Microbiology (2000), **146**, 1295–1310 Printed in Great Britain

Phylogenetic and physiological diversity of *Arthrobacter* strains isolated from unconsolidated subsurface sediments

F. H. Crocker,[1]† J. K. Fredrickson,[2] D. C. White,[3] D. B. Ringelberg[3]‡ and D. L. Balkwill[1]

Author for correspondence: D. L. Balkwill. Tel: +1 850 644 5719. Fax: +1 850 644 4865. e-mail: balkwill@bio.fsu.edu

[1] Department of Biological Science, Florida State University, 312 Nuclear Research Building, Tallahassee, FL 32306-4470, USA

[2] Pacific Northwest National Laboratory, Richland, WA 99352, USA

[3] Center for Environmental Biotechnology, University of Tennessee, Knoxville, TN 37932, USA

Forty strains of Gram-positive, aerobic, heterotrophic bacteria isolated from saturated subsurface lacustrine, paleosol and fluvial sediments at the US Department of Energy's Hanford Site in south central Washington State were characterized by phylogenetic analysis of 16S rRNA gene sequences and by determination of selected morphological, physiological and biochemical traits. Phylogenetic analyses of 16S rDNA sequences from subsurface isolates in the context of similar sequences from previously described bacterial species indicated that 38 of the subsurface strains were most closely related to *Arthrobacter*. The other two strains appeared to be most closely related to *Kocuria*. The subsurface isolates fell into seven phylogenetically coherent and distinct clusters, indicating that there was a significant degree of diversity among them. Additional diversity was detected by analysis of cellular fatty acids and physiological traits. The general morphological, physiological and biochemical traits of the subsurface strains were consistent with those of *Arthrobacter*, *Micrococcus* and genera recently separated from *Micrococcus*, such as *Kocuria*. Some of the subsurface strains were phylogenetically closely related to certain species of *Arthrobacter* (16S rDNA sequence similarities >99%). However, most of the subsurface isolates did not cluster with previously established species in phylogenetic analyses of 16S rRNA gene sequences or with hierarchical cluster analysis of cellular fatty acid profiles. Moreover, many of the subsurface isolates that were most closely related to *Arthrobacter* also differed from all established species of that genus in several of their specific physiological characteristics. Most of the subsurface isolates, then, are likely to be novel strains or species of *Arthrobacter*.

Keywords: *Arthrobacter*, subsurface microbiology, microbial phylogeny

INTRODUCTION

Microbiologists seldom studied terrestrial subsurface environments until the 1980s, when it was reported that certain shallow aquifers contained substantial numbers of culturable bacteria and other micro-organisms (for a review, see Ghiorse & Wilson, 1988). Since then, culturable microbes have been discovered in saturated and unsaturated subsurface environments, at depths ranging to 2·7 km, and at temperatures as high as 60 °C. They have also been found in a wide range of geologically distinct types of unconsolidated sediments and lithologies (Amy *et al*., 1992; Balkwill, 1989; Balkwill *et al*., 1989; Boivin-Jahns *et al*., 1995; Boone *et al*., 1995; Brockman *et al*., 1992; Fredrickson *et al*., 1991, 1993, 1995; Haldeman & Amy, 1993; Haldeman *et al*., 1993; Kieft *et al*., 1993; Pedersen & Ekendahl, 1990, 1992; Pedersen *et al*., 1996a). Little is known about the composition of most of these subsurface microbial communities or about how the organisms in them are related to one another and to established taxa.

The advent of molecular biological approaches in

†**Present address:** Center for Microbial Ecology, Michigan State University, East Lansing, MI 48824, USA.

‡**Present address:** Waterways Experiment Station, Vicksburg, MS 39180, USA.

The GenBank accession numbers for the sequences determined in this study are given in Methods.

microbial ecology and systematics has led to major changes in the ways in which microbiologists look at microbial communities and classify bacteria. The analysis of 16S rRNA sequences to establish microbial phylogenies, as pioneered by Carl Woese (Woese, 1987) and colleagues (Pace *et al*., 1986), has become an indispensable tool in many microbiology and microbial ecology laboratories. Furthermore, by extracting nucleic acids directly from environmental samples and analysing them by various fingerprinting or sequencing methods, the diversity and phylogeny of the microbial communities associated with those samples can be studied without culturing. This approach has now been applied to a wide range of natural environments, including a few subsurface environments (e.g. Chandler *et al*., 1997; Ekendahl *et al*., 1994; Pedersen *et al*., 1996b). Although analysis of 16S rRNA sequences is a powerful way to determine phylogeny, it is clear that this approach usually cannot be used to infer a microbe's full suite of morphological, physiological and genetic characteristics. Thus, it is possible to assign ecological roles to organisms detected solely by direct extraction of nucleic acids only when their 16S sequences correspond very closely to those of previously described organisms, which is often not the case. In contrast, characterization of culturable micro-organisms provides information on the ecological roles of at least some members of the microbial community, and it may enhance information on community structure that is obtained by direct molecular approaches (e.g. see Chandler *et al*., 1997). Yet, with only a few exceptions (e.g. Amy *et al*., 1992; Balkwill *et al*., 1997; Boivin-Jahns *et al*., 1995), many of the micro-organisms that have been cultured from subsurface environments remain uncharacterized.

In a recent study (Balkwill *et al*., 1997), we carried out a preliminary characterization of 169 chemoheterotrophic bacteria isolated from saturated paleosols, fluvial sands and gravels, and lacustrine sediments at the US Department of Energy Hanford Site in south central Washington State. 16S rRNA gene sequences from the subsurface isolates were compared to those in the Ribosomal Database Project (RDP) and GenBank sequence databases (using BLAST and SimilarityRank analyses, respectively; see Balkwill *et al*., 1997), to determine the sequences for established species of bacteria to which they were most similar. These analyses implied that roughly 25% of the subsurface isolates were most closely related to the genus *Arthrobacter*, making them the most numerically predominant component of the culturable aerobic microbial community. In addition, *Arthrobacter*-like bacteria were the only types of aerobic heterotrophs to be isolated from all five lithologies that were examined at this site.

Arthrobacters are considered to be ubiquitous and predominant members of culturable soil microbial communities (Hagedorn & Holt, 1975; Keddie *et al*., 1986). They have also been detected in several subsurface environments in addition to the Hanford Site sediments, including saturated Atlantic coastal plain sediments (20% of 187 heterotrophic isolates; Balkwill & Boone, 1997; Reeves *et al*., 1995), deep mine gallery clays (Boivin-Jahns *et al*., 1995), a karstic aquifer (Rusterholz & Mallory, 1994), a basalt aquifer (Zheng & Kellog, 1994) and unwelded volcanic tuffs (21% of 119 isolates; Haldeman *et al*., 1993). Because arthrobacters utilize a wide variety of organic compounds, including aromatic hydrocarbons (Hagedorn & Holt, 1975; Keddie *et al*., 1986; Stevenson, 1967), they are widely believed to play a significant role in the transformation of organic matter in natural environments.

Although arthrobacters are commonly detected in the culturable microbial communities of terrestrial subsurface environments and are sometimes the numerically predominant members thereof, little is known about their significance in the broader microbial communities that can be detected with direct molecular biological methods that do not depend on culturing. Only a few such studies have been carried out on samples from subsurface environments, and most of these have focused on very deep rock or groundwater systems (e.g. Ekendahl *et al*., 1994; Pedersen *et al*., 1996b) in which arthrobacters might not survive.

Recent 16S rDNA phylogenetic analyses have indicated that the species belonging to the genus *Arthrobacter* form a monophyletic clade (Koch *et al*., 1994, 1995). *Arthrobacter* species had previously been separated into two major clusters based on peptidoglycan composition (A3α or A4α), and the phylogenetic analyses confirmed this clustering within the genus (Koch *et al*., 1995). Individual *Arthrobacter* species can be distinguished from each other by peptidoglycan type, menaquinone composition and the degree of DNA homology (Stackebrandt & Fiedler, 1979; Stackebrandt *et al*., 1983). It has also been shown that three *Micrococcus* species are phylogenetically intermixed with various species of *Arthrobacter* (Koch *et al*., 1994, 1995), and that six other *Micrococcus* species separate into three distinct sublines within the *Arthrobacter* line of descent (Stackebrandt *et al*., 1995). As a result of these and other findings, it has been proposed that *Micrococcus* be redefined as four new genera (Stackebrandt *et al*., 1995).

Because of their predominance among the culturable aerobic heterotrophic bacteria from the sediments at the Hanford Site, the objective of this study was to examine the phylogenetic and physiological diversity and probable identities of the *Arthrobacter*-like bacterial isolates from these sediments. Phylogenetic analyses of 16S rRNA gene sequences were utilized in combination with analyses of morphological, biochemical and physiological traits in order to address these questions. We describe here the relatedness of the subsurface isolates to each other and to previously described bacterial taxa, and we present evidence that many of the isolates are novel strains or species of *Arthrobacter*.

METHODS

Subsurface core samples. Subsurface cores were obtained from the Yakima Barricade YB-02 borehole at the US Department of Energy Hanford Site in south-central

Table 1. Selected characteristics of the subsurface sediment samples

Lithology	Depth (m)	% Clay	% Silt	% Sand	Total organic C (mg kg^{-1})	Permeability (millidarcies)
Lacustrine	173·4	38	58	4	379	$<1\times10^{-9}$
	174·8	24	76	0	11067	$<1\times10^{-9}$
	176·6	38	53	9	13506	ND
	177·2	33	56	11	14719	$2{\cdot}9\times10^{-7}$
	178·0	41	57	2	14164	ND
	178·9	34	64	2	10946	ND
	179·9	45	55	0	12499	ND
	180·6	28	71	1	10098	$2{\cdot}2\times10^{-8}$
	181·4	28	72	2	107555	ND
	182·1	30	70	0	8534	ND
	182·6	29	71	0	8147	ND
	183·5	36	64	0	7040	ND
	184·7	35	63	2	16493	ND
Tephra	184·8	44	20	36	ND	$1{\cdot}1\times10^{-7}$
Upper paleosol	184·9	15	52	33	1360	ND
	185·6	35	58	7	1348	ND
	186·5	45	52	3	2171	ND
	188·0	51	34	15	7808	ND
	189·1	10	75	15	9410	$4{\cdot}5\times10^{-7}$
	192·6	22	61	17	786	ND
Fluvial sand	195·4	8	33	59	272	ND
	196·6	10	40	50	551	ND
Fluvial gravel	197·7	ND*	ND	ND	160	ND
Lower paleosol	217·6	ND	ND	ND	ND	$8{\cdot}8\times10^{-7}$

* ND, Not determined.

Washington State, as part of an overall effort to investigate the microbial, geochemical and geological properties of the subsurface at this site. All samples came from suprabasalt sediments of the Ringold formation, at depths ranging from 173 to 218 m below land surface. Cores from six lithological units within this sample interval were examined in the present study: fine-grained lacustrine sediments (173–185 m), sediments from a thin (5 cm) layer of tephra (185 m), an upper paleosol layer (185–193 m), coarse-grained fluvial sands (193–197 m), coarse-grained fluvial gravel (197–211 m) and a lower paleosol formation (211–218 m). Particle size distributions, total organic carbon content and liquid permeabilities for the individual samples are presented in Table 1. Additional chemical, physical and microbiological characteristics for three lithological units (lacustrine sediments, upper paleosol and fluvial sands) have been described previously (Fredrickson *et al.*, 1995; Kieft *et al.*, 1995; McKinley *et al.*, 1997). The sediments examined in this study were predominantly anoxic, but they were contained within an aerobic aquifer; the appearance of the upper zone of lacustrine sediment was consistent with its being oxidized (McKinley *et al.*, 1997).

Cable-tool drilling and split-spoon coring were performed as detailed previously (Kieft *et al.*, 1993), as was the use of various tracers to detect chemical and/or microbial contamination of the subcores (Kieft *et al.*, 1995). Sample handling and subcoring procedures were modified from those described by Phelps *et al.* (1989), Russell *et al.* (1992) and Colwell *et al.* (1992). Briefly, cores were transferred to anaerobic glove boxes immediately after recovery from the borehole and split lengthwise. The cores were then sectioned (perpendicular to their long axis) into subcores, and the inner portion of each subcore was retrieved and homogenized using sterile implements. Homogenized samples were designated by the mean depth of the homogenized subcore interval and shipped to participating laboratories for various analyses. All of the microbiological analyses were initiated within 24 h after cores were retrieved from the borehole.

Isolation of aerobic chemoheterotrophs. Aerobic, chemoheterotrophic bacteria were isolated from agar plates that were used for determination of viable counts. Samples were prepared for plating by blending 10 g sediment in 0·1 % (w/v) sodium pyrophosphate, followed by serial dilution in phosphate-buffered saline (PBS) as described previously (Balkwill, 1989). Dilutions were spread-plated in triplicate on the following media: PTYG and 1 % PTYG (Balkwill & Ghiorse, 1985), 5 % (w/v) trypticase soy agar, OPCA (Stevens & Holbert, 1995), hydroxyapatite agar (Sperber, 1958), B-4 (Boquet *et al.*, 1973), and a sediment extract agar (Wollum, 1982). All plates were incubated aerobically at 25 °C for 2–4 weeks.

Because viable counts were not statistically valid at the lowest dilution (10^{-2}) used (fewer than 30 colonies – usually fewer than 5 – appearing on each plate), alternative methods were used to recover additional aerobic chemoheterotrophs from the Hanford Site samples. Direct contact plates were inoculated by aseptically scattering small quantities (0·25–0·75 g) of homogenized samples over the surfaces of PTYG, 1 % PTYG and 5 % TSA plates. Alternatively, 1 g (wet wt) of sediment was added to 9 ml PBS, shaken vigorously by hand for 1 min, and then shaken for 30 min at 25 °C in a

reciprocating water bath. Serial dilutions in PBS were then plated on PTYG, 1% PTYG and 5% TSA.

All of the colonies appearing on the various types of plates described above (except for the approximately 4% that failed to transfer) were isolated by restreaking on the original plating medium and deposited in the DOE Subsurface Microbial Culture Collection (SMCC; Balkwill, 1993). All of the successfully isolated strains were included in the preliminary analysis of 16S rDNA sequences (see Introduction) carried out by Balkwill *et al*. (1997).

Bacterial strains and culture conditions. All of the subsurface isolates from the core samples described above that were closely related to *Arthrobacter globiformis* (a total of 40 strains, representing about 24% of all isolates) based on BLAST and Similarity Rank analyses of 16S rRNA gene sequences (Balkwill *et al*., 1997) were examined in this study (Table 2). The following reference strains were also included for comparative purposes: *Arthrobacter atrocyaneus* ATCC 13752^T, *Arthrobacter aurescens* ATCC 13344^T, *Arthrobacter citreus* ATCC 11624^T, *Arthrobacter crystallopoietes* ATCC 15481^T, *Arthrobacter globiformis* ATCC 8010^T, *Arthrobacter histidinolovorans* ATCC 11442^T, *Arthrobacter nicotianae* ATCC 15236^T, *Arthrobacter oxydans* ATCC 14358^T, *Arthrobacter pascens* ATCC 13346^T, *Arthrobacter protophormiae* ATCC 19271^T, *Arthrobacter ramosus* ATCC 13727^T, *Arthrobacter sulfureus* ATCC 19098^T, *Arthrobacter uratoxydans* ATCC 21749^T and *Arthrobacter ureafaciens* ATCC 7562^T. Subsurface and reference strains were maintained on 5% PTYG agar (Balkwill & Ghiorse, 1985) or grown directly from frozen stocks. Broth cultures were grown either in 5% PTYG or in E mineral medium containing 1 g glucose l^{-1} and 1 g yeast extract l^{-1} (EYG; Cure & Keddie, 1973).

Characterization of *Arthrobacter* strains. The subsurface and reference *Arthrobacter* strains were characterized by examination of 78 morphological, physiological and biochemical traits. Three cell and eight colony morphological traits were determined. The cell morphological traits were cell size (during exponential growth), Gram stain reaction and presence of a rod–coccus growth cycle (see below). The colony morphological traits were relative size, presence of pigmentation, type of pigmentation (if present), type of edge, type of surface, opacity, elevation, and presence or absence of any distinctive features (wrinkles, concentric rings, etc.). Colony morphology data were recorded after 3–5 d incubation on 5% PTYG agar at 25 °C. Cell morphologies and Gram reactions were determined from 12 h and 3-d-old cultures grown in EYG broth at 25 °C.

All strains were examined for the existence of a rod–coccus growth cycle according to the method of Cure & Keddie (1973). Cultures were grown for 3 d in EYG broth, after which the cells in 10 ml broth were harvested by centrifugation. The cell pellet was resuspended in 2 ml 0·85% (w/v) NaCl and subsequently diluted in 0·85% NaCl to a turbidity equivalent to that of a no. 5 MacFarland standard. A fresh EYG broth (50 ml) was inoculated with 2–4 drops of the diluted cell suspension, and cell morphologies were examined by phase-contrast microscopy of wet-mount preparations after 0, 6, 12, 24, 36 and 72 h incubation at 25 °C.

Forty-two compounds were tested as sole sources of carbon and energy: glucose, D-arabinose, D-ribose, DL-xylose, adonitol, D-galactose, D-fructose, L-sorbose, L-rhamnose, dulcitol, inositol, sorbitol, salicin, D-cellobiose, lactose, melibiose, sucrose, trehalose, D-melezitose, sorbic acid, D-raffinose, succinic acid, L-alanine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-serine, L-threonine, L-asparagine, L-cysteine, L-glutamine, L-glutamic acid, L-phenylalanine, glycine, L-histidine, L-arginine, L-aspartic acid, L-tyrosine, L-tryptophan, L-ornithine and casein. Aqueous stocks of these organic compounds were filter-sterilized (0·45 μm Millipore filters) and added to E mineral base supplemented with a vitamin stock (Cote & Gherna, 1994) and 0·001% (w/v) yeast extract. An increase in turbidity greater than that observed in carbon-free broth inoculated as a control was considered to represent growth on the offered carbon source.

Additional biochemical and physiological characteristics were determined with the API 20NE (formerly Rapid NFT) system (bioMérieux Vitek), which tests for nine specific enzymic capabilities (glucose fermentation, arginine dihydrolase, urease, aesculin hydrolysis, gelatinase, PNPG-β-galactosidase, nitrate reduction, oxidase and tryptophanase) and the ability to grow aerobically on 12 organic compounds as sole sources of carbon (D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-D-glucosamine, maltose, D-gluconate, caprate, adipate, L-malate, citrate and phenylacetate). Production of catalase, DNase, cellulase and amylase (starch hydrolysis) was determined using standard methods (Difco, 1984)

The Phylogenetic Analysis Using Parsimony program (PAUP* 4.0 beta version 4a; Swofford, 2000) was used to analyse a data matrix of 60 taxonomically informative strain phenotypic characteristics for maximum parsimony. The traits included in this analysis were: colony pigmentation; presence of a rod–coccus growth cycle; all of the non-API carbon source utilization tests except dulcitol, methionine, cysteine, casein and glucose (37 traits); and all of the traits tested with the API 20NE system (21 traits). The remaining 18 phenotypic traits were not included because either they were phylogentically uninformative or they yielded variable reactions in triplicate test runs. Positive and negative character states were represented by the numerals 1 and 0, respectively, in the data matrix. A heuristic search (using standard program defaults for parsimony analysis) generated a total of 22840 equally parsimonious trees. A 50% majority rule consensus tree was then generated in order to determine the frequencies at which specific clusters of strains were recovered during the parsimony analysis.

16S rRNA gene (rDNA) sequencing. Chromosomal DNA was isolated from the subsurface strains by a chloroform/isoamyl alcohol procedure (Johnson, 1981). Twenty to one hundred nanograms of genomic DNA was used as a template for PCR amplification (Sambrook *et al*., 1989) of an approximately 1500-base segment of the 16S rRNA gene (i.e. nearly the entire gene). The PCR primers were fD1 and rP2 (Weisburg *et al*., 1991), and the resulting PCR products were purified with the QIAquick PCR purification kit (QIAGEN) according to the manufacturer's instructions, except that sterile deionized water was used to elute the DNA. The purified PCR products were sequenced with an Applied Biosystems 373A sequencer, using the Taq DyeDeoxy terminator cycle sequencing method (Applied Biosystems, 1992; McBride *et al*., 1989). The sequencing primers were A, C, G, H, H-complement, P and P-complement (Balkwill *et al*., 1997; Lane *et al*., 1985; Reeves *et al*., 1995). The sequences were then assembled with the AutoAssembler DNA Sequence Assembly Software (Applied Biosystems, 1994).

The 16S rRNA sequences inferred from the 16S rDNA sequences determined as described above were aligned with 16S rRNA sequences from selected species within the *Arthrobacter* line of descent and from several related genera. Sequences of the reference strains were obtained from the Ribosomal Database Project (Maidak *et al*., 1996) and

Table 2. Hanford isolates examined in this study

Sample lithology and depth (m)	No. of *Arthrobacter*-like isolates from sample	Strain numbers of *Arthrobacter*-like isolates	No. of non-*Arthrobacter* isolates from sample
Lacustrine sediments			
173·4	10	G960, G961, G964, G965, ZAT001, ZAT002, ZAT004, ZAT005, ZAT012, ZAT013	21
174·8	1	ZAT014	0
176·6	3	G962, G969, ZAT255	3
177·2	1	G915	0
178·0	2	G963, G970	4
178·9	0	–	1
179·9	2	G966, G980	4
180·6	3	G968, G979, ZAT200	6
181·4	0	–	2
182·1	0	–	1
182·6	2	ZAT262, ZAT263	14
183·5	2	ZAT351, ZAT352	5
184·7	0	–	4
Tephra zone			
184·8	1	ZAT277	0
Upper paleosol sequence			
184·9	0	–	11
185·6	0	–	5
186·5	0	–	5
188·0	0	–	4
189·1	1	ZAT031	16
192·6	0	–	5
Fluvial sand			
195·4	0	–	4
196·6	3	G954, G986, G991	7
Fluvial gravel			
197·7	4	G919, G982, G984, G993	6
Lower paleosol sequence			
217·6	5	G950, G959, ZAT054, ZAT055, ZAT056	1

GenBank/EMBL databases. Regions of the sequence alignment in which data were missing from some of the sequences or the alignment was ambiguous (corresponding to positions 1–66, 462–470 and 1361–1542 in the 16S rRNA sequence for *Escherichia coli*; Brosius *et al*., 1979) were excluded from the phylogenetic analyses; 1279 bases were retained in the analyses. The phylogenetic positions of the subsurface strains were analysed using distance matrix, maximum-likelihood and parsimony methods. The PHYLIP computer program (Felsentein, 1993) was used for distance matrix analyses. Distances were calculated by the method of Jukes & Cantor (1963), after which phylogenies were estimated using the algorithms of DeSoete (1983) and/or Fitch & Margoliash (1967). The DNAML component of the PHYLIP package was used for maximum-likelihood analyses. In addition, sequences for a smaller subset of 38 strains were analysed for maximum parsimony with the PAUP program (Swofford, 2000) in order to construct the most parsimonious phylogenetic tree. Only the phylogenetically informative sites were considered, and alignment gaps were retained in the analysis. A heuristic search was performed with the standard program defaults.

The GenBank accession numbers for the 16S rDNA sequences of the subsurface strains examined in this study are as follows: G915, AF197020; G919, AF197021; G950, AF197022; G954, AF197023; G959, AF197024; G960, AF197025; G961, AF197026; G962, AF197027; G963, AF197028; G964, AF197029; G965, AF197030; G966, AF197031; G968, AF197032; G969, AF197033; G970, AF197034; G979, AF197035; G980, AF197036; G982, AF197037; G984, AF197038; G986, AF197039; G991, AF197040; G993, AF197041; ZAT001, AF197042; ZAT002, AF197043; ZAT004, AF197044; ZAT005, AF197045; ZAT012, AF197046; ZAT013, AF197047; ZAT014, AF197048; ZAT031, AF197049; ZAT054, AF197050; ZAT055, AF197051; ZAT056, AF197052; ZAT200, AF197053; ZAT255, AF197054; ZAT262, AF197055; ZAT263, AF197056; ZAT277, AF197057; ZAT351, AF196342; ZAT352, AF196343.

Whole-cell fatty acids. Bacterial cultures were grown on 5 % PTYG agar plates at 25 °C for 24–48 h. Cells were then scraped from the plates with a sterile loop. Saponification,

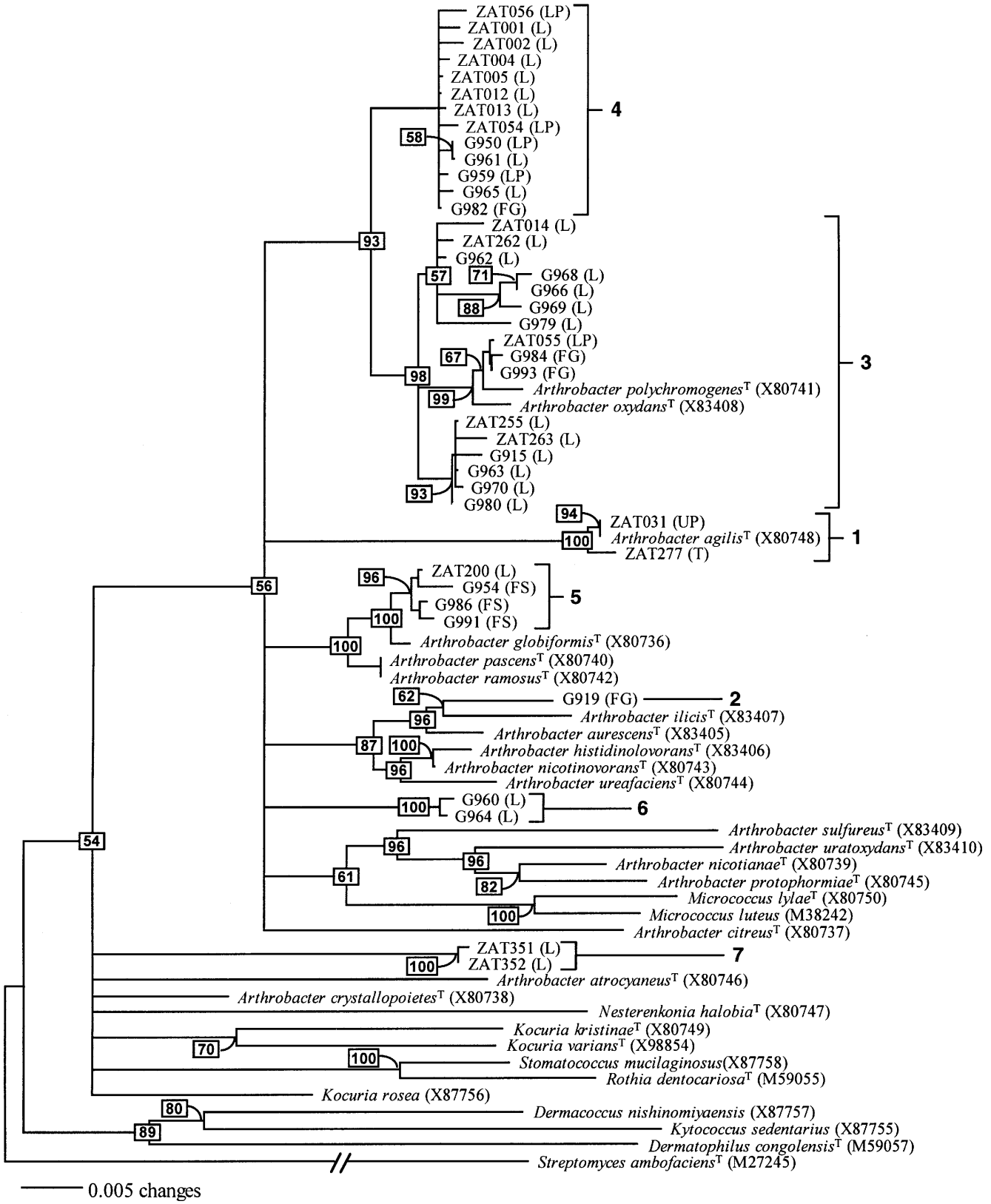

Fig. 1. For legend see facing page.

methylation, extraction of fatty acids and gas-chromatographic analysis of the fatty acid methyl esters were performed as described by Sasser (1990). A hierarchical cluster analysis was performed on the data using arcsin-transformed peak area percentages. The cluster analysis was by complete linkage.

RESULTS AND DISCUSSION

Isolation frequencies of *Arthrobacter*-like strains

The frequencies at which *Arthrobacter*-like versus non-*Arthrobacter* strains (based on preliminary analyses of 16S rRNA gene sequences; Balkwill *et al*., 1997) were isolated from each of the Hanford Site sediment samples are shown in Table 2. Overall, *Arthrobacter*-like strains accounted for 24% of the 169 isolates obtained from these samples. (All of the colonies appearing on dilution or direct contact plates – see Methods – that transferred successfully were isolated and analysed.) However, the relative proportion of *Arthrobacter*-like isolates varied from one lithology to another. Twenty-nine per cent of the 91 strains isolated from lacustrine sediment samples were closely related to *Arthrobacter*, whereas only 1 of the 47 isolates from the upper paleosol samples was *Arthrobacter*-like. *Arthrobacter* strains represented 21%, 40% and 83% of the isolates from the fluvial sands, fluvial gravel and lower paleosol, respectively, but, given the small total numbers of isolates from these three lithologies, such variations may not be significant. For the same reason, apparent variations in the proportions of *Arthrobacter* isolates from different depths within the lacustrine sequence may not be significant either (with the possible exception of the samples from 173·4 and 182·6 m).

16S rRNA phylogenetic analysis

Approximately 80% of the 16S rDNA sequence was determined for the 40 *Arthrobacter*-like subsurface isolates from the Hanford Site. Similarities among these sequences (over 1279 bases compared) ranged from 94·7 to 100%, implying that the subsurface isolates were closely related to each other. When the subsurface sequences were compared to available sequences for previously described bacterial species, it appeared that the subsurface isolates were most closely related to bacteria from the genus *Arthrobacter* (with sequence similarities ranging from 96·3 to 100%). To further assess the phylogenetic positions of the subsurface strains, their sequences were analysed with those for members of the *Arthrobacter* line of descent and several other related genera. This analysis placed the *Arthrobacter*-like subsurface isolates into seven distinct clusters that were preserved with a high degree of confidence during bootstrap analysis (Fig. 1), indicating that there was some detectable phylogenetic diversity among these strains.

Twenty-nine of the 40 subsurface strains were assigned to two large clusters (clusters 3 and 4; Fig. 1) that represented sister lineages. One of these larger clusters (cluster 3) included two previously defined *Arthrobacter* species (*A. polychromogenes* and *A. oxydans*), while the other was composed entirely of subsurface strains. Similarities ranged from 98·4 to 100% among the cluster 3 sequences, from 99·4 to 100% among cluster 4 sequences, and from 97·9 to 99·1% between cluster 3 and cluster 4 sequences. Fox *et al*. (1992) have reported that the actual relatedness between closely related bacterial strains cannot be resolved solely by analysis of 16S rRNA gene sequences when those sequences are more than 97·5% similar. DNA–DNA reassociation studies and/or other analyses are required to detect species-level differences under these circumstances. According to these guidelines, then, the phylogenetic analysis failed to detect any species-level differences among the subsurface isolates and type strains in clusters 3 and 4.

Four subsurface isolates formed a separate cluster (cluster 5) on a branch that included *A. globiformis*, *A. pascens* and *A. ramosus*. Similarities among the sequences for strains on this branch of the tree ranged from 98·9 to 99·6%, while similarities between the cluster 5 sequences and all other sequences for subsurface isolates ranged from 94·1 to 97·9%. Isolates G960 and G964 also clustered (cluster 6) on a distinct branch of the tree, and their sequences were most similar to those from *A. pascens* and *A. ramosus* (97·5%). Isolates ZAT031 and ZAT277 clustered with *Arthrobacter* (formerly *Micrococcus*) *agilis* (cluster 1) and were very closely related to this species (sequence similarities 99·7 to 100%). Subsurface isolate G919 was placed on a relatively long, separate branch of the phylogenetic tree that was part of a larger cluster including five *Arthrobacter* species. The 16S rDNA sequence from G919 was most similar (98·4%) to that from *A. aurescens*.

In distance matrix, maximum-likelihood and parsimony analyses, isolates ZAT351 and ZAT352 clustered (cluster 7) on a relatively long, distinct branch within a larger cluster that contained the three *Kocuria* species included in the analysis (data not shown). However, this larger cluster was not retained in 50% or more of the trees when bootstrap analysis (Felsenstein, 1985) was performed on the distance-matrix data (Fig. 1). The ZAT351 and ZAT352 sequences were most similar to that of *Kocuria rosea* (96·3%), making them the only subsurface isolates with sequences less than 97·5% similar to those of all other strains examined in this study.

There was significant phylogenetic diversity among strains within groups of *Arthrobacter*-like bacteria

Fig. 1. Consensus phylogenetic tree for 40 *Arthrobacter*-like subsurface isolates from the Hanford Site and selected reference strains of *Arthrobacter*, *Micrococcus*, *Kocuria* and other eubacteria, based on distance matrix analysis. The PHYLIP program (Felsenstein, 1993) was used to calculate distances by the method of Jukes & Cantor (1963), after which the FITCH (Fitch & Margoliash, 1967) option was used to estimate phylogenies from the distance matrix data. The tree shown was generated by bootstrapping at the >50% confidence limits, with 100 replications (Felsenstein, 1985). The numbers in boxes indicate the number of trees (in 100 replications) in which the indicated branch points were retained. *Streptomyces ambofaciens* was used as the outgroup.

Table 3. Major cellular fatty acids of subsurface and reference *Arthrobacter* strains

Strain	Percentage of total fatty acids represented by:							
	$iC_{14:0}$	$iC_{15:0}$	$aC_{15:0}$	$C_{16:0}$	$iC_{16:0}$	$aC_{17:0}$	$iC_{17:1}$	$C_{15:1\omega5c}$
G915		12·1	44·8		13·6			
G919		17·1	52·0		13·5			
G950	7·6		44·9		27·5			
G954		9·3	52·1		8·5			
G959			41·7	10·2	26·6			
G960			37·1	14·4	18·1			
G961			40·9	9·9	27·5			
G962		12·3	47·1		13·6			
G963			44·7		13·6	10·8		
G964			36·5	15·0	18·5			
G965			44·2	7·2	25·2			
G966			40·2	1·2	15·0			
G968			42·6	13·4	13·4			
G969		13·1	46·5		8·7			
G970			46·2		12·8	11·5		
G979			42·5	14·2	13·4			
G980		15·5	42·2		8·9			
G984		16·9	43·6		9·8			
G986			53·0		9·3	8·1		
G991		7·7	52·9		9·1			
G993		15·2	44·7		9·8			
ZAT001			44·1	8·8	24·7			
ZAT002			45·0	9·0	24·2			
ZAT004			44·3	9·3	24·2			
ZAT005			44·9	8·1	24·4			
ZAT012			42·0	10·7	25·9			
ZAT013			46·6	11·0	21·4			
ZAT014			39·3	10·0	21·4			
ZAT031		12·6	48·1		16·0			
ZAT054			42·8	9·2	26·7			
ZAT055		15·4	42·5		10·7			
ZAT056	7·7		43·3		26·0			
ZAT200		11·5	50·2		11·2			
ZAT255			39·6		14·3			11·1
ZAT262		12·9	45·7		9·1			
ZAT263		10·1	43·0		16·5			
ZAT277		8·3	40·1		22·3			
ZAT351			79·3			5·0	5·2	
ZAT352			77·6			5·0	4·7	
A. globiformis		13·3	55·2		21·0			
A. aurescens		11·5	69·8		7·2			
A. ramosus		10·8	63·2		12·1			
A. pascens		10·6	59·6			8·0		
A. ureafaciens		10·4	53·6		17·4			
A. uratoxydans		26·3	51·1		7·7			
A. oxydans		12·6	49·6		13·0			
A. histidinolovorans		12·7	63·3			8·5		
A. sulfureus		18·2	67·9			4·1		
A. atrocyaneus			47·3		18·2	14·8		
A. protophormiae		28·2	48·2		7·4			
A. crystallopoietes			67·2		13·3	5·0		

isolated from some, but not all, of the geological formations examined in this study. For example, the 26 isolates from the lacustrine sediments were sufficiently diverse to be assigned to clusters 3, 4, 5, 6 and 7. Although there were only four isolates from the fluvial gravels, these strains were placed in three separate clusters (clusters 2, 3 and 4). In contrast, four of the five lower paleosol isolates were assigned to cluster 4 and the fifth isolate was placed in cluster 3, the cluster that is most closely related phylogenetically to cluster 4.

The isolates from the fluvial sands, upper paleosol and tephra were phylogenetically distinct from nearly all of the strains isolated from other geological formations, falling into clusters 1 and 5. The total number of isolates from these three lithologies was quite small (only five strains), making comparison to isolates from other lithologies difficult. Nevertheless, the largest and most diverse group of isolates (i.e. from the lacustrine sediments) included only one strain that fell into cluster 5 and none that could be assigned to cluster 1. In addition, four strains from the lacustrine sediment and one from the fluvial gravels were placed in three clusters (clusters 2, 6 and 7) that contained no isolates from other lithologies. Given that all of the *Arthrobacter*-like strains that could be isolated from the samples were included in the analysis, these findings suggest that, to some extent, differing physical and chemical conditions in different lithologies may have selected for phylogenetically distinct arthrobacters.

Although all of the core samples from 173·4 to 183·5 m were lacustrine (lake sediments deposited 6–8 million years ago; Fredrickson *et al*., 1995), there was considerable variation in the chemical and physical properties of these samples (Table 1). For example, the uppermost sample from 173·4 m contained relatively low concentrations of organic carbon and many yellow-brown iron concretions indicative of an oxidizing environment. In contrast, the sample collected immediately below (174·8 m) contained more than 11 g organic carbon per kg sediment and was dark grey in appearance, suggesting that this sample was not oxidized. Both of these core samples had very low liquid permeabilities ($<1\times10^{-9}$ millidarcies), and thus the movement of bacteria between these formations would have been very unlikely (Fredrickson *et al*., 1995). It is not surprising that the highest proportion (10/40) of arthrobacters came from the oxidized region within the lacustrine sediment because *Arthrobacter* is a genus composed entirely of obligately aerobic organisms. It is interesting to note that 8 of 10 isolates from the 173·4 m sample were placed in cluster 4 while the remaining two were placed in cluster 6.

The two PHYLIP distance matrix methods and the maximum-likelihood method (see Methods) produced very similar results in terms of how the subsurface isolates were grouped in the resulting phylogenetic trees. There were some minor differences in branch lengths and the position of subsurface strain G919 within the clade that included *A. aurescens* and *A. ureafaciens* (data not shown), but no major discrepancies were detected. The parsimony analysis (PAUP program; see Methods) had some difficulty in resolving the order of branching within the tree and was unable to complete a single heuristic search without running out of memory. This may be an example of the difficulty that parsimony analysis sometimes has when assessing the phylogenetic relationships among very closely related strains, a problem that was observed previously by Koch *et al*. (1995). To address this problem, a heuristic search was halted after approximately 100000 trees had been saved, and a majority-rule consensus tree was generated from those trees. This analysis indicated that the seven distinct clusters containing the *Arthrobacter*-like subsurface isolates were retained in at least 91% of the trees (data not shown). The confidence that could be placed in the clusters of *Arthrobacter*-like strains was also assessed by performing a bootstrap analysis (Felsenstein, 1985) on the distance matrix data. (It was not possible to run a bootstrap analysis with parsimony because of the aforementioned computational problem.) This analysis produced the tree shown in Fig. 1, in which the clusters were again retained with a reasonably high degree of confidence.

Cellular fatty acid methyl ester analysis

The major fatty acids of the subsurface strains and *Arthrobacter* reference strains were anteiso- and iso-terminally branched saturated fatty acids, including 13-methylpentadecanoic acid (anteiso-$C_{15:0}$), 14-methylpentadecanoic acid (iso-$C_{15:0}$) and 15-methylhexadecanoic acid (iso-$C_{16:0}$) (Table 3). Somewhat smaller quantities of 15-methylheptadecanoic acid (anteiso-$C_{17:0}$) were detected in 13% of the subsurface isolates and 42% of the reference strains. The reference strains did not contain significant quantities of hexadecanoic acid ($C_{16:0}$), whereas this acid was detected in 41% of the subsurface isolates. Subsurface strains ZAT351 and ZAT352 differed from the *Arthrobacter* reference strains and the other subsurface isolates in that they contained 15-methylheptadecanoic acid (iso-$C_{17:1}$, which was not detected in any of the other strains) and comparatively large amounts of 13-methylpentadecanoic acid (anteiso-$C_{15:0}$). Moreover, they differed from all other subsurface isolates in that they did not contain 15-methylhexadecanoic acid (iso-$C_{16:0}$). In terms of the major iso- and anteiso- methyl-branched acids, these cellular fatty acid patterns are consistent with those described previously for species of the genus *Arthrobacter* (Keddie *et al*., 1986; Stackebrandt *et al*., 1995).

A hierarchical cluster analysis of fatty acid profiles produced a dendrogram (Fig. 2) in which relationships among the subsurface isolates were generally comparable to those indicated by analysis of 16S rRNA gene sequences (Fig. 1). For example, the subsurface isolates in 16S rDNA clusters 1, 4, 5, 6 and 7 were assigned to the same fatty acid clusters. Similarly, the two *Kocuria*-like subsurface strains (ZAT351 and ZAT352) and the two strains most closely related to *A. agilis* (ZAT031 and

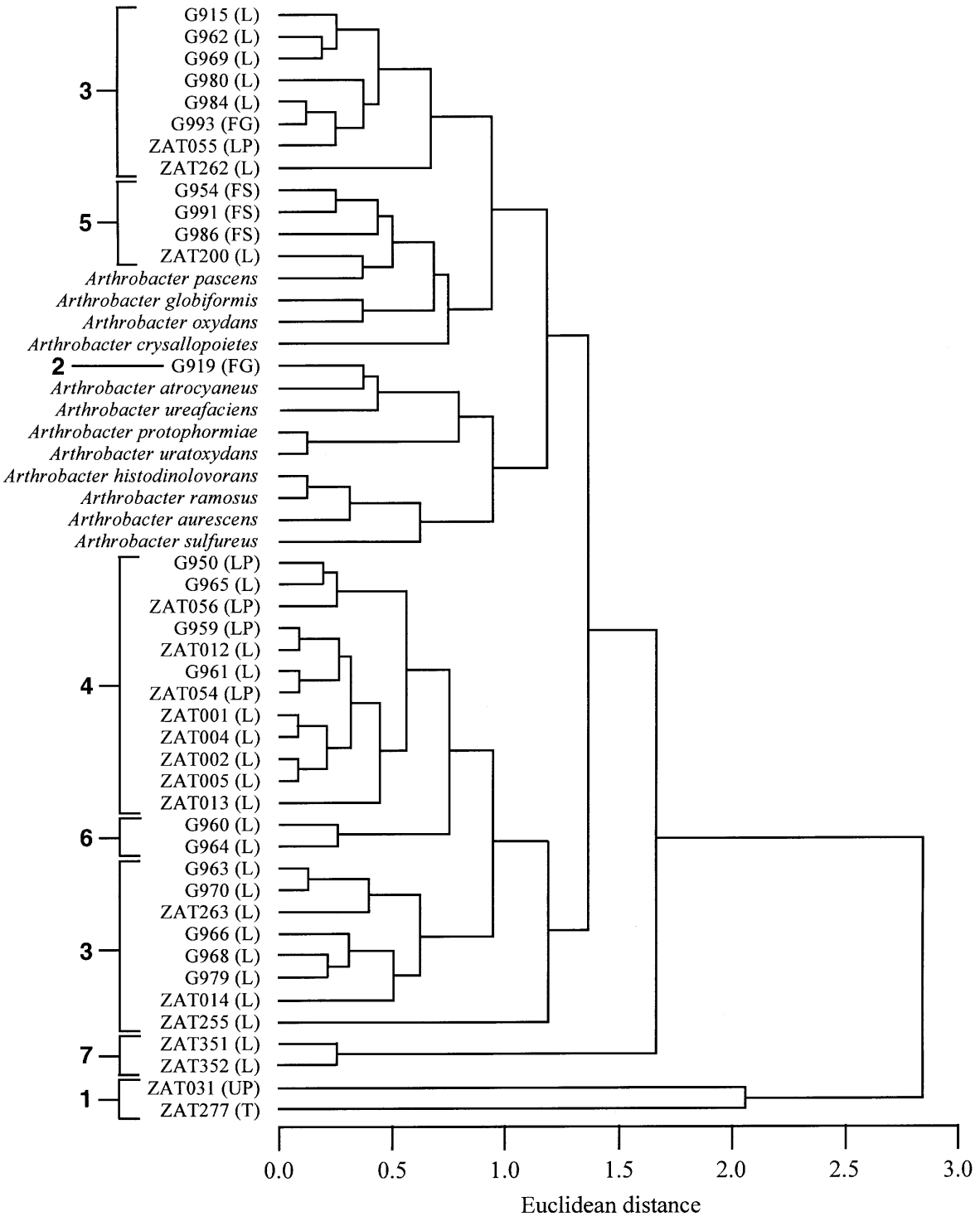

Fig. 2. Hierarchical cluster dendrogram of whole-cell fatty acid profiles of the subsurface *Arthrobacter* isolates and *Arthrobacter* type strains. *A. nicotianae*, *A. citreus* and subsurface isolate G982 were omitted from this analysis because they grew poorly on 5% PTYG agar.

ZAT277) appeared to be very distinct from the *Arthrobacter* strains included in the fatty acid analysis. However, the two analytical approaches were not in full agreement. The branching patterns joining the fatty acid clusters differed from those seen in the 16S rDNA tree. In particular, isolates belonging to 16S rDNA cluster 3 were distributed in two separate fatty acid clusters that did not correspond to the apparent subclusters (see Fig. 1) within cluster 3. There were also discrepancies in regard to how some of the subsurface strains might be

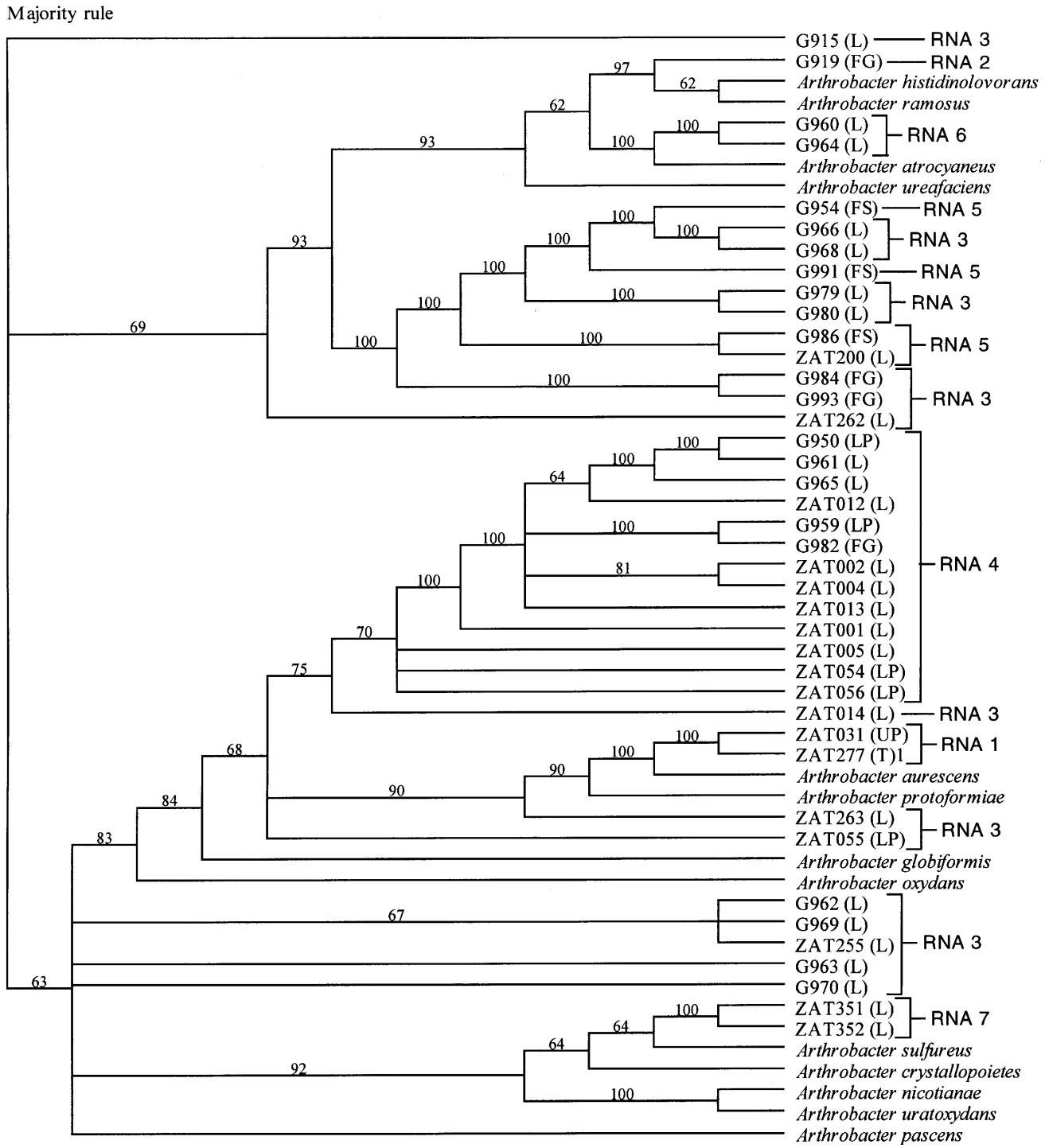

Fig. 3. Parsimony analysis of selected physiological and biochemical characteristics of the subsurface *Arthrobacter* isolates and *Arthrobacter* type strains. This cladogram was generated with the PAUP program (Swofford, 2000), which allows the use of 0 and 1 to represent character-state symbols. *A. citreus* was omitted from this analysis because of poor growth in the minimal medium used to determine substrate utilization patterns. Shown here is a 50% majority rule cladogram calculated from the 22840 equally parsimonious trees retained during a heuristic search (see Methods). The numbers above the branches indicate the percentage of trees in which the branch points to the right of the numbers were retained.

related to the *Arthrobacter* type strains. Some of these differences could be the result of variations in the expression of cell envelope fatty acids with culture age or cell density. Nevertheless, the separation of 16S rDNA cluster 3 isolates at a euclidean distance of approximately 1·3 may reflect real diversity (perhaps a species boundary) among these isolates because the subsurface strains within a 16S rDNA cluster generally cluster below a euclidean distance of 1·0.

Morphological and physiological analyses

Except for strains ZAT031, ZAT277, ZAT351 and ZAT352, the subsurface isolates were Gram-positive rods during the exponential phase of growth and exhibited a cyclic variation in cell shape. The cells shortened and became nearly spherical as they approached the stationary phase. On inoculation into fresh medium, the cells gradually elongated into rods and underwent snapping cell division. All of these morphological traits are consistent with those described for the genus *Arthrobacter* (Keddie *et al*., 1986).

Subsurface strains ZAT031, ZAT277, ZAT351 and ZAT352 were observed to be Gram-positive cocci, regardless of the age of the culture. ZAT031 and ZAT277 cells were spheres that occurred in pairs and tetrads, and ZAT031 cells were motile. Colonies on 5% PTYG agar were light pink (ZAT277) to dark rose (ZAT031), circular, smooth, slightly convex and entire. These morphological traits are consistent with the description of *Arthrobacter* (formerly *Micrococcus*) *agilis* (Koch *et al*., 1995). Cells of strains ZAT351 and ZAT352 were spheres that occurred in pairs, tetrads and clusters. Colonies on 5% PTYG agar were yellow, circular, smooth, convex and entire. These characteristics are more consistent with the description of *Kocuria* (formerly *Micrococcus*) *varians* than that of *Kocuria rosea* (formerly *Micrococcus roseus*; Stackebrandt *et al*., 1995), even though 16S rDNA sequence analysis implied that ZAT351 and ZAT352 were slightly more closely related phylogenetically to *K. rosea*.

A comparison of metabolic traits indicated that the subsurface isolates and *Arthrobacter* type strains generally had similar substrate ranges and enzymic capabilities. Most of the type and subsurface strains were β-galactosidase-positive, whereas only 50% could hydrolyse aesculin or gelatin. None of the strains exhibited tryptophanase, arginine dihydrolase or cellulase activity, nor did they ferment glucose to acidic end products. None of the strains used dulcitol, cysteine or methionine, and fewer than 15% utilized adonitol, sorbitol, sorbic acid or caproic acid. More than 90% of the strains utilized glucose, maltose, D-gluconate, citric acid, malic acid, asparagine, lysine, glutamine and casein as sole carbon sources. Despite the overall similarities, most of the subsurface isolates differed from the *Arthrobacter* type strains in at least some of the metabolic characteristics examined, suggesting that the two populations were physiologically distinct.

Some of the phylogenetic relationships suggested by 16S rRNA gene sequence analysis appeared to be maintained at the phenotypic level when a parsimony analysis was carried out on phenotypic traits (Fig. 3). Most of the isolates in 16S rDNA cluster 4 (Fig. 1) also clustered together phenotypically, as did subsurface strains in clusters 1, 6 and 7. This finding was not unexpected because fatty acid analysis also confirmed the close relatedness among the strains in these clusters (Fig. 2). Moreover, several of the subsurface strains in these clusters were isolated from the same sample depth (Table 1), and thus have a greater likelihood of being duplicate (or near-duplicate) strains.

In some cases, analysis of phenotypic traits implied more diversity among the subsurface isolates than was detected by analysis of 16S rDNA sequences or cellular fatty acids. This was especially true for the subsurface isolates in 16S rDNA cluster 3 (Fig. 1). Parsimony analysis of phenotypic traits (Fig. 3) placed these isolates on several individual branches and in several distinct clusters within the resulting tree. Moreover, some of the cluster 3 strains appeared to be physiologically related to strains in 16S rDNA clusters 4 and 5. Only two pairs of strains in 16S rDNA cluster 3 (G966 and G968; G984 and G993) were found to be as physiologically similar as might be expected from 16S rDNA and fatty acid analyses.

Some of most distinctive phenotypic differences between the subsurface strains assigned to separate 16S rDNA clusters are summarized in Table 4. Five physiological properties (urease, tryptophanase, PNPG-β-galactosidase and utilization of L-serine and sucrose) were present or absent in all members of a single 16S rDNA cluster, and thus could be used to differentiate that cluster from the others. Except for cluster 3, all strains in each of the 16S rDNA clusters differed from the strains of other clusters by at least three (sometimes more than 10) phenotypic traits. There were no distinct phenotypic differences between the strains in clusters 3 and 5 (which clustered together during parsimony analysis of phenotypic traits; Fig. 3). Moreover, the cluster 3 strains differed from those in cluster 4 by only two traits (utilization of L-ornithine and DL-xylose) and from those in cluster 5 by only a single characteristic (utilization of L-ornithine). However, the apparent lack of differences between the cluster 3 strains and those in other clusters was mostly the result of the high phenotypic diversity within cluster 3 noted earlier. There were very few phenotypic traits that all isolates in cluster 3 had in common (note the number of variable traits listed in Table 4 alone), so only a few valid comparisons could be made between this and the other clusters. The variations in phenotypic traits among the strains in 16S rDNA cluster 3 did not appear to correspond either to the subclusters detected by phylogenetic analysis (Fig. 1) or to the two subgroups defined by analysis of fatty acids (Fig. 2).

Parsimony analysis of phenotypic traits appeared to confirm that most of the subsurface isolates were physiologically distinct from the *Arthrobacter* type

Table 4. Distinctive phenotypic traits of subsurface isolates in different 16S rRNA clusters

Phenotypic trait	Characteristics of strains in these clusters:*						
	1	2	3	4	5	6	7
Morphological traits							
Colony pigmentation	+	+	−	−	−	+	+
Rod−coccus cell cycle	−	+	+	+	+	+	−
Enzymic capabilities							
Gelatinase	−	+	±	+†	−	±	−
Nitrate reductase	−	−	±	−	−	+	+
PNPG-β-galactosidase	+	+	+	+	+	+	−
Tryptophanase	−	+	−	−	−	−	−
Urease	−	−	−‡	−	−	−	+
Substrate utilization							
L-Histidine	−	+	−‡	+	−	±	−
L-Isoleucine	−	+	±	+†	+	+	−
L-Ornithine	−	+	+	−	−	±	±
Phenylacetate	−	+	±	+	+	+	−
L-Serine	−	+	+	+	+	+	+
Sucrose	−	+	+	+	+	+	+
Sorbitol	−	−	±	−†	+	−	+
Trehalose	−	+	+	+	+	+	+
L-Tyrosine	−	+	±	+	±	−	−
DL-Xylose	−	+	+	−†	+	+	±

* Cluster numbers refer to those in Figs 1–3.

† 12 of 13 strains yielded this reaction.

‡ 15 of 16 strains yielded this reaction.

strains, in that these two groups of strains were usually placed on separate branches (Fig. 3). Moreover, many subsurface isolates were phenotypically quite distinct from the phylogenetically most closely related *Arthrobacter* species. For example, the isolates in 16S rDNA cluster 5 were phylogenetically most closely related to *A. globiformis* (Fig. 1), but they differed from this organism in 10 to 12 phenotypic traits and were assigned to a very distant cluster by parsimony analysis of those traits (Fig. 3). Similarly, subsurface strains ZAT055, G984 and G993 were phylogenetically closely related to *A. oxydans*, but they differed from that species in 10 to 15 phenotypic traits.

Conclusions

All but two of the subsurface isolates examined in this study (i.e. ZAT351 and ZAT352) can be considered species of *Arthrobacter*, based on the results of phylogenetic analyses (Fig. 1) and on general similarities in morphological, physiological and biochemical (i.e. cell fatty acid profiles) characteristics. Strains ZAT351 and ZAT352 are most likely species of *Kocuria*, based on morphological similarities and the results of the phylogenetic analyses (all methods), in which they were assigned to a cluster including the three *Kocuria* type strains. However, the fact that this clustering was not retained during a bootstrap analysis (see Fig. 1) raises uncertainties as to the actual genus affiliation of these two subsurface strains.

The subsurface *Arthrobacter*-like strains examined in this study were phylogenetically and physiologically diverse, and they very likely represent several distinct species. 16S rRNA sequence similarities between strains in the seven distinct clusters defined by the phylogenetic analyses (Fig. 1) were generally too high (i.e. $>97{\cdot}5\,\%$; see Fox *et al*., 1992) for these clusters to be considered as different species on the basis of their 16S rRNA phylogeny alone. However, this is not unusual in the genus *Arthrobacter*, where well-defined species often have very high 16S rRNA sequence similarities (e.g. $>99\,\%$ among *A. globiformis*, *A. pascens* and *A. ramosus*). Moreover, with the exception of cluster 3, the strain clusters defined by phylogenetic analysis could also be differentiated by analysis of cellular fatty acids (Fig. 2) and by three or more distinct phenotypic traits (Table 4). The strains in 16S rDNA cluster 3 were not easily distinguished from those in other clusters because of their high physiological diversity. There may well be significant species-level diversity within cluster 3, but additional analyses would be needed to resolve this issue because analyses of phylogenetic, biochemical (i.e. cell fatty acid) and physiological data subdivided the cluster 3 strains in different ways. DNA–DNA reassociation experiments (beyond the scope of this study) would be helpful in this regard.

Several of the subsurface isolates examined in this study are likely to be strains of novel species. This is clearly the case for isolates ZAT351 and ZAT352, whose 16S rDNA sequences are less than 97·5 % similar to those of the most closely related established species. As noted above, however, there is some uncertainty in regard to the genus in which these isolates should be placed. Subsurface strains G960 and G964 almost certainly represent a new species as well. Their 16S rDNA sequences differed by 2·5 % from those of the most closely related *Arthrobacter* species (on the borderline of a species-level difference according to Fox *et al*., 1992), they did not cluster with any *Arthrobacter* species in fatty acid analysis (Fig. 2), and they differed from all tested *Arthrobacter* species in at least eight phenotypic traits. Similar arguments could be made for considering subsurface isolate G919 and those in 16S rDNA cluster 3 to be novel species of *Arthrobacter*. The isolates in cluster 4 were separated from established *Arthrobacter* species by phylogenetic, fatty acid and physiological analyses, so these strains may also represent one or more novel species of *Arthrobacter*. The situation within 16S rRNA cluster 3 is less clear, and DNA–DNA reassociation studies or other analyses will probably be necessary to define species-level differences (if any) within this group of strains. Moreover, additional properties commonly used to distinguish species of *Arthrobacter* (such as type of peptidoglycan, menaquinones and polar lipid content) should probably be determined for all of the subsurface strains before any of the apparent new species are named and described in more detail.

The results of this study and others (Amy *et al*., 1992; Balkwill & Boone, 1997; Boivin-Jahns *et al*., 1995; Reeves *et al*., 1995; Rusterholz & Mallory, 1994; Zheng & Kellog, 1994) indicate that *Arthrobacter* strains are often among the predominant members of the culturable microbial communities in terrestrial subsurface environments. It is not yet known whether they are also predominant members of the broader microbial communities that would be detected by direct molecular analyses of subsurface sediments. Nevertheless, this study has indicated that most of the culturable arthrobacters in the Hanford sediments are likely to be members of novel species with distinctive physiological characteristics. The ecological significance and specific functional roles of these previously uncharacterized organisms should be examined in future studies. For example, it would be interesting to know whether the disparity of organic carbon concentrations between the upper regions of the lacustrine sediments and the unoxidized zone that lies immediately beneath it at the Hanford Site is in any way related to oxidation of organic matter by subsurface arthrobacters.

ACKNOWLEDGEMENTS

We thank B. Murfee, G. Drake, D. Fair, L. Sander, L. Ecker, K. Bernabe, M. Groh, S. Padgett, J. Sutly and L. Turner III of Florida State University, and C. Spadoni of Pacific Northwest National Laboratory, for technical assistance and advice.

This research was supported by: (i) the US Department of Energy (DOE) Laboratory Technology Research Division under the terms of a Cooperative Research and Development Agreement, and (ii) DOE Grant nos DE-FG05-91ER61159 and DE-FG02-96ER62210 from the Subsurface Science Program, DOE Office of Energy Research.

REFERENCES

Amy, P. S., Haldeman, D. L., Ringelberg, D., Hall, D. H. & Russell, C. (1992). Comparison of identification systems for classification of bacteria isolated from water and endolithic habitats within the deep subsurface. *Appl Environ Microbiol* **58**, 3367–3373.

Applied Biosystems (1992). *Taq DyeDeoxy Terminator Cycle Sequencing Kit User Bulletin no. 901497, Revision E*. Foster City, CA: Applied Biosystems.

Applied Biosystems (1994). *Autoassembler DNA Sequence Assembly Software User Manual no. 903226, Revision A*. Foster City, CA: Applied Biosystems.

Balkwill, D. L. (1989). Numbers, diversity, and morphological characterization of aerobic, chemoheterotrophic bacteria in deep subsurface sediments from a site in South Carolina. *Geomicrobiol J* **7**, 33–51.

Balkwill, D. L. (1993). DOE makes subsurface cultures available. *ASM News* **59**, 504–506.

Balkwill, D. L. & Boone, D. R. (1997). Identity and diversity of microorganisms cultured from subsurface environments. In *The Microbiology of the Terrestrial Deep Subsurface*, pp. 105–117. Edited by P. S. Amy & D. L. Haldeman. New York: Lewis Publishers.

Balkwill, D. L. & Ghiorse, W. C. (1985). Characterization of subsurface bacteria associated with two shallow aquifers in Oklahoma. *Appl Environ Microbiol* **50**, 580–588.

Balkwill, D. L., Fredrickson, J. K. & Thomas, J. M. (1989). Vertical and horizontal variations in the physiological diversity of the aerobic chemoheterotrophic bacterial microflora in deep Southeast Coastal Plain subsurface sediments. *Appl Environ Microbiol* **55**, 1058–1065.

Balkwill, D. L., Reeves, R. H., Drake, G. R., Reeves, J. Y., Crocker, F. H., King, M. B. & Boone, D. R. (1997). Phylogenetic characterization of bacteria in the subsurface microbial culture collection. *FEMS Microbiol Rev* **20**, 201–216.

Boivin-Jahns, V., Bianchi, A., Ruimy, R., Garcin, J., Daumas, S. & Christen, R. (1995). Comparison of phenotypical and molecular methods for the identification of bacterial strains isolated from a deep subsurface environment. *Appl Environ Microbiol* **61**, 3400–3406.

Boone, D. R., Liu, Y., Zhao, Z.-J., Balkwill, D. L., Drake, G. R., Stevens, T. O. & Aldrich, H. C. (1995). *Bacillus infernus* sp. nov., an Fe(III)- and Mn(IV)-reducing anaerobe from the deep terrestrial subsurface. *Int J Syst Bacteriol* **45**, 441–448.

Boquet, E., Boronat, A. & Ramos-Cormenzana, A. (1973). Production of calcite (calcium carbonate) crystals by soil bacteria is a general phenomenon. *Nature* **246**, 527–528.

Brockman, F. J., Kieft, T. L., Fredrickson, J. K., Bjornstad, B. N., Li, S. W., Spangenberg, W. & Long, P. E. (1992). Microbiology of vadose zone paleosols in south-central Washington State. *Microb Ecol* **23**, 279–301.

Brosius, J., Palmer, M. L., Kennedy, P. J. & Noller, H. R. (1979). Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proc Natl Acad Sci USA* **75**, 4801–4805.

Chandler, D. P., Li, S.-M., Spadoni, C. M., Drake, G. R., Balkwill, D. L., Fredrickson, J. K. & Brockman, F. J. (1997). A molecular

comparison of culturable aerobic heterotrophic bacteria and 16S rDNA clones derived from a deep subsurface sediment. *FEMS Microbiol Ecol* **23**, 131–144.

Colwell, F. S., Stormberg, G. J., Phelps, T. J. & 11 other authors (1992). Innovative techniques for collection of saturated and unsaturated subsurface basalts and sediments for microbiological characterization. *J Microbiol Methods* **15**, 279–292.

Cote, R. J. & Gherna, R. L. (1994). Nutritional media. In *Methods for General and Molecular Bacteriology*, pp. 155–178. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.

Cure, G. L. & Keddie, R. M. (1973). Methods for morphological examination of aerobic coryneform bacteria. In *Sampling – Microbiological Monitoring of Environments* (Society for Applied Bacteriology Technical Series 7), pp. 123–135. Edited by R. G. Board & D. N. Lovelock. London: Academic Press.

DeSoete, G. (1983). A least squares algorithm for fitting additive trees to proximity data. *Psychometrica* **48**, 621–626.

Difco (1984). *Difco Manual: Dehydrated Culture Media and Reagents for Microbiology*, 10th edn. Detroit: Difco Laboratories.

Ekendahl, S., Arlinger, J., Ståhl, F. & Pedersen, K. (1994). Characterization of attached bacterial populations in deep granitic groundwater from the Stripa research mine by 16S-rRNA gene sequencing and scanning electron microscopy. *Microbiology* **140**, 1575–1583.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.

Felsenstein, J. (1993). PHYLIP (Phylogeny Inference Package), version 3.5c. Seattle: University of Washington.

Fitch, W. M. & Margoliash, E. (1967). Construction of phylogenetic trees. *Science* **155**, 279–284.

Fox, G. E., Wisotzkey, J. D. & Jurtshuk, P., Jr (1992). How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *Int J Syst Bacteriol* **42**, 166–170.

Fredrickson, J. K., Balkwill, D. L., Zachara, J. M., Li, S. W., Brockman, F. J. & Simmons, M. A. (1991). Physiological testing and distribution of heterotrophic bacteria in deep Cretaceous sediments of the Atlantic Coastal Plain. *Appl Environ Microbiol* **57**, 402–411.

Fredrickson, J. K., Brockman, F. J., Bjornstad, B. N. & 7 other authors (1993). Microbiological characteristics of pristine and contaminated deep vadose sediments from an arid region. *Geomicrobiol J* **11**, 95–107.

Fredrickson, J. K., McKinley, J. P., Nierzwicki-Bauer, S. A., White, D. C., Ringelberg, D. B., Rawson, S. A., Li, S., Brockman, F. J. & Bjornstad, B. N. (1995). Microbial community structure and biogeochemistry of Miocene subsurface sediments: implications for long-term microbial survival. *Mol Ecol* **4**, 619–626.

Ghiorse, W. C. & Wilson, J. T. (1988). Microbial ecology of the terrestrial subsurface. *Adv Appl Microbiol* **33**, 107–272.

Hagedorn, C. & Holt, J. G. (1975). A nutritional and taxonomic survey of *Arthrobacter* soil isolates. *Can J Microbiol* **21**, 353–361.

Haldeman, D. L. & Amy, P. S. (1993). Bacterial heterogeneity in deep subsurface tunnels at Ranier Mesa, Nevada Test Site. *Microb Ecol* **25**, 183–194.

Haldeman, D. L., Amy, P. S., Ringelberg, D. & White, D. C. (1993). Characterization of the microbiology within a 21 m^3 section of rock from the deep subsurface. *Microb Ecol* **26**, 145–159.

Johnson, J. L. (1981). Genetic characterization. In *Manual of Methods for General Bacteriology*, pp. 450–472. Edited by P. Gerhardt, R. G. E. Murray, R. W. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg & G. B. Phillips. Washington, DC: American Society for Microbiology.

Jukes, T. H. & Cantor, C. R. (1963). Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.

Keddie, R. M., Collins, D. & Jones, D. (1986). Genus *Arthrobacter*. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1288–1301. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.

Kieft, T. L., Amy, P. S., Brockman, F. J., Fredrickson, J. K., Bjornstad, B. N. & Rosacker, L. L. (1993). Microbial abundance and activities in relation to water potential in the vadose zone of arid and semiarid sites. *Microb Ecol* **26**, 59–78.

Kieft, T. L., Fredrickson, J. K., McKinley, J. P., Bjornstad, B. N., Rawson, S. A., Phelps, T. J., Brockman, F. J. & Pfiffner, S. M. (1995). Microbiological comparisons within and across contiguous lacustrine, paleosol, and fluvial subsurface sediments. *Appl Environ Microbiol* **61**, 749–757.

Koch, C., Rainey, F. A. & Stackebrandt, E. (1994). 16S rDNA studies on members of *Arthrobacter* and *Micrococcus*: an aid for their future taxonomic restructuring. *FEMS Microbiol Lett* **123**, 167–172.

Koch, C., Schumann, P. & Stackebrandt, E. (1995). Reclassification of *Micrococcus agilis* (Ali-Cohen 1889) to the genus *Arthrobacter* as *Arthrobacter agilis* comb. nov. and emendation of the genus *Arthrobacter*. *Int J Syst Bacteriol* **45**, 837–839.

Lane, D. J., Pace, G., Olsen, G. J., Stahl, D. A., Sogin, M. L. & Pace, N. R. (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc Natl Acad Sci USA* **82**, 6955–6959.

McBride, L. J., Koepf, S. M., Gibbs, R. A., Salser, W., Mayrand, P. E., Hunkapiller, M. W. & Kronick, M. N. (1989). Automated DNA sequencing methods involving polymerase chain reaction. *Clin Chem* **35**, 2196–2201.

McKinley, J. P., Stevens, T. O., Fredrickson, J. K., Zachara, J. M., Colwell, F. S., Wagnon, K. B., Smith, S. C., Rawson, S. A. & Bjornstad, B. N. (1997). Biogeochemistry of anaerobic lacustrine and paleosol sediments within an aerobic unconfined aquifer. *Geomicrobiol J* **14**, 23–39.

Maidak, B. L., Olsen, G. J., Larsen, N., Overbeek, R., McCaughey, M. J. & Woese, C. R. (1996). The ribosomal database project (RDP). *Nucleic Acids Res* **24**, 82–85.

Pace, N. R., Stahl, D. A., Land, D. J. & Olsen, G. J. (1986). The analysis of natural microbial populations by ribosomal RNA sequences. *Adv Microb Ecol* **9**, 1–55.

Pedersen, K. & Ekendahl, S. (1990). Distribution and activity of bacteria in deep granitic groundwaters of southeastern Sweden. *Microb Ecol* **20**, 37–52.

Pedersen, K. & Ekendahl, S. (1992). Assimilation of CO_2 and introduced organic compounds by bacterial communities in groundwater from southeastern Sweden deep crystalline bedrock. *Microb Ecol* **23**, 1–14.

Pedersen, K., Arlinger, J., Ekendahl, S. & Halbeck, L. (1996a). 16S rRNA gene diversity of attached and unattached bacteria in boreholes along the access tunnel of the Åspö hard rock laboratory, Sweden. *FEMS Microbiol Ecol* **19**, 249–262.

Pedersen, K., Arlinger, J., Hallbeck, L. & Pettersson, C. (1996b). Diversity and distribution of subterranean bacteria in groundwater at Oklo in Gabon, Africa, as determined by 16S rRNA gene sequencing. *Mol Ecol* **5**, 427–436.

Phelps, T. J., Fliermans, C. B., Garland, T. R., Pfiffner, S. M. & White, D. C. (1989). Methods for recovery of deep terrestrial

subsurface sediment for microbiological analyses. *J Microbiol Methods* **9**, 15–27.

Reeves, R. H., Reeves, J. Y. & Balkwill, D. L. (1995). Strategies for phylogenetic characterization of subsurface bacteria. *J Microbiol Methods* **21**, 235–251.

Russell, B. F., Phelps, T. J., Griffin, W. T. & Sargent, K. A. (1992). Procedures for sampling deep subsurface microbial communities in unconsolidated sediments. *Ground Water Monit Rev* **12**, 96–104.

Rusterholtz, K. J. & Mallory, L. M. (1994). Density, activity, and diversity of bacteria indigenous to a karstic aquifer. *Microb Ecol* **28**, 79–99.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sasser, M. (1990). *Technical Note 101: Identification of Bacteria by Gas Chromatography of Cellular Fatty Acids*. North Newark, DE: MIDI.

Sperber, J. I. (1958). The incidence of apatite-solubilizing organisms in the rhizosphere and soil. *Aust J Agric Res* **9**, 778–781.

Stackebrandt, E. & Fiedler, F. (1979). DNA–DNA homology studies among strains of *Arthrobacter* and *Brevibacterium*. *Arch Microbiol* **120**, 289–295.

Stackebrandt, E., Fowler, V. J., Fiedler, F. & Heiler, H. (1983). Taxonomic studies on *Arthrobacter nicotianae* and related taxa: description of *Arthrobacter uratoxydans* sp. nov. and *Arthrobacter sulfureus* sp. nov. and reclassification of *Brevibacterium protophormiae* as *Arthrobacter protophormiae* comb. nov. *Syst Appl Microbiol* **4**, 470–486.

Stackebrandt, E., Koch, C., Gvozdiak, O. & Schumann, P. (1995). Taxonomic dissection of the genus *Micrococcus*: *Kocuria* gen. nov., *Nesterenkonia* gen. nov., *Kytococcus* gen. nov., *Dermacoccus* gen. nov., and *Micrococcus* Cohn 1872 gen. emend. *Int J Syst Bacteriol* **45**, 682–692.

Stevens, T. O. & Holbert, B. S. (1995). Variability and density-dependence of bacteria in terrestrial subsurface samples: implications for enumeration. *J Microbiol Methods* **21**, 283–292.

Stevenson, I. L. (1967). Utilization of aromatic hydrocarbons by *Arthrobacter* spp. *Can J Microbiol* **13**, 205–211.

Swofford, D. L. (2000). PAUP* 4.0, beta version 4a. Sunderland, MD: Sinauer Associates.

Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal RNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.

Woese, C. (1987). Bacterial evolution. *Microbiol Rev* **51**, 221–271.

Wollum, A. G., II (1982). Cultural methods for soil microorganisms. In *Methods of Soil Analysis*, *Part 2: Chemical and Microbiological Properties*, 2nd edn, pp. 781–782. Edited by A. L. Page. Madison, WI: American Society for Agronomy.

Zheng, M. & Kellog, S. T. (1994). Analysis of bacterial populations in a basalt aquifer. *Can J Microbiol* **40**, 944–954.

Received 7 October 1999; revised 25 February 2000; accepted 1 March 2000.