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Plasmid Incidence in Bacteria from Deep Subsurface Sediments

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Bacteria were isolated from deep terrestrial subsurface sediments underlying the coastal plain of South Carolina. A total of 163 isolates from deep sediments, surface soil, and return drill muds were examined for plasmid DNA content and resistance to the antibiotics penicillin, ampicillin, carbenicillin, streptomycin, kanamycin, and tetracycline. MICs of Cu^{2+}, Cr^{3+}, and Hg^{2+} for each isolate were also determined. The overall frequency of plasmid occurrence in the subsurface bacteria was 33%. Resistance was most frequent to penicillin (70% of all isolates), ampicillin (49%), and carbenicillin (32%) and was concluded to be related to the concentrations of the individual antibiotics in the disks used for assaying resistance and to the production of low levels of β-lactamase. The frequencies of resistance to penicillin and ampicillin were significantly greater for isolates bearing plasmids than for plasmidless isolates; however, resistance was not transferable to penicillin-sensitive *Escherichia coli*. Hybridization of subsurface bacterial plasmids and chromosomal DNA with a whole-TOL-plasmid (pWWO) probe revealed some homology of subsurface bacterial plasmid and chromosomal DNAs, indicating a potential for those bacteria to harbor catabolic genes on plasmids or chromosomes. The incidences of antibiotic resistance and MICs of metals for subsurface bacteria were significantly different from those for drill mud bacteria, ruling out the possibility that bacteria from sediments were derived from drill muds.

The presence of a viable microflora, mainly bacteria, in shallow aquifers has been substantiated at numerous locations (2, 20, 23). However, the microbiota of deep unconsolidated subsurface sediments has been the topic of relatively few thorough studies (9, 29, 33), despite the importance of these aquifers as a source of industrial and domestic water supplies. Recently, a collaborative scientific endeavor was initiated by the U.S. Department of Energy to study the diversity and metabolic function of microorganisms in deep terrestrial sediments at the Savannah River plant in South Carolina (14).

The incidence of plasmids in bacterial isolates from aquatic and marine environments has been the subject of a number of studies (4, 6, 17, 22), yet little is known of the incidence and function of plasmid DNA in isolates from subsurface environments (27). Despite the widespread existence of plasmid DNA in environmental bacterial isolates, most of these plasmids remain cryptic. Known bacterial plasmid functions include resistance to antibiotics and metals (5, 16, 25, 30), metabolism of organic compounds (8), and nitrogen fixation (26).

Several studies have examined the relationship between plasmid incidence and the presence of environmental contaminants at a given site. Burton et al. (6) found no significant difference in bacterial plasmid incidence between polluted and unpolluted sites, whereas Hada and Sizemore (17) found that *Vibrio* spp. from oil-polluted water had a higher incidence of plasmid-bearing strains than did isolates from unpolluted water, and Baya et al. (4) found a similar difference between bacteria isolated from toxic waste-contaminated water and bacteria isolated from either uncontaminated or domestic sewage-affected waters.

These studies were undertaken to investigate the occurrence of plasmids and of antibiotic and metal resistance traits in bacterial isolates from deep subsurface sediments, information needed to better understand the physiology and function of deep subsurface bacteria.

MATERIALS AND METHODS

Sample collection. Subsurface sediment samples were obtained from four boreholes drilled at the Department of Energy's Savannah River plant. A total of 50 samples from sediments ranging in depth from 0.2 to 260 m were analyzed. Aseptic sampling procedures (34) were used during handling and processing of core materials (T. Phelps, J. Microbiol. Methods, in press). Samples were placed in sterile Whirl-Pak bags and shipped, on ice, by overnight express carrier to our laboratory in Richland, Wash.

Enumeration and isolation of bacteria. Subsurface sediment samples were shaken in sterile 0.85% NaCl for 20 min, diluted in sterile saline, and plated on tryptic soy agar (24). Plates were incubated for 4 days at 22°C, at which time bacterial colonies were enumerated. Individual colonies, which varied in shape and color, were picked and purified on tryptic soy agar. All bacterial isolates were preserved by freezing broth cultures mixed 1:1 with 80% glycerol at −80°C in cryotubes.

Antibiotic resistance. Resistance to antibiotics was determined by using Sensi-Discs (BBL Microbiology Systems, Cockeysville, Md.) and tryptic soy agar plates according to the instructions of the manufacturer and standard procedures (3). Subsurface isolates were tested for sensitivity to penicillin (6.3 μg), ampicillin (10 μg), carbenicillin (100 μg), kanamycin (30 μg), streptomycin (10 μg), and tetracycline (30 μg). Select isolates were assayed for β-lactamase activity by using Cefinase disks (BBL).

Metal MIC. The metal MIC for each isolate was determined by using tryptic soy agar plates and metal-impregnated filter disks similar to those used for the antibiotic sensitivity tests described above. Standard filter disks (7 mm) were prepared by applying 25 μl of the appropriate concentration of metal solution to disks and allowing the disks to air dry. Metals and standard concentrations used were as follows: Cr^{3+} as $CrCl_3 \cdot 6H_2O$, 25, 50, 125, 250, 375,

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and 500 μg per disk; Cu^{2+} as $CuCl_2 \cdot 6H_2O$, concentrations used for Cr; and Hg^{2+} as $HgCl_2$, 1, 25, 50, 250, 375, and 500 μg per disk. Standard sodium chloride disks were prepared in the same manner at concentrations ranging from 1 to 2,000 μg per disk to determine whether Cl^- could cause growth inhibition. Plates were incubated at 27°C for 24 to 48 h, and diameters of zones of inhibition were measured. These diameters were plotted against metal concentrations, and regression equations were determined for the linear portion of the curve. The MIC was determined as the amount of metal required to produce a 10-mm-diameter zone of inhibition as calculated from the regression equation.

Plasmid DNA analysis. Plasmid DNA was visualized by the method of Eckhardt (10) with modifications. After centrifugation of cells from broth cultures, medium was removed and each pellet was suspended in 0.1% sarcosyl (*N*-laurylsarcosine; Sigma Chemical Co., St. Louis, Mo.) in 50 mM Tris–25 mM EDTA (pH 8.0); the pellet was then incubated at room temperature for 10 min in a 1.5-ml microcentrifuge tube. Cells were repelleted, the sarcosyl was removed, and the pellet was suspended in a lysozyme mixture (10) and incubated at room temperature for 10 to 15 min. Cell lysis was monitored during this time by checking the viscosity of the suspension, and incubation was allowed to continue for an additional 5 to 10 min if the degree of cell lysis was not adequate. A sodium dodecyl sulfate mixture was added and mixed gently with the suspension, which was allowed to incubate for 10 min, followed by addition of 5 μl of proteinase K (5 mg/ml; International Biotechnologies Inc., New Haven, Conn.) and gentle mixing. The mixture was transferred to wells of a 0.7% agarose gel with wide-mouth pipette tips, and the wells were sealed with molten agarose. Samples were electrophoresed for 1 h at 35 V and 6 h at 100 V before being stained with ethidium bromide and visualized under UV light. Plasmid size was determined by comparing the R_f values of plasmids for the unknown subsurface culture with those of plasmids of known size (pGS9, 30.5 kilobases [kb]; pRK2013::Tn*1721*, 62 kb; pWWO, 117 kb; and plasmids carried by *Rhizobium leguminosarum* T87K3, 475, 440, 300, 255, 200, and 155 kb).

Transfer of DNA visualized by the procedure described above onto GeneScreen Plus (Dupont, NEN Research Products, Boston, Mass.) was performed by the capillary blot method according to the protocol of the manufacturer. Filters were subjected to DNA hybridization analysis to determine the relationship of subsurface bacterial plasmid DNA to TOL plasmid DNA. TOL (pWWO) DNA was isolated and purified from *Pseudomonas putida* ATCC 23973 by using a preparation procedure for large plasmids (G. R. Chaudrhy, University of Florida, Gainesville, personal communication). A ^{32}P-labeled whole-TOL-plasmid probe with specific activity of 5×10^7 dpm/μg of DNA was obtained by nick translation (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Prehybridization, hybridization, washings, and autoradiography were performed as previously described (13).

Pore water analyses. The water content of the majority of core samples was insufficient for analysis, necessitating a modification of standard methods (1) for pore water expression. Deionized water was added to near saturation, and the sediment sample was incubated at 25°C under an argon atmosphere for 3 days. A sample volume of 250 ml was packed into an argon-sparged, 76-mm cell (Amicon Corp., Lexington, Mass.) containing a prewashed XM50 ultrafiltration membrane. The sample cell was then pressurized to 80 lb/in^2 with argon to express pore water. Pore water was collected directly into an argon-sparged, prewashed 25-ml polyethylene bottle without contact with the atmosphere. The Cu and Cr concentrations were determined by inductively coupled plasma spectroscopy analysis.

RESULTS

Heterotrophic bacterial populations and plasmid frequencies. Populations of heterotrophic bacteria, as determined by plate count, ranged from below detection to 10^8 CFU/g (dry weight) of sediment (Table 1). In general, populations did not decrease with depth, and strata with low populations tended to yield samples with high amounts of clay (>20%). Sample soil depths ranged from 10 cm to approximately 250 m below the surface for the deepest sample recovered from the Middendorf formation in borehole P24.

Plasmids were observed in heterotrophic bacteria from all four boreholes and from all strata, and the frequency of occurrence did not vary greatly among either boreholes or strata (Table 1). The frequency of plasmids in bacteria tended to be less in the surface soil (Upland) and in the upper aquifer (Tobacco Road, 8 to 34 m) than in deeper sediments. Plasmid frequency for bacteria from the Williamsburg formation was determined from only two samples and therefore cannot be readily compared with frequencies determined for bacteria from other formations. The frequency was also less for bacteria from borehole MCB5 than for bacteria from the other three boreholes. This borehole was drilled near a miscellaneous chemical basin at the Savannah River plant, which in the past has received various organic chemical wastes. Since gram-positive bacteria were encountered infrequently, the data represent only gram-negative bacteria.

Although a broad range of plasmid sizes (3 to 400 kb) was observed in the subsurface bacteria examined, the majority were >150 kb in size (Fig. 1). Plasmids of between 200 and 400 kb were the most frequently encountered, observed in close to 30% of all plasmid-bearing isolates. There were no striking differences in plasmid size distributions among bacteria from the different boreholes except for the absence of plasmids of greater than 200 kb from the MCB5 bacteria. However, the deepest MCB5 sediment sample was 69 m, and the frequency values were based on determinations for only six plasmid-positive isolates. The larger plasmids tended to be associated with bacteria from the deeper sediments (>30 m), which is likely related to the greater frequency of occurrence of plasmids in the deeper sediments. Subsurface bacteria isolated from a range of depths were subjected to plasmid gel electrophoresis and are shown in relation to plasmids of known size in the three reference strains (Fig. 2). The resolution of plasmids by the modified in-well lysis procedure was good enough to identify five of six T87K3 plasmids. Only the largest (475-kb) plasmid was unresolved.

DNA hybridization analysis was conducted to determine the relationship between subsurface bacterial plasmid DNA and the catabolic TOL plasmid, using the latter as a probe. Gel electrophoresis revealed plasmid bands in 7 of 10 isolates from sediments ranging in depth from 40 to 245 m (Fig. 3). All plasmids were between 30 and 117 kb in size. Hybridization with a whole-TOL-plasmid probe indicated that there was homology with plasmids from four of the plasmid-bearing subsurface isolates and with chromosomal DNA from two of the isolates (Fig. 3). Three of these four bands were similar in size to the TOL plasmid. These isolates were all from different sediment samples and represented all three of the boreholes. Filters were washed under

TABLE 1. Plasmid frequency in subsurface bacterial isolates and heterotroph populations in subsurface sediments

Stratum	Depth (m)	Log CFU/g (dry wt) of sediment from borehole:				Plasmid frequency by stratum[a]
		P29	P28	P24	MCB5	
Upland	0.1	6.57 (0/3)[b]	6.43 (0/0)	6.24 (0/0)	6.75 (1/4)	1/7 (14)
Tobacco Road	8–34	6.23 (2/3)	3.57 (1/5)	5.27 (1/5)	3.96 (0/12)	4/25 (16)
Dry Branch	29–45	6.57 (2/3)	7.19 (3/6)	7.01 (0/4)	2.36 (1/5)	7/22 (32)
				5.75 (1/4)		
McBean	36				2.68 (0/0)	
Congaree	39–92	7.00 (1/2)	6.55 (2/6)	6.33 (2/3)	6.49 (4/13)	9/24 (38)
Williamsburg	68–72		2.34 (2/2)		NG[c] (0/0)	2/2 (100)
Ellenton	118–139			5.42 (2/4)		9/29 (31)
				4.32 (0/4)		
Pee Dee	69–180	2.79 (0/0)	6.95 (2/3)	2.11 (0/1)		5/15 (33)
		6.30 (0/4)	3.11 (0/2)	6.76 (0/0)		
			6.45 (3/5)			
Black Creek	111–203	4.04 (0/3)	6.44 (1/6)	6.90 (3/5)		9/23 (39)
		6.35 (3/4)		3.13 (2/5)		
Middendorf	148–260	7.14 (0/2)	6.37 (2/6)	NG (0/0)		15/40 (38)
		<1.00 (1/2)	5.10 (0/2)	5.86 (3/5)		
		6.93 (0/2)	7.28 (4/4)	2.88 (0/3)		
		5.51 (2/3)	7.43 (0/0)	7.39 (0/3)		
		5.72 (2/3)	6.19 (0/2)			
		8.03 (1/1)	5.15 (0/2)			
Plasmid frequency by borehole[d]		14/32 (44)	20/51 (39)	14/46 (31)	6/34 (18)	

[a] Expressed as number of plasmids/number of isolates examined. Numbers in parentheses are percentages.
[b] Ratios in parentheses indicate number of isolates with plasmids/number of isolates examined.
[c] NG, No growth.
[d] Plasmid frequencies (number of isolates with plasmids/number of isolates examined) in surface soils were not included in subsurface frequency totals. Numbers in parentheses are percentages.

high-stringency conditions (low salt, 65°C) to prevent spurious homology. It is also unlikely that the degree of homology arose from differences in the amount of DNA, because all chromosomal bands were very heavy yet there were extremes in the extent of TOL probe hybridization to these bands, from essentially none (Fig. 3, lane A) to a high degree (lane F). In addition, only single plasmid bands were homologous with TOL DNA for the four isolates showing homology, yet all of these isolates had more than one plasmid band.

Antibiotic resistance. Antibiotic resistance and frequencies of plasmid occurrence for isolates from individual boreholes are presented in Table 2. On the basis of the standardized disk method of determining antibiotic resistance, resistance was found most frequently to penicillin, followed by ampicillin and carbenicillin. Resistance to kanamycin, streptomy-

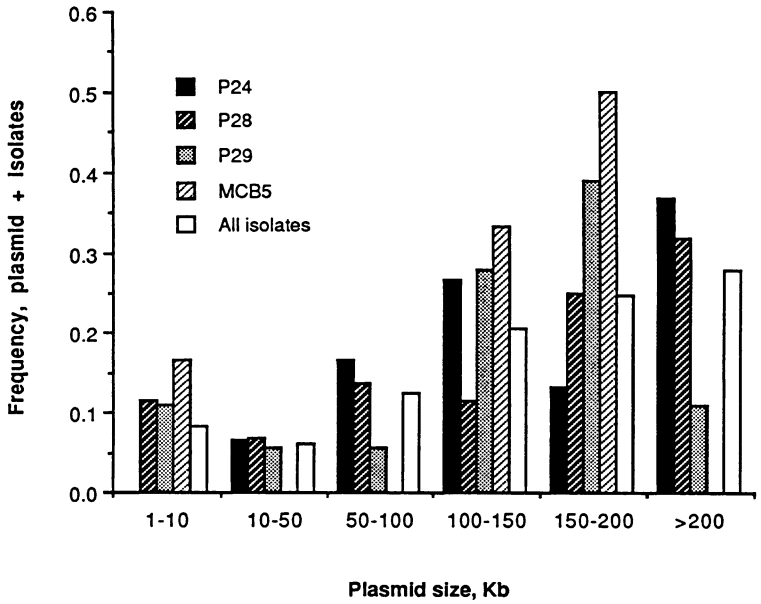

FIG. 1. Plasmid size distributions for all isolates of bacteria from Savannah River subsurface sediments by individual borehole.

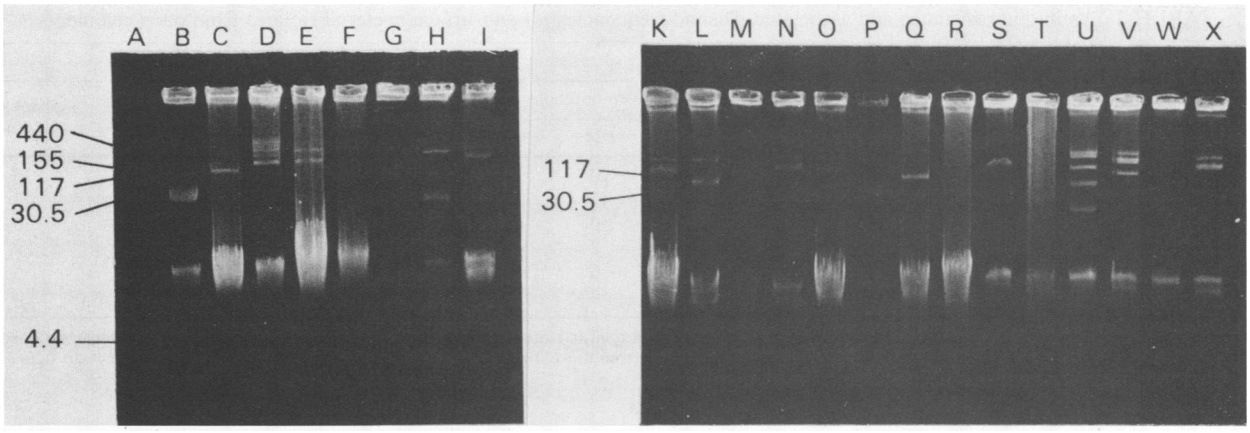

FIG. 2. Visualization of plasmids in subsurface bacteria by modified Eckhardt-type gel electrophoresis. Plasmid molecular markers are pBR322 (A), pGS9 (B, P), pWWO (C, Q), and plasmids of *R. leguminosarum* (D, R). Subsurface bacteria identified by the borehole and depth (in feet; 1 ft = 30.48 cm) from which they were isolated are P28-589A (E), P28-589B (F), P28-589E (G), P28-628A (H), P28-628D (I), P28-193D (K), P28-193B (L), P28-628B (M), P28-628D (N), P28-367F (O), P29-655A (S), P24-860B (T), P24-657C (U), P24-802E (V), P24-802A (W), and P24-103C (X).

cin, and tetracycline was much less frequent. There was little difference in overall antibiotic resistance patterns among isolates from the three deep boreholes (P24, P28, and P29), but there was a significant difference between these isolates and those from the relatively shallow borehole MCB5 and an even greater difference in resistance patterns among all subsurface bacteria and those isolated from the MCB5 return drill muds. Antibiotic-resistant isolates were evenly distributed among the substratum samples within each borehole. The MCB5 subsurface isolates were more often resistant to carbenicillin, streptomycin and, to a lesser degree, tetracycline. Bacteria from the MCB5 return drill muds were resistant to all antibiotics except tetracycline at a greater frequency; none of the 13 drill mud isolates was resistant to tetracycline.

The percentage of antibiotic-resistant isolates that contained plasmids was greater than the overall frequency for all subsurface bacteria (33%) only with respect to resistance to streptomycin (36% [slightly greater]), penicillin (38% [slightly greater]), and ampicillin (47% [significantly greater]) (Table 2). Chi-square tests for association between plasmids and antibiotic resistance were performed for each antibiotic by using the data from all subsurface isolates. Only for penicillin and ampicillin was the hypothesis of independence rejected ($P = 0.001$), indicating a relationship between plasmid occurrence and antibiotic resistance. For plasmid-bearing subsurface bacterial isolates, the ratio of penicillin-resistant to penicillin-sensitive isolates was 9.2:1; the ratio of ampicillin-resistant to ampicillin-sensitive isolates was less, 2.3:1. Resistant-to-sensitive ratios for isolates without plasmids were 1.7:1 and 0.6:1 for penicillin and ampicillin, respectively. A random sample of penicillin-resistant and -sensitive subsurface bacteria assayed for β-lactamase activity revealed that 22 of 22 penicillin-resistant bacteria and 9 of 27 penicillin-sensitive bacteria were positive for β-lactamase activity. In an experiment to determine whether penicillin resistance was transferable, a random sample of 41 penicillin-resistant subsurface isolates with and without plasmids was mated with *Escherichia coli* HB101. No penicillin-resistant *E. coli* transconjugants were ever observed.

Metal MICs. The mean metal MICs for the bacterial isolates tested with Cr^{3+} and Cu^{2+} tended to increase with increasing distance downdip in the sedimentary profile, from P29 to P24 (Fig. 4). The MICs of Cr and Cu for MCB5 bacteria were similar to those for P24 and P28 bacteria. Mean MICs for drill mud bacteria were highest in compari-

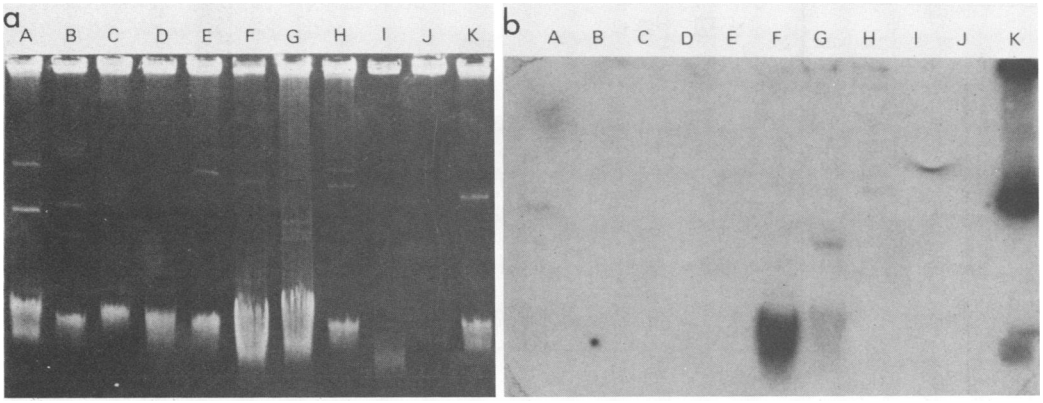

FIG. 3. (a) Subsurface bacterial plasmids determined by agarose gel electrophoresis; (b) autoradiograph of a Southern blot hybridized with pWWO whole-plasmid probe and washed under high-stringency conditions. Bacterial subsurface isolates are P28-367F (A), P24-482A (B), P29-700A (C), P29-655C (D), P29-463C (E), P29-128B (F), P24-802F (G), P28-440A (H), P28-589E (I), P8-709C (J), and pWWO (K).

TABLE 2. Antibiotic resistance and associated plasmid frequencies for subsurface bacterial isolates from deep sediments

Antibiotic	% of resistant isolates among:						
	All subsurface isolates	Isolates from given borehole				Isolates from MCB5 return drill muds[a]	Isolates with plasmids
		P29	P28	P24	MCB5		
Penicillin	70	74	63	70	76	92	38
Ampicillin	49	41	52	47	56	85	47
Carbenicillin	32	26	19	30	51	69	30
Kanamycin	6	5	8	2	7	15	29
Streptomycin	13	15	6	6	24	69	36
Tetracycline	6	5	6	4	7	0	30

[a] Based on mean inhibition zone diameters for all antibiotics; the return drill mud bacteria were from a different population than were all subsurface isolates ($P = 0.01$), and values for P29, P28, and P24 bacteria are significantly different from those for MCB5 subsurface bacteria ($P = 0.02$) by the Wilcoxon signed-rank test.

son with values obtained for isolates from the other three boreholes. The mean MIC of Cu, 87 μg/ml, for the drill mud bacteria was the highest of any of the three metals tested and for any group of isolates tested and was significantly different ($P = 0.05$) from any of the mean borehole values based on a one-way analysis-of-variance test. The mean MIC of Hg was nearly identical for bacteria from the individual deep boreholes but was significantly higher for both MCB5 and MCB5 drill mud bacteria. The MIC of Cu was highest overall, followed by Cr and Hg, for the subsurface bacterial isolates. Bacterial isolates resistant to the highest concentrations of metals tended to be randomly distributed throughout the sediment samples and did not appear to be associated with any particular strata. Bacterial growth inhibition by Cl^- was not observed at the concentrations associated with those of the metal salts for MIC determinations.

Mean pore water concentrations of Cu did not vary greatly but tended to increase with increasing distance downdip, from 0.063 μg/ml at P29 to 0.089 μg/ml at P28 to 0.103 μg/ml at P24. However, Cu in pore waters from MCB5 sediments was below detection (0.004 μg/ml), and in drill muds the concentration was low at 0.007 μg/ml, likely because Cu has low solubility at the relatively high pH (9.1) of the muds. Pore water concentrations of Cr were below detection (0.02 μg/ml) in MCB5 muds and in all but 5 of 51 sediment samples. Pore water Hg concentrations were not determined.

DISCUSSION

The relatively even distribution and greater frequency of plasmid occurrence in bacteria isolated from relatively deep subsurface strata in contrast to the more shallow aquifers suggest there may be differences in the compositions of the bacterial communities of these two systems. Ogunseitan et al. (27) found plasmid incidences ranging from 0 to 8% in bacteria isolated from pristine, relatively shallow aquifers, whereas incidences for isolates from polycyclic aromatic hydrocarbon-contaminated sites tended to be higher (up to 35%). Bacteria isolated from strata deeper than Tobacco Road had plasmid frequencies (31 to 39%) comparable to the 30 to 42% incidence for bacteria from Antarctic sea ice or sediment (22) or the 45 to 55% incidence in marine bacteria at the air-water interface (19). Evidence exists both pro (4, 17, 27) and con (6) that plasmid frequencies are greater in bacteria from polluted sites than in bacteria from similar pristine environments. The sediment samples in this study

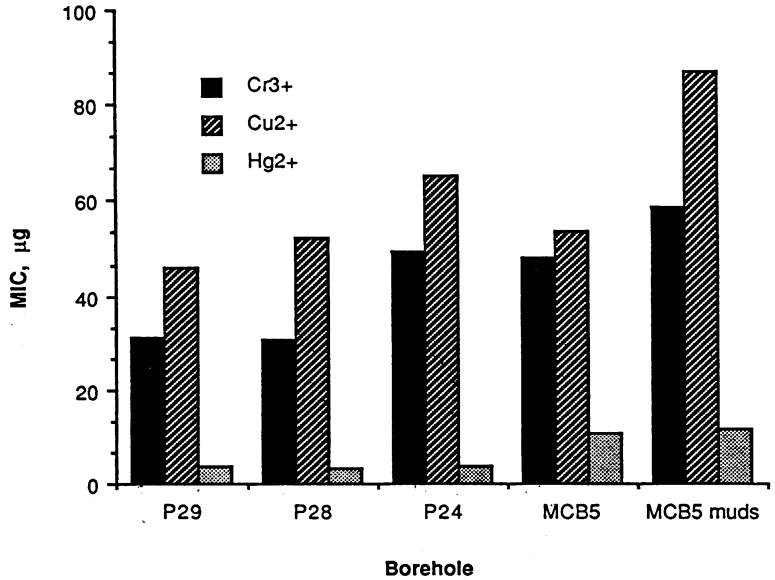

FIG. 4. Mean MICs of Cr^{3+}, Cu^{2+}, and Hg for subsurface bacteria by individual borehole.

are believed to be uncontaminated by Savannah River plant activities, except for possibly the MCB5 samples and the upper stratum samples in P28, which are located adjacent to waste sites and established wells. However, the incidence of plasmids in MCB5 bacteria, as determined for isolates from samples no deeper than 63 m, is less than those of bacteria from the other three boreholes.

A high proportion (53%) of the plasmids associated with subsurface bacteria were greater than 150 kb in size, which contrasts with findings of other studies that have investigated plasmids in natural microbial assemblages. The frequency of large plasmids in deep subsurface bacteria is considerably greater than those reported for other bacteria in shallower subsurface systems (27), bacteria from Antarctic environs (22), and marine bacteria from unpolluted sources (4). Glassman and McNicol (15) found plasmids in bacteria from a polluted site to be larger than those in bacteria from clean water; however, plasmid sizes of greater than 45 kb were not specified. One potential explanation for the high frequency of large plasmids is that the modified in-well lysis procedure used in this study readily resolved plasmids of up to 440 kb (Fig. 2).

Although the majority of plasmids associated with bacteria from aquatic or sedimentary environments remain cryptic, there has been speculation (22) that plasmids in bacteria from pristine, low-nutrient environments may encode oligotrophic traits beneficial to resident bacteria. The dissolved organic carbon levels in the pore waters of the subsurface sediments from which the bacteria in this study were isolated ranged from 0.54 to 7.3 mg/liter (data not shown) and therefore would be considered oligotrophic (2). Subsurface bacteria were isolated and maintained on agar containing 0.3% tryptic soy, which would not be considered an oligotrophic medium (28), but total heterotrophic populations obtained on this medium were similar to those obtained on more dilute medium or water agar (D. Balkwill, Florida State University, Tallahassee, personal communication). The majority of plasmids in these deep subsurface bacteria are large enough to carry genes necessary for transfer, catabolism of recalcitrant compounds, and other functions.

Several of the subsurface bacterial isolates contain plasmid and/or chromosomal DNAs which have homology with TOL plasmid DNA. Although these isolates were not evaluated for ability to degrade toluene or related compounds, the hybridization results indicate that these subsurface bacteria may have the potential to degrade aromatic organic compounds. Evidence from studies conducted in our laboratory (J. K. Fredrickson and R. J. Hicks, submitted for publication) demonstrated the biodegradation of the aromatic organic compounds phenol, 4-methoxybenzoate, aniline, quinoline, nitrobenzene, and aminonaphthalene in the same subsurface sediments, or by bacteria isolated from those sediments, from which bacteria in this study were isolated. The TOL plasmid has been well characterized and is known to contain enzyme-structural and -regulatory genes involved in the catalysis of toluene and related hydrocarbons as well as genes involved in conjugal transfer and replication (12). Further studies are required to identify the exact nature of the relationship between TOL DNA and subsurface bacterial plasmid DNA.

One intriguing hypothesis is that large plasmids associated with subsurface bacteria carry plasmids capable of catabolizing aromatic organic compounds. Organic compounds identified in groundwaters consist mainly (88%) of recalcitrant humic and fulvic acids, tannins, and lignins (32), all of which are condensed polyaromatic structures. As groundwater and associated dissolved organic carbon is separated in time and distance from the point of infiltration, the more readily available carbon is metabolized, leaving the more recalcitrant humic and fulvic materials. It would therefore be advantageous for bacteria in deeper or more isolated sediments to harbor catabolic genes for aromatic organic fractions. The residence of these genes on plasmids would require that the genes be needed only occasionally and that the positive contribution from these genes outweigh their maintenance cost (7). Homology of TOL DNA with DNA of bacteria from relatively shallow aquifer sediments has not been observed (27).

The high frequency of antibiotic resistance in subsurface bacteria, particularly to β-lactam antibiotics, could be due to the fact that (i) bacteria in the sediments originate in or are derived from organisms associated with sewage or sludge (4); (ii) the organisms are in contact with antimicrobial agents produced by plants, animals, or other microorganisms (19); or (iii) the bacteria have an intrinsic resistance. Frequency of resistance to the β-lactam antibiotics ampicillin and penicillin has been shown to be higher for bacteria isolated from environmental samples affected by sewage than in bacteria from clean samples (4, 31). Alternatively, frequencies of resistance to ampicillin and penicillin were found to be high (44 to 85%) for bacteria representing a variety of genera from different environmental sources (21). Ampicillin resistance has been found even in bacteria from Antarctic environmental samples (22). Resistance to β-lactam antibiotics is often the result of inactivation by β-lactamase, with low levels of chromosome-encoded enzyme being produced in most, if not all, gram-negative bacteria while high levels of resistance are generally plasmid encoded (11). In this study, β-lactamase activity was associated with 100% of the penicillin-resistant isolates and 33% of the penicillin-sensitive isolates tested. Also, antibiotic resistance was determined by using commercially available Sensi-Discs. The frequency of β-lactam antibiotic resistance may reflect the concentrations of antibiotics on the disk. The concentrations were 6.3, 10, and 100 μg per disk for penicillin, ampicillin, and carbenicillin, respectively, and the associated frequencies of resistance in subsurface bacteria were 70, 49, and 32% (Table 2).

A significantly greater proportion of the bacteria that harbored plasmids were resistant to penicillin and ampicillin than were sensitive. For bacteria without plasmids, however, the proportions of resistant and sensitive isolates were similar, suggesting that some of these plasmids may encode genes for penicillin inactivation. Kobori et al. (22) found a greater frequency of ampicillin resistance in plasmid-bearing bacteria than in bacteria without plasmids from Antarctic samples. These bacteria had in common their origin in sediments and a single small plasmid of approximately 1.5 megadaltons. Ampicillin resistance was not transferred to or transformed into sensitive strains in their studies. Mating experiments with penicillin-resistant subsurface bacteria and *E. coli* HB101 did not result in any penicillin-resistant *E. coli* transconjugants, providing further evidence for chromosome-encoded β-lactamase activity in these subsurface bacteria. The majority of plasmids in this study were considerably larger than most R-factor plasmids and therefore would be unlikely to encode only resistance genes.

Although metal resistance is often encoded on plasmids (11), no relationship was obvious between the presence of plasmids in subsurface bacteria and MICs of Cr, Cu, and Hg. One interesting observation was that the mean MICs of Cu and Cr for bacteria isolated from the various boreholes

tended to increase with distance downdip in the substrata and distance from the recharge zone. This finding may reflect the natural maturation of groundwaters with distance from the recharge zone. During this process the dissolution of minerals occurs, which increases the concentrations of anions and cations in the water. The increasing mean concentrations of pore water Cu for boreholes P29, P28, and P24 suggest that increases in MICs of Cu for bacteria may reflect groundwater chemistry. However, levels of Cr were generally below detection in sediment samples from all boreholes. The MICs of Hg were relatively high only in sediment samples from MCB5 and the return drill muds from this borehole, possibly reflecting the history of this site as a miscellaneous chemical waste basin.

To obtain a representative picture of the subsurface microflora in unconsolidated sediments, it is necessary to obtain core samples rather than just well water. Harvey et al. (18) showed that approximately 95% of subsurface bacteria in a Cape Cod, Mass., aquifer were attached to particles. However, there are inherent problems with drilling to obtain core samples for microbiological analysis, mainly because of the potential for contamination with drill muds and sediments from overlying strata. Although problems associated with retrieving subsurface sediment samples for microbiological analysis have been identified (34), the patterns of antibiotic and metal resistance observed in this study indicate that not all of the bacteria isolated from subsurface sediment samples can be attributed to intrusion of drill muds into unconsolidated sediments.

These studies suggest that plasmids are common among deep subsurface bacteria and may encode genes for degradation of aromatic compounds. Although plasmid-bearing bacteria were observed to have a high frequency of resistance to ampicillin and penicillin, we suggest that the mechanism for resistance to β-lactam antibiotics is intrinsic because of low levels of β-lactamase production associated with chromosomal genes. Although the question of the origin of deep subsurface bacteria remains unresolved, it is evident from these studies that the subsurface bacterial population is diverse and maintains physiological traits that could benefit microbial maintenance and survival in contaminated environments.

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