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Vertical and Horizontal Variations in the Physiological Diversity of the Aerobic Chemoheterotrophic Bacterial Microflora in Deep Southeast Coastal Plain Subsurface Sediments

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Aerobic chemoheterotrophic bacteria were isolated from surface soils and coastal plain subsurface (including deep aquifer) sediments (depths to 265 m) at a study site near Aiken, S.C., by plating on concentrated and dilute media. Morphologically distinct colonies were purified, and their responses to 21 selected physiological tests were determined. These isolates were quite diverse; 626 physiologically distinct types (i.e., types with a unique pattern of responses to the 21 tests) were detected among the 1,112 isolates obtained. Physiologically distinct types were isolated on concentrated and dilute media (only 11% overlap between the groups); isolates from surface soils and subsurface sediments were also quite different (only 3% overlap). The surface soil isolates more readily utilized all but 1 of 12 carbon sources offered, and a significantly larger proportion of them hydrolyzed esculin and gelatin. Only 4% of the subsurface isolates fermented glucose, even though 82% of them could use it aerobically. L-Malate and D-gluconate were utilized by at least 75% of the subsurface isolates, and seven other carbon sources were used by at least 40% of them. Subsurface isolates from different geological formations (depths) and, to a lesser extent, from the same geological formation at different boreholes differed distinctly in their group responses to certain physiological tests. Moreover, sediments from different depths and boreholes contained physiologically distinct types of bacteria. Thus, considerable bacterial diversity was observed in coastal plain subsurface sediments, even within defined geological formations.

The microbiology of aquifers and other subsurface sediments has recently become the subject of rapidly expanding interest, in part because aquifers are the major source of fresh water in many countries (8, 22) and are now becoming contaminated with the toxic by-products of human activities (5, 15). If sufficient numbers of microorganisms reside in subsurface sediments, they may play (or, through nutrient amendment or genetic manipulation, be made to play) an important role in the degradation of toxic groundwater pollutants.

Most recent studies have focused on shallow subsurface sediments (depths less than 30 to 35 m), in which substantial numbers of microorganisms were found (1–3, 9, 12, 13, 16, 17, 20, 24). These and other investigations have shown or implied that the shallow subsurface microflora (i) is predominantly procaryotic, (ii) appears to be specially adapted for growth and survival in nutrient-poor conditions, (iii) includes strains that can function throughout a wide range of nutrient concentrations, and (iv) may sometimes exert a significant effect on groundwater chemistry (for a recent review, see reference 11).

Deeper subsurface sediments (depths greater than 30 to 35 m) have received comparatively little attention, primarily owing to the high cost and difficulties associated with sampling. However, reports during the past 60 years (for reviews, see references 6 and 11) and the finding that microbial numbers do not decrease with depth in the shallow subsurface (2, 3, 13, 16) imply that significant microbial populations occur in the deeper subsurface as well.

Only recently has it become apparent that deep subsurface sediments are, indeed, occupied by a substantial microflora. In 1982, Hoos and Schweisfurth (14) found 10^3 to 10^6 bacteria per g to a depth of 90 m in aquifer sediments from a site in the Federal Republic of Germany. Chapelle et al. (4) then reported the presence of similar numbers of bacteria to a depth of 182 m in Maryland coastal plain sediments. In June 1986, the U.S. Department of Energy began a collaborative research program (7) in which aseptically handled sediment cores (depths to 265 m) were collected from the site of the Savannah River Plant (SRP) near Aiken, S.C., and examined by investigators at several universities and national laboratories. Our specific task in this program was to enumerate, isolate, and partially characterize aerobic chemoheterotrophic bacteria in the deep SRP sediments.

In an earlier paper (D. L. Balkwill, Geomicrobiol. J., in press), it was reported that substantial numbers of aerobic chemoheterotrophic bacteria (10^5 to 10^8 viable cells per g) were present in the SRP sediments. These bacteria (most of which were gram-negative rods) were the numerically predominant components of the deep subsurface microflora. They appeared, on the basis of their cell and colony morphology characteristics, to be a very diverse group of microorganisms. Neither the number of chemoheterotrophs nor their apparent diversity decreased with increasing depth.

The purpose of the present study was to obtain physiological data about the chemoheterotrophic bacteria that were isolated from the SRP sediments, specifically data that would be of use in further quantifying the diversity of the microflora and the extent to which its specific composition varies spatially throughout the subsurface environment. We report here that most of the subsurface chemoheterotrophs were metabolically oxidative (rather than fermentative) and could assimilate a variety of carbon sources. We also describe (i) how the physiological traits of the subsurface bacteria differed from those of bacteria in the surface soil and (ii) how the subsurface microbial populations in different

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geological formations at the SRP site differed in their specific composition and their group physiological traits.

MATERIALS AND METHODS

Soil and subsurface sediment samples. Surface soils and subsurface sediments were acquired from the SRP, Aiken, S.C. The SRP is situated in the Upper Atlantic Coastal Plain, on a comparatively flat plateau that is dissected by several small tributaries of the Savannah River. The plant site is underlain by approximately 300 m of unconsolidated material (primarily sands, sandy clays, and clayey sands), which, in turn, is underlain by metamorphic rock or consolidated mudstone. Several aquifers are situated beneath the SRP site. These aquifers are separated by nontransmissive, clay confining layers that retard, but do not fully prevent, the flow of water between the aquifers. The ambient groundwater temperature at the site is 23 to 25°C year round.

Samples for microbiological analysis were obtained in June and July 1986. One surface soil sample (from immediately beneath the rooting zone of the surface vegetation) and 14 to 15 subsurface samples were collected at each of the three sites P24, P28, and P29. The sample sites were situated approximately along a straight line; site P28 was 3.9 km east southeast of site P29, and site P24 was 11.8 km southeast of site P28. When possible, samples were recovered from the same geological formations at each site (Table 1). In all cases, subsurface samples came from the vadose zone; from several transmissive, aquifer zones; and from several relatively nontransmissive, clay confining layers. The depth to the water table was 41 m at P24, 32 m at P28, and 36 m at P29.

Numerous precautions were taken to limit microbial contamination of subsurface sediment samples from surface soil, overlying sediments, or drilling muds (sodium bentonite viscosifying fluid). Details of the methods used to collect and pare the sediment cores are described by Phelps et al. (T. J. Phelps, C. B. Fliermans, T. R. Garland, S. M. Pfiffner, and D. C. White, J. Microbiol. Methods, in press). Briefly, cored sediments in polyvinyl chloride core liners were placed in an N_2-flushed glove bag and extruded or pared (19) to remove the outer portions of the cores (approximately one-half) that were more likely to be contaminated with drilling muds. The remaining core material was homogenized, packed in sterile Whirl-Pak bags (NASCO, Inc., Ft. Atkinson, Wis.), placed in quart canning jars, and removed from the glove bag. The canning jars were packed on ice, shipped to this laboratory via overnight express service (within 16 h after sampling), and stored at 4°C until they could be examined as described below.

Isolation of bacterial strains for physiological testing. The bacterial cultures examined in this study were isolated from plates originally used for enumeration of viable, aerobic, chemoheterotrophic bacteria in soil and sediment samples. These plate counts were performed within 72 h after receipt of each sample. (Viable counts do not increase during this period [10].) The samples were prepared for plating by blending in 0.1% $Na_4P_2O_7 \cdot 10H_2O$ (pH 7) (1). Serial (10-fold) dilutions of the blended samples were then prepared in phosphate-buffered saline (8.3 mM Na_2HPO_4, 16 mM NaH_2PO_4, 0.15 M NaCl [pH 7.2]).

For plate counting, serial dilutions were spread-plated in triplicate on one concentrated, nutrient-rich medium (peptone-tryptone-yeast extract-glucose medium [PTYG] [1]) and one dilute medium (1% PTYG medium; a 1:100 dilution of PTYG, except that the concentrations of agar,

TABLE 1. Characteristics of surface soil and subsurface sediment samples collected at the SRP site

Sample site	Geological formation[a]	Physical characteristics[b]				Viable counts[c] (log CFU/g [dry wt] of sediment) at 23°C on[d]:	
		Depth (m)	Moisture content (%)	% Clay/ % sand	Pore water pH	1% PTYG	PTYG
P24	UP	0[e]	2.2	12/85	5.0	6.49	6.31
P24	TR	34	18.7	12/85	6.6	4.09	4.13
P24	DB	45[f]	31.9	9/59	6.1	6.37	5.51
P24	MB	58	15.1	14/72	7.6	4.81	4.61
P24	CG	91	15.9	7/87	8.0	6.48	5.68
P24	EL	118	34.3	10/83	6.9	5.76	5.06
P24	EL	139	16.3	9/90	6.4	3.22	3.12
P24	PD	145[g]	18.5	45/45	6.2	3.24	3.32
P24	PD	180	11.4	10/83	6.9	6.65	5.96
P24	BC	200	22.4	5/90	7.3	4.97	5.71
P24	BC	204	23.8	32/48	4.6	4.98	4.80
P24	BC	234[g]	24.3	67/17	4.7	2.12	NG[h]
P24	MD	244	16.5	8/87	5.4	4.40	4.46
P24	MD	255[g]	11.7	52/26	5.1	NG	NG
P24	MD	259	24.5	11/84	6.4	6.46	5.94
P24	MD	265[g]	ND[i]	ND	ND	2.48	2.90
P28	UP	0[e]	5.3	2/96	5.3	6.38	6.48
P28	TR	14	21.7	7/93	6.5	2.88	3.05
P28	MB	31[f]	18.5	6/93	4.2	5.58	5.29
P28	CG	59	21.7	3/94	7.6	7.01	6.42
P28	EL	72[g]	24.7	20/64	5.6	3.84	3.86
P28	PD	112	19.4	10/85	6.9	7.29	6.09
P28	PD	115[g]	0	43/27	6.0	NG	NG
P28	BC	134	17.3	6/91	6.6	5.16	4.40
P28	BC	162	19.0	7/88	6.0	3.27	3.88
P28	MD	180[g]	39.9	48/40	5.7	5.72	5.67
P28	MD	182[g]	21.9	63/23	4.7	3.68	3.70
P28	MD	191	30.2	20/74	6.6	7.16	5.86
P28	MD	203	19.0	16/81	4.9	7.61	6.70
P28	MD	214	13.9	11/84	6.9	6.40	6.10
P28	MD	216	15.9	14/79	6.2	3.90	3.74
P29	TR	0[e]	3.2	9/86	5.7	6.59	6.29
P29	TR	8	14.2	8/92	5.7	5.67	4.02
P29	DB	29	24.5	8/90	5.7	6.20	5.46
P29	CG	39[f]	21.7	4/93	7.4	6.72	4.84
P29	EL	69[g]	23.6	72/14	3.9	NG	NG
P29	PD	94	23.0	7/90	5.7	4.74	5.08
P29	BC	111[g]	27.0	80/9	4.3	NG	<2
P29	BC	141	18.2	10/85	5.5	5.54	5.39
P29	MD	151	17.8	8/89	6.4	6.56	5.91
P29	MD	176	16.5	32/48	5.8	6.28	5.79
P29	MD	181[g]	11.7	40/47	4.1	2.25	<2
P29	MD	187	14.4	13/83	6.1	5.90	4.67
P29	MD	193	18.3	10/87	6.1	5.20	4.50
P29	MD	200	17.0	6/93	7.1	5.93	4.02
P29	MD	213	16.6	11/84	6.0	6.89	5.74

[a] Abbreviations: UP, Upland; TR, Tobacco Road; DB, Dry Branch; MB, McBean; CG, Congaree; EL, Ellenton; PD, Pee Dee; BC, Black Creek; MD, Middendorf.

[b] Data provided by T. Garland, K. McFadden, J. K. Fredrickson, and R. J. Hicks, Pacific Northwest Laboratory.

[c] All data are reported as the average count from triplicate spread plates.

[d] Data are from Balkwill, in press.

[e] Surface soil sample, taken from just beneath the rooting zone of the surface vegetation.

[f] Depth to water table: P24, 41 m; P28, 32 m; P29, 36 m.

[g] Clay confining layer.

[h] NG, No growth on any of the lowest-dilution plates (10^{-2}).

[i] ND, No data.

$MgSO_4 \cdot 7H_2O$, and $CaCl_2 \cdot 2H_2O$ were the same as in PTYG). The plates were incubated aerobically at 23°C. PTYG plates were examined after 5 days and again after 2 weeks; 1% PTYG plates were examined after 2 weeks and again after 4 weeks. The viable counts (Table 1) (Balkwill, in press) were calculated in the usual manner.

To select strains for isolation from each set of PTYG or 1% PTYG plates (i.e., the plates for each sample), all of the recognizably distinct types of bacterial colonies (based on morphological characteristics [21]) present at the countable dilution (or higher) were numbered and described in detail. Each of the colony types was then isolated by streaking and restreaking on the original enumeration medium. A total of 1,112 bacterial strains were successfully isolated from the surface soil and sediment samples in this way.

Preservation of isolated bacterial strains. To minimize genetic changes that might occur during repeated transfer of the soil and subsurface bacterial isolates on laboratory media, we preserved all isolates by freezing shortly after isolation. In each case, cell material from the restreak plate (see above) was transferred to 50 ml of broth (a liquid version of the plating medium) and incubated at 20 to 25°C. The broth cultures were grown to mid-exponential phase, concentrated by centrifugation, suspended in fresh growth medium containing 7% sterile dimethyl sulfoxide, and frozen at −75°C. All cultures were stored at −75°C until they were examined as described below.

Physiological testing of isolated bacterial strains. A preliminary morphological and physiological examination of selected strains showed that the majority (perhaps as many as 80%) of the isolates from subsurface sediments were nonfermentative, gram-negative rods. Consequently, API Rapid NFT kits (Analytab Products, Plainview, N.Y.), which were originally designed for the identification of nonfermentative, gram-negative rods from clinical samples, were used to test each of the isolates for 21 selected physiological traits. The tested characteristics included seven specific metabolic traits and the ability to aerobically utilize 12 different compounds as sole sources of carbon (see Table 2). The API Rapid NFT kits were used primarily as a means of rapidly obtaining physiological data for a large number of isolates rather than as a means of identifying the isolated strains; it was understood that the kits were not designed for the identification of nonclinical isolates.

Isolates were activated for physiological testing by streaking from the frozen stocks described above. The isolates were always cultivated on their original isolation medium (either PTYG or 1% PTYG) for API testing because some of them would not grow on the other medium (thus precluding the use of a common medium for all isolates). Tests conducted with a randomly selected subset of isolates that could grow on either PTYG or 1% PTYG indicated that the same API results were obtained after growth on either medium.

The plates streaked from frozen stocks (see above) were incubated at 23°C until visible growth appeared, after which the cultures were restreaked on fresh medium. Restreak plates were incubated at 23°C for 2 to 4 days (for PTYG) or 5 to 7 days (for 1% PTYG) prior to inoculation of the API test strips. The API test results did not change during these ranges of incubation times (D. L. Balkwill, unpublished results of time course experiments performed on randomly selected isolates).

The API Rapid NFT kits were inoculated, incubated (at 30°C), and scored as specified by the manufacturer. Incubation of the kits at 30°C (as opposed to 23°C) did not affect the results (Balkwill, unpublished data). The resulting data were recorded, entered into a microcomputer, and organized with a commercially supplied database management program (dBASE III PLUS; Ashton-Tate, Torrance, Calif.). The data were then analyzed to determine how various groups of isolates (e.g., all PTYG isolates, all isolates from a particular sample site, or all isolates from a particular geological formation) reacted to the 21 API tests. The data were also analyzed to determine how many physiologically distinct types (i.e., strains with a unique pattern of responses to the 21 API tests) were isolated from each soil and sediment sample (as a measure of microbial diversity). The vertical and lateral distributions of distinct physiological types were then assessed by determining the number of depths and sample sites at which each type was detected. The data for each isolate were also checked against the *API Rapid NFT Identification Codebook*, Analytab Products, Plainview, N.Y., 1986, and the possible identities of strains that keyed out (certainty of identification, >90%) were noted.

RESULTS

Group physiological traits of subsurface bacterial isolates. Only 4% of the 1,032 aerobic chemoheterotrophs isolated from the SRP subsurface sediments on PTYG and 1% PTYG media fermented glucose, even though 82% of them could use it aerobically as a carbon source (Table 2). Only 1% and 11% of these bacteria tested positive for tryptophanase and arginine dihydrolase, respectively. All of the API test carbon sources except caprate and *N*-acetyl-D-glucosamine were assimilated by at least 40% of the isolates, and three of them (glucose, L-malate, and D-gluconate) were utilized by at least 75% of the isolates.

The individual data for PTYG and 1% PTYG subsurface isolates indicated that the physiological traits of these two groups of bacteria were somewhat different (Table 2). A statistically significant difference ($P < 0.05$) between the responses of PTYG and 1% PTYG isolates to a number of the physiological tests was observed. A significantly larger proportion of the PTYG isolates tested positive for arginine dihydrolase, gelatin hydrolysis, and oxidase, whereas a larger proportion of the 1% PTYG isolates were positive for urease and *p*-nitrophenyl-beta-galactopyranoside (PNPG) β-galactosidase. The two groups reacted similarly to 8 of the 12 carbon assimilation tests, but a significantly larger proportion of the PTYG isolates assimilated D-gluconate, caprate, citrate, and malate (Table 2).

Group physiological traits of surface soil bacterial isolates. The group physiological traits of the aerobic chemoheterotrophs isolated from SRP surface soils on both plating media (Table 2) differed markedly from those of the subsurface isolates. The surface soil bacteria more readily utilized most of the carbon sources offered in the assimilation tests; 8 of the 12 sources were used by at least 75% (often by more than 85%) of the surface soil isolates (compared with 3 of the 12 for the subsurface isolates). Caprate, which was used by only 9% of the surface soil isolates, was the obvious exception to the rule. Surface and subsurface bacteria responded similarly to the other API tests, except that a significantly larger proportion of surface soil isolates hydrolyzed esculin and gelatin, whereas a significantly larger percentage of subsurface isolates reduced nitrate ($P < 0.05$; exact binomial test [18]).

The individual data for PTYG and 1% PTYG surface soil isolates implied that the physiological traits of organisms isolated on concentrated and dilute (i.e., oligotrophic) media were somewhat more similar than were those of the same

TABLE 2. Group physiological characteristics of bacteria isolated from surface soils and subsurface sediments on concentrated (PTYG) and dilute (1% PTYG) media[a]

Physiological test	% Subsurface isolates testing positive on:			Surface soil isolates testing positive on:		
	Both[b] (1,032)[c]	PTYG (602)	1% PTYG (430)	Both (80)	PTYG (50)	1% PTYG (30)
Specific metabolic traits						
Nitrate reductase	53	50	58*[d]	39	16	77**
Tryptophanase	<1	<1	<1	0	0	0
Arginine dihydrolase	11	15	5**	20	12	33*
Urease	32	24	42**	30	28	33
Esculin hydrolysis	50	47	54*	70	64	80
Gelatin hydrolysis	38	43	32**	71	74	67
PNPG β-galactosidase	50	45	56**	59	56	63
Oxidase	48	54	39**	39	54	13**
Glucose fermentation	4	7	1**	0	0	0
Aerobic assimilation tests						
D-Glucose	82	78	86**	96	96	97
L-Arabinose	46	43	51*	69	72	63
D-Mannose	53	51	56	85	84	87
D-Mannitol	60	59	61	85	90	77
N-Acetyl-D-glucosamine	34	33	35	90	90	90
Maltose	65	61	69*	83	86	77
D-Gluconate	75	79	70**	89	92	83
Caprate[e]	28	37	15**	9	12	3
Adipate[e]	48	44	53*	56	60	50
L-Malate	82	87	76**	88	86	90
Citrate	57	66	44**	79	82	73
Phenylacetate	41	45	36*	39	48	23**

[a] Combined data for all three sample sites (P24, P28, and P29).
[b] Both, Combined results for PTYG and 1% PTYG isolates.
[c] Numbers in parentheses indicate the sample size for the group of organisms described in each column.
[d] Symbols: *, the PTYG and 1% PTYG percentage values are very close to being significantly different ($P < 0.05$), as determined by the exact binomial test (1); **, the PTYG and 1% PTYG percentage values are significantly different ($P < 0.01$ to 0.05); the remaining PTYG and 1% PTYG percentage pairs are not significantly different.
[e] Caprate (hexanoic acid) and adipate (1,4-butanedicarboxylic acid) are short-chain fatty acids; the remaining compounds used in the assimilation tests are common sugars or organic acids.

two groups (i.e., PTYG and 1% PTYG) of subsurface isolates (see above). The two groups of surface soil isolates differed significantly in their responses to only three physiological tests (nitrate reductase, oxidase, and phenylacetate assimilation [Table 2]). It is possible, however, that fewer differences were seen with the surface soil isolates because the sample size was smaller (80 surface soil isolates versus 1,032 subsurface isolates), thereby reducing the statistical power to detect them.

Subsurface microbial physiological traits at different sample sites. The individual data for PTYG isolates from different sample sites (Table 3) indicated that these three groups of organisms differed significantly in only five of the tested traits. A significantly larger ($P < 0.01$) proportion of isolates from site P28 were positive for maltose assimilation and arginine dihydrolase (compared with the isolates from sites P29 and P24). In contrast, a significantly smaller proportion of isolates from site P28 were positive for phenylacetate assimilation, adipate assimilation, and urease (compared with those from site P29 and, except for urease, those from site P24). Except for these differences, the responses to the physiological tests were quite consistent from one sample site to another.

The 1% PTYG isolates from sites P24, P28, and P29 also

TABLE 3. Physiological characteristics of bacteria isolated from subsurface sediments at three sample sites on concentrated (PTYG) and dilute (1% PTYG) media

Physiological test	% Positive isolates on PTYG			% Positive isolates on 1% PTYG		
	P24 (197)[a]	P28 (198)	P29 (207)	P24 (144)	P28 (153)	P29 (133)
Specific metabolic traits						
Nitrate reductase	49	50	51	58	55	62
Tryptophanase	<1	0	0	0	1	0
Arginine dihydrolase	8	23	13**[b]	4	6	3
Urease	21	18	34**	31	44	52**
Esculin hydrolysis	46	51	45	63	54	45
Gelatin hydrolysis	38	45	45	47	29	20**
PNPG β-galactosidase	44	48	44	61	54	53
Oxidase	48	58	56	40	26	52**
Glucose fermentation	6	5	9	1	2	1
Aerobic assimilation tests						
D-Glucose	78	85	72	91	81	87
L-Arabinose	48	41	39	54	49	49
D-Mannose	52	51	50	60	58	51
D-Mannitol	52	66	59	57	52	77**
N-Acetyl-D-glucosamine	29	36	33	40	35	29
Maltose	58	73	52**	72	74	61
D-Gluconate	73	77	87	76	64	71
Caprate	36	36	39	21	13	12
Adipate	44	33	56**	51	49	59
L-Malate	86	83	92	83	65	83
Citrate	58	67	73	42	42	49
Phenylacetate	39	37	59**	33	30	46*

[a] Numbers in parentheses indicate the number of isolates that received each test in that column.
[b] Symbols: *, statistically significant difference between the values from different sites ($P < 0.05$); **, highly significant difference ($P < 0.01$); the remaining sets of results are not significantly different; (determined by the chi-square test in which the expected value is the mean number observed).

differed significantly in their responses to five of the physiological tests (Table 3), and two of these differences (urease and phenylacetate assimilation) paralleled those detected among the PTYG isolates from different sites. A significantly larger proportion of P29 isolates tested positive for urease, oxidase, mannitol assimilation, and phenylacetate assimilation, but a significantly larger proportion of P24 isolates tested positive for gelatin hydrolysis. Responses to the other physiological tests were fairly consistent from one sample site to another.

Subsurface microbial physiological traits in different geological formations. Data for aerobic chemoheterotrophs isolated from specific geological formations at the SRP site on PTYG medium were compared to determine the extent to which the group physiological traits of the subsurface microorganisms varied from one formation to another. The results from the Pee Dee formation generally exhibited the highest standard deviation, resulting from variations within the formation across the three sample sites, whereas the data from the Middendorf and Ellenton formations were the most consistent across the three sample sites.

Variations within single formations across the three sample sites aside, the PTYG isolates from the Congaree and Ellenton formations differed markedly from those from the other formations in their responses to many physiological tests. For example, 43 to 62% of the PTYG isolates from the other formations hydrolyzed esculin, compared with 18% of those from Congaree and 85% of those from Ellenton; 26 to 67% of the isolates from other formations tested positive for

PNPG β-galactosidase, compared with 15% of those from Congaree and 97% of those from Ellenton; and 22 to 54% of the isolates from other formations utilized phenylacetate, compared with 12% of those from Congaree and 73% of those from Ellenton. In contrast, isolates from the three deepest formations (Pee Dee, Black Creek, and Middendorf) that are all part of a single aquifer responded similarly to most physiological tests. The physiological characteristics of these three groups of isolates were generally more like those of bacteria from shallower sediments (such as Dry Branch and McBean) than like those of bacteria from the Congaree and Ellenton formations.

The physiological traits of the 1% PTYG isolates also differed from one geological formation to another. However, the differences were not as distinct as with the PTYG isolates, because there was more variation within each formation across the three sample sites.

Diversity of physiologically distinct forms in surface soils and subsurface sediments. At least 626 physiologically distinct types (i.e., isolates that differed from all other isolates in their responses to at least 1 of the 21 tests in the API kits [see Materials and Methods]) were detected among the 1,112 surface soil and subsurface isolates (on PTYG and 1% PTYG) tested. A more detailed analysis of the 210 PTYG isolates that were obtained from site P24 indicated that 145 of them differed from all other isolates in their response to at least 1 of the 21 API tests (i.e., were physiologically distinct). Of these 145 distinct types, 85 differed from all other types in the group by at least 2 of the 21 tests, 36 differed by at least 3 tests, and 10 differed by at least 4 tests. Further analysis indicated that the average distinct type differed from 0.7 of the other 144 types in the group by only 1 of 21 tests, from 2.5 of them by 2 tests, from 4.8 of them by 3 tests, from 7.5 of them by 4 tests, and from 128.5 of them by 5 or more tests. The degree of diversity in this group of isolates, then, appeared to be quite high.

A comparison of the specific types present in each group implied that somewhat different organisms were isolated on 1% PTYG and PTYG media. Only about 11% of the PTYG isolates had response patterns (to the API tests) that matched those of any of the 1% PTYG isolates. In another comparison, only 3% of the surface soil isolates (on either medium) were found to have response patterns matching those of any of the subsurface isolates.

An analysis of the data for individual sediment samples indicated that the microflora in most of these samples was quite diverse. Fifteen or more distinct types of bacteria (total number of types from PTYG and 1% PTYG) were isolated from 34 of the 43 subsurface samples; 25 or more types were isolated from 16 samples. Microbial diversity (as reflected by the number of distinct types) was low only in samples from the relatively nontransmissive confining layers (with clay contents above 40%), in which case the numbers of colonies present on the plates were also quite small (Table 1).

Although subsurface microbial diversity varied somewhat among geological formations at the SRP site (Table 4), there was no tendency for diversity on PTYG or 1% PTYG agar to decrease sharply with increasing depth. In fact, the deepest sediments at the site often yielded as many physiologically distinct types of isolates as did the shallower sediments and the surface soil above them.

Vertical and lateral distribution of physiologically distinct bacterial types. The physiological characteristics (response patterns to the 21 physiological tests) of the PTYG isolates from different depths at each sample site were compared to determine the number of depths at which each physiologically distinct type was detected (Table 5). Most of the types (84%) at each sample site were detected at only one depth. Only 5 to 7% of them were found at three or more depths, and no type was detected at more than seven depths. Similar results were obtained for the 1% PTYG isolates at each site (Table 5).

TABLE 4. Microbial diversity in SRP subsurface sediments as reflected by the number of physiologically distinct types isolated from enumeration plates

Geological formation[a]	Site P24			Site P28			Site P29		
	Depth (m)	Diversity[b] on:		Depth (m)	Diversity on:		Depth (m)	Diversity on:	
		PTYG	1% PTYG		PTYG	1% PTYG		PTYG	1% PTYG
UP	0[c]	13/13	8/8	0[c]	17/21	11/12			
TR	34	7/7	7/7	14	9/10	10/12	0[c]	16/16	9/10
TR							8	25/28	9/9
DB	45	13/16	7/7				29	26/34	10/10
MB	58	17/19	10/10	31	8/9	15/15			
CG	91	10/11	9/9	59	9/17	19/20	39	9/11	10/10
EL	118	16/19	10/10	72	15/19	7/8	69	0/0	0/0
EL	139	13/14	13/14						
PD	145	6/7	3/3	112	14/15	15/15	94	9/12	10/10
PD	180	15/22	8/9	115	0/0	1/1			
BC	200	14/18	17/17	134	19/21	9/10	111	3/3	0/0
BC	204	13/13	23/26	162	22/28	10/13	141	22/27	17/19
BC	234	0/0	0/0						
MD	244	11/13	14/17	180	12/13	15/15	151	10/17	13/16
MD	255	0/0	0/0	182	11/11	5/5	176	15/15	10/10
MD	259	23/28	10/12	191	16/19	7/9	181	0/0	4/4
MD	265	9/10	2/2	203	11/14	13/13	187	19/22	12/15
MD				214	7/7	10/11	193	6/7	12/12
MD				216	10/11	6/6	200	11/12	8/9
MD							213	16/18	8/9

[a] For definitions, see Table 1, footnote *a*.

[b] Diversity is presented as the number of physiologically distinct types/number of isolates tested. Physiologically distinct types are defined as those that differ in at least one (but usually several) of the 21 physiological characteristics assayed with the API Rapid-NFT kits.

[c] Surface soil sample.

The physiological traits (response patterns) of the isolates from different sample sites were compared to determine the number of sites at which each physiologically distinct type was detected. Most (84%) of the 364 types isolated on PTYG were detected at only one site, and only 4% were detected at all three sites. Of the 325 distinct types isolated on 1% PTYG medium, 90% were detected at only one site and only 2% were detected at all three sites.

Possible identities of subsurface bacterial isolates. Only about 11% of the PTYG and 1% PTYG isolates from subsurface sediments keyed out to specific taxa (Table 6) when their responses to the 21 physiological tests were matched to those listed in the *API Rapid-NFT Identification Codebook*. Of those that keyed out (certainty of identification, 90% or better), most were in the genera *Pseudomonas* (69 isolates), *Acinetobacter* (30 isolates), and *Agrobacterium* (14 isolates). However, these identifications must be considered tentative (especially at the species level), because the isolates were not of clinical origin (which is the intended use of the API Rapid NFT system).

DISCUSSION

The subsurface sediments at the SRP are believed to contain large numbers of microorganisms (10^5 to 10^8 viable

TABLE 5. Extent to which distinct physiological types of bacteria isolated on PTYG and 1% PTYG media were detected at different depths in SRP sediments[a]

No. of depths	No. of types (% of total) detected					
	PTYG isolates			1% PTYG isolates		
	P24	P28	P29	P24	P28	P29
1	122 (84%)	119 (84%)	124 (84%)	105 (86%)	125 (91%)	90 (86%)
2	16 (11%)	15 (11%)	14 (9%)	15 (12%)	10 (7%)	8 (7%)
3	4 (3%)	3 (2%)	8 (5%)	2 (2%)	0	4 (4%)
4	2 (1%)	3 (2%)	1 (1%)	0	2 (2%)	1 (1%)
5	1 (1%)	2 (1%)	0	0	0	2 (2%)
7	0	0	1 (1%)	0	0	0

[a] This table includes data for surface soil and subsurface sediment isolates; the results are not significantly different if surface soil data are excluded.

cells per g), the majority of which are aerobic or facultative chemoheterotrophic bacteria (Balkwill, in press). The present study has shown that most of these chemoheterotrophs (i.e., most of those that can be isolated on PTYG or 1% PTYG agar) are oxidative rather than fermentative (82% assimilating glucose aerobically but only 4% fermenting it). Moreover, pore water chemical analyses (J. K. Fredrickson, T. R. Garland, R. J. Hicks, J. M. Thomas, S. W. Li, and K. M. McFadden, Geomicrobiol. J., in press) indicated that the SRP sediments examined in this study were not highly reduced (e.g., reduced sulfur species and Fe^{2+} were, for the most part, absent).

The SRP subsurface chemoheterotrophs were metabolically versatile with respect to their ability to utilize the 12 compounds offered as substrates in the API tests, but not nearly as versatile as were the chemoheterotrophs isolated from surface soils (Table 2). These results suggest that chemoheterotrophs in the subsurface may be restricted to a narrower range of carbon sources than are their surface soil counterparts. More likely, they indicate that the subsurface microorganisms use carbon sources that are different from those utilized by heterotrophic bacteria in soil. (Presumably, most of the easily metabolized carbon in surface soils is consumed or partially degraded before reaching the subsurface [11].) Extensive testing will be required to define the full range of compounds that can be assimilated by bacteria deep below the surface.

TABLE 6. List of recognized taxa that may be among the SRP bacterial isolates according to physiological analysis with the API Rapid NFT system

Taxon	No. of isolates keying out[a]
Achromobacter xylosoxidans	1
Acinetobacter calcoaceticus subsp. *anitratus*	27
Acinetobacter calcoaceticus subsp. *lwoffi*	3
Aeromonas hydrophila	1
Agrobacterium radiobacter	14
Chromobacterium violaceum	1
Flavobacterium multivorum	1
Pasteurella haemolytica	2
Pasteurella spp.	2
Pseudomonas acidovorans	2
Pseudomonas cepacia	21
Pseudomonas fluorescens	2
Pseudomonas maltophilia	1
Pseudomonas paucimobilis	4
Pseudomonas pickettii	2
Pseudomonas stutzeri	22
Pseudomonas testosteroni/alcaligenes	3
Pseudomonas vesicularis	12

[a] Isolates were considered to key out only if the certainty of identification, according to the *Rapid NFT Identification Codebook*, was greater than 90%.

The subsurface sediments and surface soils at the SRP site were clearly populated by different types of microorganisms (i.e., strains with different response patterns to the 21 API tests), whose group physiological characteristics also differed in several ways (Table 2). Similar results have been reported for shallow aquifer sediments at a site in Oklahoma, in which case analysis of colony morphologies on enumeration plates indicated that most of the microbial types detected in the subsurface were not detected in the surface soil (2, 3). Such findings imply that subsurface environments are not inhabited by microorganisms that drift down from the overlying surface soils, at least not by the numerically predominant components of the surface soil flora that can be detected by plating or other isolation procedures.

Viable counts for SRP sediments on dilute media were usually higher (up to 10-fold higher) than those on concentrated media (Table 1) (Balkwill, in press), implying that many of the organisms seen on dilute media were not detected on the concentrated media. The present results confirm that largely different groups of organisms (organisms with different response patterns [only 11% overlap] to the API tests) were isolated on one concentrated (PTYG) and one dilute (1% PTYG) medium. Hirsch and Rades-Rohkohl (13) recently obtained similar results when they isolated (from a shallow aquifer) morphologically distinct groups of bacteria on two types of oligotrophic media. Such findings evince the importance of using a variety of media to isolate microorganisms if one wishes to study microbial diversity in the environment. The present findings also indicate that one might want to include a concentrated medium in such a study, even when examining a nutrient-poor environment.

Analysis of colony morphologies on enumeration plates has indicated that the chemoheterotrophic bacterial portion of the SRP subsurface microflora is quite diverse; e.g., 172 distinct colony morphologies could be found among the 210 strains isolated from site P24 on PTYG agar (Balkwill, in press). The present study has shown that many of the distinct colony types defined during that analysis also differ in their physiological traits (626 distinct API response patterns among 1,112 strains). The chemoheterotrophic microflora, then, does appear to be diverse, but the extent of its diversity cannot be defined without more information. (For example, the API results do not permit us to say whether the isolates included a large number of unrelated genera or just an assemblage of similar strains from closely related genera.) Physiologically and morphologically diverse populations of chemoheterotrophic bacteria have been reported to occur in

most shallow aquifers as well (12, 13, 16, 17), a notable exception being the aquifer at a frequently studied site in Oklahoma (1–3, 10, 24).

Different geological formations at the SRP site contained distinct types of bacteria (strains with distinct API response patterns). In addition, the group physiological traits of the bacteria in some formations (e.g., Congaree) differed markedly from those in the other formations. Therefore, the specific composition and physiological traits of the subsurface microflora varied with depth, probably in response to physical and chemical differences among geological formations at the SRP. Kölbel-Boelke et al. (16, 17) recently reported similar findings for shallow aquifer sediments (depths to 35 m) at a site in the Federal Republic of Germany, concluding that the microbial community at each depth was both very diverse and distinctly different from those at other depths.

Extensive precautions were taken to limit the contamination of subsurface sediment samples with drilling muds, but it cannot be stated conclusively that the cored sediment samples were entirely free of microorganisms arising from drilling-mud intrusion. Drilling fluids were recirculated through the entire borehole and most probably contained a mixture of microorganisms from the overlying sediments and soil. Consequently, one would expect a core sample from a given depth to contain microorganisms from the overlying strata and soil if it were extensively contaminated with drilling fluids. The fact that 84% of the isolates were unique to one sample or stratum (Table 5) indicates that extensive contamination did not occur. Moreover, the proportion of physiologically distinct types isolated from each sample did not diminish with depth (Table 4), as would be expected if the cores were extensively contaminated with drilling fluids. Nevertheless, a closer examination of the potential for contamination of cored sediments with drilling fluids should be included in future studies of this nature.

Although the group physiological traits of the bacteria in specific geological formations were reasonably consistent from one sample site to another (with a few distinct exceptions), the different sample sites appeared to contain distinct bacterial types (with distinct API response patterns). Kölbel-Boelke et al. (16, 17) found even greater differences from one sample site to another in a shallow aquifer. Such findings suggest that the composition and characteristics of the subsurface microflora vary laterally, even within a single stratum. This possibility has implications with respect to the potential use of microorganisms for the remediation of groundwater contamination. For example, if one wants to stimulate the in situ microflora to degrade contaminants (by nutrient amendment, etc.), one might have to develop different approaches that are suitable for the microflora at each contaminated site or stratum. Research on the spatial heterogeneities of the subsurface heterotrophic bacterial population and on the possible relationships between these heterogeneities and the physical and chemical properties of the sediments could assist in the development of new methods to stimulate the subsurface microflora to remediate contaminated deep aquifers.

The present data are not sufficient to permit identification of the subsurface bacterial isolates. However, the API results, the fact that many of the isolates are oxidative, and the fact that many of them are gram-negative rods (Balkwill, in press) suggest that the SRP microflora contains pseudomonads. This is an interesting possibility, considering the abilities of this group to degrade complex organic compounds (including a number of environmental pollutants). A more definitive identification of the isolates and a more detailed study of their degradative abilities are obvious priorities for future research efforts.

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LITERATURE CITED

1. **Balkwill, D. L., and W. C. Ghiorse.** 1985. Characterization of subsurface bacteria associated with two shallow aquifers in Oklahoma. Appl. Environ. Microbiol. **50**:580–588.
2. **Beloin, R. M., J. L. Sinclair, and W. C. Ghiorse.** 1988. Distribution and activity of microorganisms in subsurface sediments of a pristine study site in Oklahoma. Microb. Ecol. **16**:85–97.
3. **Bone, T. L., and D. L. Balkwill.** 1988. Morphological and cultural comparison of microorganisms in surface soil and subsurface sediments at a pristine study site in Oklahoma. Microb. Ecol. **16**:49–64.
4. **Chapelle, F. H., J. L. Zelibor, D. J. Grimes, and L. L. Knobel.** 1987. Bacteria in deep coastal plain sediments of Maryland: a possible source of CO_2 to groundwater. Water Resour. Res. **23**:1625–1632.
5. **Craun, G. F.** 1984. Health aspects of groundwater pollution, p. 135–195. *In* G. Bitton and C. P. Gerba (ed.), Groundwater pollution microbiology. John Wiley & Sons, Inc., New York.
6. **Dunlap, W. J., and J. F. McNabb.** 1973. Subsurface biological activity in relation to ground water pollution. EPA-6601/273-014, National Environmental Research Center, Office of Research Monitoring, U.S. Environmental Protection Agency, Corvallis, Wash.
7. **Fredrickson, J. K., and R. J. Hicks.** 1987. Probing reveals many microbes beneath earth's surface. ASM News **53**:78–79.
8. **Freeze, R. A., and J. A. Cherry.** 1979. Groundwater. Prentice-Hall, Englewood Cliffs, N.J.
9. **Ghiorse, W. C., and D. L. Balkwill.** 1983. Enumeration and morphological characterization of bacteria indigenous to subsurface environments. Dev. Ind. Microbiol. **24**:213–224.
10. **Ghiorse, W. C., and D. L. Balkwill.** 1985. Microbiological characterization of subsurface environments, p. 387–401. *In* C. H. Ward, W. Giger, and P. L. McCarty (ed.), Ground water quality. John Wiley & Sons, Inc., New York.
11. **Ghiorse, W. C., and J. T. Wilson.** 1988. Microbial ecology of the terrestrial subsurface. Adv. Appl. Microbiol. **33**:107–172.
12. **Hirsch, P., and E. Rades-Rohkohl.** 1983. Microbial diversity in a groundwater aquifer in northern Germany. Dev. Ind. Microbiol. **24**:183–200.
13. **Hirsch, P., and E. Rades-Rohkohl.** 1988. Some special problems in the determination of viable counts of groundwater microorganisms. Microb. Ecol. **16**:99–113.
14. **Hoos, E., and R. Schweisfurth.** 1982. Untersuchungen über die Verteilung von Bakterien von 10 bis 90 Meter unter Boden-Oberkante. Vom Wasser **58**:103–112.
15. **Keswick, B. H.** 1984. Sources of groundwater pollution, p. 39–64. *In* G. Bitton and C. P. Gerba (ed.), Groundwater pollution microbiology. John Wiley & Sons, Inc., New York.
16. **Kölbel-Boelke, J., E.-M. Anders, and A. Nehrkorn.** 1988. Microbial communities in the saturated groundwater environment. II. Diversity of bacterial communities in a pleistocene sand aquifer and their *in vitro* activities. Microb. Ecol. **16**:31–48.
17. **Kölbel-Boelke, J., B. Tienken, and A. Nehrkorn.** 1988. Microbial

communities in the saturated groundwater environment. I. Methods of isolation and characterization of heterotrophic bacteria. Microb. Ecol. **16**:17–29.

18. **Mainland, D., L. Herrera, and M. I. Sutcliffe.** 1956. Statistical tables for use with binomial samples—contengency tests, confidence limits and sample size estimates. Department of Medical Statistics, New York University, New York.
19. **McNabb, J. F., and G. E. Mallard.** 1984. Microbiological sampling in the assessment of groundwater pollution, p. 235–259. *In* G. Bitton and G. P. Gerba (ed.), Groundwater pollution microbiology. John Wiley & Sons, Inc., New York.
20. **Sinclair, J. L., and W. C. Ghiorse.** 1987. Distribution of protozoa in subsurface sediments of a pristine groundwater study site in Oklahoma. Appl. Environ. Microbiol. **53**:1157–1163.
21. **Smibert, R. M., and N. R. Krieg.** 1981. General characterization, p. 409–443. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
22. **U.S. Environmental Protection Agency.** 1977. The report to Congress: waste disposal practices and their effects on ground water. Office of Water Supply and Office of Solid Waste Management Programs, U.S. Environmental Protection Agency, Washington, D.C.
23. **White, D. C., G. A. Smith, M. J. Gehron, J. H. Parker, R. H. Findlay, R. F. Martz, and H. L. Fredrickson.** 1983. The groundwater aquifer microbiota: biomass, community structure, and nutritional status. Dev. Ind. Microbiol. **24**:201–211.
24. **Wilson, J. T., J. F. McNabb, D. L. Balkwill, and W. C. Ghiorse.** 1983. Enumeration and characterization of bacteria indigenous to a shallow water-table aquifer. Ground Water **21**:134–142.