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Biodegradation of polycyclic aromatic hydrocarbons by *Sphingomonas* strains isolated from the terrestrial subsurface

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Several strains of *Sphingomonas* isolated from deep Atlantic coastal plain aquifers at the US Department of Energy Savannah River Site (SRS) near Aiken, SC were shown to degrade a variety of aromatic hydrocarbons in a liquid culture medium. *Sphingomonas aromaticivorans* strain B0695 was the most versatile of the five strains examined. This strain was able to degrade acenaphthene, anthracene, phenanthrene, 2,3-benzofluorene, 2-methylnaphthalene, 2,3-dimethylnaphthalene, and fluoranthene in the presence of 400 mg l^{-1} Tween 80. Studies involving microcosms composed of aquifer sediments showed that *S. aromaticivorans* B0695 could degrade phenanthrene effectively in sterile sediment and could enhance the rate at which this compound was degraded in nonsterile sediment. These findings indicate that it may be feasible to carry out (or, at least, to enhance) *in situ* bioremediation of phenanthrene-contaminated soils and subsurface environments with *S. aromaticivorans* B0695. In contrast, strain B0695 was unable to degrade fluoranthene in microcosms containing aquifer sediments, even though it readily degraded this polynuclear aromatic hydrocarbon (PAH) in a defined liquid growth medium. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 283–289.

Keywords: polycyclic aromatic hydrocarbons; PAH degradation; *Sphingomonas*; subsurface bacteria; fluoranthene; phenanthrene

Introduction

Fredrickson *et al* [9] found that five strictly aerobic strains of bacteria isolated from the US Department of Energy Savannah River Site (SRS) aquifers were able to degrade several low-molecular-weight aromatic compounds (including toluene, benzoate, dibenzothiophene, naphthalene, *p*-cresol, and all isomers of xylene) when these compounds were supplied as sole carbon sources in a defined growth medium. It was later shown that this group of isolates represented three novel species of *Sphingomonas* [4]. *Sphingomonas* species isolated from topsoils have also been found to degrade aromatic compounds, and the degradative genes and/or pathways in some of these species have been characterized [17–19,23,25,26]. However, the subsurface aromatic-degrading sphingomonads differ markedly from most of the surface-derived strains in their phylogenetic and genetic traits [10,26,28].

Degradative capabilities of the *Sphingomonas* isolates from the SRS aquifers have been studied mostly using laboratory media. However, Fredrickson *et al* [10] showed that one of these strains, *Sphingomonas aromaticivorans* SMCC B0695, could degrade naphthalene effectively in sediment-based laboratory microcosms designed to simulate the conditions in shallow Atlantic coastal plain aquifers. This strain also survived and retained its ability to degrade naphthalene effectively for an extended time in sterile subsurface sediments.

The *Sphingomonas* strains isolated from the SRS aquifers have been studied only in regard to degradation of relatively simple, low-molecular-weight aromatic compounds. Yet, their potential ability to degrade a broader range of aromatic compounds, including polynuclear aromatic hydrocarbons (PAHs), is of interest because of the toxic nature and relatively high recalcitrance of these compounds. The purpose of this study, then, was twofold: (i) to determine whether the *Sphingomonas* isolates from the SRS could degrade selected PAHs, and (ii) to assess the capacity of one isolate (*S. aromaticivorans* SMCC B0695) to degrade phenanthrene and fluoranthene in microcosms composed of Atlantic coastal plain aquifer sediments. The latter experiments were part of an ongoing effort to evaluate the potential usefulness of the *Sphingomonas* isolates from the SRS in attempts to bioremediate organic subsurface contaminants *in situ*.

Materials and methods

Bacterial strains

Five strains of *Sphingomonas* isolated from Atlantic coastal plain aquifers at the SRS were examined in this study: *S. aromaticivorans* strains F199^T, B0522, and B0695; *Sphingomonas subterranea* strain B0478^T; and *Sphingomonas stygia* strain B0712^T (see Ref. [4]). All strains were obtained from the DOE Subsurface Microbial Culture Collection (SMCC) at Florida State University [2] and cultured in 1% PTYG medium [3], King's medium B [20], or Stanier's mineral salt broth (MSB [27]).

Aromatic hydrocarbon substrates and stock solutions

Seven PAHs were tested as possible substrates for biodegradation and were of the highest purity (>98%) available. Stock solutions (23.5 g l^{-1}) of 2,3-dimethylnaphthalene, 2-methylnaphthalene, acenaphthene, fluoranthene, and phenanthrene (Aldrich, Milwau-

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kee, WI) were prepared in acetone. Stocks (23.5 g l^{-1}) of 2,3-benzofluorine and anthracene (Sigma, St. Louis, MO) were prepared by heating them to 60°C in benzene and chloroform, respectively.

PAH-containing nutrient media

The ability of the *Sphingomonas* strains to use PAHs under laboratory conditions was tested by attempting to grow them in MSB containing one of the PAHs as the sole source of carbon. The MSB media with selected PAHs were prepared by adding an aliquot of stock solution containing 2.0 mg of PAH to each of several sterile, dry, 125-ml hypovials (Pierce Chemical, Rockford, IL). The solvent from the stock solution was allowed to evaporate into cotton plugs for 24 h at 25°C, after which the cotton plugs were discarded and any residual solvent was allowed to evaporate in the dark for several hours. After adding 20 ml of sterile MSB (with or without Tween 80; see below) to each hypovial, the vials were immediately capped with crimp-sealed Teflon™-lined septa and placed on a rotary shaker (at 200 rpm) for 48 h at 25°C (to mix their contents). MSB containing Tween 80 was prepared by adding a filter-sterilized concentrated solution of Tween 80 (in MSB) to bulk-sterilized MSB to a final Tween 80 concentration of 400 mg l^{-1}.

Assay for bacterial growth on PAHs under laboratory conditions

To test their ability to grow on PAHs as sole carbon and energy sources, cultures of the five *Sphingomonas* isolates were grown in 5% PTYG broth and washed twice by centrifugation in 50 mM phosphate buffer. Aliquots (1.0 ml) of washed cells suspended in phosphate buffer were inoculated into hypovials containing MSB (or MSB amended with Tween 80) and one of the seven PAHs listed above (or no PAH, as a control). The initial cell density was approximately 1×10^6 cells ml^{-1}. The cultures were incubated on a rotary shaker in the dark at 25°C. Aeration was provided every other day by opening the caps for 10 min in a sterile, laminar flow hood. The ability of the *Sphingomonas* strains to grow on each PAH was evaluated after 7 days by measuring the turbidity of the culture at 600 nm and comparing it to that of a control culture in PAH-free medium.

Microcosm experiments

The capacity of *S. aromaticivorans* B0695 to degrade phenanthrene and fluoranthene under conditions similar to those in a shallow coastal plain aquifer was tested in microcosms consisting of aquifer sediments from a DOE study site near Oyster, VA. The sediment was collected aseptically from a depth of 6 to 8 m below land surface and maintained at 4°C until used. Sediment pH was measured from a 1:2 suspension of dried sediment in distilled water (wt/vol), after shaking the suspension for 1 h on a rotary shaker [8]. Water holding capacity (WHC) was determined gravimetrically by drying sediment at 105°C to a constant weight (about 12 h). The amount of water retained per g (dry wt) of sediment was calculated and expressed as percent (w/w) [8]. Sediment nitrogen and phosphorous were analyzed using US EPA Methods 351.4 and 365.4 (March 1983 Version), respectively.

Microcosm experiments were performed with sterile or non-sterile sediment (see Results). For those experiments using sterile sediment, the aquifer sediment was air-dried and passed through a 2.0-mm sieve. Twenty-gram portions of the dried sediment were transferred to 125-ml hypovials and autoclaved at 121°C for 30 min on three consecutive days. A small portion of sediment was then inoculated directly onto trypticase soy agar plates and into trypticase soy broth (Beckton Dickinson, Cockeysville, MD) and incubated at 25°C for 7 days as a sterility check. For experiments based on nonsterile sediment, the microcosms were prepared by simply adding an amount of wet sediment (approximately 13.8% water content or 60% WHC) that was equivalent to 20 g dry wt to each hypovial. PAHs were added to the microcosms by placing 100 μl of phenanthrene or fluoranthene stock solution (23.5 mg ml^{-1}) directly onto the sterile or nonsterile sediment in each vial to produce a final concentration of approximately 100 mg kg^{-1}. The contents of the hypovials were mixed thoroughly by hand shaking, after which the ethyl acetate was allowed to evaporate for 24 h at 25°C.

To provide an inoculum, *S. aromaticivorans* B0695 was grown aerobically in 5% PTYG broth [3] at 25°C for 18 h. Exponential-phase cells were harvested by centrifugation, washed twice with 50 mM phosphate buffer (pH 7.0), and resuspended in either MSB or MSB amended with Tween 80. For experiments using sterile sediment, hypovials were inoculated with 3.5 ml of *S. aromaticivorans* B0695 cells in MSB or 3.5 ml of cells in MSB containing 600 mg l^{-1} Tween 80. The MSB in the inoculum brought the inorganic nitrogen and phosphorous concentrations in the microcosm sediment to 27 and 4595 mg kg^{-1}, respectively.

Microcosm experiments using nonsterile sediments included treatments with and without inoculation with *S. aromaticivorans* strain B0695. For the inoculated treatments, each hypovial received 0.88 ml of cells (prepared as described above) in double-strength MSB or 0.88 ml of cells in double-strength MSB amended with 2400 mg l^{-1} Tween 80. The final concentrations of inorganic nitrogen and phosphorous in the sediments were 15 and 2300 mg kg^{-1}, respectively. The final water content of the sediments was 18.4%, or 80% WHC. Initial cell density was approximately 10^8 cells g^{-1} (dry wt). For uninoculated treatments, 0.88 ml of double-strength MSB or 0.88 ml of double-strength MSB amended with 2400 mg l^{-1} Tween 80 was added to each hypovial.

Triplicate microcosms were prepared for each analytical point in each treatment. The microcosms were incubated at 25°C in the dark and aerated by opening their caps for 5 min (in a sterile laminar flow hood) every 3 days. The vials were also hand-shaken every 3 days, to obtain reasonably homogeneous conditions within each microcosm. Zero and end-time abiotic controls were prepared by adding the appropriate amount of MSB (or MSB amended with Tween 80) to hypovials containing sterile sediment.

Assay for biodegradation of fluoranthene in mineral salts broth

S. aromaticivorans B0695 was grown in MSB containing Tween 80 (400 mg l^{-1}) and fluoranthene to confirm that it could degrade this PAH as a sole carbon source under laboratory conditions. Fluoranthene was added to sterile, empty hypovials as described above. A 50-ml aliquot of MSB (with Tween 80) was added to each vial, and the vials were placed on a rotary shaker for 48 h at 25°C. Undissolved fluoranthene was then removed by passing the fluid from each vial through a sterile 0.2-μm Nylon filter (Nalge, Rochester, NY). The vials were inoculated with 0.1 ml of a suspension of washed, exponential-phase *S. aromaticivorans* cells (see above; initial density of approximately 10^6 cells ml^{-1}) and incubated on a rotary shaker at 25°C in the dark. One-milliliter aliquots of medium were removed from duplicate vials at various time points and filtered through 0.2-μm Nylon filters. The fluoranthene concentration in each filtrate was determined by HPLC analysis as described below.

HPLC analyses

Fluoranthene and phenanthrene were extracted from microcosm sediments with 20 ml of methanol by shaking the hypovials on a rotary shaker (at 250 rpm) for 30 min. Three replicate microcosms were extracted for each analytical time point for each set of conditions tested. Hypovials were tightly sealed with Teflon-lined septa and aluminum crimp caps during the extraction procedure, to prevent evaporation of the PAHs. One-milliliter aliquots of the extracts were filtered through a 0.2-μm pore size, solvent-resistant filter (Millipore, Bedford, MA), and 10 to 100 μl of the filtrate was analyzed as described below.

Fluoranthene and phenanthrene concentrations in filtrates from sediment extracts or MSB cultures (above) were determined by reversed-phase HPLC on a Supelcosil LC-PAH analytical column (4.6×250 mm), with Milli-Q deionized water and acetonitrile as mobile phases A and B, respectively. Variations in the linear gradient elution protocols were as follows. For phenanthrene, there was an initial isocratic step at 40% acetonitrile for 5 min, after which the column was developed with a linear increase from 40% to 100% acetonitrile over 25 min, kept at 100% acetonitrile for 5 min, and reduced to 40% acetonitrile over 3 min. For fluoranthene, there was an initial isocratic step at 50% acetonitrile for 5 min, after which the column was developed with a linear increase from 50% to 100% acetonitrile over 25 min, kept at 100% acetonitrile for 5 min, and reduced to 50% acetonitrile over 3 min. The flow rate was held constant at 1 ml min^{-1}, and all analyses were carried out at ambient temperature. The column effluent was analyzed for PAH by measuring absorbance at 210 and 254 nm. The HPLC system consisted of a Beckman 126 solvent delivery system, a Beckman 168 diode array UV detector, an Altex 210A sample injection valve, and the Beckman System Gold software. Quantitation was done using an external standard curve made from dilutions of a standard phenanthrene or fluoranthene solution in methanol.

Enumeration of culturable cells

Bacterial cells were extracted from microcosm sediments by shaking the sediment on a rotary shaker at 250 rpm for 30 min in 50 mM phosphate buffer (pH 7.2). Serial dilutions were prepared in 50 mM phosphate buffer and spread-plated in triplicate on 5% PTYG agar. The plates were incubated aerobically at 25°C and counted after 4 days. Three replicate microcosms were analyzed at each time point (see Results).

Statistical analysis

Standard errors of the means of percentages of PAH degraded in the microcosms from triplicate assays were calculated. A two-way analysis of variance (ANOVA) model was used, with mathematical factors of treatment level and time and interactions between time and treatment level to determine whether the treatment, time, or interaction between treatment and time had a significant effect on biodegradation. To determine specifically if differences in the biodegradation rates among levels of each treatment were significant, mean value comparisons between levels of treatment were made by *post hoc* tests, using Bonferroni. All of the data were analyzed statistically at the 95% confidence level and all statistical analyses were performed using SYSTAT, Version 5.2 (Systat, Evanston, IL) and JMP version 3.1 (SAS Institute, Cary, NC).

Results and discussion

Sediment properties

The aquifer sediment used in the microcosm studies was composed mostly of fine sands. It had a pH of 6.8 to 7.0 and its WHC was 23%. Total nitrogen and phosphorous in the sediment were 130 and 47 mg kg^{-1}, respectively.

Growth of Sphingomonas strains on PAHs under laboratory conditions

The growth of the five subsurface *Sphingomonas* strains from deep aquifers at the SRS on selected PAHs is shown in Table 1. *S. aromaticivorans* strains B0695 and B0522 and *S. stygia* strain B0712 grew very slightly on Tween 80 as the sole source of carbon and energy (i.e., control with Tween 80). All five strains grew reasonably well on 2,3-dimethylnaphthalene and phenanthrene in the absence of Tween 80, but growth on these two compounds was enhanced by the presence of 400 mg l^{-1} Tween 80 in all cases.

Table 1 Growth of subsurface *Sphingomonas* strains on selected PAHs, with and without Tween 80[a]

Compound	*S. subterranea* SMCC B0478		*S. aromaticivorans* SMCC B0522		*S. aromaticivorans* SMCC B0695		*S. aromaticivorans* SMCC F199		*S. stygia* SMCC B0712	
	w/ T80[b]	w/o T80[c]	w/ T80	w/o T80	w/ T80	w/o T80	w/ T80	w/o T80	w/ T80	w/o T80
Acenaphthene	–	–	++	+	++	+	+	–	++	+
Anthracene	+	–	+	–	++	–	–	–	++	–
2,3-Benzofluorene	–	–	+	–	++	–	–	–	++	–
2,3-Dimethylnaphthalene	+++	++	+++	++	+++	++	++	+	+++	++
Fluoranthene	+	–	+	–	++	–	–	–	+	–
2-Methylnaphthalene	–	–	++	–	++	–	–	–	+	–
Phenanthrene	++	+	+++	++	+++	++	++	+	+++	++
No PAH (control)	–	–	+	–	+	–	–	–	+	–

[a]Growth was measured as the increase in turbidity of liquid cultures after 7 days incubation on a rotary shaker at 25°C. – = O.D. increase 0–0.05; + = O.D. increase 0.06–0.10; ++ = O.D. increase 0.11–0.15; +++ = O.D. increase 0.16–0.20.
[b]With 400 mg l^{-1} Tween 80.
[c]Without Tween 80.

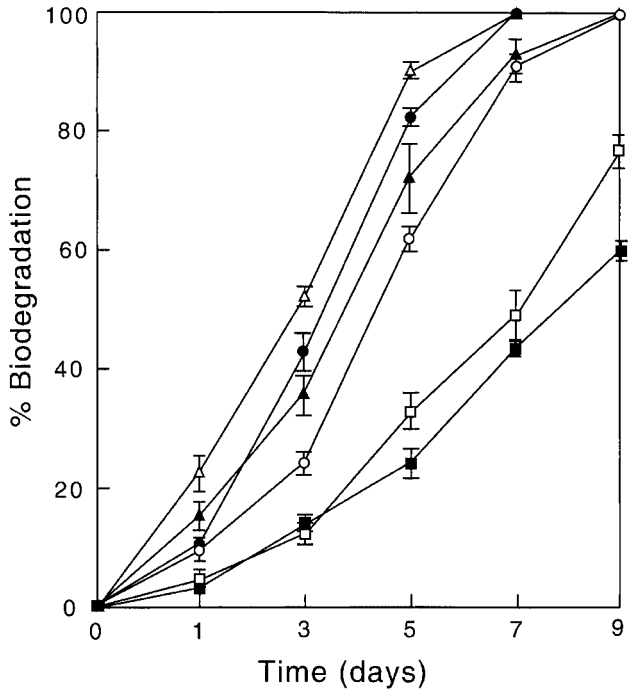

Figure 1 Biodegradation of phenanthrene in subsurface sediments under different conditions. (–■––■–) Unamended nonsterile sediment; (–□––□–) nonsterile sediment amended with approximately 100 mg kg^{-1} Tween 80; (–○––○–) nonsterile sediment inoculated with *S. aromaticivorans* strain B0695; (–●––●–) nonsterile sediment inoculated with strain B0695 and amended with approximately 100 mg kg^{-1} Tween 80; (–▲––▲–) = sterile sediment inoculated with strain B0695; (–△––△–) sterile sediment inoculated with strain B0695 and amended with approximately 100 mg kg^{-1} Tween 80.

Growth on phenanthrene was often accompanied by a color change in the medium, which may have been caused by the accumulation of partially degraded metabolic intermediates. *S. stygia* B0712 and *S. aromaticivorans* B0522 and B0695 grew on acenaphthene without Tween 80, but their growth was enhanced by Tween 80. *S. aromaticivorans* F199 could grow (very slightly) on acenaphthene, but only in the presence of Tween 80. None of the *Sphingomonas* strains grew on anthracene, 2,3-benzofluorene, 2-methylnaphthalene, or fluoranthene without Tween 80, but several of them were able to grow on one or more of these compounds in the presence of Tween 80. In fact, *S. aromaticivorans* B0695 grew on all four PAHs in the presence of Tween 80, and growth was clearly more extensive than that observed on Tween 80 alone. Mueller *et al* [25] reported that *Pseudomonas* (now *Sphingomonas*) *paucimobilis* strain EPA505 could utilize 2,3-dimethylnaphthalene and phenanthrene as carbon sources in a liquid mineral medium only after cells were induced by growth on fluoranthene. In contrast, the subsurface *Sphingomonas* strains could grow on 2,3-dimethylnaphthalene or phenanthrene without prior induction with these or other aromatic compounds, a characteristic that could be of considerable practical significance.

Enhancement of growth on PAHs by Tween 80 might indicate that the rather low solubility of these compounds inhibits their utilization by microorganisms that are able to degrade them. The Tween compounds are nonionic detergents and have been reported to enhance bioavailability of PAHs in biodegradation studies based on growth in laboratory media [11,25]. Willumsen *et al* [29] found that fluoranthene degradation rates observed with two *Sphingomonas* strains, EPA505 and 10-1, were more than doubled when Tween 80 was supplied in liquid media. Mueller *et al* [25] reported that the aqueous solubility of fluoranthene in mineral salt broth increased from 0.2 to 5.7 mg l^{-1} when 200 mg l^{-1} Tween 80 was added. In the present study, the concentration of fluoranthene increased from 0.1 to 8.36 mg l^{-1} in MSB (based on HPLC analyses) when 400 mg l^{-1} Tween 80 was added to the broth. The growth (or enhanced growth) of *S. aromaticivorans* F199 and *S. subterranea* B0478 on some PAHs in the presence of Tween 80 (Table 1) was probably the result of a Tween-induced increase in PAH solubility because neither of these strains could use Tween 80 as a sole carbon source. In contrast, the other strains grew (albeit poorly) on Tween 80 alone, so the compound might serve to stimulate catabolism of the PAHs, perhaps in addition to increasing, bioavailability.

The results summarized in Table 1 indicate that the *Sphingomonas* strains from deep aquifers at the SRS can degrade an even broader variety of aromatic compounds (under laboratory conditions) than has been reported. The findings also show that the strains vary considerably in their ability to do this, with *S. aromaticivorans* B0695 and *S. stygia* B0712 being the most versatile in regard to the range of compounds they are able to degrade. Strain F199, the type strain of *S. aromaticivorans* [4], displayed notably less capacity for utilizing the PAHs tested in this study than did either of the other two strains of this species (B0522 and B0695).

Table 2 Numbers of culturable bacterial cells in subsurface sediment amended with phenanthrene

Microcosm conditions			Cell numbers[a] ($\times 10^7$) g^{-1} at these incubation times		% Increase in cell numbers
Type of sediment	Tween 80 amendment	Inoculum	0 days	5 days	
Nonsterile	None	None	0.51±0.02	4.77±1.77	952
Nonsterile	100 mg kg^{-1}[b]	None	0.53±0.02	9.93±0.17	1410
Nonsterile	None	*S. aromaticivorans* B0695	4.8±0.06	21.03±3.32	440
Nonsterile	100 mg kg^{-1}[b]	*S. aromaticivorans* B0695	4.9±0.12	25.9±0.45	533
Sterile	None	*S. aromaticivorans* B0695	4.8±0.08	26.33±0.79	547
Sterile	100 mg kg^{-1}[b]	*S. aromaticivorans* B0695	4.6±0.21	29.77±0.58	647

[a]Total cell numbers for nonsterile sediment; number of *S. aromaticivorans* B0695 cells for sterilie sediment. Data are means of three replicates±SEM.
[b]Approximate final concentration of Tween 80 in the microcosm sediment.

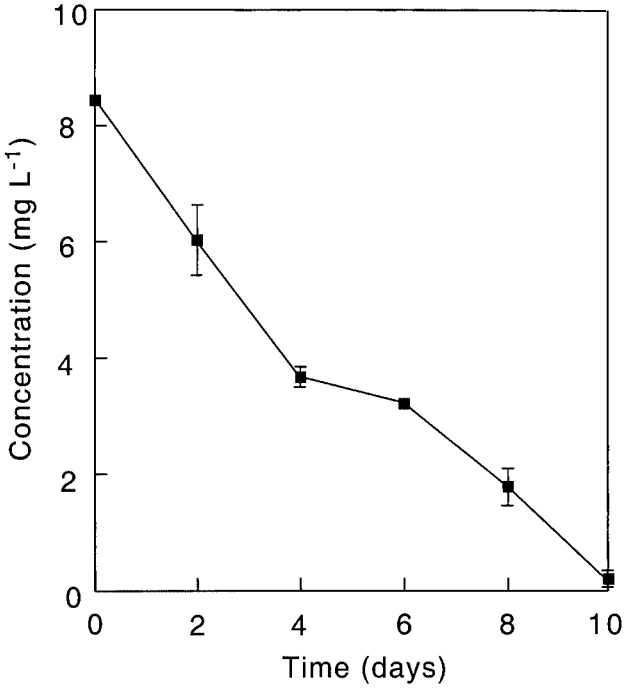

Figure 2 Biodegradation of fluoranthene by *S. aromaticivorans* strain B0695 in mineral salt broth with 400 mg l^{-1} Tween 80. Data are means of duplicate assays.

Biodegradation of phenanthrene in subsurface sediment

Based on the above findings, we utilized *S. aromaticivorans* B0695 to investigate the ability of the *Sphingomonas* isolates from the SRS to degrade PAHs under conditions similar to those in the subsurface. The results of an experiment to test this strain's ability to degrade phenanthrene in microcosms with either sterile or nonsterile aquifer sediment (see Methods), with or without the addition of Tween 80, are shown in Figure 1. In nonsterile sediments that were not inoculated with strain B0695, indigenous microbes degraded phenanthrene, removing approximately 60% of it after 9 days of incubation. Degradation by the indigenous microbes was enhanced slightly by addition of Tween 80 (nearly 80% removal after 9 days). However, removal of phenanthrene was more rapid if the nonsterile sediment was inoculated with strain B0695, both with and without addition of Tween 80 (100% and 95% removal after 7 days, respectively). The rates at which phenanthrene was degraded when strain B0695 was inoculated into sterile aquifer sediment with or without Tween 80 were slightly, but significantly, higher than in the equivalent treatments in nonsterile sediment (Figure 1). These results suggest there may have been antagonism between stain B0695 and the indigenous organisms. Perhaps indigenous species that did not degrade phenanthrene competed with strain B0695 for the mineral nutrients that were added in the MSB (see Methods).

The microcosm study results imply that *S. aromaticivorans* B0695 can degrade phenanthrene effectively under conditions similar to those occurring in shallow Atlantic coastal plain aquifers and can degrade this compound more rapidly than microorganisms indigenous to the aquifer sediment. These findings suggest that strain B0695 might also be effective in attempts to bioremediate phenanthrene-contaminated soils and subsurface sediments *in situ*. The addition of Tween 80 (or a similar surfactant) could enhance its utility in this regard, as the degradation rates in the microcosms were consistently higher in the presence of Tween 80. Aronstein *et al* [1] reported that phenanthrene degradation in mineral and organic soils was enhanced by addition of nonionic surfactants Alfonic 810-60 and Novel II 1412-56 at 10 mg l^{-1}, a concentration several times lower than that of the Tween 80 utilized in this study. Similarly, Jahan *et al* [13] demonstrated that mineralization of phenanthrene in sands by a mixed microbial culture was promoted in the presence of 25 mg kg^{-1} of Tween 40 or Corexit o600, and that the aqueous solubility of phenanthrene was directly proportional to the concentration of the surfactant, increasing from 1 mg l^{-1} to about 10 mg l^{-1} in the presence of Tween 40 at a concentration of 400 mg l^{-1}.

The numbers of culturable microorganisms in microcosm sediments 0 and 5 days after addition of phenanthrene are shown in Table 2. In all cases, cell numbers increased markedly as phenanthrene was degraded, indicating that degradation was associated with microbial growth. This was true for both *S. aromaticivorans* and the microbes that were indigenous to the sediment in the microcosms. Similarly, Moller and Ingvorsen [24] noted a greater than 100-fold increase in cell numbers of an introduced *Alcaligenes* sp. during biodegradation of phenanthrene in soil microcosms.

Biodegradation of fluoranthene in subsurface sediment

Increasing numbers of studies on the use of high-molecular-weight PAHs as sole sources of carbon and energy by pure cultures of

Table 3 Degradation of fluoranthene in microcosms of subsurface sediment under different conditions

Microcosm conditions			Fluoranthene concentration[a] (ppm) at these incubation times			% Degradation
Type of sediment	Tween 80 amendment	Inoculum	0 days	15 days	30 days[b]	
Nonsterile	None	None	92.42±0.44	34.03±2.74	N/A	63.2
Nonsterile	100 mg kg^{-1}[c]	None	91.75±0.26	0.19±0.09	N/A	99.8
Sterile	None	*S. aromaticivorans* B0695	92.37±0.47	91.11±1.10	N/A	1.36
Sterile	100 mg kg^{-1}[c]	*S. aromaticivorans* B0695	92.84±0.16	88.97±1.41	91.02±0.35	4.17

[a]Data are means of three replicate assays±SEM.
[b]Data from a separate experiment that was run for 30 days to confirm that fluoranthene was not being degraded.
[c]Approximate final concentration of Tween 80 in the microcosm sediment.

Table 4 Numbers of culturable bacterial cells in subsurface sediment amended with fluoranthene

Microcosm conditions			Cell numbers[a] ($\times 10^7$) g^{-1} at these incubation times		% Increase
Type of sediment	Tween 80 amendment	Inoculum	0 days	15 days	
Nonsterile	None	None	0.94±0.07	13.27±0.35	1316
Nonsterile	100 mg kg^{-1}[b]	None	0.88±0.06	14.17±0.58	1504
Sterile	None	*S. aromaticivorans* B0695	43.73±0.94	8.0±0.55	−82
Sterile	100 mg kg^{-1}[b]	*S. aromaticivorans* B0695	44.37±0.35	9.73±0.35	−78

[a]Total cell numbers for nonsterile sediment; numbers of *S. aromaticivorans* B0695 cells for sterile sediment. Data are means of three replicates±SEM.
[b]Approximate final concentration of Tween 80 in the microcosm sediment.

bacteria have appeared in recent years [5–7,12,14–16,21,22]. The results of the pure culture experiments summarized in Table 1 suggested that *S. aromaticivorans* strain B0695 degraded fluoranthene under laboratory conditions. To confirm this, B0695 was grown in fluoranthene-saturated MSB supplemented with Tween 80 (see Methods), and the concentration of fluoranthene was monitored by HPLC analysis. This strain degraded fluoranthene in a liquid medium (Figure 2), although the degradation rate was slow. When strain B0695 was inoculated into sterile sediment amended with fluoranthene (in microcosms); however, little or no degradation of fluoranthene was detected in 15 days in the absence of Tween 80, or within 30 days in the presence of Tween 80 (Table 3). In contrast, indigenous microbes degraded almost all the fluoranthene in 15 days when fluoranthene and Tween 80 were added concurrently to nonsterile sediment. Moreover, the indigenous organisms removed 63% of the fluoranthene within 15 days in the absence of Tween 80.

It is not known why *S. aromaticivorans* B0695 failed to degrade fluoranthene in microcosms consisting of sterile aquifer sediments. Adsorption of fluoranthene to the sediment may have interfered with degradation, but this seems unlikely in view of its rapid degradation by indigenous microbes in nonsterile sediment. In any case, these results demonstrate that the ability to degrade a particular aromatic hydrocarbon in the laboratory in liquid media does not necessarily translate to a practical ability to degrade that compound under field conditions in the subsurface. Studies that attempt to simulate environmental conditions through the use of microcosms or other means, then, are critical for a realistic assessment of the potential usefulness of contaminant-degrading bacterial strains in applications involving *in situ* bioremediation of contaminants.

The numbers of culturable cells of *S. aromaticivorans* B0695 decreased after this organism was added to sterile sediment amended with fluoranthene (Table 4), perhaps because it was unable to utilize fluoranthene as the carbon source effectively. In contrast, the numbers of culturable cells in nonsterile sediment increased by 1300% to 1500% after addition of fluoranthene. As was seen with phenanthrene, fluoranthene degradation was linked to microbial growth. A single colony type was numerically predominant on the plates used to enumerate culturable bacterial cells in the nonsterile sediment 15 days after addition of fluoranthene. Because this organism was probably responsible for much of the fluoranthene degradation, it was isolated and characterized by analysis of its 16S rRNA gene sequence and physiological traits (data not shown). The isolate appears to be very closely related to *Pseudomonas aeruginosa* strain NIH, and is obviously a member of the genus *Pseudomonas*. Although *S. aromaticivorans* B0695 appeared to have little potential for *in situ* bioremediation of environments contaminated with fluoranthene, the new *Pseudomonas* isolate could prove to be useful in this regard and merits further study.

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