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Distribution of plasmids in groundwater bacteria

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SUMMARY

Bacteria isolated from groundwater aquifer core materials of pristine aquifers at Lula and Pickett, OK, and from a site with a history of aromatic hydrocarbon contamination and natural renovation located at Conroe, TX, were screened for the presence of plasmid DNA by alkaline or enzyme lysis and agarose gel techniques. Some of the isolates were also subjected to taxonomic tests in addition to screening for resistance to antibiotics, tolerance to heavy metal salts, and bacteriocin production. There was no significant difference in the distribution of the traits usually associated with plasmid occurrence in isolates from the three sites. These traits, which occurred at low frequencies, were not restricted to plasmid-bearing strains of the communities. Plasmids were found in isolates from all three sites, but on the average there was a significantly higher percentage of isolates containing plasmids in the samples from Conroe (19.4%) than from either Lula (1.8%) or Pickett (7.7%). The sizes of the plasmids found ranged between 3.5 and 202 kilobases but, for the Conroe samples, many more isolates (67%) contained smaller plasmids (< 10 kb) rather than larger ones. No plasmids were found in bacteria recovered from naturally renovated aquifer material at the Conroe site.

INTRODUCTION

Although groundwater contamination has occurred for centuries, increased industrialization, population density and agricultural activities in recent years have exacerbated the problem in several regions. As the dependence on subterranean supplies of water increases, the quality and efficiency of renovation of this resource becomes an important issue. Organic pollutants in groundwater systems are generally inaccessible to natural physiochemical reactions like photolysis, oxidation and evaporation that would reduce the burden on the subsurface environment. For this reason, it is desirable to improve and to develop efficient in situ microbiological decontamination processes for this remote system.

Contrary to previous results, several recent studies have revealed the existence of an almost exclusive bacterial population, up to 10^7 organisms/g subsurface material, in this environment [25,27,29]. With increased recognition of the role of plasmids in biodegradation, several techniques are being developed for maximizing the efficiency of bacteria in

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biodegradation within specific environmental systems [8,14]. The literature on the influence of incidence and transfer of plasmids, selective pressure and environmental conditions promoting plasmid maintenance is extensive for bacterial populations in a few well characterized ecological milieux [3]. However, this information may not be extrapolated directly to the deeper subsurface environment where substantiated data on the genotypic and phenotypic characteristics of the biota are not yet available.

This study was undertaken to investigate the incidence, distribution and function of plasmids in the bacterial populations of three selected groundwater environments. Due to the cost and difficulties of subsurface sampling, extensive sampling of subsurface aquifer materials was not possible and the three sites examined thus permit a preliminary assessment of plasmid occurrence in bacterial populations from the groundwater environment. However, populations from these sites were found to contain differential levels of strains harboring plasmid DNA. These data support the general observation of a correlation between plasmid frequency and degree of environmental contamination.

MATERIALS AND METHODS

Aquifer samples

Bacteria examined for the presence of plasmid DNA were recovered from aseptically collected aquifer core samples provided by John Wilson, R.S. Kerr Environmental Research Laboratory (Ada, OK). The core sampling device has been previously described [29]. Fresh and aged experimental samples were shipped on ice within 2 days. Aquifer material examined originated from sample aquifers at Conroe, TX, Lula, OK, and Pickett, OK, and have been previously described [13,30]. Sample site characteristics are given in Table 1. As indicated, Lula and Pickett samples originated from pristine aquifer material, while samples from Conroe, TX represent polyaromatic hydrocarbon-contaminated samples and naturally renovated (in situ) samples (Table 1).

Bacterial enumeration and cultivation

Aquifer samples were mixed by vortexing in phosphate-buffered saline (1.2 g Na_2HPO_4, 0.8 g NaH_2PO_4, and 8.5 g NaCl per litre of distilled H_2O, final pH 7.6) and diluted in the same buffer. Aliquots for enumeration and isolation of representative organisms were plated on 1/10 strength peptone glucose (YEPG) agar [20] or dilute peptone yeast extract glucose (PYG) agar [7], or soil extract (SE) agar [29]. One group of samples was incubated anaerobically on YEPG agar by the technique of Adler and Crow [1] as previously described [19]. Colony counts were recorded after 1 and 2 weeks' incubation at 25°C and single colony isolates were randomly picked and purified on YEPG or PYG. In addition, representatives of each colony type were isolated. No isolates were subjected to more than two passages on laboratory media prior to initial plasmid screening. Where required, cells were harvested from liquid medium by centrifugation, using a table top centrifuge.

Table 1

Sample site characteristics for aquifer core samples

Sample site	Sample depth (m)	pH	Relevant site characteristics
Lula, OK	2.7–5.6	5.5	Saturated zone – no contamination
Pickett, OK	3.8	6.0	Saturated zone – no contamination
Conroe, TX	7.0–8.5	< 5.0	
well No. 16			Saturated zone – creosote process waste contamination
well No. 5			Saturated zone – naturally renovated area

Escherichia coli V517 containing eight cryptic plasmids [15] and *Pseudomonas putida arvilla* containing the toluene degradative plasmid, pWWO [17], were used as control strains for the plasmid detection procedure as well as sources for plasmid molecule size markers. In addtion *Streptococcus faecalis* strain 39-5 (containing five plasmids) and DS 16 (containing two plasmids) [23] were used as gram-positive control strains for plasmid detection. These strains were maintained on YEPG medium.

Physical characterization

Pigmentation, colony morphology and growth rate were used to select isolates for purification and examination. Other taxonomic tests (Table 3) were performed for further identification by standard methods [6]. Antibiotic sensitivity discs (BBL) were used to detect resistance to nalidixic acid, penicillin, streptomycin, chloramphenicol, clindamycin, trimethoprim, novobiocin, ampicillin, kanamycin and erythromycin. Expression of resistance was recorded after incubation on YEPG agar for up to 7 days [6]. Bacteriocin production was examined by the cross-streaking technique of Waksman [24]. Laboratory strains of *E. coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Bacillus subtilis* were used as indicators.

Tolerance for heavy metal ions was examined by the method of Mergeay et al. [16]. Minimum inhibitory concentrations were evaluated for isolates that grew in the presence of 100 mg/l concentration of the following salts: $CdCl_2$, $HgCl_2$ (10 mg/l), $NiCl_2$, $Pb(NO_3)_2$, $CoCl_2$, and $ZnCl_2$.

Statistical analysis

A statistical test of the differences in proportions of bacteria containing plasmids at the different sites and wells was performed. A normal approximation using the arcsine transformation method was used since the sample sizes are large enough [21]. The statistical test is defined by the following equation: $t_s = \arcsin\sqrt{p1} - \arcsin\sqrt{p2}/\sqrt{820.8(1/n1 + 1/n2)}$ where $p1$ and $p2$ are the proportions of bacteria with plasmids at sites or wells one and two, respectively, and $n1$ and $n2$ are the numbers of isolates examined for each corresponding location. The test has the standard normal distribution under the null hypothesis that the proportion of plasmid-harboring bacteria in two different samples are similar.

Plasmid isolation and analysis

Plasmids were isolated by the alkaline extraction procedure of Kado and Liu [12] and analyzed by vertical agarose gel electrophoresis in 0.5% agarose (ME, Seakem Corp.) at 100 V in 0.04 M Tris-acetate, 0.002 M EDTA buffer for 3–5 h. Selected strains (30% of total) were also examined using the lysozyme-SDS lysis procedure of Anderson and McKay [2]. In addition, select gram-positive isolates were examined by the method of Clewell and Helinski [5].

Two groups of samples were examined for the presence of bacteria containing DNA related to TOL plasmid (pWWO) DNA. The colony hybridization procedure of Hanahan and Meselson [11] was employed as previously described [21]. Colonies were directly transferred from primary enumeration media to replica Genescreen Plus (New England Nuclear) hybridization filters. The colonies were lysed with 0.5 N NaOH and the DNA was fixed to the filter as previously described [21]. Nick-translated ^{32}P-labeled whole TOL plasmid DNA was used as probe and was isolated from the positive control, *Pseudomonas putida* TOL, by the method of Wheatcroft and Williams [26]. Hybridization washings were conducted under low-salt conditions (95% stringency) and detection was by autoradiography as previously described [21].

RESULTS

Results of plate count studies revealed that population density of bacteria in the aquifer core materials ranged between 10^3 and 10^5 CFU/gram dry weight (Table 2). These results are in good agreement with previous estimates, although values were lower than those obtained by direct acridine orange counts on similar subterranean samples [29]. A greater number of bacteria were found in aquifer samples from well 16 of the Conroe site (a contaminated aquifer) than any other sample. There was

Table 2

Bacterial density and plasmid occurrence in groundwater aquifer material samples

ND, not determined.

Site	ID No.	Wells	Date obtained	CFU (g^{-1} dry wt.)	No. colonies examined	Plasmid-positive colonies	
						number	proportion
Lula	9D-2		5/7/83	$< 1 \cdot 10^4$	16	0	0%
	9G-2		10/13/83	$9.1 \cdot 10^4$	181	1	0.5%
	9G-5		10/13/83	$6.7 \cdot 10^4$	134	5	4.5%
	9F-6		10/13/83	$7.2 \cdot 10^4$	ND		
Pickett	17F-3		6/29/83	$5.0 \cdot 10^3$	246	19	7.7%
Conroe	5A-2		11/4/82	$5.0 \cdot 10^3$	5	1	20%
	266	16	10/14/83	$8.0 \cdot 10^5$	137	49	35.8%
	273	16	10/14/83	$9.5 \cdot 10^5$	143	13	9.1%
	287	16	10/14/83	$3.4 \cdot 10^5$	97	22	22.7%
	356	5	10/14/83	$1.2 \cdot 10^4$	52	0	0%

no substantial difference in the CFU in samples that received sterile, dissolved oxygen-saturated water prior to shipment and examination (Nos. 266 and 273) compared to one that did not (No. 287). Differences were noted in the number of distinguishable colony types, which is reflected in the number of colonies selected for examination of plasmid content and physiological characteristics. The samples obtained from Conroe were taken from a greater depth and were generally more acidic than those from Lula or Pickett (Table 1). The detectable plasmid content of the total population in the samples from Lula was significantly lower than those from Pickett and Conroe. Only one isolate from sample 9G-2, out of 181 colonies examined, harbored a plasmid, 3.32 kb in size (Fig. 1). Other plasmid-containing isolates from the Lula site were recovered from sample 9G-5. Three isolates harbored a 78 kb plasmid, while two plasmids (83 kb and 4.5 kb) were found in one isolate. Seven out of the 19 isolates from the sample taken from Pickett contained two to four plasmids of different sizes, while another group of seven isolates contained a 90 kb sized plasmid (Fig. 1).

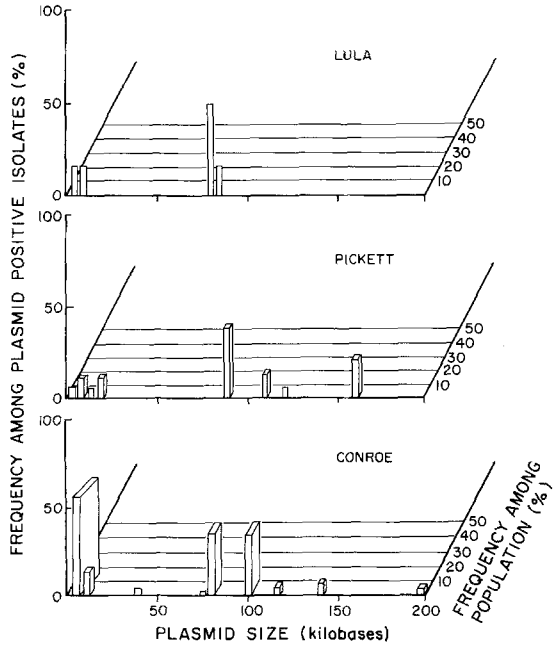

Fig. 1. Comparative plasmid occurrence, size and frequency in groundwater bacterial populations.

Three core samples were collected from well 16 at Conroe. Although the bacterial populations were within the same range, the proportion of isolates bearing plasmids was significantly higher in sample 266 (35.8%) than in either samples 273 (9.1%) or 287 (22.7%) (Table 2). Compared to the sample

from Pickett the percentage of plasmid-positive colonies in Conroe sample 273 was not significantly higher but averages taken with isolates from samples 266 and 287 produced a significantly higher percentage (19.58%) of plasmid bearer strains in aquifer samples from this well. Of the plasmid-containing isolates from the Conroe site, 54% contained one or two small plasmids between 3.5 and 5.5 kb in size. Another group of 29 isolates from well 16 also produced two bands on agarose gels, one corresponding to 79.5 kb and the other to 103.5 kb. Three isolates from Conroe sample number 266 harbor the largest (approx. 200 kb) plasmid recorded in this study (Fig. 1). None of the 52 isolates examined from a naturally renovated well (in situ) located at Conroe (sample 356, well No. 5) was found to contain plasmids. In addition, none of the colonies probed under conditions of high stringency colony hybridization revealed any homology with the TOL plasmid (117 kb) (data not shown).

Although both gram-positive and gram-negative bacteria were isolated, gram-positive rod-like prevailed in the samples from Lula and Pickett while gram-negative, coccobacilli forms were abundant in the Conroe samples examined (Table 3). More *Pseudomonas*-like isolates (motile, gram-negative

Table 3

Salient physiological characteristics of groundwater bacterial population

15% of the total population examined for plasmid content were randomly selected for these tests from the Lula and Conroe samples, while 25% of the population from the Pickett sample was tested. ND, not determined.

Test	Lula	Pickett	Conroe
Gram reaction: +	64%	56%	43%
−	28%	34%	57%
+/−	8%	10%	0%
Motility	2.5%	18%	6%
Oxidase present	0%	10%	68%
Catalase present	30%	74%	96%
DNase present	0%	12%	ND
Phenylalanine deaminase present	0%	12%	2%
Acid from:			
glucose	45%	30%	11%
xylose	0%	26%	2%
maltose	0%	38%	14%
Hydrolysis of:			
starch	ND	8%	7%
casein	ND	26%	7%
urea	ND	38%	31%
Citrate utilized	57%	18%	3%
Nitrate reduced	30%	48%	36%
Sulfide produced	33%	44%	11%
Bacteriocin produced	0%	13%	4.3%
Heavy metal tolerance	Pb, 2.5%	Pb, 8.3%	Pb, 7%; Zn, 1.4%
Antibiotic resistance	7.5%	21%	22.8%

rods, oxidase- and catalase-positive) were found in Conroe samples than either in the Lula or Pickett samples.

Bacteriocin production, heavy metal tolerance or antibiotic resistance did not occur at significant levels in bacteria from any of the three sites. Although there was no correlation between these traits and plasmid occurrence, a few isolates that expressed multiple antibiotic resistance were found to contain at least two plasmids. Tolerance to lead salt (at 200 mg/l) was observed for five isolates from the Pickett sample and for one isolate from Lula. The lead-tolerant isolate from Lula did contain plasmids but no similar plasmids were found in the Pickett isolates.

DISCUSSION

Results of these studies clearly indicate that plasmids are present in indigenous bacterial populations within the microbial community of the aquifer environment. Although plasmids may be widespread in pristine aquifer samples, this trait appears to be amplified in populations recovered from contaminated sources. The distribution of plasmids in populations examined here is slightly lower but comparable to studies conducted on aerobic, heterotrophic bacterial populations of rivers and sediments. Hada and Sizemore [10] examined planktonic marine *Vibrio* isolates from an oil field and a control area, and found a higher incidence of plasmids in isolates from the oil field area (35%) than in isolates from the control area (23%). However, Burton et al. [3] did not consider the difference between the frequency of occurrence of plasmids at a polluted site in a South Wales River (15%) and an unpolluted section of the same river (9.4%) significant. Glassman and McNicol [9] reported that up to 46% of the estuarine heterotrophs from the Chesapeake Bay sediment and water column carry plasmids. They reported that bacteria from cleaner sites tend to harbor small plasmids (< 5 kb), in contrast to isolates from a polluted site which harbor multiple plasmids that are larger than 45 kb. Similar size distribution patterns cannot be inferred from the present study of groundwater bacteria. A significant proportion of plasmids found in samples from any of the three sites were greater than 50 kb but samples from polluted Conroe wells also contained a high proportion (67%) of small plasmids (< 10 kb) (Fig. 1).

Although the plasmids identified in these studies are cryptic, it is probable that the plasmids contribute phenotypically to a variety of physiological activities of the host cells in their natural habitat. According to Lee et al. [13], wood-creosoting waste contains naphthalene, dibenzofuran, fluorene, anthracene and pentachlorophenol, at the mg $\cdot$ l^{-1} level. The biodegradation of some of these organic pollutants in subsurface material has also been demonstrated by Wilson et al. [30]. The extent to which such catabolic adaptations involve the selection of plasmid-containing bacteria in the population cannot be easily quantified but several reports have implied a correlation between plasmid occurrence and environmental selective pressures [4,10,16]. In this study, the probe for catabolic genes was restricted to a toluene degradative plasmid, and identical or closely related DNA sequences could not be detected. This does not preclude the presence of organisms able to use toluene as a carbon source since such metabolic activities may be chromosomally controlled [4].

Although care was taken in choosing the media on which bacterial populations were enumerated and selected for examination, it is known that very few bacteria in samples from low-nutrient sites are capable of growth [7], perhaps as few as 10% being metabolically active [25]. In addition, some active cells may not form colonies on solid, media, which precludes enumeration and analysis of plasmid distribution.

For this environment, it is recommended that study of organisms which are difficult to grow should be undertaken, using methods specifically developed for the study of oligotrophs [7,18]. Since some of the plasmids found in bacteria from the samples were large enough to carry genes for conjugal transfer [28], the potential for gene transfer among these organisms should be investigated.

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