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Ecology, physiology, and phylogeny of deep subsurface *Sphingomonas* sp

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Several new species of the genus *Sphingomonas* including *S. aromaticivorans, S. stygia*, and *S. subterranea* that have the capacity for degrading a broad range of aromatic compounds including toluene, naphthalene, xylenes, *p*-cresol, fluorene, biphenyl, and dibenzothiophene, were isolated from deeply-buried (>200 m) sediments of the US Atlantic coastal plain (ACP). In *S. aromaticivorans* F199, many of the genes involved in the catabolism of these aromatic compounds are encoded on a 184-kb conjugative plasmid; some of the genes involved in aromatic catabolism are plasmid-encoded in the other strains as well. Members of the genus *Sphingomonas* were common among aerobic heterotrophic bacteria cultured from ACP sediments and have been detected in deep subsurface environments elsewhere. The major source of organic carbon for heterotrophic metabolism in ACP deep aquifers is lignite that originated from plant material buried with the sediments. We speculate that the ability of the subsurface *Sphingomonas* strains to degrade a wide array of aromatic compounds represents an adaptation for utilization of sedimentary lignite. These and related subsurface *Sphingomonas* spp may play an important role in the transformation of sedimentary organic carbon in the aerobic and microaerobic regions of the deep aquifers of the ACP.

Keywords: *Sphingomonas aromaticivorans*; *S. stygia*; *S. subterranea*; deep subsurface; aromatic compound; plasmid

Introduction

Over the past few decades, there has been increasing scientific and practical interest in the microbiology of deep terrestrial subsurface environments. Microorganisms of diverse phylogeny and physiology [7,8,34,36] inhabit deeply buried rocks and sediments [5,24,45,57], often surviving on low concentrations of nutrients but carrying out a broad array of geochemical reactions. These reactions typically involve oxidation of organic carbon, or reduced inorganic compounds such as H_2 in the case of lithotrophic bacteria, coupled to reduction of electron acceptors. Thus, microorganisms exert a major influence on groundwater geochemistry via their metabolic activities. This is evidenced by changes in redox (Eh) along aquifer flow paths [13,63] or in localized 'microsites' within aquifers [40] and changes in isotope abundance of such elements as C and S that are fractionated by microorganisms during metabolism [18,38,46].

During the mid-1980s, the US Department of Energy (DOE) initiated efforts to investigate the presence, distribution, and function of microorganisms in deeply buried sediments of the Atlantic Coastal Plain beneath the Savannah River Site (SRS) [28]. At the time, scientific information was unavailable and the DOE was interested in whether such microorganisms, if present, were capable of degrading organic compounds that were contaminating groundwater at the site as a result of past activities. These activities involved production of weapons grade nuclear materials. To this end, multiple bore holes were drilled across the SRS where a series of cores were collected with depth using methods designed to limit and measure contamination during drilling and after their collection [47,53]. Microbiological analyses of the cores revealed a relatively diverse and active microbial population in aquifer sediments in contrast to the low-permeability, fine-grained strata where microbial populations were at or below limits of detection [5,26,49]. Somewhat unusual was the high percentage of the total population of bacteria, as determined by direct microscopic counts, in some of the aquifer sand samples that could be cultured on aerobic heterotrophic media [57]. Bacteria that were isolated in this manner were physiologically [6,24] and phylogenetically characterized [8]. Recognizing the potential scientific and commercial importance of microorganisms cultured from these samples, the DOE established the Subsurface Microbial Culture Collection (SMCC), at Florida State University [3] and Portland State University.

Because of DOE's interest in the ability of indigenous subsurface microorganisms to degrade organic contaminants, some of the organisms in the SMCC were evaluated for this ability. As a result of these efforts, a bacterium was identified from the Middendorf Formation (410 m depth), strain F199, that could use a broad range of aromatic compounds, including toluene, naphthalene, dibenzothiophene, salicylate, benzoate, *p*-cresol, and all isomers of xylene as sole sources of carbon and energy [25]. Phylogenetic and lipid analyses revealed that this organism and five other isolates with similar catabolic properties from deep ACP sediments were members of the genus *Sphingomonas* [23]. Detailed taxonomic analyses, including 16S rRNA gene sequence analysis, DNA–DNA reassociation, BOX-PCR genomic fingerprinting, and cellular lipid composition of these isolates indicated that they were distinct from pre-

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viously described species of *Sphingomonas* [7]. Among these strains, three new species were established: *Sphingomonas aromaticivorans* (type strain, SMCC F199), *S. subterranea* (type strain, SMCC B0478) and *S. stygia* (type strain, SMCC B0712).

Herein, we review the characteristics of the subsurface environments from which these strains were derived to provide insights into their function in and adaptation to such environments. We also describe the physiological and phylogenetic characteristics of subsurface *Sphingomonas* spp, the extent of their distribution in the subsurface (especially in deep ACP sediments), and investigations into the survival and maintenance of catabolic activity in *S. aromaticivorans* in aquifer sediment microcosms.

The Atlantic Coastal Plain subsurface environment

The ACP sediments from which the *Sphingomonas* spp described above were derived are geologically complex and represent diverse depositional environments that range from upper to lower delta plain [54]. The upper delta plain consists of fluvial sediments deposited in a relatively high energy environment and are generally coarse textured and contain low concentrations of organic matter (lignite). In contrast, the lower delta plain consists of fine-grained (clay- and silt-sized particles) sediments; lignite and iron sulfides are common. The formation names, lithologies and textures of sediment core samples collected from borehole P24 on DOE's SRS are shown in Table 1. Since the southeast region of the US is relatively stable from a tectonic standpoint, these sediments have not been subjected to extremes in temperature or pressure which can occur at greater depth or in tectonically active regions. Hence, nutrients, including sedimentary organic carbon and microorganisms associated with the sediments would not have been exposed to high temperatures which would have promoted thermal maturation of organic matter and death of microorganisms. The lack of significant physical change suggests that organisms originally associated with the sediments at the time of deposition could be 'ancestors' of microorganisms recently cultured from the cored sediments, especially if they were adapted to using scarce endogenous nutrients. Other researchers suggested that subsurface microorganisms may be descendants of microorganisms associated with sediments at the time of deposition [9,17,27], but such a hypothesis has proven difficult to test rigorously.

Viable aerobic heterotrophic bacteria were common throughout the ACP sediments, but their population size varied from below detection to 6.7 log CFU g^{-1} (Table 1; [5,6]). Their population density was negatively correlated with clay content [26], but also varied significantly within a given formation [24]. Intact bacterial cells were present in all samples and their density ranged from 6.5 to 7.5 log cells g^{-1} (Table 1; [57]); in some cases the population of viable aerobic heterotrophs was nearly the same as the population determined by AODC, such as in the sample from the Dry Branch Formation. In other samples, such as those from 255 and 265 m depths in the Middendorf Formation, the proportion of the total population that could be cultured aerobically on agar media was very low or zero.

The sources of organic carbon that could potentially support heterotrophic microbial populations in ACP sediments are dissolved organic carbon (DOC), transported with infil-

Table 1 Lithology and characteristics of core samples collected from borehole P24 on DOE's Savannah River Site in South Carolina, USA

Formation[a]	Lithology	Depth (meters)	% Clay/% sand	Viable counts, log CFU g^{-1}[b]	Direct counts, log cells g^{-1}[c]
Tobacco Road	Laminated or mottled, clayey, very fine to fine sands and sandy clays	34	12/85	4.1	7.0
Dry Branch	Laminated clays and clayey and clean, fine to medium sands	45	9/59	6.4	6.5
McBean	Glauconite to very fine to medium, clean 'beach' sand and calcareous sand	58	14/72	4.8	nd
Congaree	Well-sorted, fine to very coarse, clean sands	91	7/87	6.5	7.2
Ellenton	Laminated, dark, lignitic clays, silts, and very fine sands to medium to coarse sands	118	10/83	5.8	7.1
Ellenton	″	139	9/90	3.2	7.1
Pee Dee	Medium to coarse sands with thick interbedded clay layers	145	45/45	3.2	7.0
Pee Dee	″	180	10/83	6.7	7.2
Black Creek	Lignitic and sulfidic sands with thick layers of clay and silt	200	5/90	5.0	7.2
Black Creek	″	204	32/48	5.0	7.0
Black Creek	″	234	67/17	2.1	6.5
Middendorf	Angular, slightly silty fine to very coarse sands	244	8/87	4.4	7.5
Middendorf	″	255	52/26	ng[d]	6.9
Middendorf	″	259	11/84	6.5	7.5
Middendorf	″	265	nd[e]	2.5	7.2

[a]For more detailed descriptions of these formations see Sargent and Fliermans [54].
[b]From dilution plate counts on 1% PTYG agar [6].
[c]Acridine Orange direct microscopic counts [57].
[d]ng = no growth.
[e]nd = not determined.

trating meteoric water, or sedimentary organic carbon. In the Middendorf aquifer, the origin of *S. aromaticivorans* strains F199 and B0695 and *S. stygia* B0712, the dissolved organic carbon concentrations are generally very low, <0.5 mg L^{-1}, in groundwater samples collected from wells [40]. Once removed, probably by microbial metabolism, the DOC in groundwater can only be replenished from sedimentary organic matter as water travels along a flowpath. Sedimentary organic carbon is present in low permeability confining beds and in aquifers of the ACP at concentrations ranging from 0.02 to 1.7% (wt/wt) [17]. Sedimentary organic carbon also occurs as lignite inclusions in ACP aquifer sediments and has been hypothesized to be an important source of organic carbon for *in situ* heterotrophic metabolism [40]. Rates of bacterial CO_2 production in deep ACP sediments ranged from below detection to 2750 nmol of CO_2 g^{-1} of sediments day^{-1} [38]. Unamended anaerobic slurries of ACP deep subsurface sediments, including some of those listed in Table 1, accumulated acetate and methane in quantities far exceeding those added in amendments or present as DOC [31], providing additional evidence for the presence of metabolizable sedimentary organic carbon.

Murphy *et al* [40] analyzed particulate organic carbon from a 'black sand' sample and from a lignite inclusion from the Middendorf Formation. Carbon 13 NMR analyses of these materials revealed major proportions of both aliphatic and aromatic structures. The lignite contained 2-, 3-, and 4-ring partially-hydrogenated aromatic compounds, some of which were alkylated (ketone and alcohol groups) as determined by GC-MS analysis of CO_2 isopropanol extracts. In order for extant heterotrophic microorganisms to utilize effectively such complex and heterogeneous material as a carbon and energy source, catabolic enzymes with relaxed substrate specificity, as observed among aromatic-degrading *Sphingomonas* strains from ACP sediments [23], would be of use.

One of the interesting and yet puzzling aspects of *Sphingomonas* spp being present (and relatively common; see following section) in deep ACP sediments that are mostly anoxic is their apparent strict aerobic metabolism. There are several potential explanations for this, although all are currently speculative. Along the flow path of many aquifers, including the Middendorf, there is a clear change in oxidation-reduction potential with distance from the recharge region that reflects the progressive utilization of electron acceptors with an increasingly lower E_0 [40], including O_2, Fe(III), SO_4^{2-}, and CO_2. In the subsurface near the recharge zone, Murphy *et al* [40] measured DO in Middendorf groundwater ranging from 9.4 to 3.9 mg L^{-1}; in groundwater collected from wells that were significantly further downgradient the DO was <0.1 mg L^{-1}. A contributing factor to longevity of DO (and sedimentary organic carbon) in ACP aquifers is the extremely slow rate of *in situ* microbial respiration. Estimates of *in situ* microbial metabolism based on geochemical modeling of CO_2 evolution indicate the respiration rates in the Middendorf range from 2.9×10^{-5} to 6.6×10^{-6} mg CO_2 L^{-1} $year^{-1}$ [17]. These low rates of metabolism make deep ACP sediments among the most oligotrophic environments known where microorganisms are actively respiring [17]. These slow rates of metabolism are consistent with an estimated average doubling time for bacteria in ACP aquifer sands on the order of centuries [48]. Hence, the *in situ* rate of O_2 consumption via microbial respiration would also be extremely slow and may, in part, explain how bacteria with strictly aerobic respiratory metabolisms may survive and function in the deep ACP sediments.

Although the *Sphingomonas* strains isolated from the ACP are apparently strict aerobes, *S. aromaticivorans* F199 preferred microaerobic conditions (40–80 μM) for growth in mineral salts medium with aromatic hydrocarbons, glucose, acetate, or lactate as the sole carbon sources [25]. These results suggest that *S. aromaticivorans* F199 is adapted to the low DO concentrations that occur over extended areas of deep ACP aquifers or, alternatively, to low concentrations of organic substrates and slow rates of metabolism. Although none of the *Sphingomonas* strains from the subsurface are able to grow anaerobically with nitrate as the electron acceptor (J Fredrickson, unpublished results), the subsurface strains of *Sphingomonas* isolated from ACP sediments and characterized have not been exhaustively tested for their ability to utilize alternative electron acceptors. Recent studies have demonstrated that some bacteria, such as members of the genus *Thermus*, previously believed to have a predominantly aerobic metabolism, can grow under anaerobic conditions with NO_3^- [51], Fe(III) or S^0 [34] as the sole electron acceptors. Thus, until these *Sphingomonas* strains have been thoroughly tested, it is not possible to state conclusively that they have a strictly aerobic metabolism.

Distribution of *Sphingomonas* spp in the deep subsurface

Most of what is currently known about the occurrence and distribution of *Sphingomonas* strains in the subsurface is based on analysis or screening of bacterial strains that were cultured from various subsurface environments. *Sphingomonas* typically produces rather distinctive dark yellow or yellow-orange colonies when grown on media such as R2A or 1% PTYG agar that have been routinely used to culture aerobic heterotrophic bacteria from subsurface sediments [4,29]. Therefore, this characteristic can be used as an initial screen to detect *Sphingomonas* strains among large numbers of isolates. Methanol-extracted *Sphingomonas* pigments display a characteristic absorption spectrum [50], providing a relatively easy and rapid method to verify which yellow-pigmented colonies are members of the genus *Sphingomonas*. The phylogenetic relatedness of candidate isolates to the genus *Sphingomonas* (and previously described species thereof) is most easily determined by analysis of 16S ribosomal RNA gene sequences [7,61,62]. Another defining characteristic of members of the genus *Sphingomonas* is the presence of sphingolipids [65].

Modern molecular biological methods make it possible to detect *Sphingomonas* strains in subsurface environmental samples directly, without having to culture and analyze individual isolates. One approach that has worked successfully involves direct extraction of nucleic acids from sediment, rock, or water samples, followed by PCR amplification of 16S rRNA genes, cloning of amplification products, and phylogenetic analysis of cloned sequences

[14–16,43,44]. Cloned sequences closely related to the genus *Sphingomonas* were detected by this approach in a low biomass paleosol (ancient soil) in a sample obtained from 188 m beneath the ground surface at DOE's Hanford Site in south-central Washington [14]. It may also be possible to detect the presence of *Sphingomonas* strains directly with 16S rRNA-based oligonucleotide probes.

To date, *Sphingomonas* spp have been found to occur in several geochemically and hydrologically distinct subsurface environments (Table 2). *Sphingomonas* strains have been isolated or detected among clones of 16S rDNA extracted from sediment, from Atlantic coastal plain sediments (mostly sands) at the Savannah River Site near Aiken, South Carolina [23], the Hanford Site in south-central Washington [15] and in shallow subsurface sediments from a site near Oyster, Virginia (D Balkwill, unpublished results). At the former site, *Sphingomonas* isolates were obtained from four different boreholes located several kilometers apart, as well as from several different depths and geological formations within these boreholes (Table 2).

Phylogeny of subsurface *Sphingomonas* strains

Analysis of 16S ribosomal RNA gene sequences has become a standard method for studying phylogenetic relationships among bacteria. To date, only a limited number of *Sphingomonas* strains isolated from subsurface environments have been examined in this way. A phylogenetic tree indicating how these isolates are related to one another and to previously described species of *Sphingomonas* is shown in Figure 1.

Analyses of 16S rRNA sequences indicate that the subsurface *Sphingomonas* isolates examined thus far are phylogenetically distinct from previously described species of the genus, often appearing in distinct clusters and/or on separate branches of the phylogenetic tree. Five isolates from ACP sediments at the Savannah River Site, for example, formed a distinct and well-defined cluster (Figure 1) within the overall tree. This cluster also contained two strains of *Sphingomonas capsulata*, but the *S. capsulata* and subsurface strains were assigned to distinct branches within the cluster. These results indicate that the subsurface isolates are likely to be novel species of *Sphingomonas*. However, it is necessary to carry out additional analyses, such as DNA-DNA reassociation studies and/or characterization of cell lipids, to confirm this. When such analyses were performed on the five Savannah River Site subsurface *Sphingomonas* isolates [7], it was shown that these isolates represent three new species: *S. aromaticivorans*, *S. subterranea* and *S. stygia*. These strains were examined in detail because they degrade a variety of aromatic compounds that frequently occur as environmental contaminants. Interestingly, the subsurface strains are not closely related to strains of *Sphingomonas* such as *S. yanoikuyae*

Table 2 List of *Sphingomonas* strains identified from deep terrestrial subsurface environments

Organism	SMCC strain(s)[a]	Characterization method[b]	Site/formation	Borehole/depth (m)[c]
Sphingomonas sp	A0035	pigment	ACP/McBean	P28/31
Sphingomonas sp	D0092,D0102	pigment	ACP/Ellenton	C10/194
Sphingomonas sp	D0117, D0194	pigment	ACP/Ellenton	C10/213
Sphingomonas sp	A1033, A1034	pigment	ACP/Congaree	P28/59
Sphingomonas sp	A0463	pigment	ACP/Pee Dee	P28/112
Sphingomonas sp	B0477	16S, lipids, pigment	ACP/Pee Dee	P24/180
Sphingomonas subterranea	B0478[d]	16S, lipids, pigment	ACP/Pee Dee	P24/180
Sphingomonas sp	F0086	pigment	ACP/Pee Dee	C10/290
Sphingomonas aromaticivorans	B0522	16S, lipids, pigment	ACP/Black Creek	P24/204
Sphingomonas sp	A0225, A0229, A0242, A0243	pigment	ACP/Black Creek	P28/134
Sphingomonas sp	C0419, C0422, C0427	pigment	ACP/Middendorf	P29/151
Sphingomonas sp	C0480	pigment	ACP/Middendorf	P29/176
Sphingomonas sp	C0563	pigment	ACP/Middendorf	P29/187
Sphingomonas sp	A0564	pigment	ACP/Middendorf	P28/180
Sphingomonas sp	A0691, A0742, A0743, A0757, A0858,	pigment	ACP/Middendorf	P28/191
Sphingomonas sp	C0662, C0667, C0669, C0672	pigment	ACP/Middendorf	P29/213
Sphingomonas sp	B0615	pigment	ACP/Middendorf	P24/244
Sphingomonas aromaticivorans	B0695	16S, lipids, pigment	ACP/Middendorf	P24/259
Sphingomonas stygia	B0712[d]	16S, lipids, pigment	ACP/Middendorf	P24/259
Sphingomonas sp	B0713	pigment	ACP/Middendorf	P24/259
Sphingomonas sp	F0203	pigment	ACP/Middendorf	C10/375
Sphingomonas aromaticivorans	F199[d]	16S, lipids, pigment	ACP/Middendorf	C10/407
Sphingomonas sp	F0116	pigment	ACP/Middendorf	C10/407
Sphingomonas sp	F0069	pigment	ACP/Middendorf	C10/408
Sphingomonas sp	F0042	pigment	ACP/Middendorf	C10/413
Sphingomonas sp	H0349	pigment	Hanford/Ringold	YB/102
Sphingomonas sp	Clone group Q, S	16S (direct extraction)	Hanford/paleosol	YB/188

[a]SMCC = US Department of Energy Subsurface Microbial Culture Collection at Florida State University.
[b]16S = 16s rRNA gene sequence analysis; lipid = presence or predominance of one or more lipids that are characteristic of *Sphingomonas*; pigment = pigment profile characteristic of *Sphingomonas*.
[c]For information on boreholes P24, P28 and P29, see reference [5]; for the C10 borehole, see reference [40].
[d]Type strain.

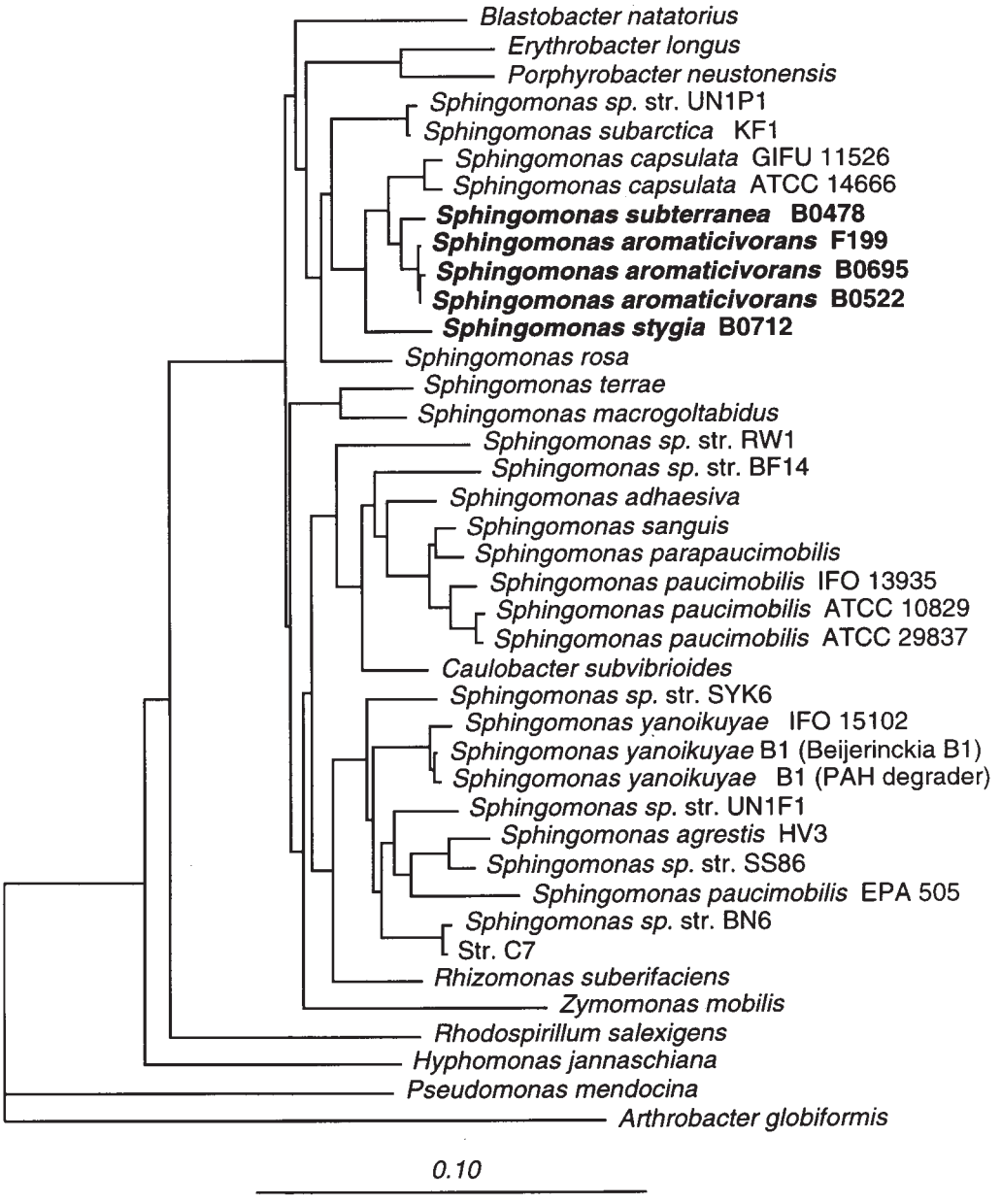

Figure 1 Phylogenetic tree for subsurface *Sphingomonas* isolates and selected strains of eubacteria based on a distance matrix analysis. The PHYLIP program [21] was used to calculate distances by the method of Jukes and Cantor [32], after which the FITCH option [22] was used to estimate phylogenies from distance matrix data. The analysis included a region of the 16S rRNA gene corresponding to *E. coli* positions 30–1375 [12], from which a few small segments of sequence were excluded because the alignment was ambiguous in those regions. A total of 1292 bases were retained for analysis. *Arthrobacter globiformus* was used as the outgroup. Scale bar = 10 substitutions per 100 bases. Parsimony analysis followed by bootstrapping produced virtually identical results in regard to the phylogenetic positions of the subsurface *Sphingomonas* strains [7].

B1 [33] or *S. paucimobilis* Q1 [37] that degrade a similar range of aromatic compounds (Figure 1), indicating that they most likely have a distinct evolutionary history. Additional evidence for a distinct evolutionary history has been found by detailed analysis of degradative genes in the subsurface isolates and the plasmids on which those genes are situated [52,59]; homologous genes are located on the chromosome in *S. yanoikuyae* B1 [35], *S. agrestis* HV3, *S. paucimobilis* Q1, and *Pseudomonas* sp DJ77 [52].

Catabolic characteristics of ACP subsurface *Sphingomonas* strains

Members of the genus *Sphingomonas* are well-recognized for their capacity to degrade a wide range of 'exotic' organic compounds that includes: pentachlorophenol [65] (Crawford and Ederer, this issue); dibenzo-*p*-dioxin and dibenzofuran [64]; hexachlorocyclohexane [30] (Nagata *et al*, this issue); chlorinated biphenyls [60]; halogenated diphenyl ethers [55] (Keim *et al*, this issue); naphthalene

Table 3 Growth on, or catabolism of, aromatic organic compounds by select subsurface *Sphingomonas* strains

Compound	Relative growth[a] or ability to clear agar plates[b] of strain:					
	F199	B0477	B0478	B0522	B0695	B0712
Toluene[a]	+	–	+	+	–	+
Naphthalene[a]	+	–	+	+	+	–
p-Cresol[a]	++	–	–	++	++	–
o-Xylene[a]	+	–	+	–	+	+
m-Xylene[a]	++	–	–	++	++	++
p-Xylene[a]	++	–	++	–	–	–
Salicylate[a]	++	+	++	++	++	++
Benzoate[a]	+	+	+	+	+	+
Acenaphthene[a]	–	nd[c]	–	+	+	+
Anthracene[a]	–	nd	–	–	–	–
Phenanthrene[a]	+	nd	+	++	++	++
2,3-Dimethylnaphthalene[a]	+	nd	++	++	++	++
Fluorene[a]	+	–	+	+	+	+
Biphenyl[a]	+	–	+	+	+	+
Dibenzothiophene[a]	+	–	+	+	+	+
Syringate[a]	++	++	++	++	++	–
Vanillate[a]	++	++	++	++	++	–

[a]Symbols: –, no growth; + and ++, relative robustness of growth compared with that of a control consisting of cells on mineral salts agar without an exogenous carbon source.
[b]Relative ability to clear mineral salts agar plates sprayed with ethereal solutions of target compound. Symbols: –, no clearing; +, significant clearing around colonies.
[c]nd = not determined.

sulfonic acids [37] (Stolz, this issue); methyl-substituted naphthalenes [19]; polyaromatic hydrocarbons [39] (Ho *et al*; Kim and Zylstra, this issue); nonylphenol [62]; polyethylene glycol [61]; and dimeric model compounds of lignin such as syringate and vanillate [41]. In addition, a number of bacteria previously classified as belonging to genera other than *Sphingomonas* that have been studied because of their interesting or novel catabolic activities have been reclassified as *Sphingomonas* [33,42,65].

S. aromaticivorans strain F199, originally identified and studied because of its ability to grow on a wide range of aromatic compounds as sole sources of carbon and energy [25], is one of several strains of *Sphingomonas* isolated from ACP subsurface sediments that can catabolize a wide range of aromatic compounds [23]. Examples of the aromatic compounds that these strains can grow on or can catabolize are shown in Table 3. Interestingly, among the subsurface *Sphingomonas* strains listed in Table 2, A0035, A0229, A0463, A0564, A0691, A0742, A0743, A0858, A1033, A1034, B0615, B0713, C0419, C0422, C0622, C0667, and C0669, in addition to those listed in Table 3, also have *meta*-cleavage dioxygense activity. Catechol is a common intermediate produced during bacterial catabolism of many aromatic compounds. Hence, it appears that the ability to degrade aromatic compounds is relatively widespread among *Sphingomonas* isolates from ACP subsurface sediments.

A novel aspect of the genes encoding enzymes involved in the degradation of aromatic compounds in *S. aromaticivorans* F199 is that they are encoded on a 184-kilobase conjugative plasmid, pNL1 [59], that has now been completely sequenced and annotated [52]. Evidence presented by Kim *et al* [35] indicate that genes for aromatic hydrocarbon degradation are also plasmid-encoded in other subsurface strains, including *S. subterraneae* B0478, *S. stygia* B0712, and *S. aromaticivorans* B0522 and B0695. *S. aromaticivorans* B0695 harbors two plasmids that share some homology with strain F199 plasmid-encoded catabolic genes [35]. Homologous genes are also plasmid-encoded in *Sphingomonas* sp strain HV3 [65] but, in contrast, homologous genes in *S. yanoikuyae* strain B1 are located on the chromosome. Having relaxed specificity, catabolic genes that are encoded on conjugative plasmids may reflect a general strategy in subsurface bacteria to maximize the ability to utilize such complex sediment-associated organic carbon as lignite while minimizing energy requirements of the community for maintaining such capabilities. All the subsurface *Sphingomonas* strains, except for B0712, exhibited extensive growth on defined mineral medium with the lignin monomers vanillate and syringate (Table 3), indicating that these strains are adapted for utilization of naturally-occurring aromatic compounds.

In contrast to procedures typically used for enrichment and isolation of biodegradative bacteria, subsurface *Sphingomonas* strains described here were isolated on a low-nutrient medium, 1% PTYG, formulated for culturing aerobic heterotrophic bacteria [4]. More typically, organisms are enriched in medium that contains the target organic compound as the dominant or sole organic carbon compound for growth and energy, often using sediments or soil that have been exposed to the compound as the inoculum source. Such procedures often select for bacteria that can grow rapidly, potentially outcompeting other organisms that may grow more slowly under the specific enrichment conditions. Bacteria enriched in such a manner may not be the most environmentally competitive or the most effective at degrading the target contaminant(s) *in situ*.

Survival of *S. aromaticivorans* B0695 and maintenance of its catabolic activity in subsurface sediments

As noted earlier, several *Sphingomonas* isolates from subsurface environments have been shown to degrade a range of aromatic compounds under laboratory conditions. However, these organisms would have to survive and maintain their degradative abilities under typical subsurface conditions for extended periods of time in order to be of significance in regard to influencing the fate of aromatic contaminants in subsurface environments. To address this issue, Shi [56] studied the ability of *S. aromaticivorans* strain B0695 (Table 2), to survive and maintain its degradative activities under conditions simulating those in Atlantic coastal plain aquifers.

Survival of *S. aromaticivorans* B0695 was initially studied by inoculating washed, exponential-phase cells into microcosms consisting of sterilized Atlantic coastal plain aquifer sediments (inoculum density 5×10^8 cells g^{-1}) and then monitoring the population of viable cells over time by dilution plate count. The microcosms were adjusted to 80% of the maximum water holding capacity but were not

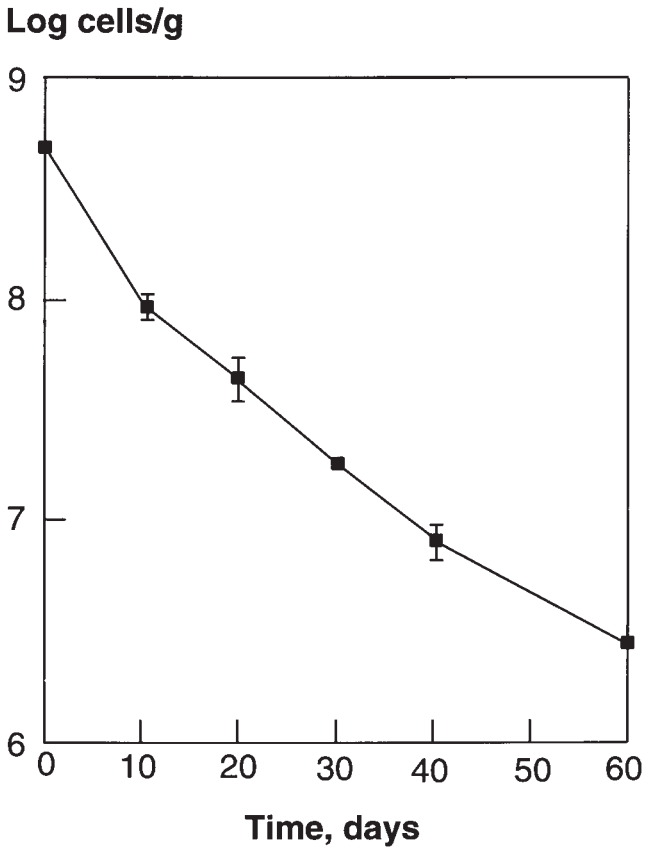

Figure 2 Numbers of viable cells of *S. aromaticivorans* B0695 during extended incubation in unamended sterilized subsurface sediment. Abiotic conditions in the microcosms were: pH 7, 80% WHC, and incubation temperature 25°C. Error bars represent standard errors of the means from three replicates and are shown only when they are larger than the size of the markers for the data points.

amended with nutrients. They were incubated in the dark at 25°C for up to 60 days and hand-shaken every 3 days. Viable cell populations decreased by approximately two orders of magnitude over the 60-day incubation period, with the sharpest decline occurring during the first 10 days (Figure 2). These results indicate that the relatively large cell populations used as the inoculum could not be sustained in the unamended sediment. However, the population of viable cells appeared to level off after 40 days, suggesting that the *S. aromaticivorans* population approached a sustainable level. The decrease in cell numbers followed a pattern that was similar to that observed by Amy and Morita [1], who studied the starvation survival patterns of 16 marine bacterial isolates in buffered salts medium. Moreover, the 50% survival time calculated from the change in cell numbers over 60 days was 8.1 days, which is longer than those reported for a variety of different bacterial species subjected to extreme starvation [10]. It would appear that *S. aromaticivorans* B0695 is reasonably well adapted to survival under nutrient-poor conditions.

To assess the ability of *S. aromaticivorans* B0695 to degrade naphthalene, as measured by the loss of parent compound, after storage (and survival) in unamended Atlantic coastal plain sediments, microcosms were inoculated and incubated as described above. Naphthalene ($30\ mg\ L^{-1}$) and a small amount of Stanier's mineral salt broth [58] as a source of mineral nutrients were added to the microcosms after 0, 10, 20, 30, 40, or 60 days of incubation, after which the residual naphthalene concentrations were monitored by HPLC analysis. Naphthalene was readily degraded, and at similar rates, regardless of how long the *S. aromaticivorans* cells were incubated in unamended sediments prior to naphthalene addition (Figures 3 and 4). Thus, *S. aromaticivorans* survived in a nutrient-poor environment for extended periods of time and retained the ability to degrade and respond quickly to the presence of naphthalene.

It is interesting that *S. aromaticivorans* B0695 responded to naphthalene quickly during this experiment because there was no attempt to induce the enzymes required for naphthalene degradation prior to inoculating the bacterium into unamended sediment. Rather, it was cultured in 5% PTYG [4], a medium that is rich in such simple organic compounds as glucose, but that does not contain naphthalene. With some microorganisms used for bioremediation, efficient degradation of an organic contaminant in the field is obtained only if the microbe is grown on or exposed to the contaminant to induce the necessary enzymes before it is applied in the field [2]. For *S. aromaticivorans* B0695, either enzyme induction is rapid under low nutrient conditions or the enzymes involved in naphthalene catabolism are constituitively expressed. In strain *S. aromaticivorans* F199, the enzymes involved in naphthalene mineralization are not constitutive [25].

At some sites, low levels of organic contaminants are

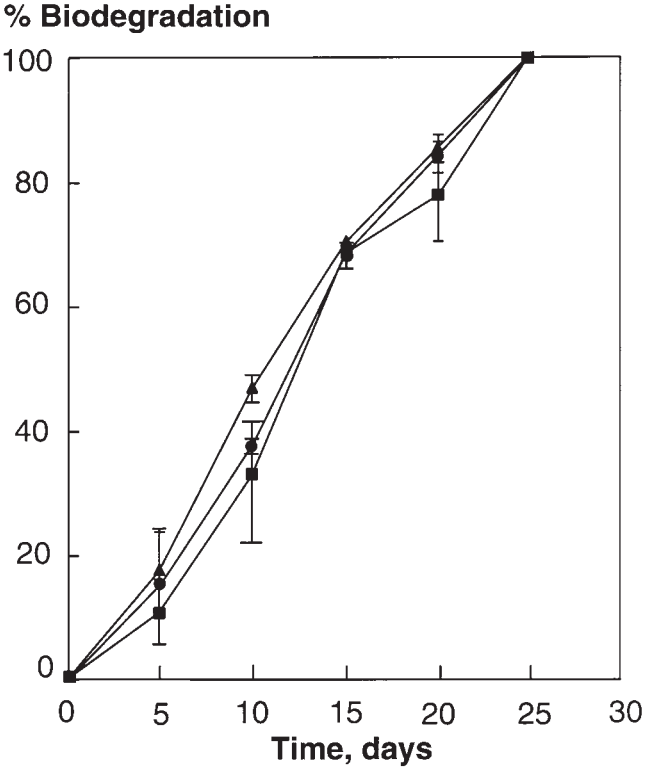

Figure 3 Biodegradation of naphthalene added after incubation of *S. aromaticivorans* B0695 in unamended sterilized subsurface sediment for 0 (—▲—), 10 (—●—), and 20 (—■—) days.

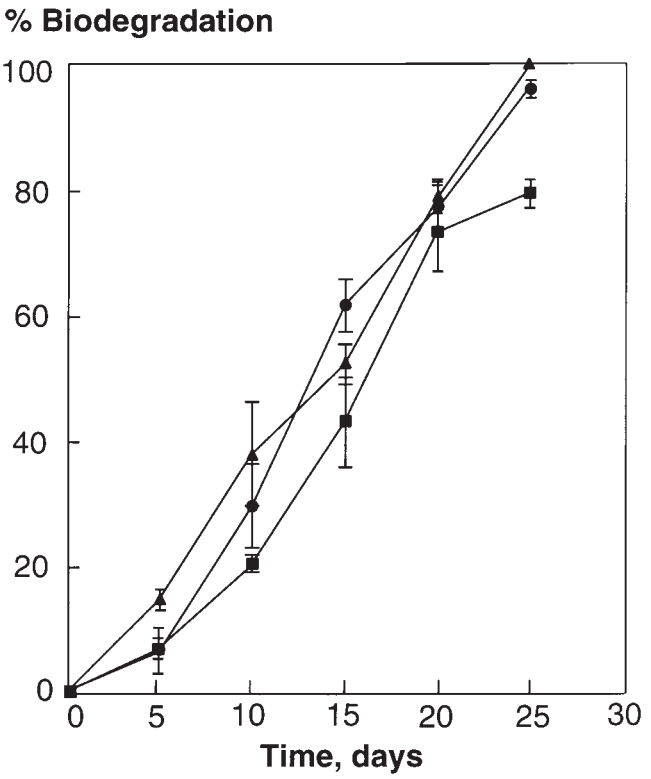

Figure 4 Biodegradation of naphthalene added after incubation of *S. aromaticivorans* B0695 in unamended sterilized subsurface sediment for 30 (—▲—), 40 (—●—), and 60 (—■—) days.

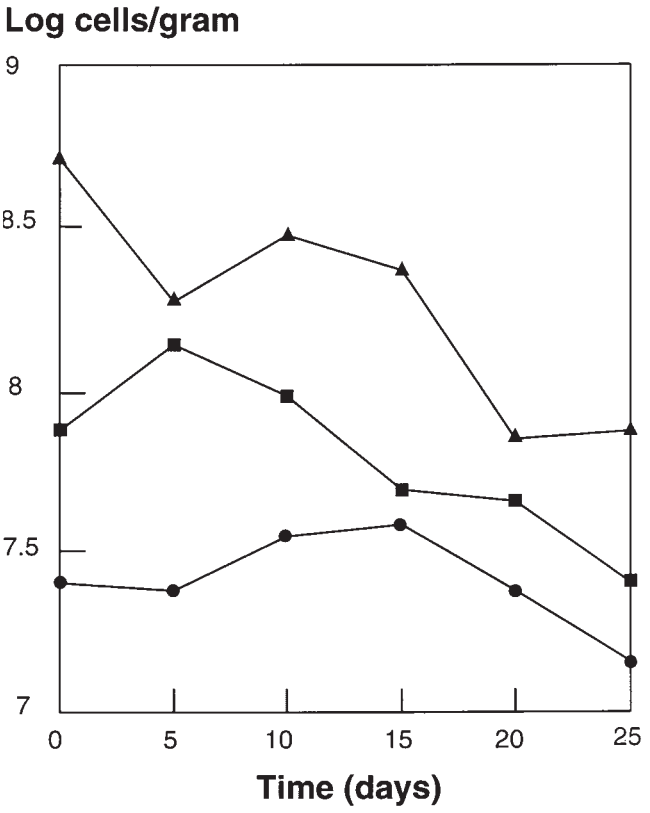

Figure 5 Changes of cell numbers of *S. aromaticivorans* B0695 in microcosm of sterilized subsurface sediment amended with napthalene three times in three consecutive 25-day periods. (—▲—) 0–25 days; (—■—) 26–50 days; (—●—) 51–75 days. Data are means of six or more counts. Conditions in microcosms were: pH 7, 80% WHC, 30 ppm naphthalene, incubation temperature 25°C with nutrient amendment.

released into a subsurface environment periodically, either from occasional accidental discharges from production facilities or as the result of leaching from adjacent (and more heavily contaminated) zones after rainfall events. Therefore, it is of interest to know how well an introduced degradative microorganism survives and maintains its degradative ability in an environment that is subjected to periodic influxes of contaminants. This was investigated by monitoring the survival and naphthalene-degrading activities of *S. aromaticivorans* B0695 for a 75-day period in microcosms of sterile Atlantic coastal plain sediments (inoculated and incubated as described above) to which naphthalene (30 mg L^{-1}) was added after 0, 25, and 50 days of incubation. Cell populations decreased by approximately 1.5 orders of magnitude during the 75-day duration of the experiment (Figure 5), which was smaller than the decrease seen when *S. aromaticivorans* was incubated in unamended sediment for 60 days (Figure 2). Cell numbers increased slightly before continuing to decrease after naphthalene addition at 25 days and 50 days (Figure 5). The largest decrease in cell population occurred during the first 25 days. Decreases following naphthalene addition at 25 and 50 days were progressively smaller, implying that the population of strain B0695 approached a reasonably constant density (about 10^6 cells g^{-1}) that could be maintained by periodic addition of naphthalene to the sediment. The 50% survival time calculated from the change in cell numbers over 75 days was 14.4 days. This is shorter than that of some species of *Arthrobacter* (a genus with unusually high starvation-survival capabilities [11]), but longer than those of many other bacteria (<5 days) tested by Boylen and Ensign [10]. The 50% survival time was also longer than that observed when *S. aromaticivorans* was incubated in the sediment for 60 days without addition of naphthalene (above).

The extent to which *S. aromaticivorans* B0695 degraded naphthalene after it was added to the microcosm sediments at 0, 25, and 50 days in the above experiment is shown in Figure 6. Naphthalene was degraded readily, and at similar rates, after each amendment. Degradation of naphthalene added at day 0 was slightly faster than that observed after subsequent additions, but there was no significant difference between the degradation rates observed after addition of naphthalene at 25 and 50 days. These results demonstrate that *S. aromaticivorans* survived and retained the ability to degrade naphthalene rapidly in a subsurface sediment for an extended period of time, if supplied with small amounts of naphthalene at 25-day intervals. Such abilities are generally considered to be useful in microorganisms that are to be used for *in situ* bioremediation of soils or sediments [2].

Summary

Members of the genus *Sphingomonas* were common among aerobic heterotrophic bacteria cultured from deeply-buried sediments of the US Atlantic coastal plain and have been detected in other subsurface environments. Among the ACP

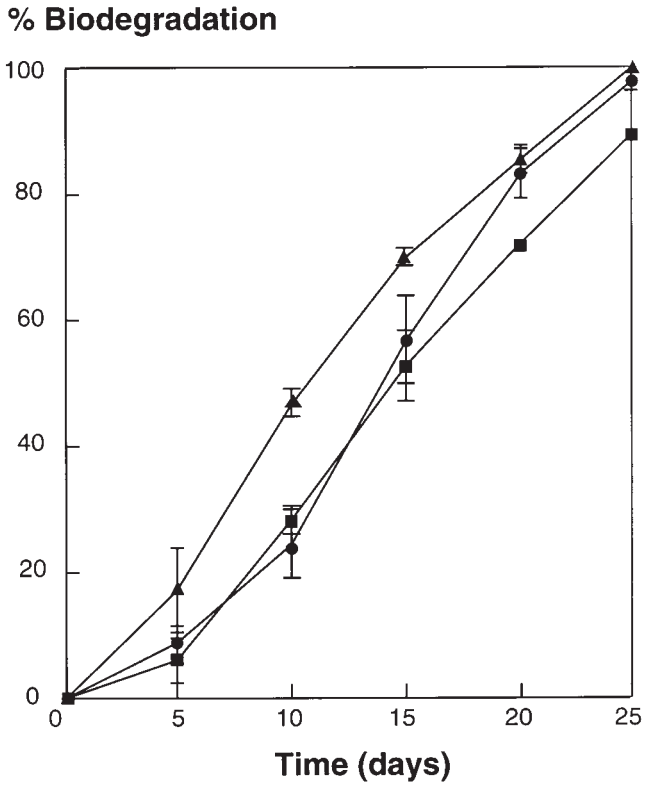

Figure 6 Biodegradation of naphthalene by *S. aromaticivorans* B0695 after incubation in sterilized subsurface sediment amended with MSB and naphthalene (30 ppm) at 0 (—▲—), 25 (—●—), and 50 (—■—) days.

strains were several new species including *S. aromaticivorans*, *S. stygia*, and *S. subterranea* which have the capacity for degrading a broad range of aromatic compounds. Many of the genes involved in catabolism of aromatic compounds in *S. aromaticivorans* F199, and probably the other subsurface strains, are encoded on conjugative plasmids. We speculate that the ability to degrade a wide array of aromatic compounds may be related to the composition of the major source of organic carbon in the ACP aquifers, sedimentary lignite. These and related subsurface *Sphingomonas* spp may play an important role in the transformation of sedimentary organic carbon in the aerobic and microaerobic regions of the deep aquifers of the ACP. The fact that many of the subsurface strains can degrade components of lignin, such as syringic and vanillic acids, supports this hypothesis.

S. aromaticivorans B0695 survived for extended periods in relatively high numbers under nutrient-poor conditions in aquifer sediment microcosms and was able to respond rapidly to contaminant (naphthalene) input up to 60 days after inoculation. These studies indicate that the SCP subsurface *Sphingomonas* isolates may be well adapted to survive under oligotrophic conditions and can rapidly respond to nutrients as they become available. Marine *Sphingomonas* spp, such as strain RB2256, represent a dominant class of ultramicrobacteria ($<0.1\ \mu m^3$) in oligotrophic marine waters [20], providing evidence that other members of the genus *Sphingomonas* are adapted to life under oligotrophic conditions. Additional research is needed to fully resolve the ability of these strains to metabolize natural sources of complex organic carbon such as lignite and to survive under low nutrient conditions, and the mechanisms that allow them to do so.

Acknowledgements

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