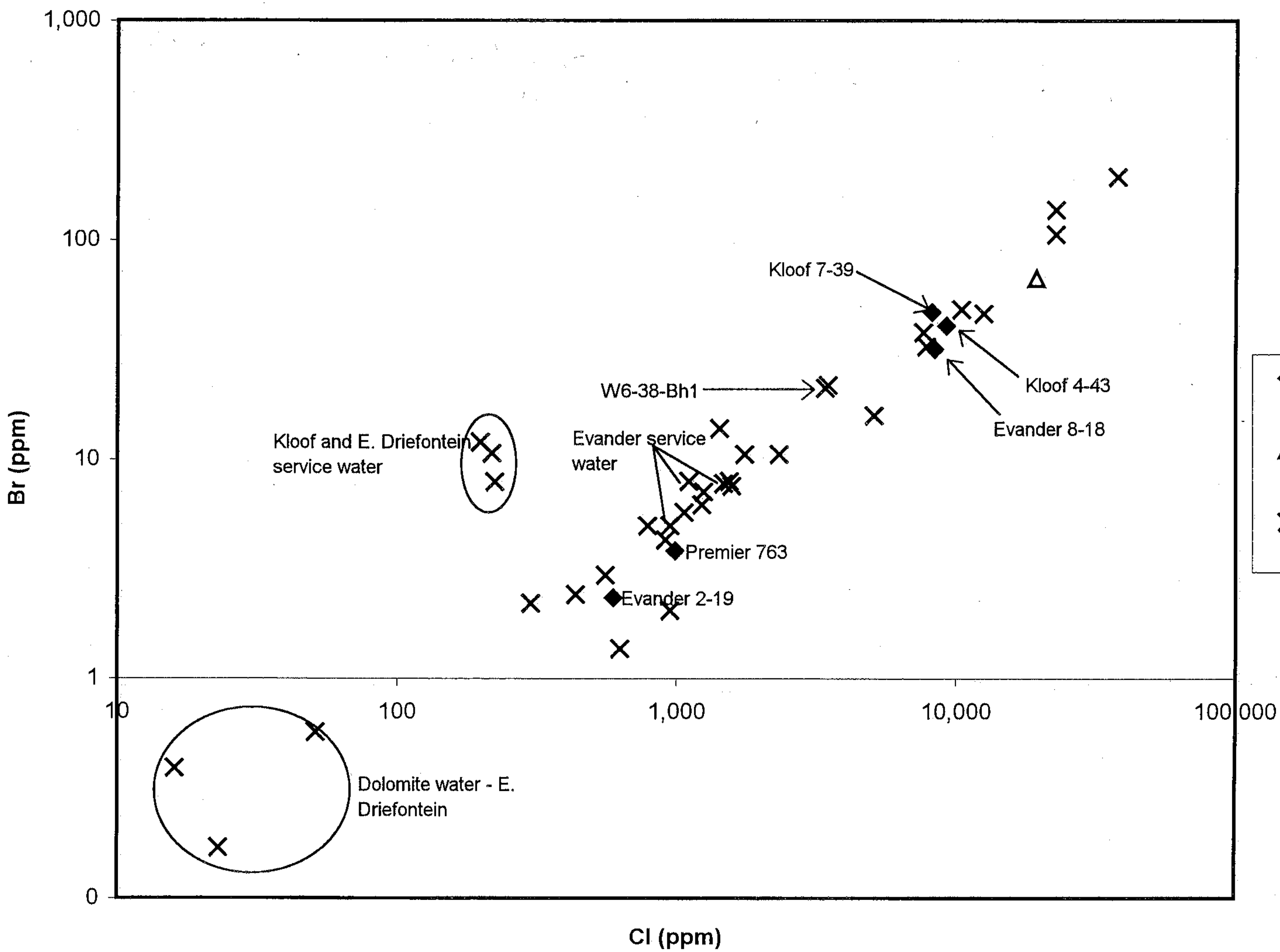

Br vs. Cl
Br (ppm)
Cl (ppm)
1,000
100
10
1
0
10
100
1,000
10,000
100,000
Kloof 7-39
Kloof 4-43
Evander 8-18
W6-38-Bh1
Kloof and E. Driefontein service water
Evander service water
Premier 763
Evander 2-19
Dolomite water - E. Driefontein
This study
Seawater
South African mine waters

Figure 2. Ca versus Na for South African mine waters and South African thermal springs. Seawater included as reference.

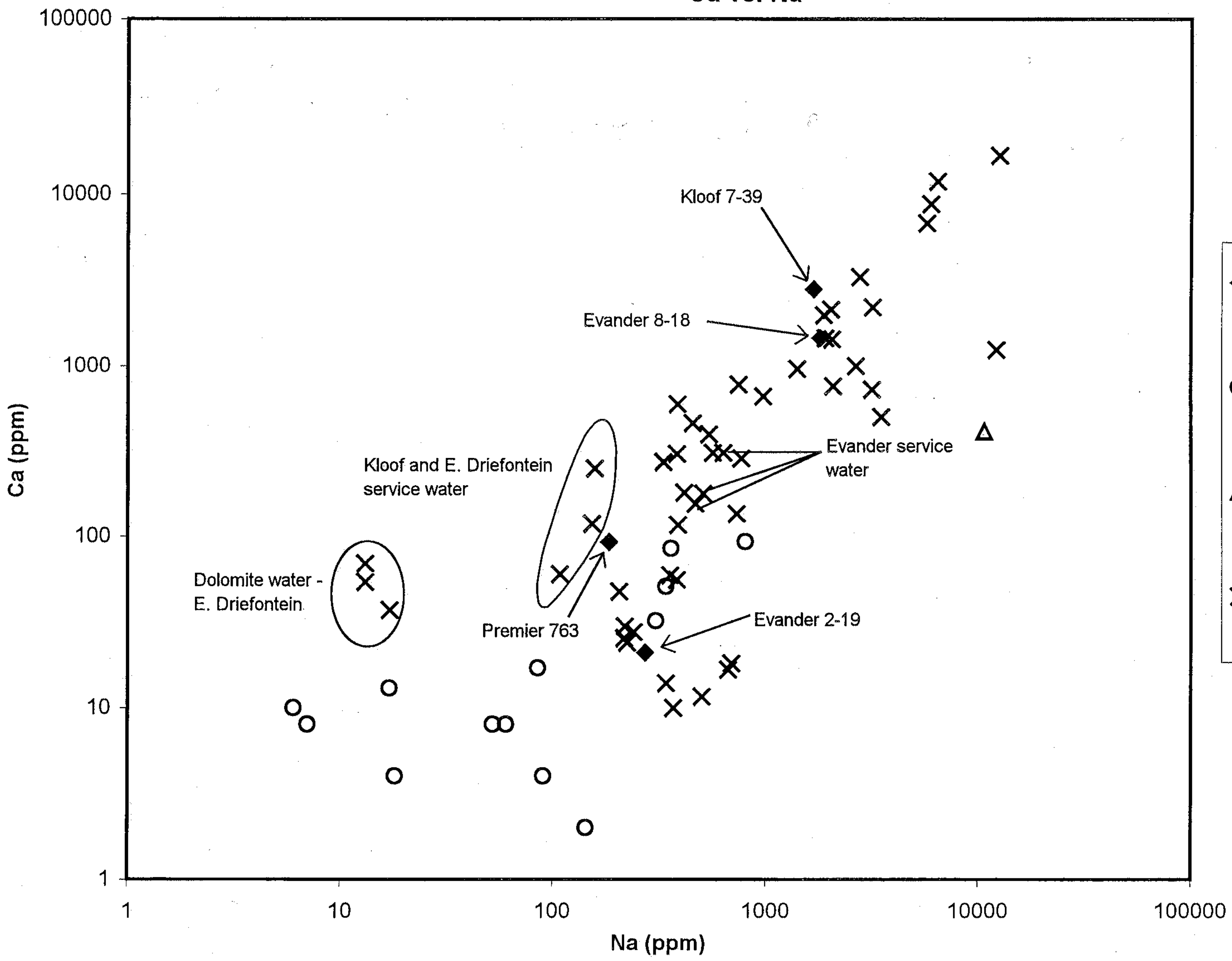

Ca vs. Na
Ca (ppm)
Na (ppm)
100000
10000
1000
100
10
1
Kloof 7-39
Evander 8-18
Evander service water
Kloof and E. Driefontein service water
Dolomite water - E. Driefontein
Premier 763
Evander 2-19
This study
South African thermal springs
Seawater
South African mine waters

Figure 3. δD versus $\delta^{18}O$ for South African gold mine waters (Duane et al., 1997; Onstott et al., 1997; Takai et al., 2001a; Lippmann et al., in preparation; unpublished Evander data (Onstott); unpublished Kloof data (Onstott)), South African thermal springs (Mazor and Verhagen, 1983), and South African rivers (Mazor and Verhagen, 1983). Pretoria Mean Meteoric Water (IAEA/WMO, 2001) included as reference. Global meteoric water line and local meteoric water line (Mazor and Verhagen, 1983) are also shown.

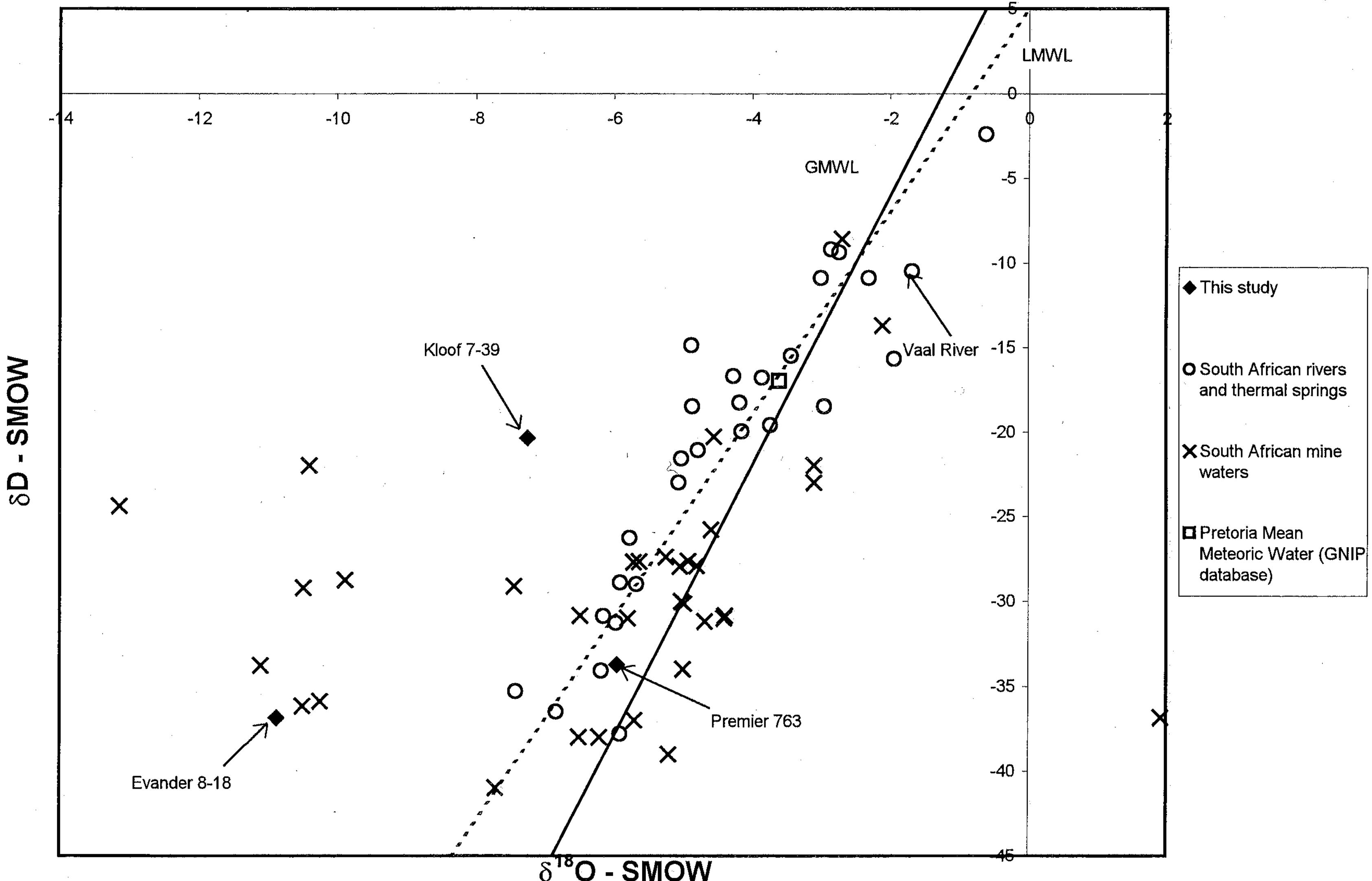
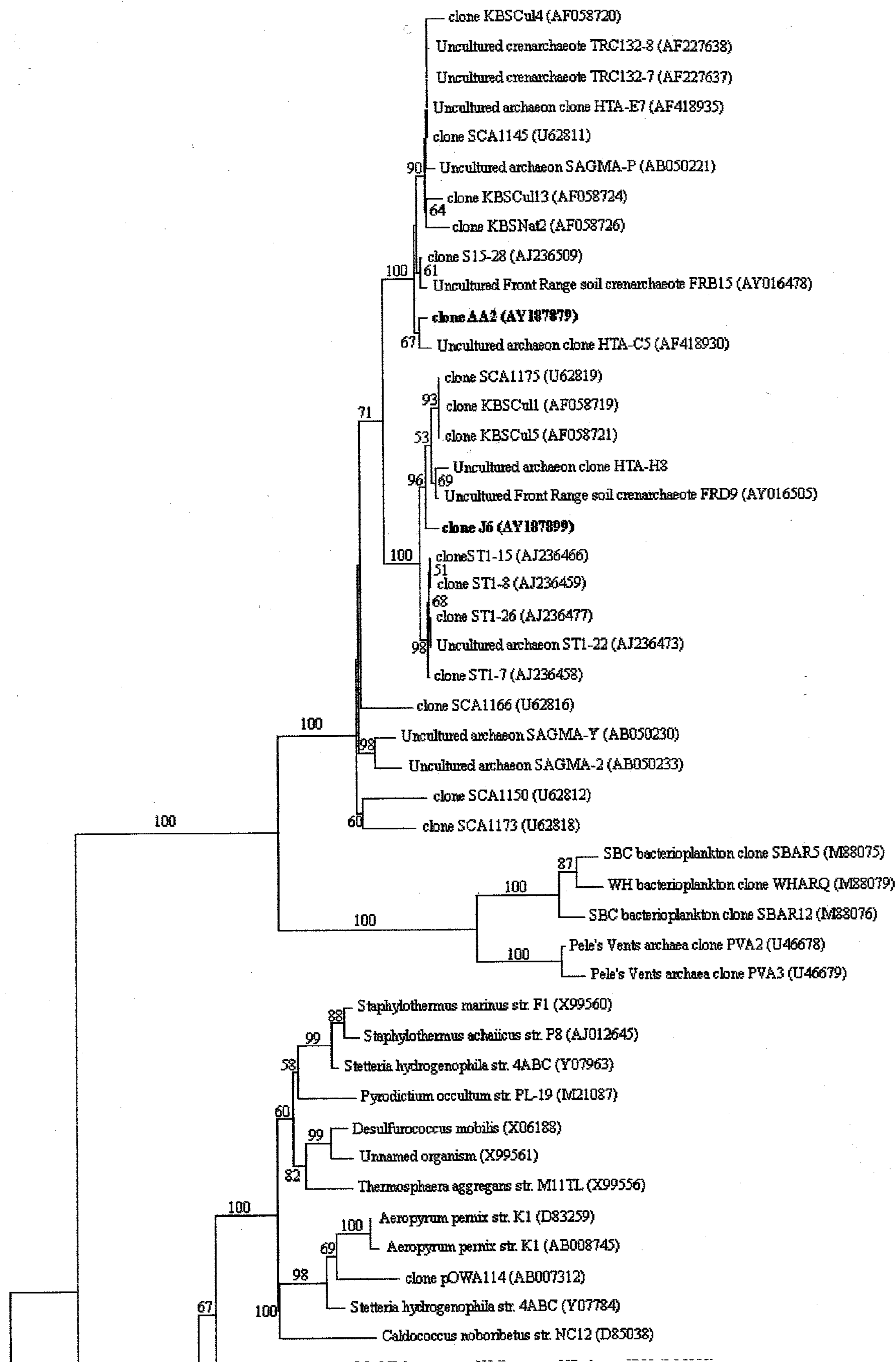

δD - SMOW
δ18O - SMOW
-14
-12
-10
-8
-6
-4
-2
0
2
5
0
-5
-10
-15
-20
-25
-30
-35
-40
-45
LMWL
GMWL
Vaal River
Kloof 7-39
Premier 763
Evander 8-18
This study
South African rivers and thermal springs
South African mine waters
Pretoria Mean Meteoric Water (GNIP database)

Figure 4. Phylogenetic tree for Archaea. The tree was inferred by a neighbor-joining analysis of 670 homologous positions of the 16S rDNA sequence. One hundred bootstrap replications were performed at the greater than 50% confidence limit. Clones from this study are in boldface.


clone KBSCul4 (AF058720)
Uncultured crenarchaeote TRC132-8 (AF227638)
Uncultured crenarchaeote TRC132-7 (AF227637)
Uncultured archaeon clone HTA-E7 (AF418935)
clone SCA1145 (U62811)
Uncultured archaeon SAGMA-P (AB050221)
clone KBSCul13 (AF058724)
clone KBSNat2 (AF058726)
clone S15-28 (AJ236509)
Uncultured Front Range soil crenarchaeote FRB15 (AY016478)
clone AA2 (AY187879)
Uncultured archaeon clone HTA-C5 (AF418930)
clone SCA1175 (U62819)
clone KBSCul1 (AF058719)
clone KBSCul5 (AF058721)
Uncultured archaeon clone HTA-H8
Uncultured Front Range soil crenarchaeote FRD9 (AY016505)
clone J6 (AY187899)
cloneST1-15 (AJ236466)
clone ST1-8 (AJ236459)
clone ST1-26 (AJ236477)
Uncultured archaeon ST1-22 (AJ236473)
clone ST1-7 (AJ236458)
clone SCA1166 (U62816)
Uncultured archaeon SAGMA-Y (AB050230)
Uncultured archaeon SAGMA-2 (AB050233)
clone SCA1150 (U62812)
clone SCA1173 (U62818)
SBC bacterioplankton clone SBAR5 (M88075)
WH bacterioplankton clone WHARQ (M88079)
SBC bacterioplankton clone SBAR12 (M88076)
Pele's Vents archaea clone PVA2 (U46678)
Pele's Vents archaea clone PVA3 (U46679)
Staphylothermus marinus str. F1 (X99560)
Staphylothermus achaiicus str. P8 (AJ012645)
Stetteria hydrogenophila str. 4ABC (Y07963)
Pyrodictium occultum str. PL-19 (M21087)
Desulfurococcus mobilis (X06188)
Unnamed organism (X99561)
Thermosphaera aggregans str. M11TL (X99556)
Aeropyrum pernix str. K1 (D83259)
Aeropyrum pernix str. K1 (AB008745)
clone pOWA114 (AB007312)
Stetteria hydrogenophila str. 4ABC (Y07784)
Caldococcus noboribetus str. NC12 (D85038)
90
64
100
61
67
93
53
71
96
69
100
51
68
98
100
98
100
60
87
100
100
100
88
99
58
60
99
82
100
100
69
98
67
100

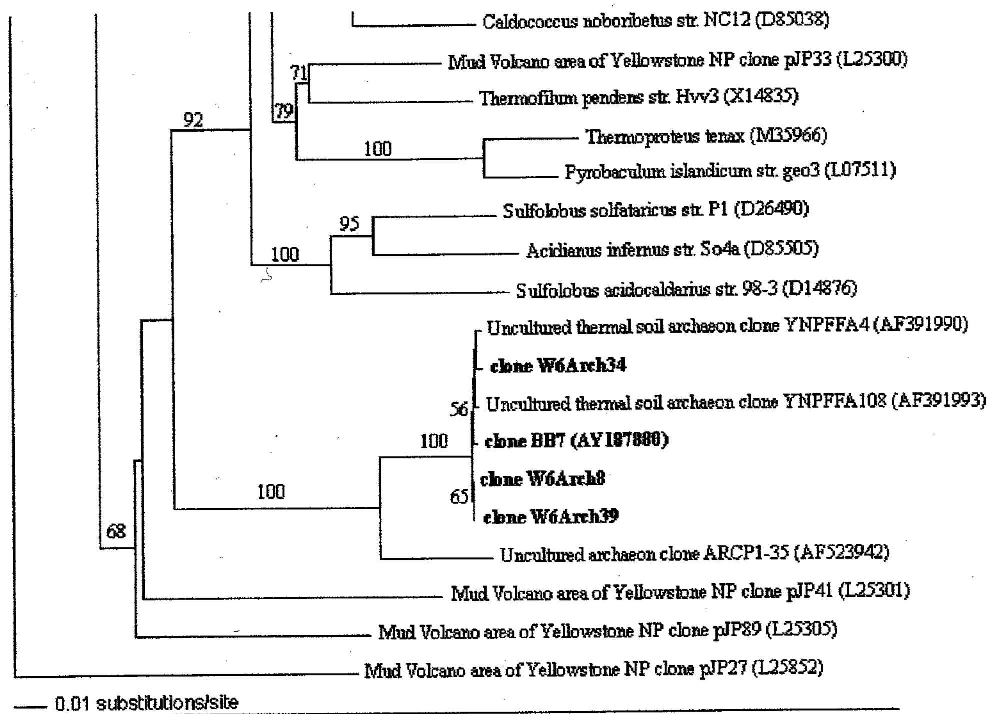
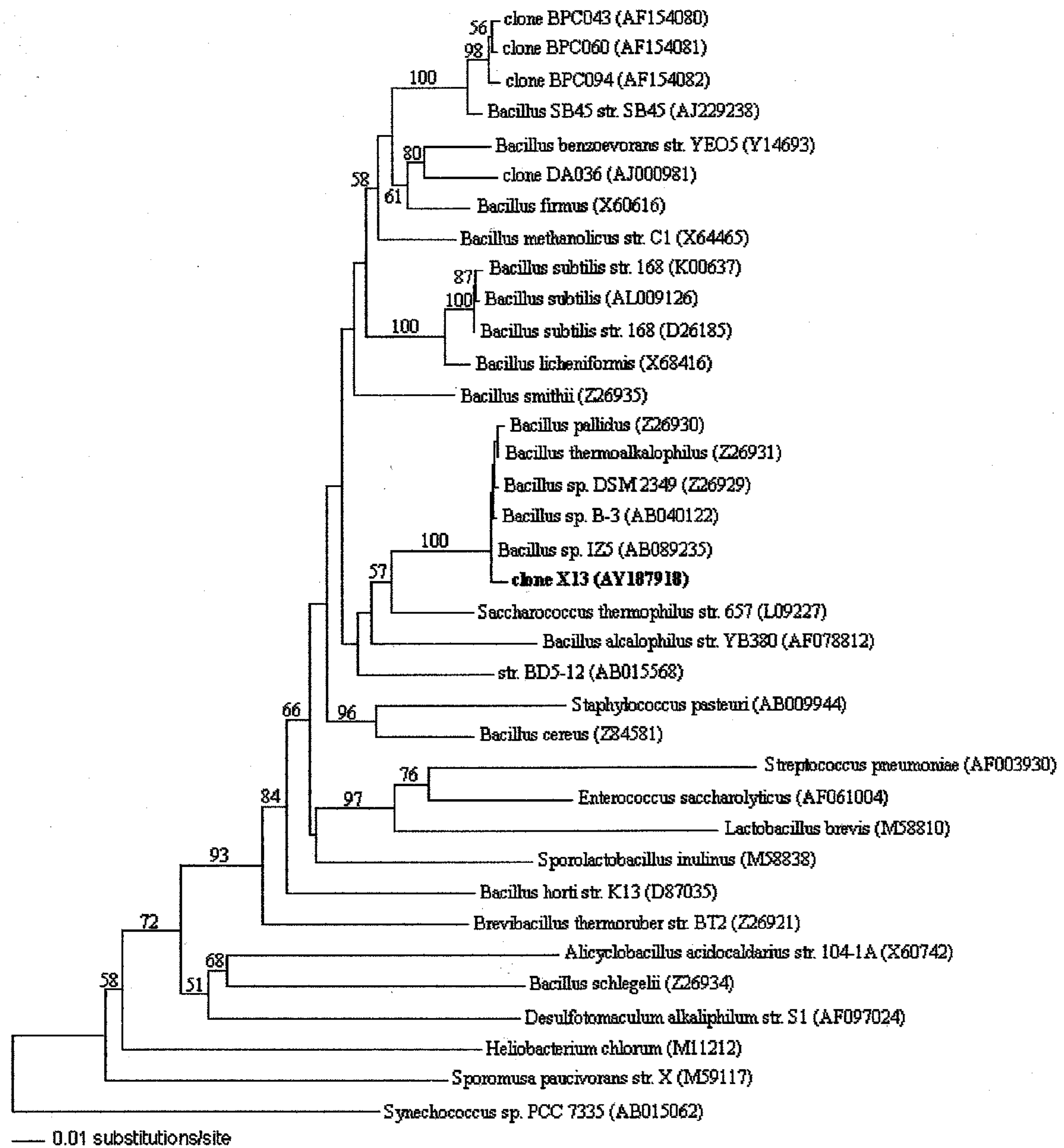
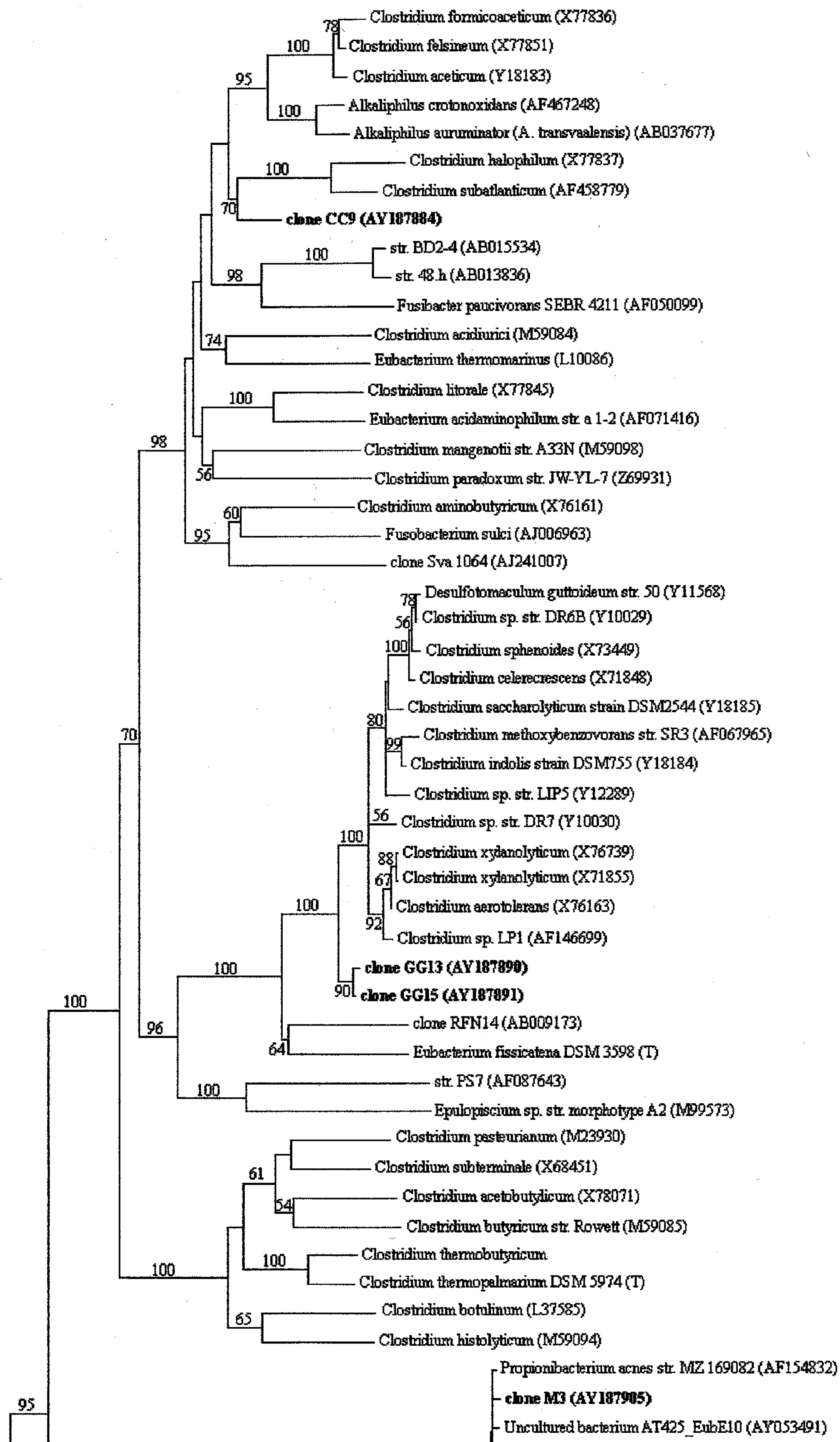

Caldococcus noboribetus str. NC12 (D85038)
Mud Volcano area of Yellowstone NP clone pJP33 (L25300)
71
Thermofilum pendens str. Hvv3 (X14835)
79
92
Thermoproteus tenax (M35966)
100
Pyrobaculum islandicum str. geo3 (L07511)
95
Sulfolobus solfataricus str. P1 (D26490)
100
Acidianus infernus str. So4a (D85505)
Sulfolobus acidocaldarius str. 98-3 (D14876)
Uncultured thermal soil archaeon clone YNPFFA4 (AF391990)
clone W6Arch34
56
Uncultured thermal soil archaeon clone YNPFFA108 (AF391993)
100
clone BB7 (AY187880)
clone W6Arch8
100
65
clone W6Arch39
68
Uncultured archaeon clone ARCP1-35 (AF523942)
Mud Volcano area of Yellowstone NP clone pJP41 (L25301)
Mud Volcano area of Yellowstone NP clone pJP89 (L25305)
Mud Volcano area of Yellowstone NP clone pJP27 (L25852)
0.01 substitutions/site

Figure 5. Phylogenetic tree for Gram positive bacteria (forward inserts). The tree was inferred by a neighbor-joining analysis of 990 homologous positions of the 16S rDNA sequence. One hundred bootstrap replications were performed at the greater than 50% confidence limit. The clone from this study is in boldface.



clone BPC043 (AF154080)
clone BPC060 (AF154081)
clone BPC094 (AF154082)
Bacillus SB45 str. SB45 (AJ229238)
Bacillus benzoevorans str. YEO5 (Y14693)
clone DA036 (AJ000981)
Bacillus firmus (X60616)
Bacillus methanolicus str. C1 (X64465)
Bacillus subtilis str. 168 (K00637)
Bacillus subtilis (AL009126)
Bacillus subtilis str. 168 (D26185)
Bacillus licheniformis (X68416)
Bacillus smithii (Z26935)
Bacillus pallidus (Z26930)
Bacillus thermoalkalophilus (Z26931)
Bacillus sp. DSM 2349 (Z26929)
Bacillus sp. B-3 (AB040122)
Bacillus sp. IZ5 (AB089235)
clone X13 (AY187918)
Saccharococcus thermophilus str. 657 (L09227)
Bacillus alcalophilus str. YB380 (AF078812)
str. BD5-12 (AB015568)
Staphylococcus pasteuri (AB009944)
Bacillus cereus (Z84581)
Streptococcus pneumoniae (AF003930)
Enterococcus saccharolyticus (AF061004)
Lactobacillus brevis (M58810)
Sporolactobacillus inulinus (M58838)
Bacillus horti str. K13 (D87035)
Brevibacillus thermoruber str. BT2 (Z26921)
Alicyclobacillus acidocaldarius str. 104-1A (X60742)
Bacillus schlegelii (Z26934)
Desulfotomaculum alkaliphilum str. S1 (AF097024)
Heliobacterium chlorum (M11212)
Sporomusa paucivorans str. X (M59117)
Synechococcus sp. PCC 7335 (AB015062)
56
98
100
80
58
61
87
100
100
100
57
66
96
76
84
97
93
72
68
58
51
0.01 substitutions/site

Figure 6. Phylogenetic tree for Gram positive bacteria (reverse inserts). The tree was inferred by a neighbor-joining analysis of 882 homologous positions of the 16S rDNA sequence. One hundred bootstrap replications were performed at the greater than 50% confidence limit. Clones from this study are in boldface.


Clostridium formicoaceticum (X77836)
Clostridium felsineum (X77851)
Clostridium aceticum (Y18183)
Alkaliphilus crotonoxidans (AF467248)
Alkaliphilus auruminator (A. transvaalensis) (AB037677)
Clostridium halophilum (X77837)
Clostridium subatlanticum (AF458779)
clone CC9 (AY187884)
str. BD2-4 (AB015534)
str. 48.h (AB013836)
Fusibacter paucivorans SEBR 4211 (AF050099)
Clostridium acidiurici (M59084)
Eubacterium thermomarinus (L10086)
Clostridium litorale (X77845)
Eubacterium acidaminophilum str. a 1-2 (AF071416)
Clostridium mangenotii str. A33N (M59098)
Clostridium paradoxum str. JW-YL-7 (Z69931)
Clostridium aminobutyricum (X76161)
Fusobacterium sulci (AJ006963)
clone Sva 1064 (AJ241007)
Desulfotomaculum guttoideum str. 50 (Y11568)
Clostridium sp. str. DR6B (Y10029)
Clostridium sphenoides (X73449)
Clostridium celerecrescens (X71848)
Clostridium saccharolyticum strain DSM2544 (Y18185)
Clostridium methoxybenzovorans str. SR3 (AF067965)
Clostridium indolis strain DSM755 (Y18184)
Clostridium sp. str. LIP5 (Y12289)
Clostridium sp. str. DR7 (Y10030)
Clostridium xylanolyticum (X76739)
Clostridium xylanolyticum (X71855)
Clostridium aerotolerans (X76163)
Clostridium sp. LP1 (AF146699)
clone GG13 (AY187890)
clone GG15 (AY187891)
clone RFN14 (AB009173)
Eubacterium fissicatena DSM 3598 (T)
str. PS7 (AF087643)
Epulopiscium sp. str. morphotype A2 (M99573)
Clostridium pasteurianum (M23930)
Clostridium subterminale (X68451)
Clostridium acetobutylicum (X78071)
Clostridium butyricum str. Rowett (M59085)
Clostridium thermobutyricum
Clostridium thermopalmarium DSM 5974 (T)
Clostridium botulinum (L37585)
Clostridium histolyticum (M59094)
Propionibacterium acnes str. MZ 169082 (AF154832)
clone M3 (AY187905)
Uncultured bacterium AT425_EubE10 (AY053491)

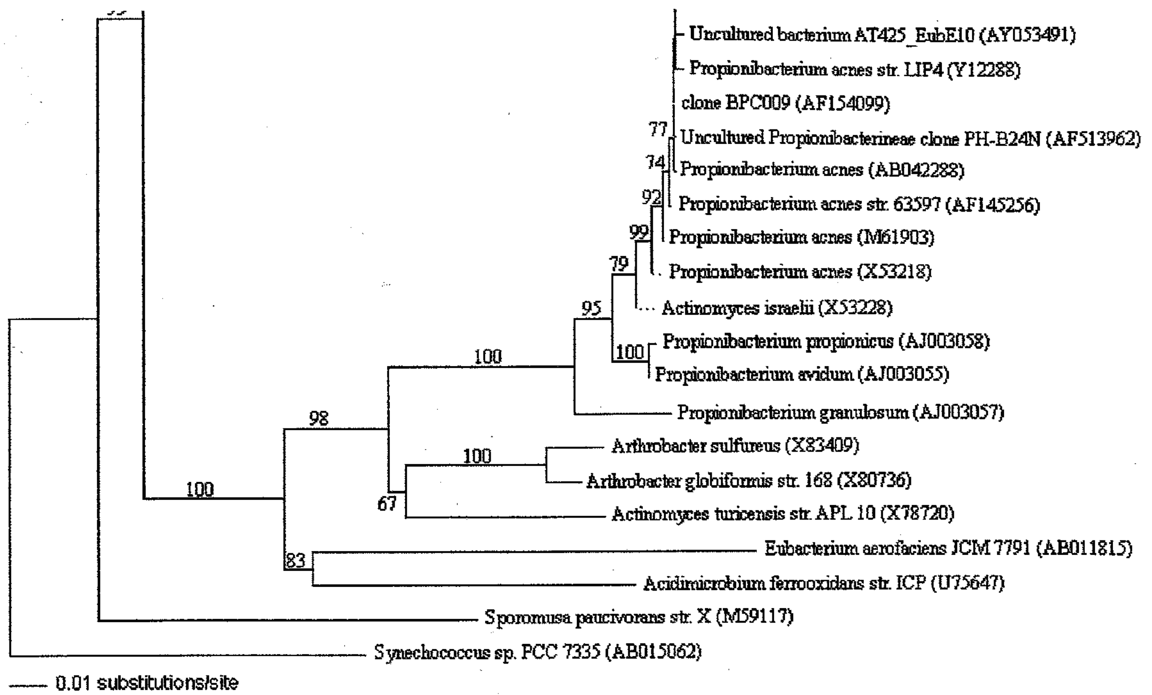

Uncultured bacterium AT425_EubE10 (AY053491)
Propionibacterium acnes str. LIP4 (Y12288)
clone BPC009 (AF154099)
77
Uncultured Propionibacterineae clone PH-B24N (AF513962)
74
Propionibacterium acnes (AB042288)
92
Propionibacterium acnes str. 63597 (AF145256)
99
Propionibacterium acnes (M61903)
79
Propionibacterium acnes (X53218)
95
Actinomyces israelii (X53228)
100
Propionibacterium propionicus (AJ003058)
Propionibacterium avidum (AJ003055)
98
Propionibacterium granulosum (AJ003057)
100
Arthrobacter sulfureus (X83409)
Arthrobacter globiformis str. 168 (X80736)
100
67
Actinomyces turicensis str. APL 10 (X78720)
83
Eubacterium aerofaciens JCM 7791 (AB011815)
Acidimicrobium ferrooxidans str. ICP (U75647)
Sporomusa paucivorans str. X (M59117)
Synechococcus sp. PCC 7335 (AB015062)
0.01 substitutions/site

Figure 7. Phylogenetic tree for the *Cytophagales/Flavobacteria/Bacteroides* (CFB) group bacteria (forward inserts). The tree was inferred by a neighbor-joining analysis of 946 homologous positions of the 16S rDNA sequence. One hundred bootstrap replications were performed at the greater than 50% confidence limit. Clones from this study are in boldface.

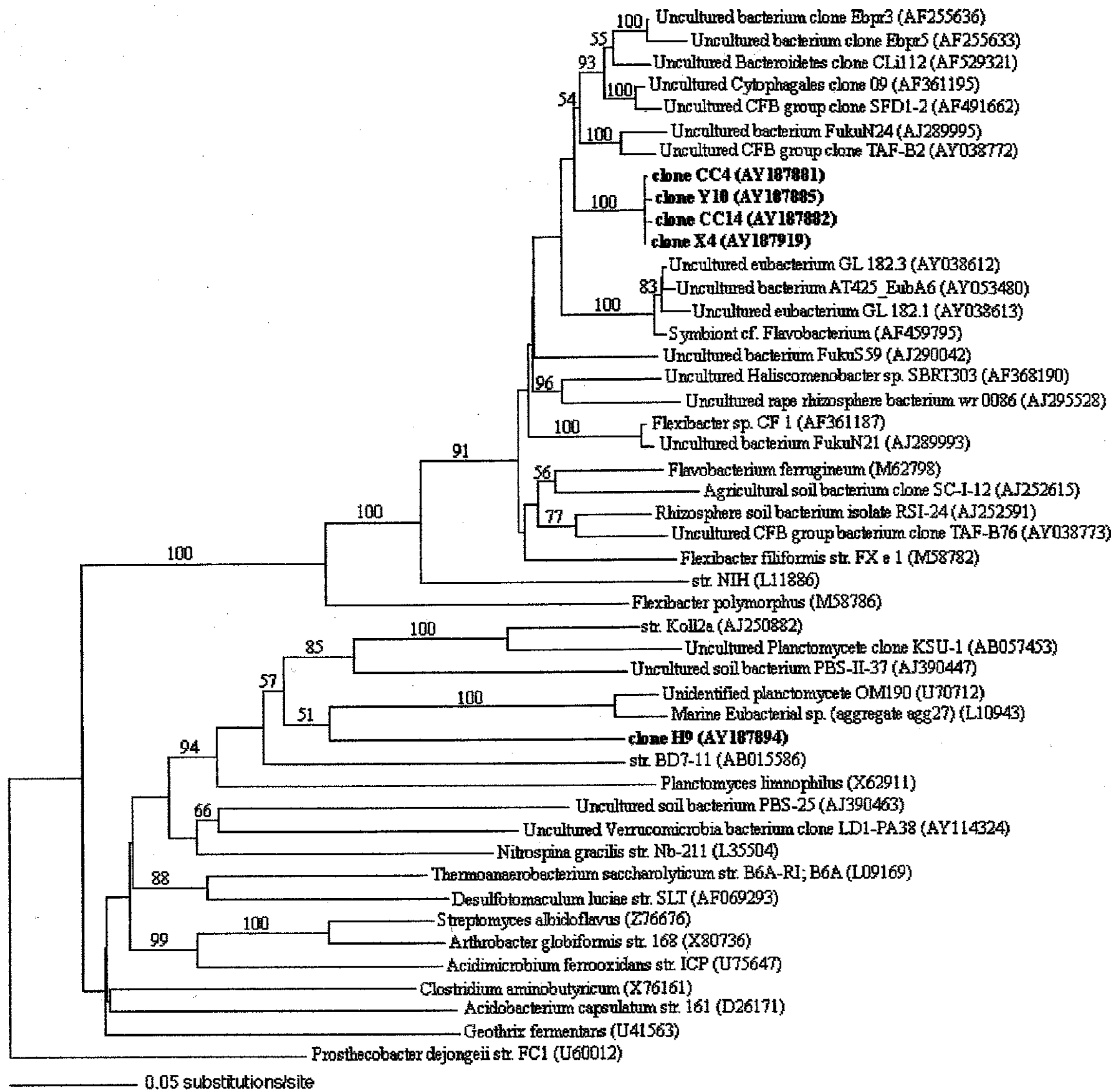
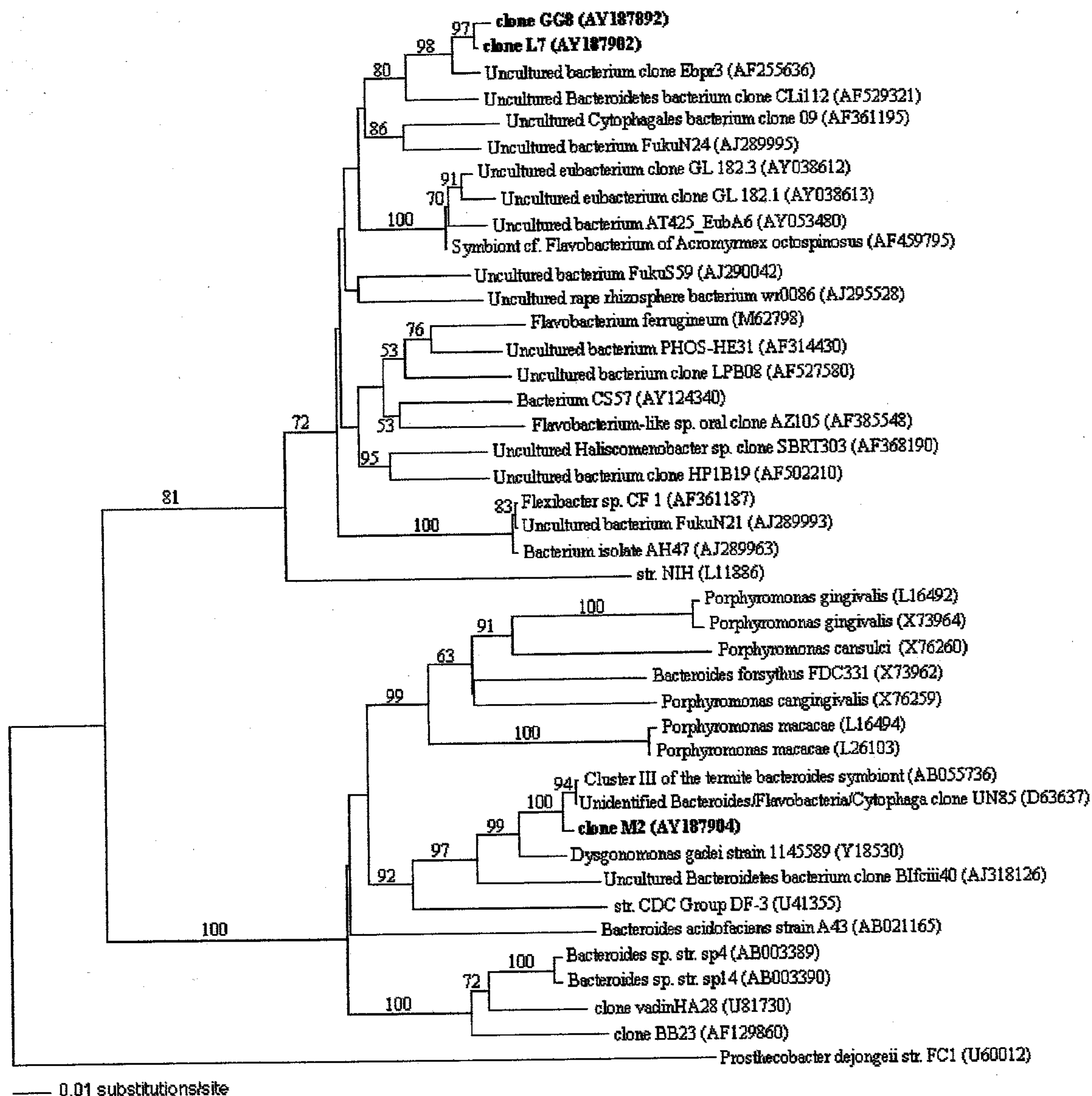

Uncultured bacterium clone Ebpr3 (AF255636)
Uncultured bacterium clone Ebpr5 (AF255633)
Uncultured Bacteroidetes clone CLi112 (AF529321)
Uncultured Cytophagales clone 09 (AF361195)
Uncultured CFB group clone SFD1-2 (AF491662)
Uncultured bacterium FukuN24 (AJ289995)
Uncultured CFB group clone TAF-B2 (AY038772)
clone CC4 (AY187881)
clone Y10 (AY187885)
clone CC14 (AY187882)
clone X4 (AY187919)
Uncultured eubacterium GL 182.3 (AY038612)
Uncultured bacterium AT425_EubA6 (AY053480)
Uncultured eubacterium GL 182.1 (AY038613)
Symbiont cf. Flavobacterium (AF459795)
Uncultured bacterium FukuS59 (AJ290042)
Uncultured Haliscomenobacter sp. SBRT303 (AF368190)
Uncultured rape rhizosphere bacterium wr 0086 (AJ295528)
Flexibacter sp. CF 1 (AF361187)
Uncultured bacterium FukuN21 (AJ289993)
Flavobacterium ferrugineum (M62798)
Agricultural soil bacterium clone SC-I-12 (AJ252615)
Rhizosphere soil bacterium isolate RSI-24 (AJ252591)
Uncultured CFB group bacterium clone TAF-B76 (AY038773)
Flexibacter filiformis str. FX e 1 (M58782)
str. NIH (L11886)
Flexibacter polymorphus (M58786)
str. Koll2a (AJ250882)
Uncultured Planctomycete clone KSU-1 (AB057453)
Uncultured soil bacterium PBS-II-37 (AJ390447)
Unidentified planctomycete OM190 (U70712)
Marine Eubacterial sp. (aggregate agg27) (L10943)
clone H9 (AY187894)
str. BD7-11 (AB015586)
Planctomyces limnophilus (X62911)
Uncultured soil bacterium PBS-25 (AJ390463)
Uncultured Verrucomicrobia bacterium clone LD1-PA38 (AY114324)
Nitrospina gracilis str. Nb-211 (L35504)
Thermoanaerobacterium saccharolyticum str. B6A-RI; B6A (L09169)
Desulfotomaculum luciae str. SLT (AF069293)
Streptomyces albidoflavus (Z76676)
Arthrobacter globiformis str. 168 (X80736)
Acidimicrobium ferrooxidans str. ICP (U75647)
Clostridium aminobutyricum (X76161)
Acidobacterium capsulatum str. 161 (D26171)
Geothrix fermentans (U41563)
Prosthecobacter dejongeii str. FC1 (U60012)
100
55
93
100
54
100
100
100
83
96
100
91
56
77
100
100
100
100
85
57
51
94
66
88
100
99
0.05 substitutions/site

Figure 8. Phylogenetic tree for the *Cytophagales/Flavobacteria/Bacteroides* (CFB) group bacteria (reverse inserts). The tree was inferred by a neighbor-joining analysis of 912 homologous positions of the 16S rDNA sequence. One hundred bootstrap replications were performed at the greater than 50% confidence limit. Clones from this study are in boldface.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Uncultured Bacteroidetes bacterium clone CLi112 (AF529321)
Uncultured Cytophagales bacterium clone 09 (AF361195)
Uncultured bacterium FukuN24 (AJ289995)
Uncultured eubacterium clone GL 182.3 (AY038612)
Uncultured eubacterium clone GL 182.1 (AY038613)
Uncultured bacterium AT425_EubA6 (AY053480)
Symbiont cf. Flavobacterium of Acromyrmex octospinosus (AF459795)
Uncultured bacterium FukuS59 (AJ290042)
Uncultured rape rhizosphere bacterium wr0086 (AJ295528)
Flavobacterium ferrugineum (M62798)
Uncultured bacterium PHOS-HE31 (AF314430)
Uncultured bacterium clone LPB08 (AF527580)
Bacterium CS57 (AY124340)
Flavobacterium-like sp. oral clone AZ105 (AF385548)
Uncultured Haliscomenobacter sp. clone SBRT303 (AF368190)
Uncultured bacterium clone HP1B19 (AF502210)
Flexibacter sp. CF 1 (AF361187)
Uncultured bacterium FukuN21 (AJ289993)
Bacterium isolate AH47 (AJ289963)
str. NIH (L11886)
Porphyromonas gingivalis (L16492)
Porphyromonas gingivalis (X73964)
Porphyromonas cansulci (X76260)
Bacteroides forsythus FDC331 (X73962)
Porphyromonas cangingivalis (X76259)
Porphyromonas macacae (L16494)
Porphyromonas macacae (L26103)
Cluster III of the termite bacteroides symbiont (AB055736)
Unidentified Bacteroides/Flavobacteria/Cytophaga clone UN85 (D63637)
clone M2 (AY187904)
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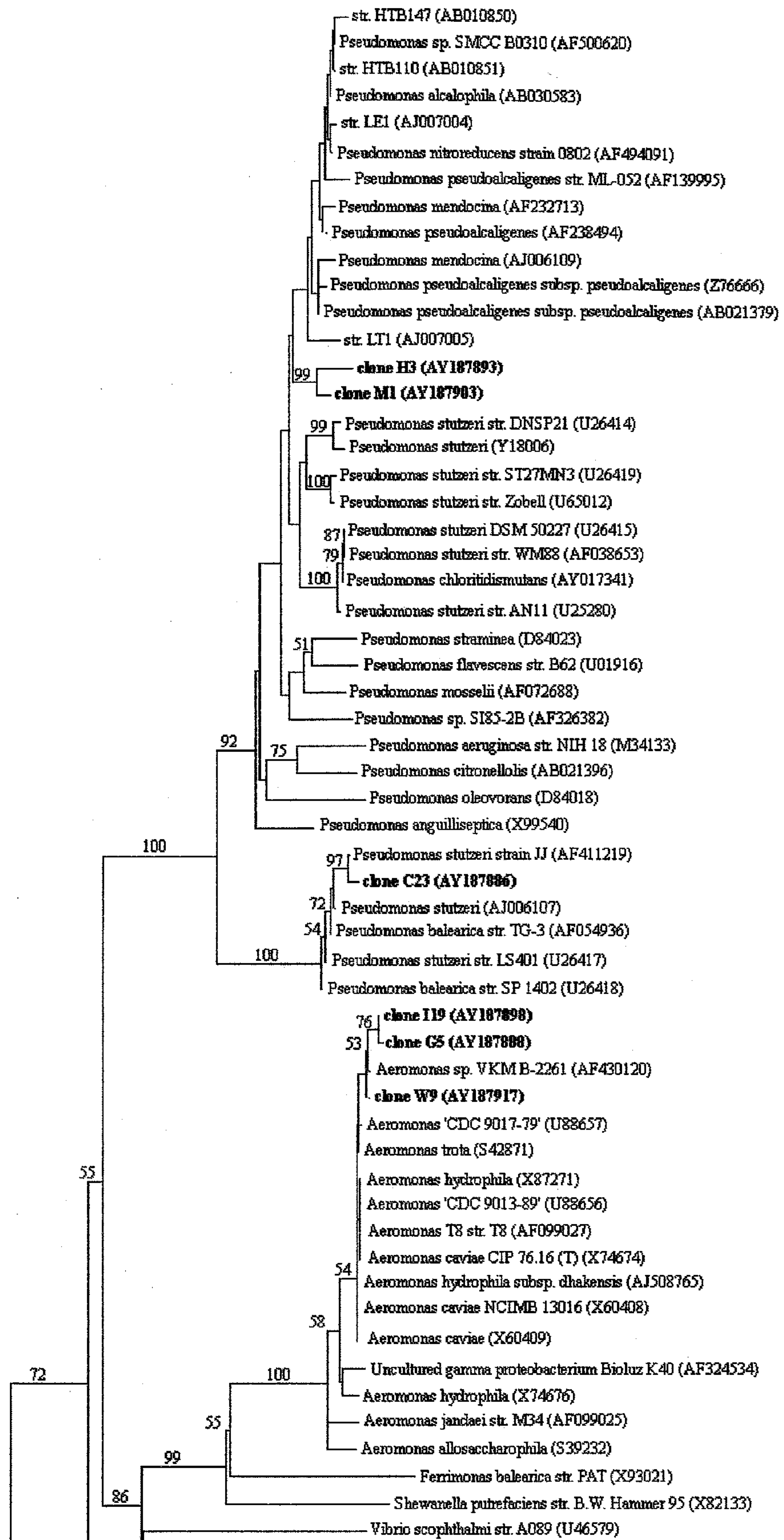
Uncultured Bacteroidetes bacterium clone BIfciii40 (AJ318126)
str. CDC Group DF-3 (U41355)
Bacteroides acidofaciens strain A43 (AB021165)
Bacteroides sp. str. sp4 (AB003389)
Bacteroides sp. str. sp14 (AB003390)
clone vadinHA28 (U81730)
clone BB23 (AF129860)
Prosthecobacter dejongeii str. FC1 (U60012)
0.01 substitutions/site

Figure 9. Phylogenetic tree for the Proteobacteria (forward inserts). The tree was inferred by a neighbor-joining analysis of 901 homologous positions of the 16S rDNA sequence. One hundred bootstrap replications were performed at the greater than 50% confidence limit. Clones from this study are in boldface.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Pseudomonas mendocina (AF232713)
Pseudomonas pseudoalcaligenes (AF238494)
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Pseudomonas pseudoalcaligenes subsp. pseudoalcaligenes (Z76666)
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str. LT1 (AJ007005)
clone H3 (AY187893)
clone M1 (AY187903)
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Pseudomonas stutzeri (Y18006)
Pseudomonas stutzeri str. ST27MN3 (U26419)
Pseudomonas stutzeri str. Zobell (U65012)
Pseudomonas stutzeri DSM 50227 (U26415)
Pseudomonas stutzeri str. WM88 (AF038653)
Pseudomonas chloritidismutans (AY017341)
Pseudomonas stutzeri str. AN11 (U25280)
Pseudomonas straminea (D84023)
Pseudomonas flavescens str. B62 (U01916)
Pseudomonas mosselii (AF072688)
Pseudomonas sp. SI85-2B (AF326382)
Pseudomonas aeruginosa str. NIH 18 (M34133)
Pseudomonas citronellolis (AB021396)
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Pseudomonas anguilliseptica (X99540)
Pseudomonas stutzeri strain JJ (AF411219)
clone C23 (AY187886)
Pseudomonas stutzeri (AJ006107)
Pseudomonas balearica str. TG-3 (AF054936)
Pseudomonas stutzeri str. LS401 (U26417)
Pseudomonas balearica str. SP 1402 (U26418)
clone I19 (AY187898)
clone G5 (AY187888)
Aeromonas sp. VKM B-2261 (AF430120)
clone W9 (AY187917)
Aeromonas 'CDC 9017-79' (U88657)
Aeromonas trota (S42871)
Aeromonas hydrophila (X87271)
Aeromonas 'CDC 9013-89' (U88656)
Aeromonas T8 str. T8 (AF099027)
Aeromonas caviae CIP 76.16 (T) (X74674)
Aeromonas hydrophila subsp. dhakensis (AJ508765)
Aeromonas caviae NCIMB 13016 (X60408)
Aeromonas caviae (X60409)
Uncultured gamma proteobacterium Bioluz K40 (AF324534)
Aeromonas hydrophila (X74676)
Aeromonas jandaei str. M34 (AF099025)
Aeromonas allosaccharophila (S39232)
Ferrimonas balearica str. PAT (X93021)
Shewanella putrefaciens str. B.W. Hammer 95 (X82133)
Vibrio scophthalmi str. A089 (U46579)
99
99
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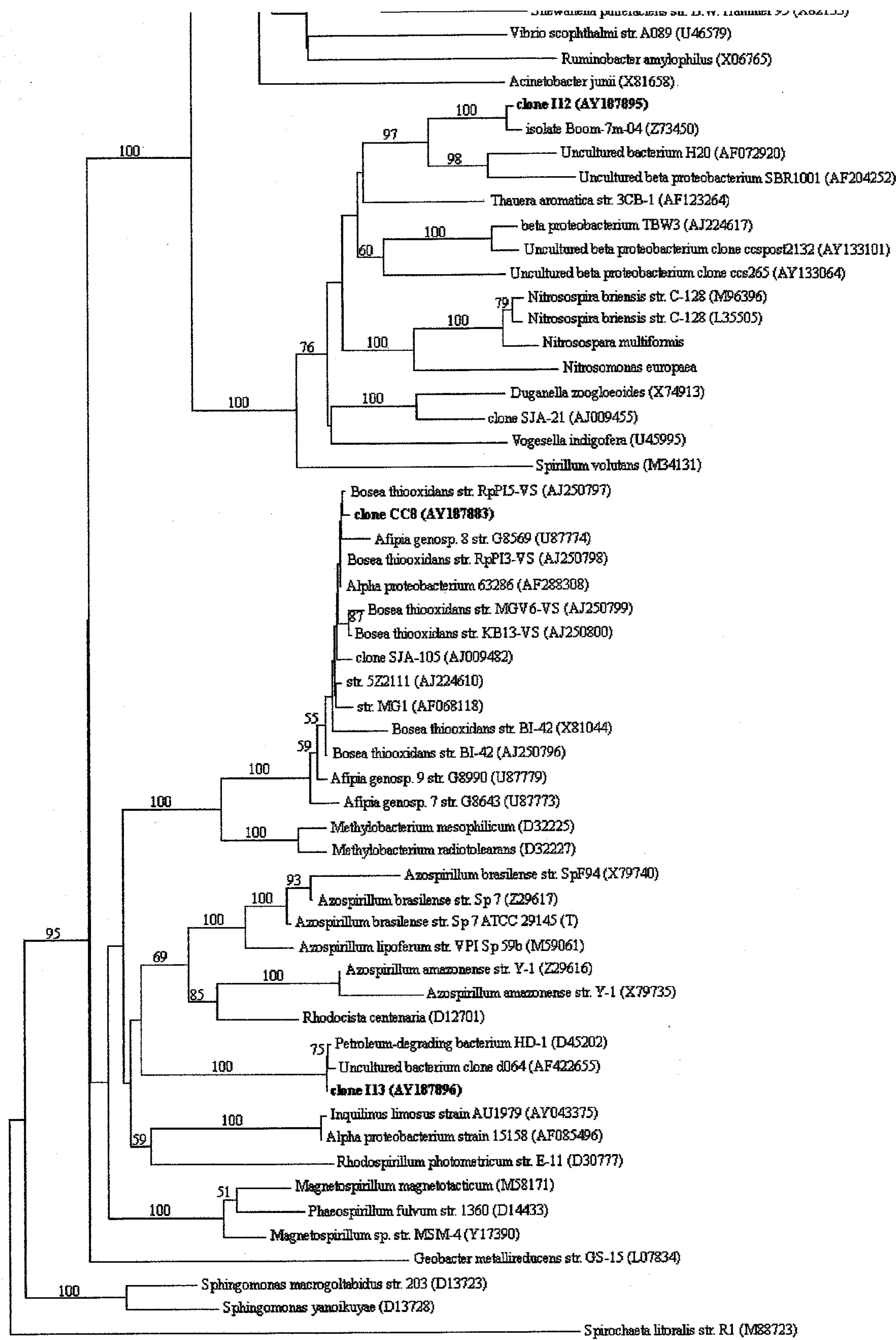
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Acinetobacter junii (X81658)
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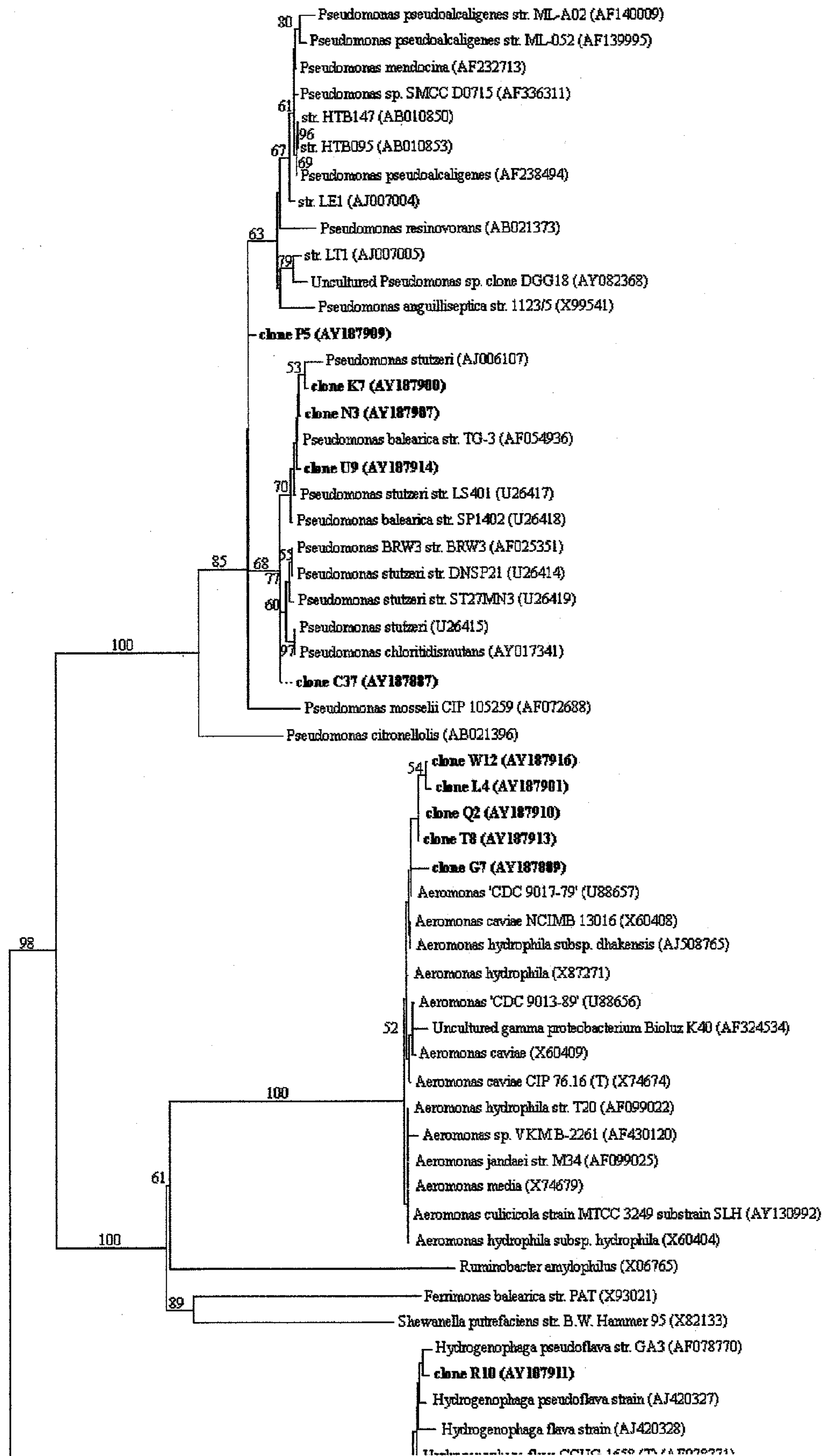
isolate Boom-7m-04 (Z73450)
Uncultured bacterium H20 (AF072920)
Uncultured beta proteobacterium SBR1001 (AF204252)
Thauera aromatica str. 3CB-1 (AF123264)
beta proteobacterium TBW3 (AJ224617)
Uncultured beta proteobacterium clone ccspost2132 (AY133101)
Uncultured beta proteobacterium clone ccs265 (AY133064)
Nitrosospira briensis str. C-128 (M96396)
Nitrosospira briensis str. C-128 (L35505)
Nitrosospara multiformis
Nitrosomonas europaea
Duganella zoogloeoides (X74913)
clone SJA-21 (AJ009455)
Vogesella indigofera (U45995)
Spirillum volutans (M34131)
Bosea thiooxidans str. RpPI5-VS (AJ250797)
clone CC8 (AY187883)
Afipia genosp. 8 str. G8569 (U87774)
Bosea thiooxidans str. RpPI3-VS (AJ250798)
Alpha proteobacterium 63286 (AF288308)
Bosea thiooxidans str. MGV6-VS (AJ250799)
Bosea thiooxidans str. KB13-VS (AJ250800)
clone SJA-105 (AJ009482)
str. 5Z2111 (AJ224610)
str. MG1 (AF068118)
Bosea thiooxidans str. BI-42 (X81044)
Bosea thiooxidans str. BI-42 (AJ250796)
Afipia genosp. 9 str. G8990 (U87779)
Afipia genosp. 7 str. G8643 (U87773)
Methylobacterium mesophilicum (D32225)
Methylobacterium radiotolearans (D32227)
Azospirillum brasilense str. SpF94 (X79740)
Azospirillum brasilense str. Sp 7 (Z29617)
Azospirillum brasilense str. Sp 7 ATCC 29145 (T)
Azospirillum lipoferum str. VPI Sp 59b (M59061)
Azospirillum amazonense str. Y-1 (Z29616)
Azospirillum amazonense str. Y-1 (X79735)
Rhodocista centenaria (D12701)
Petroleum-degrading bacterium HD-1 (D45202)
Uncultured bacterium clone d064 (AF422655)
clone I13 (AY187896)
Inquilinus limosus strain AU1979 (AY043375)
Alpha proteobacterium strain 15158 (AF085496)
Rhodospirillum photometricum str. E-11 (D30777)
Magnetospirillum magnetotacticum (M58171)
Phaeospirillum fulvum str. 1360 (D14433)
Magnetospirillum sp. str. MSM-4 (Y17390)
Geobacter metallireducens str. GS-15 (L07834)
Sphingomonas macrogoltabidus str. 203 (D13723)
Sphingomonas yanoikuyae (D13728)
Spirochaeta litoralis str. R1 (M88723)
0.01 substitutions/site

Figure 10. Phylogenetic tree for the Proteobacteria (reverse inserts). The tree was inferred by a neighbor-joining analysis of 942 homologous positions of the 16S rDNA sequence. One hundred bootstrap replications were performed at the greater than 50% confidence limit. Clones from this study are in boldface.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str. HTB095 (AB010853)
Pseudomonas pseudoalcaligenes (AF238494)
str. LE1 (AJ007004)
Pseudomonas resinovorans (AB021373)
str. LT1 (AJ007005)
Uncultured Pseudomonas sp. clone DGG18 (AY082368)
Pseudomonas anguilliseptica str. 1123/5 (X99541)
clone P5 (AY187909)
Pseudomonas stutzeri (AJ006107)
clone K7 (AY187900)
clone N3 (AY187907)
Pseudomonas balearica str. TG-3 (AF054936)
clone U9 (AY187914)
Pseudomonas stutzeri str. LS401 (U26417)
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Pseudomonas BRW3 str. BRW3 (AF025351)
Pseudomonas stutzeri str. DNSP21 (U26414)
Pseudomonas stutzeri str. ST27MN3 (U26419)
Pseudomonas stutzeri (U26415)
Pseudomonas chloritidismutans (AY017341)
clone C37 (AY187887)
Pseudomonas mosselii CIP 105259 (AF072688)
Pseudomonas citronellolis (AB021396)
clone W12 (AY187916)
clone L4 (AY187901)
clone Q2 (AY187910)
clone T8 (AY187913)
clone G7 (AY187889)
Aeromonas 'CDC 9017-79' (U88657)
Aeromonas caviae NCIMB 13016 (X60408)
Aeromonas hydrophila subsp. dhakensis (AJ508765)
Aeromonas hydrophila (X87271)
Aeromonas 'CDC 9013-89' (U88656)
Uncultured gamma proteobacterium Bioluz K40 (AF324534)
Aeromonas caviae (X60409)
Aeromonas caviae CIP 76.16 (T) (X74674)
Aeromonas hydrophila str. T20 (AF099022)
Aeromonas sp. VKM B-2261 (AF430120)
Aeromonas jandaei str. M34 (AF099025)
Aeromonas media (X74679)
Aeromonas culicicola strain MTCC 3249 substrain SLH (AY130992)
Aeromonas hydrophila subsp. hydrophila (X60404)
Ruminobacter amylophilus (X06765)
Ferrimonas balearica str. PAT (X93021)
Shewanella putrefaciens str. B.W. Hammer 95 (X82133)
Hydrogenophaga pseudoflava str. GA3 (AF078770)
clone R10 (AY187911)
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Hydrogenophaga flava strain (AJ420328)
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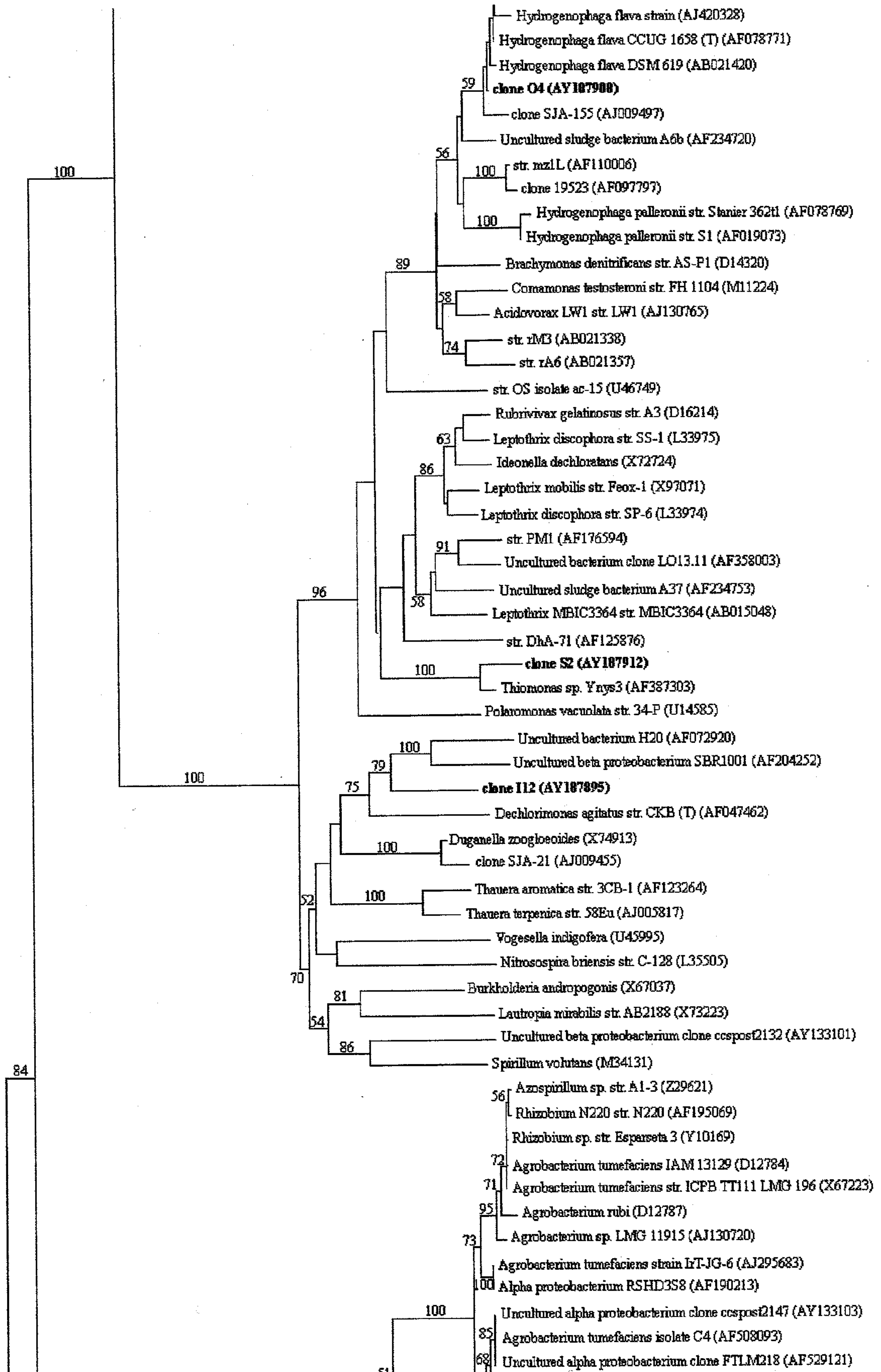
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Uncultured sludge bacterium A6b (AF234720)
str. mz1L (AF110006)
clone 19523 (AF097797)
Hydrogenophaga palleronii str. Stanier 362t1 (AF078769)
Hydrogenophaga palleronii str. S1 (AF019073)
Brachymonas denitrificans str. AS-P1 (D14320)
Comamonas testosteroni str. FH 1104 (M11224)
Acidovorax LW1 str. LW1 (AJ130765)
str. rM3 (AB021338)
str. rA6 (AB021357)
str. OS isolate ac-15 (U46749)
Rubrivivax gelatinosus str. A3 (D16214)
Leptothrix discophora str. SS-1 (L33975)
Ideonella dechloratans (X72724)
Leptothrix mobilis str. Feox-1 (X97071)
Leptothrix discophora str. SP-6 (L33974)
str. PM1 (AF176594)
Uncultured bacterium clone LO13.11 (AF358003)
Uncultured sludge bacterium A37 (AF234753)
Leptothrix MBIC3364 str. MBIC3364 (AB015048)
str. DhA-71 (AF125876)
clone S2 (AY187912)
Thiomonas sp. Ynys3 (AF387303)
Polaromonas vacuolata str. 34-P (U14585)
Uncultured bacterium H20 (AF072920)
Uncultured beta proteobacterium SBR1001 (AF204252)
clone I12 (AY187895)
Dechlorimonas agitatus str. CKB (T) (AF047462)
Duganella zoogloeoides (X74913)
clone SJA-21 (AJ009455)
Thauera aromatica str. 3CB-1 (AF123264)
Thauera terpenica str. 58Eu (AJ005817)
Vogesella indigofera (U45995)
Nitrosospira briensis str. C-128 (L35505)
Burkholderia andropogonis (X67037)
Lautropia mirabilis str. AB2188 (X73223)
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Azospirillum sp. str. A1-3 (Z29621)
Rhizobium N220 str. N220 (AF195069)
Rhizobium sp. str. Esparseta 3 (Y10169)
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Agrobacterium rubi (D12787)
Agrobacterium sp. LMG 11915 (AJ130720)
Agrobacterium tumefaciens strain IrT-JG-6 (AJ295683)
Alpha proteobacterium RSHD3S8 (AF190213)
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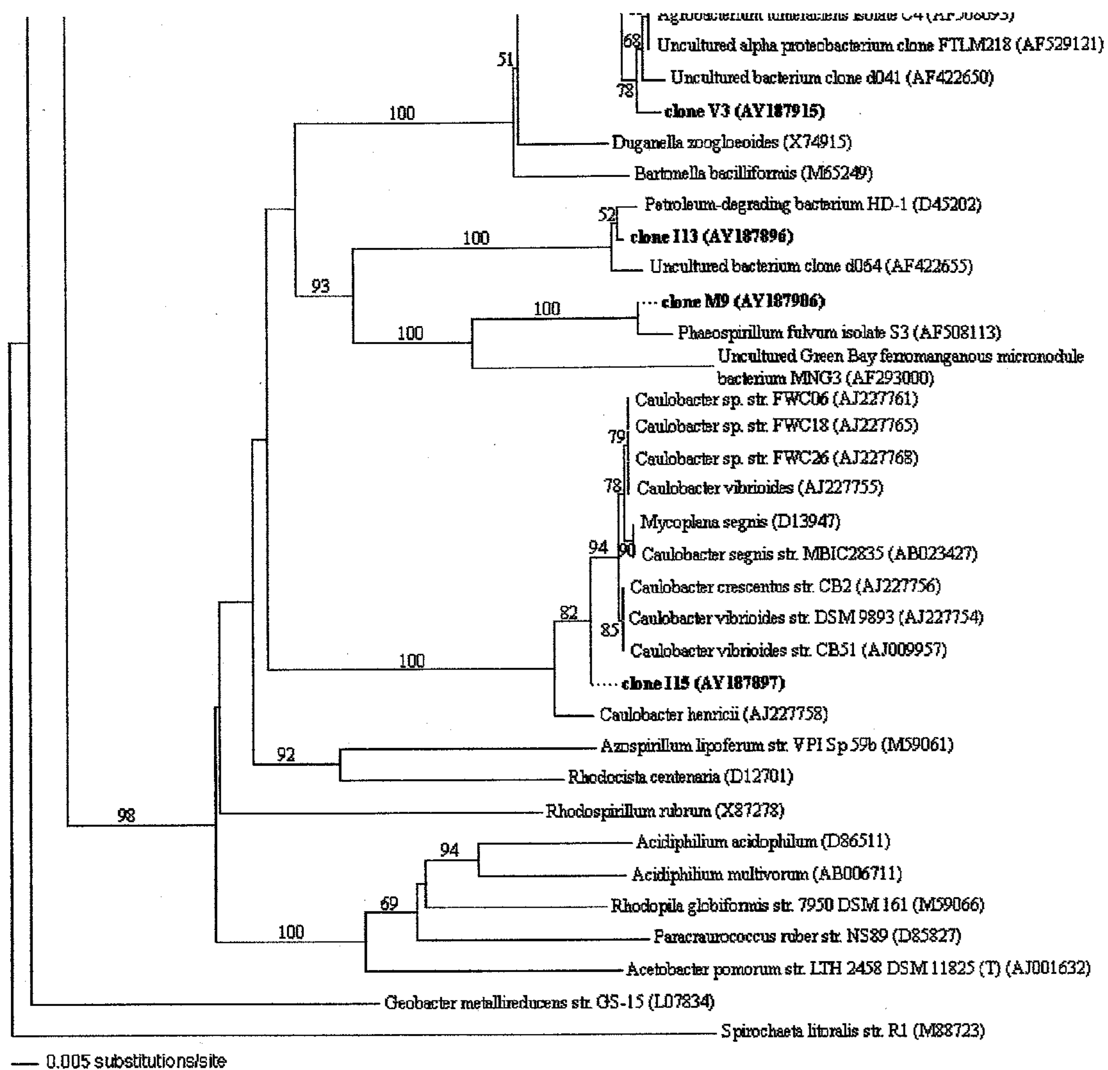

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Bartonella bacilliformis (M65249)
Petroleum-degrading bacterium HD-1 (D45202)
clone I13 (AY187896)
Uncultured bacterium clone d064 (AF422655)
clone M9 (AY187906)
Phaeospirillum fulvum isolate S3 (AF508113)
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Mycoplana segnis (D13947)
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Caulobacter vibrioides str. DSM 9893 (AJ227754)
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Acetobacter pomorum str. LTH 2458 DSM 11825 (T) (AJ001632)
Geobacter metallireducens str. GS-15 (L07834)
Spirochaeta litoralis str. R1 (M88723)
0.005 substitutions/site

Appendix 1. Compositions of media and amendments.

Medium	Ingredient	Amount
Basal salts	KH_2PO_4	0.42 g
	K_2HPO_4	0.22 g
	NH_4Cl	0.2 g
	KCl	0.38 g
	NaCl	0.36 g
	$CaCl_2 \cdot 2H_2O$	0.04 g
	$MgSO_4 \cdot 7H_2O$	0.10 g
	$NaHCO_3$	1.8 g
	Na_2CO_3	0.5 g
	Na_2SeO_4	1 ml of a 1mM solution
	H_2O (deionized)	800 ml
TYG/NO_3^-	Tryptone	5 g
	Yeast extract	3 g
	Glucose	1 g
	KNO_3	1 g
	H_2O (deionized)	1000 ml

Amendment	Ingredient	Amount
Wolfe's mineral solution	NTA (nitrilotriacetic acid)	2.14 g
	$MnCl_2 \cdot 4H_2O$	0.1 g
	$FeSO_4 \cdot 7H_2O$	0.3 g
	$CoCl_2 \cdot 6H_2O$	0.17 g
	$ZnSO_4 \cdot 7H_2O$	0.20 g
	$CuCl_2 \cdot 2H_2O$	0.03 g
	$AlK(SO4)_2 \cdot 12H_2O$	0.005 g
	H_3BO_3	0.005 g
	Na_2MoO_4	0.09 g
	$NiSO_4 \cdot 6H_2O$	0.11 g
	$Na_2WO_4 \cdot 2H_2O$	0.02 g
	H_2O (deionized)	1000 ml

Appendix 1 (continued).

Amendment	Ingredient	Amount
Wolfe's vitamin solution	Biotin	2.0 mg
	Folic acid	2.0 mg
	Pyridoxine HCl	10.0 mg
	Riboflavin	5.0 mg
	Thiamine	5.0 mg
	Nicotinic acid	5.0 mg
	Pantothenic acid	5.0 mg
	B-12	0.1 mg
	P-aminobenzoic acid	5.0 mg
	Thioctic acid	5.0 mg
	H_2O (deionized)	1000 ml

Appendix 2. GenBank accession numbers for clones in this study.

Clone	Accession number	Clone	Accession number
AA2	AY187879	K7	AY187900
BB7	AY187880	L4	AY187901
CC4	AY187881	L7	AY187902
CC14	AY187882	M1	AY187903
CC8	AY187883	M2	AY187904
CC9	AY187884	M3	AY187905
Y10	AY187885	M9	AY187906
C23	AY187886	N3	AY187907
C37	AY187887	O4	AY187908
G5	AY187888	P5	AY187909
G7	AY187889	Q2	AY187910
GG13	AY187890	R10	AY187911
GG15	AY187891	S2	AY187912
GG8	AY187892	T8	AY187913
H3	AY187893	U9	AY187914
H9	AY187894	V3	AY187915
I12	AY187895	W12	AY187916
I13	AY187896	W9	AY187917
I15	AY187897	X13	AY187918
I19	AY187898	X4	AY187919
J6	AY187899		

Appendix 3a. Field data.

Sample	Sample Date	Mine	Shaft Number	Level Number	Sample Depth (kmbls.)	Fissure Depth (kmbls.)	Geological Formaton	Sample Type	pH	Eh (mv)	pe
1	08/16/01	Evander	2	19	1.474	1.474	Footwall Sill-Ventersdorp	Borehole	7.6	N.A.	N.A.
2	06/21/01	Evander	8	18	1.830	1.950	Quartzites	Borehole	7.8	-240	-3.80
3	08/21/01	Evander	8	21	2.055	2.055		Fissure Water	N.A.	N.A.	N.A.
4	07/13/01	Kloof	4	43	3.400	3.400	Ventersdorp	Fissure Water	N.A.	N.A.	N.A.
5	06/29/01	Kloof	7	39	3.100	3.100	Ventersdorp	Fissure Water	5.8?	-220	-3.39
6	09/05/01	Premier		763	0.763	0.763	Premier kimberlite	Borehole	10.3	N.A.	N.A.

Appendix 3a (continued).

Sample	Conductivity (mS/cm)	T°C	O_2 (ppm)	Fe^{2+} (ppm)	Fe Total (ppm)	Sulfide (ppm)	NH_3 (ppm)	Alkalinity (ppm)	Total CO_2 (ppm)	Water Flow Rate (ml/min)	Gas Flow Rate (ml/min)
1	N.A.	25	> 1	0	0	> 10	6	N.A.	14	450	N.A.
2	7.01	45	0	0.4	0.4	>10	7	30	<10	N.A.	N.A.
3	N.A.	42?	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
4	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
5	N.A.	54	0	<1	N.A.	N.A.	3	N.A.	N.A.	9,000	10,000
6	12.3	33.9	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.

Appendix 3b. Carbon species (ppm).

Sample	Total Organic Carbon (ppm-C)	Diss. Organic Carbon (ppm-C)	acetic acid	propionic acid	butyric acid	Total Dissolved Inorganic Carbon	acetate	formate	propanoate
1	N.A.	N.A.	N.A.	N.A.	N.A.	143.40	0.018, 0.2894	0.049, 0.0656	<d.l.
2	3.1	N.A.	N.A.	N.A.	N.A.	9.12	0.9628, 1.8054	<d.l., 0.0222	0.0237
3	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
4	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	3.042, 23.1682	<d.l.	<d.l., 1.2050
5	5.9	N.A.	4.2	<d.l.	<d.l.	0.83	3.5859, 0.05	1.056, 0.0128	<d.l.
6	1.0	1.00	N.A.	N.A.	N.A.	1.30	0.368, 0.328	0.077, 0.1350	0.2649, 0.0248

Appendix 3c. Anions (ppm).

Sample	**F**	**Cl**	**NO_2^-**	**SO_4^{2-}**	**HS^-**	**Br**
1	3.78, 3.1532	594.10	<d.l.	5.87, 24.6708	1.47	2.33
2	0.46, 0.801	8,358.87	<d.l.	57.83, 52.4547	>10	31.76
3	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
4	0.027, 0.2067	9,231.00	<d.l., 0.0028	50.02, 124.9831	0.32	40.76
5	<d.l.	8,184.92	<d.l.	6.27, 10.3509	N.A.	47.04
6	1.03, 0.3926	987?	<d.l., 0.0313	40.2, 45.153	4.13	3.80

Appendix 3c (continued).

Sample	NO_3^-	PO_4^{2-}	Total P -ICP as PO_4^{2-}	PO_4^{2-} MAGIC+Mblue	NH_3 as N	$S_2O_3^{2-}$
1	<d.l., 0.1468	<d.l.	N.A.	<d.l.	0.45	<d.l.
2	<d.l., 0.0275	0.17, <d.l.	3.100	N.A.	0.39	N.A.
3	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
4	<d.l., 0.0267	<d.l.	N.A.	N.A.	0.89	N.A.
5	<d.l., 0.0602	<d.l.	4.650	N.A.	0.20	N.A.
6	<d.l., 0.0654	<d.l.	N.A.	0.0199	0.36	<d.l.

Appendix 3d. Cations (ppm).

Sample	Li	Na	Mg	K	Rb	Ca	Sr	Ba	Al	Si
1	0.210	272.0	0.137	1.57	0.109	21.1	0.425	0.513	<d.l.	3.176
2	1.050	1800.0	8.050	13.50	0.110	1460.0	18.400	1.590	0.053	8.600
3	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
4	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
5	1.360	1680.0	0.146	41.40	0.173	2800.0	90.000	2.090	0.360	12.200
6	0.414	184.0	0.644	2.73	<d.l.	92.7	1.120	0.263	0.001	3.190

Appendix 3d (continued).

Sample	Mn	Fe	Cr	Co	Ni	Cu	Zn	As	W	U
1	0.010	0.008	<d.l.	<d.l.	0.010	<d.l.	0.001	N.A.	0.189	0.002
2	0.105	0.019	0.017	0.002	0.021	0.025	0.017	0.080	N.A.	0.115
3	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
4	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
5	0.007	0.010	0.016	0.003	0.040	0.009	0.019	0.011	N.A.	0.200
6	0.011	0.009	<d.l.	<d.l.	0.003	<d.l.	0.002	0.048	0.018	0.015

Appendix 3e. Isotopic results.

Sample	$d^{34}S\ S^{2-}$ (CDT)	$d^{34}S-SO4^{2-}$ (CDT)	$dD-H_2O$ (SMOW)	$d^{18}O-H_2O$ (SMOW)	$d^{13}C$-DIC (PDB)
1	N.A.	8.5	N.A.	N.A.	-10.99+/-.04
2	-0.8	21.8	-36.85	-10.87	N.A.
3	N.A.	N.A.	N.A.	N.A.	N.A.
4	N.A.	N.A.	N.A.	N.A.	N.A.
5	-0.8	18.1	-20.35	-7.24	-14.67+/-.03
6	N.A.	N.A.	-33.73	-5.95	N.A.

Appendix 4. Direction of 16S rDNA insert in clones.

Clone	Alignment grou	Insert	Clone	Alignment grou	Insert
CC4	CFB and H9	Forward	G5	Proteobacteria	Forward
CC14	CFB and H9	Forward			
X4	CFB and H9	Forward	I15	Proteobacteria	Reverse
Y10	CFB and H9	Forward	O4	Proteobacteria	Reverse
H9	CFB and H9	Forward	R6	Proteobacteria	Reverse
GG8	CFB and H9	Reverse	R10	Proteobacteria	Reverse
L7	CFB and H9	Reverse	M9	Proteobacteria	Reverse
M2	CFB and H9	Reverse	P5	Proteobacteria	Reverse
			G7	Proteobacteria	Reverse
X13	Gram positives	Forward	S2	Proteobacteria	Reverse
CC9	Gram positives	Reverse	C37	Proteobacteria	Reverse
GG13	Gram positives	Reverse	U9	Proteobacteria	Reverse
GG15	Gram positives	Reverse	K7	Proteobacteria	Reverse
M3	Gram positives	Reverse	N3	Proteobacteria	Reverse
			V3	Proteobacteria	Reverse
I12	Proteobacteria	Both	W12	Proteobacteria	Reverse
I13	Proteobacteria	Both	T8	Proteobacteria	Reverse
I19	Proteobacteria	Forward	Q2	Proteobacteria	Reverse
H3	Proteobacteria	Forward	L4	Proteobacteria	Reverse
M1	Proteobacteria	Forward			
C23	Proteobacteria	Forward	BB7	Archaea	Both
CC8	Proteobacteria	Forward	J6	Archaea	Both
W9	Proteobacteria	Forward	AA2	Archaea	Reverse

Forward = 5' end of 16S rDNA molecule at T3 primer site in vector (front end of molecule sequenced).
Reverse = 3' end of 16S rDNA molecule at T3 primer site in vector (back end of molecule sequenced).
Both = Both ends of the molecule sequenced.

Appendix 5. Site descriptions for sampling locations yielding positive enrichments.

Evander 8-18. North-east prospect drive. Vertical borehole located above the Kimberley reef at depth of 1.83 kmbls. Penetrates the quartzite and intersects water-bearing fault at 1.95 kmbls. Borehole was situated in a pool of water about 1 to 2 feet deep. High flow rate.

Evander 2-19. Pilot hole drilled to explore for water. Drilled 20-30 years ago. Borehole is horizontal and located 2 to 3 meters above tunnel floor. Depth unknown, but may penetrate rock face 3 to 60 meters. Intersects a sill in the Ventersdorp formation.

Evander 8-21. Located in a cubby near the end of a development tunnel. Borehole is in quartzite and intersects a fault zone. Site is about 3 km from Evander 8-18 borehole and about 225 m deeper. Drill was still in place. Water collected as it dripped from a pipe attached to drill. Water-bearing fracture intersected the previous day.

Kloof 7-39. Development end in new part of mine. Hanging-wall drive associated with dyke. Water-bearing fracture intersected during drilling in the Ventersdorp lavas about three days prior to sampling. High water and gas flow rates. Water flowed out of long metal pipe (5 to 7 meters?) connected to the borehole, which was located near the top of the development end, several meters above the tunnel floor. Methane was detected emanating from pipe.

Kloof 4-43. Exploratory borehole drilled into Ventersdorp formation. Borehole was rough and curved and prevented packers from being used. Low water flow rate. Site was high on wall, near end of development tunnel; had to stand on platform to sample.

Premier 763. Exploratory borehole drilled into the kimberlite pipe from the adjacent intruded rock. Site was at 25 Pass South, just off main tunnel. Borehole located on tunnel floor and drilled at 82 degrees towards the kimberlite pipe. Borehole penetrated 25 m into the kimberlite pipe.

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This thesis is accepted on behalf of the
Faculty of the Institute by the following committee:

Andrew Campbell
Advisor

Jan 31, 2003
Date

I release this document to the New Mexico Institute of Mining and Technology.

Student's Signature

January 31, 2003
Date