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Degradation of Polycyclic Aromatic Hydrocarbons at Low Temperature under Aerobic and Nitrate-Reducing Conditions in Enrichment Cultures from Northern Soils

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The potential for biodegradation of polycyclic aromatic hydrocarbons (PAHs) at low temperature and under anaerobic conditions is not well understood, but such biodegradation would be very useful for remediation of polluted sites. Biodegradation of a mixture of 11 different PAHs with two to five aromatic rings, each at a concentration of 10 μg/ml, was studied in enrichment cultures inoculated with samples of four northern soils. Under aerobic conditions, low temperature severely limited PAH biodegradation. After 90 days, aerobic cultures at 20°C removed 52 to 88% of the PAHs. The most extensive PAH degradation under aerobic conditions at 7°C, 53% removal, occurred in a culture from creosote-contaminated soil. Low temperature did not substantially limit PAH biodegradation under nitrate-reducing conditions. Under nitrate-reducing conditions, naphthalene, 2-methylnaphthalene, fluorene, and phenanthrene were degraded. The most extensive PAH degradation under nitrate-reducing conditions at 7°C, 39% removal, occurred in a culture from fuel-contaminated Arctic soil. In separate transfer cultures from the above Arctic soil, incubated anaerobically at 7°C, removal of 2-methylnaphthalene and fluorene was stoichiometrically coupled to nitrate removal. Ribosomal intergenic spacer analysis suggested that enrichment resulted in a few predominant bacterial populations, including members of the genera *Acidovorax*, *Bordetella*, *Pseudomonas*, *Sphingomonas*, and *Variovorax*. Predominant populations from different soils often included phylotypes with nearly identical partial 16S rRNA gene sequences (i.e., same genus) but never included phylotypes with identical ribosomal intergenic spacers (i.e., different species or subspecies). The composition of the enriched communities appeared to be more affected by presence of oxygen, than by temperature or source of the inoculum.

Biodegradation of polycyclic aromatic hydrocarbons (PAHs) is a possible way to clean up polluted soils and water systems (4, 5). Biological treatments are cheaper than alternatives such as incineration, storage, or soil washing (12). PAHs are often found in oil spills and in soil at old gasworks sites and sites formerly used for wood preservation (creosote spills). Some PAHs are potential carcinogenic and mutagenic substances and are therefore on the pollutant priority lists of most countries' environmental protection agencies. Degradation of PAHs in situ is often slow, and research over the last decades has shown that these compounds very often are persistent (4, 9, 25). This persistence may be due to several factors such as nutrients, bioavailability of PAHs (sorption to particles), temperature, oxygen, and presence of PAH-degrading microorganisms. The water solubilities of most PAHs are in the lower parts-per-million range, and this is a major problem when studying and implementing aerobic degradation of PAHs. The use of surfactants may increase PAH solubility but may also be toxic to microorganisms (13, 43). In some Arctic and temperate regions, soil temperature remains below 10°C year-round, and wet conditions limit oxygen availability. The cost of increasing the temperature may be prohibitive, so it is desirable to optimize a treatment system for low temperature. The cost of aeration may also be prohibitive, and it may be more practical and economical to add nitrate, which is very water-soluble, as an electron acceptor. Bioagumentation with PAH-degrading bacteria and fungi has been tried with both successes and failures (4, 23), and it is still not clear why inoculation sometimes fails.

Despite the potential applications, very little is known about low-temperature degradation of PAHs (26), and even less is known about anaerobic degradation at low temperatures. There are reports of low-temperature degradation of jet fuel hydrocarbons and straight-chain aliphatic compounds by psychrotolerant organisms (47) and by polar soil communities (2, 6, 31, 32). There are only a few reports of growth on PAHs or PAH biodegradation at low temperature (1, 27, 41, 45, 46). Reports concerning anaerobic degradation of PAHs under sulfate-reducing (11, 29, 37, 40) and nitrate-reducing conditions (3, 20, 28, 30, 36, 37, 40) exist, but these processes were studied at temperatures between 20 and 30°C.

The purpose of this study was to evaluate the possibility of obtaining enrichment cultures capable of efficient PAH degradation at low temperature under aerobic or anaerobic condi-

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tions. Four different northern soils were used to enrich for mixed communities of PAH degraders at 7 and 20°C under both aerobic and anaerobic (nitrate-reducing) conditions. A mixture of 11 PAHs was used. Complex mixtures of PAHs typically occur at polluted sites and may permit cometabolic PAH degradation. The populations enriched in the cultures were characterized and compared by analysis of ribosomal intergenic spacers and 16S rRNA gene sequences.

MATERIALS AND METHODS

Soils. Four different soils were collected from two Arctic sites and two other northern sites to inoculate enrichment cultures. Alert soil was from the Canadian Forces Station Alert, on Ellesmere Island, Nunavut, Canada (82°30′N, 62°19′W). Alert soil was contaminated with Arctic diesel fuel at a concentration of ~2,000 mg/kg of soil (42). Saglek soil was from a radar installation at Saglek, Labrador, Canada (58°30′N, 63°0′W). Saglek soil was contaminated with PCBs (>50 mg/kg) and associated oil (32). Värta soil was from the former gasworks site, Värtagasverket, Husarviken, Stockholm, Sweden (59°20′N, 18°3′E). Värta soil was contaminated with creosote-PAH (~300 mg/kg) (15). Wesbrook soil was from near the Wesbrook Building, University of British Columbia, Vancouver, Canada (49°16′N, 123°7′W). Wesbrook soil was not contaminated with PAHs and is not known to be contaminated by other pollutants. All soils were sandy with low organic content and had a similar texture and particle size (<4 mm), a water content of approximately 10 to 15%, and a pH between 7 and 8. The pollutants in the contaminated soils were present in the soils for periods of years, but the exact histories of the soils are unknown.

Chemicals. The following chemicals were used (purities indicated in parentheses): naphthalene (99%), 2-methylnapthtalene (97%), 1,4-dimethylnaphthalene (95%), fluorene (99%), phenanthrene (99%), 9,10-dimethylanthracene (99%), fluoranthene (98%), pyrene (99%), 1,2-benzanthracene (99%), chrysene (98%), benzo[*a*]pyrene (98%), anthraquinone (97%), phenanthrenequinone (99%), phenanthrene-9-carboxaldehyde (97%), 9-anthracenecarboxylic acid (99%), 2-naphthyl acetic acid (99%), 2-methylanthracene (98%), 2-methylphenanthrene (95%), sulfanilamide (99%), zinc dust (<10 μm; 98%), and *N*-(1-naphthyl)ethylenediamine dihydrochloride (98%) (all from Aldrich Chemical Co.). Methylene chloride and ethyl acetate (both high-performance liquid chromatography grade) were from Fisher Scientific.

Enrichment cultures. Aerobic cultures were grown in Bushnell-Haas mineral medium (Difco). Anaerobic cultures were grown in Bushnell-Haas medium plus nitrate as an electron acceptor (0.75 g/liter of KNO_3). Primary enrichment cultures were prepared in 240-ml dark bottles with Teflon-septum-sealed screw caps (Supelco) by adding 2.0 g of soil to 20 ml of mineral medium containing a mixture of 11 different PAHs as the only organic substrates. The 11 PAHs were naphthalene, 2-methylnapthtalene, 1,4-dimethylnaphthalene, fluorene, phenanthrene, 9,10-dimethylanthracene, fluoranthene, pyrene, 1,2-benzanthracene, chrysene, and benzo[*a*]pyrene. The PAHs were added at final concentrations of 100 to 500 mg/liter in pure crystal or liquid form to avoid solvents as potential substrates. Anaerobic cultures were purged with sterile filtered (pore size, 0.4 μm) nitrogen gas for 5 min. The enrichment cultures were incubated at 7 or 20°C on shakers at 150 rpm in darkness. Thus, there were four incubation conditions, aerobic and anaerobic cultures, each at 7 and 20°C. After 45 days, secondary cultures were established by transferring 200 μl from each primary culture to fresh, homologous medium. After an additional 45 days, tertiary cultures were established and monitored for PAH degradation as described below.

The tertiary enrichment cultures were established in the same way as the secondary cultures, except 10.0 ml of medium was used. These cultures had initial cell densities of 5×10^6 to 2×10^7 cells/ml, estimated by total count under a light microscope. Sterile controls were prepared as described above but without inoculum and with 3.0 mg/ml of NaN_3 added. Sufficient replicate cultures were established to permit whole cultures to be extracted for PAH analysis to avoid potential loss of PAHs adsorbed to the bottles. Single cultures were analyzed on days 0, 15, and 60; duplicate cultures, on day 30; and triplicate cultures, on day 90. Additional replicate cultures of both 7°C treatments were established to analyze metabolites by solid-phase microextraction (SPME) (see below). For each time point, there were two additional replicate cultures of these treatments.

Anaerobic nitrate consumption at 7°C. PAH degradation under nitrate-reducing conditions was verified in the anaerobic, 7°C, Alert soil enrichment culture. These quaternary cultures were established in the same way as the tertiary cultures, except the individual PAHs, 2-methylnaphthalene or fluorene (in methylene chloride), were each added to duplicate cultures to a final concentration of 40 μg/ml. Bottles were incubated as described above at 7°C for 40 days. Then, nitrate, nitrite, and remaining PAHs were analyzed. Sterile controls were medium without inoculum.

Ethyl acetate extraction. Whole cultures (10 ml) were acidified with 1.0 ml of 3 M H_2SO_4 and extracted with 4.0 ml of ethyl acetate in the culture bottles by shaking for 24 h at 22°C. Extracts were dried over anhydrous sodium sulfate before analysis by gas chromatography-flame ionization detection (GC-FID) and GC-mass spectrometry (GC-MS). An internal standard of 2-methylanthracene in methylene chloride was added to all samples before analysis, to a final concentration of 5.0 μg/ml.

Analysis of metabolites by SPME. Samples of 2.0 ml were removed from cultures with a sterile syringe (flushed with nitrogen gas) and placed in 5-ml vials with 0.20 ml of 3 M H_2SO_4. These samples were frozen at −20°C until analysis was done. A manual SPME 85-μm polyacrylate fiber (Supelco) was immersed in each vial for 10 min with stirring at room temperature. Before injection, the fibers were held in deionized water for 10 s to remove salt from the medium and blotted on a tissue paper to remove the water droplet remaining from washing. The fiber was then injected immediately in the GC-MS for desorption and analysis. The fibers were routinely monitored for degradation and possible carryover of analytes to other samples by injecting blank runs between the samples. 2-methylphenanthrene in methylene chloride was added to the 2.0-ml samples as an internal standard before analysis, to a final concentration of 0.50 μg/ml.

GC-FID. A Hewlett-Packard GC 5890 series II was used with an FID and a Hewlett-Packard HP-5 column (length, 25 m; inner diameter [i.d.], 0.32 mm; film thickness, 0.17 μm). The carrier gas was H_2 at a pressure of 7.5 lb/in^2 and a flow rate of 1.8 ml/min. The temperature program was as follows: 40°C for 3 min, 30°C/min to 300°C, hold for 10 min. The injector was 290°C, and the detector was 300°C. Samples of 2.0 μl were injected in splitless mode for 1 min. Analytical standards of PAHs and their metabolites were prepared in methylene chloride, at a concentration of 5.0 mg/ml for each compound. Standard deviations for replicate samples (including variability in extraction and analysis) were from 2.4 to 7.6%, with the exceptions of dibenzanthracene (9.3%), benzo[*a*]pyrene (12.5%), and 9,10-dimethylanthracene (20.7%). Differences of less than 20% were not considered substantial.

GC-MS. A Varian 3400Cx gas chromatograph was used with a Saturn 4D ion trap MS detector and a J&W Scientific DB5-MS column (length, 30 m; i.d., 0.25 mm; film thickness, 0.25 μm). The carrier gas was helium at 10 lb/in^2. The temperature program was as follows: 40°C for 5 min, 10°C/min to 245°C, hold for 30 min. The injector, with a 0.8-mm-i.d. liner, was 240°C, and the transfer line was 250°C. The ion trap was operated at 70 eV with a scan range of *m*/*z* 90 to 400. Samples of 1.0 μl were injected in splitless mode for 30 s.

Nitrate and nitrite analysis. Nitrate and nitrate were analyzed using the methods described in *Methods for General and Molecular Bacteriology* (19). Nitrate is reduced to nitrite by zinc and the nitrite then reacts with *N*-(1-naphthyl)ethylenediamine to form a colored complex. The amount of nitrite is analyzed in a spectrophotometer at 543 nm. Calibration was done by analyzing known amounts of sodium nitrite in sterile water. Samples of 50 μl were withdrawn from the culture bottles for analysis.

Ribosomal intergenic spacer analysis. DNA was extracted and purified as previously described (16). A previously described (48) composite method was used for ribosomal intergenic spacer analysis. Universal bacterial PCR primers were used to amplify ribosomal intergenic spacers plus approximately 500 bp of the 16S rRNA gene (RIS-rDNA). Ribosomal intergenic spacer length polymorphism (RIS-LP) was analyzed by electrophoresis of the RIS-rDNA amplicons. The samples were analyzed twice, and the replicates yielded nearly identical fingerprints (not shown). Gelcompar II (version 2.5; Applied Maths) was used to analyze the RIS-LP fingerprints. The similarity of entire fingerprints was determined by the Pearson correlation method (2.00%). Similarity dendrograms were constructed by the unweighted-pair group method using arithmetic averages. For selected samples, clone libraries of the RIS-rDNA amplicons were prepared. From each library, 20 clones were analyzed for restriction fragment length polymorphism (RIS-RFLP). For clones representing selected RIS-RFLP phylotypes, the rDNA fragment was sequenced.

Nucleotide sequence accession numbers. The partial rDNA sequences determined were deposited in the GenBank under the accession numbers AF532132 to AF532137 and AY136514 to AY136545.

RESULTS

PAH removal. Biological removal of PAHs occurred under all experimental conditions in tertiary enrichment cultures (Fig. 1; Table 1). Predictably, the greatest PAH removal con-

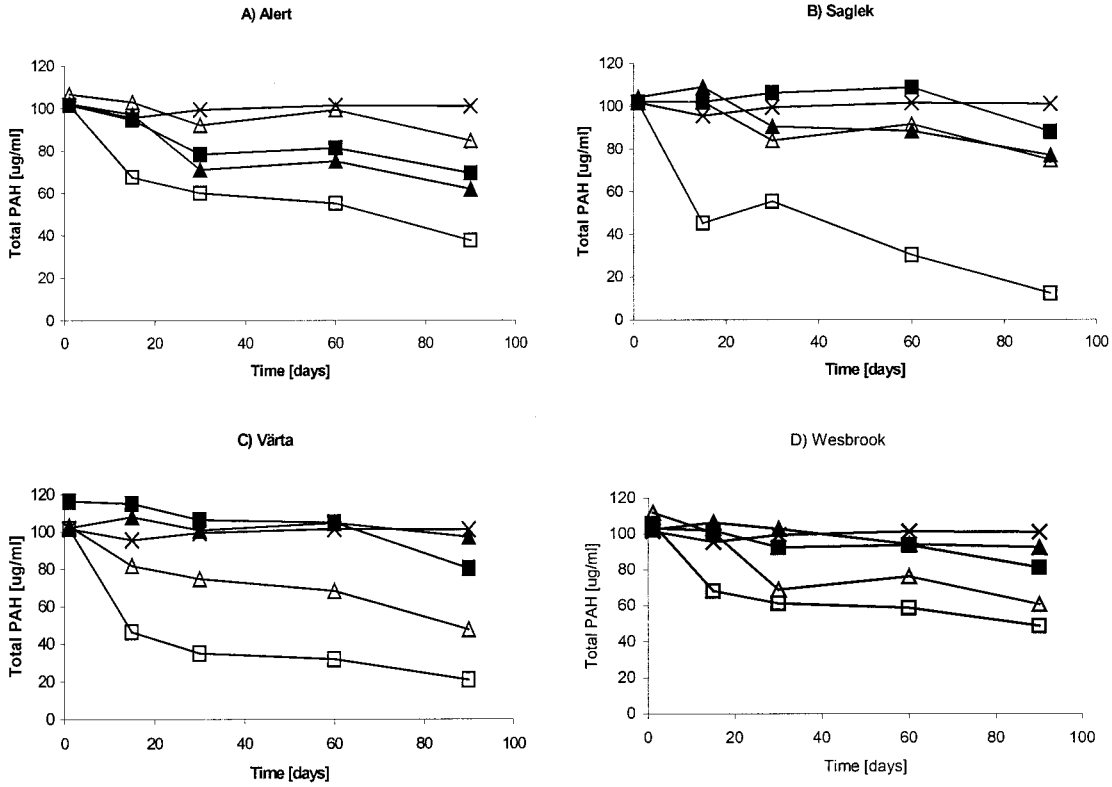

FIG. 1. Total PAH degradation in cultures inoculated with soils from Alert (A), Saglek (B), Värta (C), and Wesbrook (D). Symbols: △, aerobic conditions, 7°C; ▲, nitrate-reducing conditions, 7°C; □, aerobic conditions, 20°C; ■, nitrate-reducing conditions, 20°C; ×, killed control.

sistently occurred in aerobic, 20°C treatments, with the highest total PAH removal being 88%. Naphthalene and 2-methylnaphthalene were completely removed in all cultures, and temperature had little effect on their removal rates (not shown). With notable exceptions, reducing the temperature to 7°C reduced rates and extents of removal of the other PAHs. Aerobic degradation of 1,4-dimethylnaphthalene was particularly affected by the lower temperature, being eliminated in cultures of three soils. In the aerobic cultures inoculated with Värta soil, the removal rate for 1,4-dimethylnaphthalene was reduced at the lower temperature much more dramatically than were the rates for other PAHs (not shown).

Anaerobic conditions limited PAH removal even more drastically than lowering temperature, particularly for PAHs with three or more aromatic rings (Fig. 1; Table 1). One exception was the 7°C cultures inoculated with Alert soil, in which fluorene and phenanthrene were degraded under anaerobic, but not under aerobic, conditions. In anaerobic cultures inoculated with all soils, the extents of removal of all PAHs were very similar at the low and high temperatures.

TABLE 1. Percent removal of PAHs from enrichment cultures during 90-day incubations

PAH	% Removal from culture															
	Aerobic								Anaerobic							
	20°C				7°C				20°C				7°C			
	Alert	Saglek	Värta	Wesbrook	Alert	Saglek	Värta	Wesbrook	Alert	Saglek	Värta	Wesbrook	Alert	Saglek	Värta	Wesbrook
Naphthalene	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
2-Methylnaphthalene	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
1,4-Dimethylnaphthalene	100	100	100	100	0	0	96	0	12	3	5	6	14	9	12	6
Fluorene	92	98	97	97	3	29	97	96	96	13	21	25	93	33	17	15
Phenanthrene	80	93	92	91	0	10	94	95	92	0	7	9	87	11	13	1
Fluoranthene	48	82	74	66	0	0	34	6	0	0	0	0	8	0	0	0
Pyrene	25	87	71	0	0	0	17	3	0	0	0	0	2	0	0	0
9,10-Dimethylanthracene	15	84	58	0	0	0	0	9	0	0	0	0	7	0	0	0
Dibenzanthracene	51	85	75	25	19	18	26	25	7	4	17	10	19	17	1	0
Chrysene	36	69	60	11	12	11	18	19	2	0	8	4	13	16	0	0
Benzo[*a*]pyrene	68	76	60	27	33	31	34	37	10	10	26	23	26	22	6	3
Total PAHs	63	88	79	52	11	22	53	40	32	13	21	20	39	24	16	13

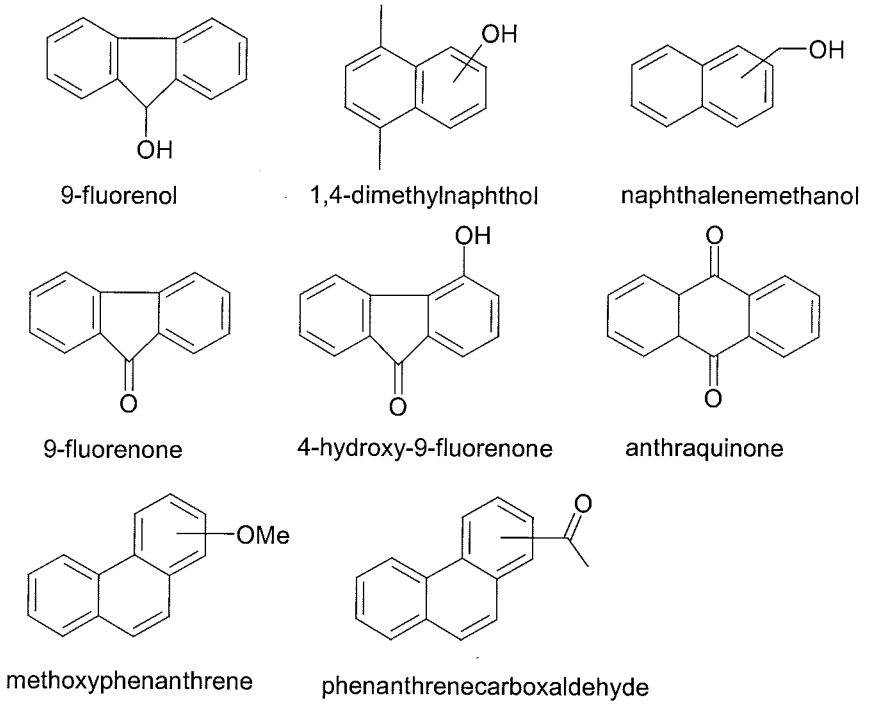

FIG. 2. Metabolites detected during degradation of PAHs at 7°C under aerobic and nitrate-reducing conditions.

The different soil inocula substantially affected removal of total PAHs in the enrichment cultures (Fig. 1; Table 1). Soils from Värta and Wesbrook yielded cultures that were relatively active in aerobic, 7°C treatments, with final removals of 53 and 45%, respectively. The soil from Alert yielded a culture that was relatively active under anaerobic conditions and actually removed more PAHs at 7°C than at 20°C (39 and 31% removal, respectively).

PAH metabolites at 7°C. The metabolites detected during PAH degradation at 7°C, under both aerobic and anaerobic conditions, were similar in the various enrichment cultures, despite the differences in PAH removal kinetics (not shown). Major metabolites detected in most or all cultures included 1,4-dimethylnaphthol, 9-fluorenone, fluorenol, naphthalenemethanol, phenanthrenecarboxaldehyde, methoxyphenanthrene, and anthraquinone (Fig. 2). The last three of these were most abundant in the anaerobic cultures. In both aerobic and anaerobic cultures, fluorenone was detected in cultures that degraded fluorene efficiently, whereas fluorenol was detected in cultures with little fluorene removal. Concentrations of the metabolites ranged from a few parts per billion (trace levels) to up to 2 ppm. Low concentrations of 4-hydroxy-9-fluorenone were detected, mainly in the anaerobic cultures. Low concentrations of phenanthrenol were detected under anaerobic conditions, but as shown by Ho et al. (22), phenanthrenol could be a GC artifact (thermal decomposition) during the GC analysis of 10-hydroxy-1-phenanthroic acid obtained from pyrene degradation. Most of the detected compounds had a maximum concentration after 15 days and then declined slowly over the remaining 75 days.

Coupling of PAH and nitrate removal at 7°C. Quaternary enrichment cultures from Alert soil incubated anaerobically tested the coupling of PAH degradation to denitrification at 7°C. Expected nitrate removal was calculated for oxidation of each PAH to carbon dioxide plus water coupled to nitrate reduction to nitrogen gas. Nitrate was present in twice the amounts required for PAH oxidation. Nitrate removal was consistent with 2-methylnaphthalene and fluorene removal in the cultures (Table 2). Trace amounts of nitrite were detected in the active samples after 40 days of incubation. The presence of nitrite confirms nitrate-reducing activity. The small amounts of nitrite accumulating suggest that the reduction process was denitrification (i.e., reduction to nitrous oxide or dinitrogen gases). These results strongly support the conclusion that anaerobic 2-methylnaphthalene and fluorene oxidation was coupled to denitrification at 7°C.

RIS-LP. The RIS-LP fingerprint of each tertiary enrichment culture at the end of the 90-day incubation was characterized by a few RIS-rDNA bands (Fig. 3). Frequently, fingerprints from cultures with different enrichment conditions inoculated with the same soil had bands of common sizes. In several cases, enrichment cultures inoculated with different soils also had bands of common sizes. Cluster analysis of fingerprint similar-

TABLE 2. PAH and nitrate consumption in anaerobic transfer cultures (7°C enrichment) inoculated with Alert soil during 40-day incubations

Culture	Concn (mM) of:			
	PAH removed	NO_3^- removed	Expected NO_3^- removal	NO_2^- produced
2-Methylnaphthalene	0.28	2.3	3.02	0.048
Fluorene	0.13	1.3	1.61	0.0085
Sterile control	0.0	0.0		0.0

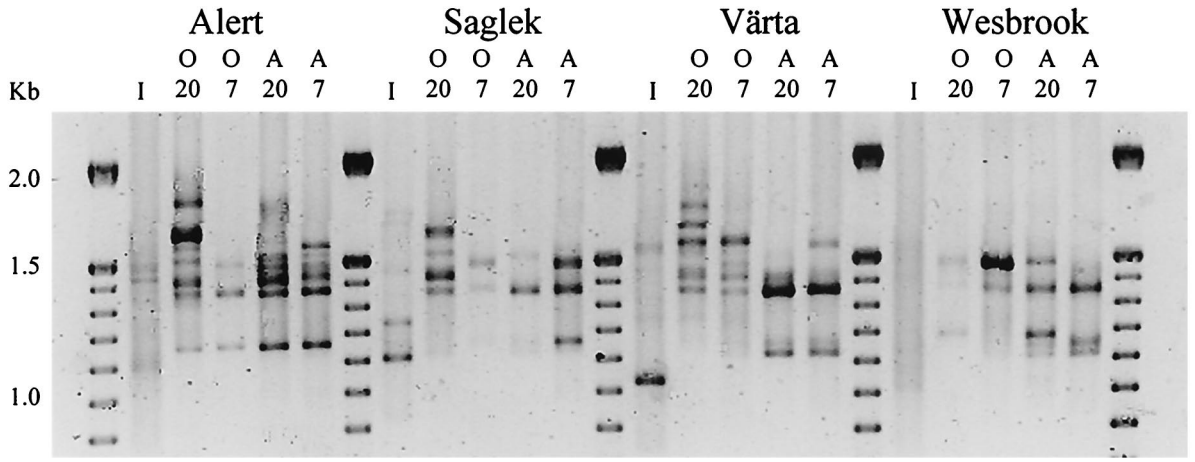

FIG. 3. RIS-LP banding patterns from enrichment cultures after 90 days of incubation. Lane identifications: I, initial soil sample; O, aerobic; A, anaerobic; 7, 7°C; 20, 20°C.

ity (Fig. 4) did not consistently indicate greatest similarity between fingerprints from cultures with common inocula, nor between fingerprints from cultures incubated at a common temperature. The most clear trend is a cluster containing all of the fingerprints from anaerobic cultures, which seems to be based on the presence of two predominant bands and, in many cases, the absence of many other bands. Another cluster contains four of the aerobic cultures. The initial fingerprints from soils prior to their use as inocula were distinct. In two cases, the initial fingerprints lacked predominant bands, and in two cases the initial fingerprints had predominant bands which were not present in final fingerprints from cultures inoculated with those soils.

RIS-RFLP and rDNA sequences. At the end of the 90-day incubation, clone libraries of RIS-rDNA amplicons were prepared from all of the tertiary cultures inoculated with Alert soil

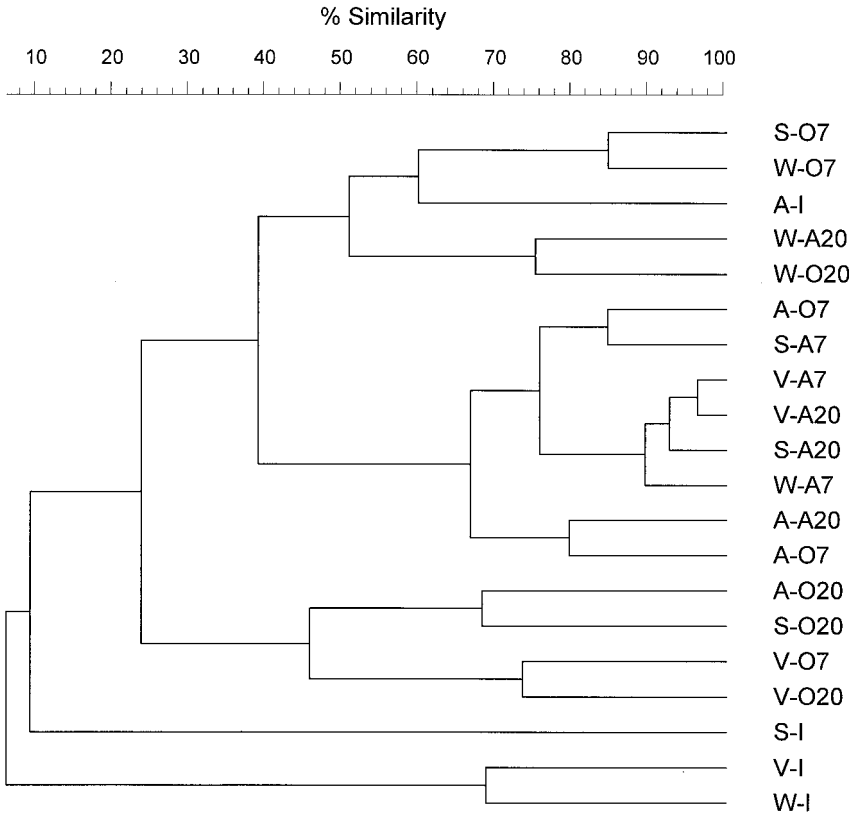

FIG. 4. Similarity analysis of RIS-LP banding patterns. Sample identification: letters preceding the hyphen indicate soil (A, Alert; S, Saglek; V, Värta; W, Wesbrook); letters following the hyphen are as explained in the legend to Fig. 3.

TABLE 3. RIS-RFLP phylotypes found in libraries from selected enrichment cultures[a]

RIS-RFLP phylotype	Insert size (kb)[b]	No. of clones	rDNA sequence affiliation	Sequence similarity (%)
A-A7-7	1.1	5	*Pseudomonas*	99
A-A7-5	1.3	4	*Pseudomonas*	99
A-A7-3	1.3	2	*Ultramicrobacterium*	97
A-A7-1		1		
A-A7-2	1.4	1	*Bordetella*	96
A-A7-4	1.4	1	*Bordetella*	96
A-A7-12		1		
A-A7-13		1		
A-A7-14		1		
A-A7-16		1		
A-A7-18		1		
A-A7-19	1.4	1	*Variovorax*	99
A-A20-3	1.3	4	*Pseudomonas*	99
A-A20-1	1.4	3	*Bordetella*	96
A-A20-5	1.3	3	*Variovorax*	99
A-A20-7	1.1	2	*Pseudomonas*	99
A-A20-2	1.3	1	*Pseudomonas*	99
A-A20-4	1.1	1	*Variovorax*	99
A-A20-6		1		
A-A20-11		1		
A-A20-13		1		
A-A20-14		1		
A-A20-17		1		
A-A20-19	0.9	1	*Pseudomonas*	99
A-O7-1	1.3	3	*Pseudomonas*	98
A-O7-3	1.5	3	*Acidovorax*	99
A-O7-10	1.1	2	*Pseudomonas*	99
A-O7-11	1.3	2	*Pseudomonas*	99
A-O7-2		1		
A-O7-4		1		
A-O7-6		1		
A-O7-8		1		
A-O7-12	0.9	1	*Pseudomonas*	99
A-O7-13	1.4	1	*Pseudomonas*	99
A-O7-14		1		
A-O7-16		1		
A-O7-18	1.0	1	*Ultramicrobacterium*	98
A-O7-20	1.1	1	*Acidovorax*	93
A-O20-1	1.7	7	*Sphingomonas*	97
A-O20-2	1.7	6	*Sphingomonas*	97
A-O20-4		1		
A-O20-6		1		
A-O20-7		1		
A-O20-10		1		
A-O20-12		1		
A-O20-15	1.1	1	*Pseudomonas*	99
A-O20-20		1		
V-O7-1	1.4	4	*Bordetella*	96
V-O7-4	1.5	3	*Sphingomonas*	97
V-O7-5	1.3	3	*Variovorax*	99
V-O7-10	1.3	2	*Variovorax*	99
V-O7-2		1		
V-O7-3	1.1	1	*Pseudomonas*	99
V-O7-6	1.5	1	*Sphingomonas*	97
V-O7-7	1.3	1	*Pseudomonas*	99
V-O7-11		1		
V-O7-17		1		
V-O7-19		1		
V-O7-20	1.5	1	*Pseudomonas*	99
S-O7-3	1.5	7	*Acidovorax*	99
S-O7-7	1.5	3	*Acidovorax*	99
S-O7-1		1		
S-O7-4		1		
S-O7-11	1.3	1	*Pseudomonas*	99
S-O7-13		1		
S-O7-15		1		
S-O7-16		1		
S-O7-17	1.4	1	*Acidovorax*	99
S-O7-19		1		
S-O7-20		1		
S-O7-21		1		
W-O7-2	1.5	12	*Acidovorax*	99
W-O7-6	1.5	5	*Acidovorax*	99
W-O7-4		1		
W-O7-12		1		
W-O7-17		1		

[a] Phylotype designations refer to samples as explained in legends to Fig. 3 and 4.
[b] Insert sizes correspond to bands in Fig. 3.

and from the cultures inoculated with the other soils and incubated aerobically at 7°C. From each library, 20 clones were screened for RIS-RFLP patterns. The libraries consistently had two to four predominant RIS-RFLP phylotypes, with the remainder being singletons (Table 3). The sequence of the rDNA fragment in the RIS-rDNA amplicon was determined for a representative of each predominant RIS-rDNA phylotype plus some singletons. The rDNA phylotypes were affiliated with six genera among three subdivisions, all within the *Proteobacteria* (Fig. 5). The predominant rDNA phylotypes were affiliated with the genera *Pseudomonas*, *Sphingomonas*, *Bordetella*, and *Acidovorax.* In many cases, a library had two predominant RIS-RFLP phylotypes affiliated with the same genus. The RIS-rDNA libraries were consistent with the RIS-LP analysis in that, for each culture analyzed, the most abundant library clones had inserts that corresponded in size to major bands in the RIS-LP fingerprint (compare Table 3 to Fig. 3). However, there were not clone inserts corresponding to every visible band. Only two RIS-RFLP phylotypes occurred in more than one library from the cultures inoculated with Alert soil. The RFLP patterns were identical for A-A20-2 and A-O7-11, as well as for A-A20-19 and A-O7-12. The rDNA sequences were identical for both phylotypes in each pair, and both pairs were affiliated with the same genus, *Pseudomonas*. There were no RIS-RFLP phylotypes that occurred in libraries from cultures inoculated with different soils.

DISCUSSION

Aerobic PAH degradation at low temperature. The capacity of the cultures for aerobic PAH biodegradation at 7°C, relative to biodegradation at 20°C, depended very much on the soil inoculum used (Fig. 1; Table 1). These results suggest that the abundance of psychrotolerant PAH degraders in the soils was a factor in the outcome of the enrichment cultures. All soils yielded cultures that degraded naphthalene and 2-methylnaphthalene at 7°C. The severe effect of low temperature on 1,4-dimethylnaphthalene degradation was unexpected, as this is not considered a particularly recalcitrant PAH. Only Värta and Wesbrook soils yielded cultures that at 7°C degraded PAHs

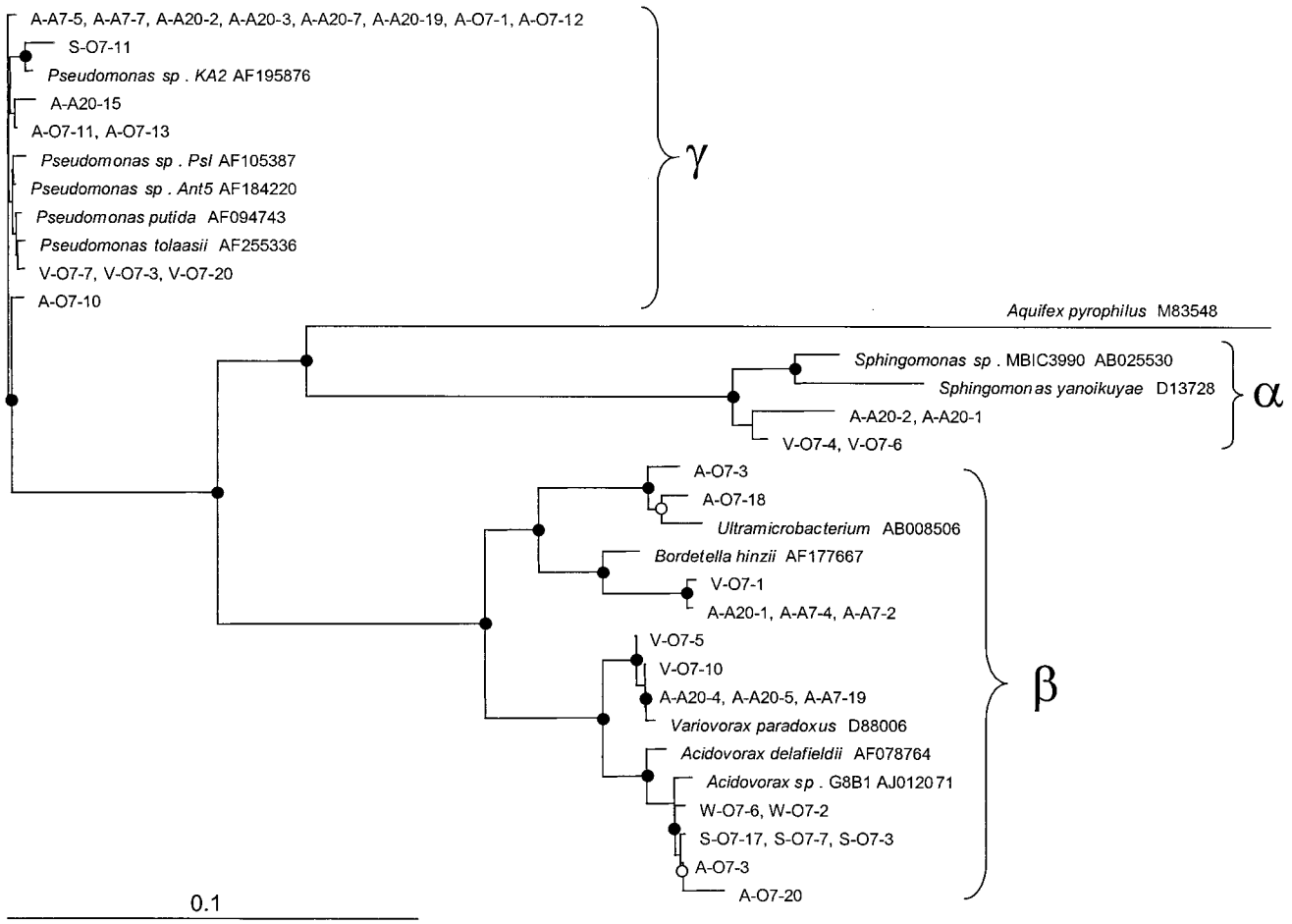

FIG. 5. Affiliations of the partial 16S rDNA sequences (*Escherichia coli* positions 910 to 1360) from cloned RIS-rDNA amplicons. Reference strains are from the Ribosomal Database Project. Phylotype designations correspond to those in Table 3. Solid circles indicate branch points with >75% bootstrap values, and open circles indicate branch points with >50% bootstrap values. The scale bar corresponds to 0.1 mutation per nucleotide position.

with three or more rings, including fluorene, phenanthrene, fluoranthene and dibenzanthracene. RISA suggests that predominant populations enriched from the two soils belong to different genera. Of the four soils, only the Värta soil came from a site with extensive PAH contamination. The Värta and Wesbrook soils come from less cold regions than do the Alert and Saglek soils; however, the regions of the former two soils are cold (<10°C) for a substantial period each year. Removal of benzo[*a*]pyrene at 7°C was anomalous, as it occurred to a similar extent (31 to 37% removal) in treatments inoculated with all inocula. This removal may represent partial degradation, possibly cometabolic, although no benzo[*a*]pyrene metabolite was detected. This removal does not appear to be abiological, since it did not occur in the uninoculated controls, nor did it occur in all of the anaerobic treatments at 7°C. This and other studies (22, 34) suggest that mesophilic PAH degraders are relatively ubiquitous in soils. However, this study suggests that psychrotolerant PAH degraders may be less ubiquitous or may require a very long time to enrich. Thus, the source of organisms may be critical for PAH biodegradation applications at low temperature.

Temperature may also affect PAH biodegradation via its affect on the bioavailability of PAHs. This explanation is consistent with the fact that low temperature mainly affected biodegradation of less-soluble, larger PAHs, having three or more aromatic rings (Table 1). However, low temperature inhibited degradation of individual PAHs to very different extents under aerobic and anaerobic conditions, which is not consistent with an effect on bioavailability. The balance of the evidence indicates that low temperature primarily limits PAH biodegradation via an effect on biological activity.

Accumulation of PAH metabolites at 7°C. The metabolites detected during the low-temperature PAH degradation, both aerobic and anaerobic, confirm biological transformation of the PAHs. The metabolites provided no evidence for substantially different degradation processes associated with different soil inocula. Naphthalenemethanol indicates oxidation of the methyl group of methyl-naphthalene under both aerobic and anaerobic conditions. Fluorene was reported to be transformed by an *Arthrobacter* sp. to 9-fluorenone and then 4-hydroxy-9-fluorenone as a dead-end metabolite (7). Fluoranthene was also proposed to be transformed to 9-fluorenone (39). Thus, the 9-fluorenone detected in this study may have come from either or both fluorene and fluoranthene. Since

some of the metabolites remained in the system for a long period of time (90 days) they should be considered as possible inhibitors of growth and PAH degradation. 9-Fluorenone, for example, was shown to be inhibitory to denitrification at concentrations of 10 ppm and higher for pure cultures of *Pseudomonas* strains (14). Very little is known about other inhibitory or possible stimulatory effects that metabolites may have on PAH degradation.

Metabolites detected in the anaerobic cultures indicate transformations, which were not observed under aerobic conditions. These transformations may be part of the primary pathways for anaerobic degradation of the PAHs or may account for transformation of only a small fraction of the substrates. Phenanthrenecarboxaldehyde has not previously been reported as an anaerobic metabolite of phenanthrene, but carboxylation has been reported as the initial step in PAH degradation under sulfate-reducing conditions (49). Since anthracene was not provided to the cultures, the anthraquinone detected probably came from oxidation of the methyl groups of 9,10-dimethylantracene. Similar reactions have been reported before where anthraquinone was a metabolite from anthracene (4). Methoxylation of PAHs has been reported to be catalyzed by fungi (38) and cyanobacteria (35), which can explain the detection of methoxyphenanthrene under anaerobic conditions.

PAH degradation coupled to denitrification at low temperature. This study demonstrated for the first time anaerobic PAH degradation at a low temperature. This degradation was limited to naphthalene, 2-methylnaphthalene, fluorene, phenanthrene, and perhaps, benzo[*a*]pyrene (Table 1). For 2-methylnaphthalene and fluorene, degradation was shown to be coupled to denitrification on the basis of the stoichiometry of removal of these compounds and removal of nitrate (Table 2). The Arctic soils, from Alert and Saglek, showed the greatest potential for anaerobic PAH degradation at low temperature, despite their relatively poor potential for aerobic PAH removal at low temperature (Fig. 1; Table 1). The capacity for anaerobic degradation may be related to anoxic conditions where those soils were collected. We do not know whether the particular source areas for the Arctic soils are frequently anoxic, but we have observed that soils from these and other Arctic sites often are wet and drain poorly, in part due to the effect of permafrost on water flow. The Värta and Wesbrook soils came from well-drained areas. In general, most successful enrichment cultures and isolates that degrade PAHs under anaerobic conditions were obtained from contaminated sediments, and not from contaminated soils (11, 36, 40). A low abundance of anaerobic PAH degraders in aerobic soils might be the reason for the lack of anaerobic PAH degradation in other experiments (40, 44). This study and that of Hayes et al. (21) indicate that anaerobic PAH degraders can be found in environments without PAH contamination.

Organisms capable of degrading hydrocarbons at low temperature tend to be psychrotolerant, rather than psychrophilic (46). Consistent with this, hydrocarbons were degraded in Arctic soils at increasing rates from 7 to 20°C (31). Therefore, it is surprising that the higher temperature did not stimulate anaerobic PAH removal by the cultures inoculated with Alert and Saglek soils (Fig. 1; Table 1). This could be because anaerobic PAH removal at low temperature was catalyzed by psychrophilic organisms. The RIS-LP fingerprints of the anaerobic Saglek soil cultures at low and high temperatures have different predominant bands (Fig. 3 and 4), which is consistent with enrichment of psychrophilic organisms at low temperature. On the other hand, the RIS-LP fingerprints of the anaerobic Alert soil cultures at low and high temperatures generally have the same predominant bands, suggesting that the same psychrotolerant organisms were enriched at both temperatures. Our results indicate that increased temperature will not always stimulate PAH biodegradation in soils from cold regions and that the reasons for this may be multiple.

Populations enriched. The RIS-LP fingerprints suggest that a few predominant populations were enriched in the cultures. We have obtained more complex RIS-LP fingerprints from wastewater treatment systems (48) and much more complex fingerprints from soil (unpublished data). Comparison of initial and final RIS-LP banding patterns (Fig. 3 and 4), suggests that the populations enriched were not abundant prior to incubation. The most intense RIS-LP bands likely represent predominant populations, but it is important to note that additional predominant populations may not have been detected for reasons such as unequal DNA recovery from different organisms, failure of the primers to amplify certain RIS-rDNA sequences, variability in *rrn* copy number and PCR bias.

It is also important to realize that one population may yield more than one RIS-LP band, as the multiple *rrn* operons of a single organism can yield distinct RIS-rDNA amplicons of different length or RIS sequences. This is consistent with the fact that, in most clone libraries, we found two predominant RIS-RFLP phylotypes with identical or nearly identical rDNA sequences (Table 3; Fig. 5). Thus, for example, the two predominant phylotypes affiliated with the genus *Acidovorax* in the library from the aerobic, 7°C culture inoculated with Wesbrook soil may represent two distinct populations or one population with at least two distinct RISs of approximately the same length but of different sequences, which yielded different RIS-RFLP patterns. We have previously obtained from one to three RIS-rDNA amplicons of different sizes from individual pure cultures (16). There is little information available to suggest the extent to which single organisms have RISs of common sizes that yield distinct RFLP patterns.

Phylotypes affiliated with only a few genera were detected as predominant populations in the enrichment cultures (Table 3). These phylotypes included probable members of *Acidovorax*, *Pseudomonas*, and *Variovorax* as well as phylotypes less closely affiliated with *Bordetella* and *Sphingomonas* (Fig. 5). All of the phylotypes identified by rDNA sequence analysis are members of the Proteobacteria. Previously (48), we have detected in wastewater treatment systems members of the *Cytophagales*, *Fexistipes*, and low-G+C gram-positive bacteria, using the method and PCR primers used in the present study. Thus, it appears that *Proteobacteria* were selectively enriched in the present study.

Our analyses suggest that members of a few proteobacterial genera are widely distributed and share characteristics that caused their enrichment in the aerobic, 7°C cultures. Members of *Acidovorax* were predominant in those cultures inoculated with Alert, Saglek, and Wesbrook soils (Table 3). Members of *Pseudomonas* were predominant in the Alert culture and detected in the Värta and Saglek cultures. Members of these

genera from the different soils often yielded RIS-rDNA amplicons of identical or nearly identical sizes. For example, phylotypes affiliated with *Acidovorax* consistently yielded 1.5-kb amplicons, and phylotypes affiliated with *Pseudomonas* yielded both 1.1- and 1.3-kb amplicons. Further, in several cases, the rDNA fragment sequences (ca. 500 bp) were identical for phylotypes originating from different soils. This may indicate that such phylotypes represent a common species. However, such a short 16S rDNA fragment cannot conclusively indicate a common species, particularly for the genus *Pseudomonas* (33). In all cases, the RIS-RFLP phylotypes affiliated with a common genus, but derived from different soils, were distinct. The RIS size and sequence is known to vary among strains of the same species (17, 24), so the RIS-RFLP analysis probably resolves strains at the species or subspecies level. Thus, the soils from distant regions appear to harbor members of the above genera that can occupy a common niche, but the species or subspecies of these genera occupying this niche may be endemic. To determine whether this is the case, it would be necessary to assay the presence of these phylotypes (as with a PCR assay) in environmental samples, rather than in enrichment cultures. The endemicity of microorganisms in natural environments has not been well studied, but there are a few reports suggesting endemicity of species or subspecies (8, 10, 18).

Similarly, members of the same genera were enriched from Alert soil under the different incubation conditions. Members of *Pseudomonas* were predominant in all cultures inoculated with Alert soil, except the aerobic, 20°C culture, in which a *Pseudomonas* strain was detected, but *Sphingomonas* was predominant (Table 3). Again, most RIS-RFLP phylotypes were found only in one enrichment culture. However, two RIS-RFLP phylotypes affiliated with the genus *Pseudomonas* were found in both the aerobic, 7°C cultures and the anaerobic, 20°C cultures inoculated with Alert soil (A-O7-11 = A-A20-2 and A-O7-12 = A-A20-19). This suggests that the one or two species or subspecies represented by these phylotypes were well adapted to both culture conditions.

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