# Isolation and Characterization of Mobile Genetic Elements from Microbial Assemblages Obtained from the Field Research Center Site

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**Abstract**

Considerable knowledge has been gained from the intensive study of a relatively limited group of bacterial plasmids. Recent efforts have begun to focus on the characterization of, at the molecular level, plasmid populations and associated mobile genetic elements (e.g., transposons, integrons) occurring in a wider range of aquatic and terrestrial habitats. Surprisingly, however, little information is available regarding the incidence and distribution of mobile genetic elements extant in contaminated subsurface environments. Such studies will provide greater knowledge on the ecology of plasmids and their contributions to the genetic plasticity (and adaptation) of naturally occurring subsurface microbial communities.

We requested soil cores from the DOE NABIR Field Research Center (FRC) located on the Oak Ridge Reservation. The cores, received in February 2003, were sampled from four areas on the Oak Ridge Site: Area 1, Area 2, Area 3 (representing contaminated subsurface locales) and the background reference sites. The average core length (24 in) was subdivided into three profiles and soil pH and moisture content were determined. Uranium concentration was also determined in bulk samples. Replicate aliquots were fixed for total cell counts and for bacterial isolation. Four different isolation media were used to culture aerobic and facultative microbes from these four study areas. Colony forming units ranged from a minimum of 100 per gram soil to a maximum of 10,000 irrespective of media composition used. The vast majority of cultured subsurface isolates were gram-positive isolates and plasmid characterization was conducted per methods routinely used in the Sobecky laboratory. The percentage of plasmid incidence ranged from 10% to 60% of all isolates tested. This frequency appears to be somewhat higher than the incidence of plasmids we have observed in other habitats and we are increasing the number of isolates screened to confirm this observation. We are also characterizing the plasmid populations at the molecular level. Isolates cultured from the background control site exhibited the lowest occurrence of plasmids (10%). Aliquots of samples were also used in enrichment assays to isolate metal resistant subsurface isolates. Samples were subjected to three different metals (chromium, mercury and cadmium) at two different concentrations and incubated following a conditioning period in which samples were amended with a carbon, nitrogen and phosphorus source. Isolates were plated on metal selection, purified to single isolates and plasmid content determined.

Hypotheses to be tested:

(1). Plasmid occurrence will be significantly reduced in the subsurface

(2).Expect new *inc/rep* plasmid groups

(3). Limited molecular diversity in plasmid populations

(4). Potential for lateral gene transfer is significantly reduced in the subsurface

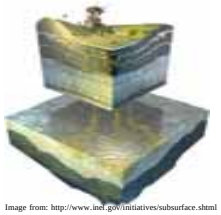

Image from: http://www.inel.gov/initiatives/subsurface.shtml

**Table 1:** Specific coring locations for sediment cores collected in February 2003

| Sample ID | Location | Location Group | Sampling Date | Comment |
|---|---|---|---|---|
| FB051-01-10 | FB051 | Area 2 | 2/18/2003 | near DP16S |
| FB053-01-08 | FB053 | Area 2 | 2/18/2003 | near DP16S |
| FB055-01-15 | FB055 | Area 3 | 2/19/2003 | near FW009 |
| FB057-10-00 | FB057 | Area 3 | 2/19/2003 | near FW024 |
| FB058-01-02 | FB058 | Area 1 | 2/20/2003 | near FW015 |
| FB059-01-00 | FB059 | Area 1 | 2/20/2003 | near FW018 |
| FB608-01-00 | FB608 | Background | 2/24/2003 | near FW301 |

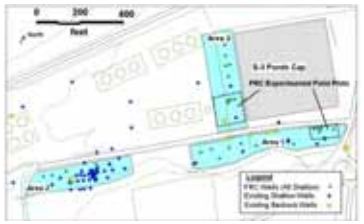


**Figure 1.** Map of U.S. Department of Energy-NABIR Field Research Center in Oak Ridge, TN from which sediment cores from contaminated Areas 1, 2 and 3 were sampled on February 18-24, 2003. Sediment cores from the background area (not shown) were obtained during the same sampling period.
Figure from http://public.ornl.gov/images/Figure A4.jpg

**Table 2:** Sediment characteristics of contaminated and background core samples

| Sample | Location | pH | Dry/wet ratio | [U] pCi/gram | Direct Cell Counts |
|---|---|---|---|---|---|
| FB059-01-00 | Area 1 | 4.2 | 0.80 | 0.83 | 7.18 _ $10^3$ |
| FB053-01-08 | Area 2 | 7.5 | 0.80 | 0.47 | 2.00 _ $10^3$ |
| FB055-01-15 | Area 3 | 4.0 | 0.82 | 1.97 | 6.13 _ $10^3$ |
| FB608-01-00 | Background | 6.7 | 0.88 | 0.43 | 6.93 _ $10^2$ |

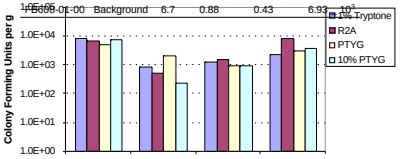


**Figure 2.** Determination of aerobic colony forming units per gram (wet wt) soil of (chemoheterotrophic) bacteria from the contaminated and background FRC sites. Four different media types (high and low nutrient) were used (1) 1% tryptone (10 g tryptone per liter); (2) R2A (0.5 g yeast extract, 0.5 g peptone and 0.5 g glucose per liter); (3) PTYG [10 g glucose, 10 g yeast extract, 5 g peptone and 5 g Trystic Soy Broth (TSB) per liter]; (4) 10% PTYG (1 g glucose, 1g yeast extract, 0.5 g peptone and 0.5 g TSB per liter). Viable cell counts, while low, were comparable for all media types tested.

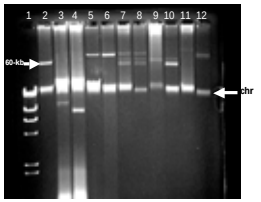


**Figure 3.** Agarose gel electrophoresis of representative plasmid-bearing subsurface isolates obtained from: Area 1 (Lanes 10-12); Area 2 (Lanes 5-8); Area 3 (Lanes 3, 4 and 9); *Escherichia coli* TG1 (Lane 2; RK2; 60-kb broad-host-range R-factor plasmid); Lambda *Hind*III ladder (Lane 1) used to determine position of chromosomal (chr) DNA.

Covalently closed circular plasmid DNA was isolated from 5 ml of stationary phase liquid cultures as described in Sobecky et al. (1997) using a modified alkaline lysis procedure. (5-300-kb). This method is suitable for the isolation of circular, low-copy number large plasmids from gram negative and gram positive bacteria.

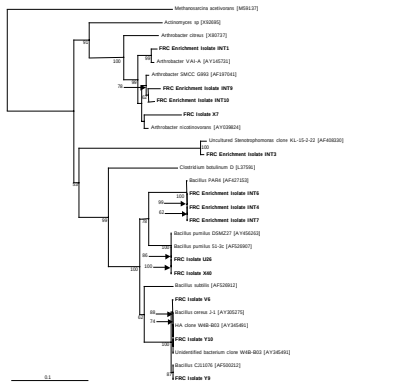


**Figure 4.** Phylogenetic tree of relationships of 16S rDNA gene sequence as determined by distance Jukes-Cantor analysis of cultured FRC subsurface microorganisms (in boldface) to selected cultured isolates. GenBank accession numbers are in parentheses. One thousand bootstrap analyses were conducted and percentages greater than 50% are reported. *Methanosarcina acetivorans* was used as the outgroup. The scale bar represents the expected number of changes per nucleotide position.

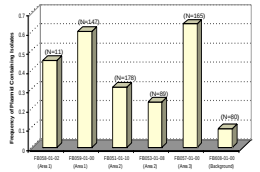


**Figure 5.** Incidence of indigenous plasmids in culturable subsurface bacteria. A total of 579 bacterial isolates were tested for the occurrence of plasmids. The vast majority of the FRC isolates were gram-positive bacteria belonging to the genera *Arthrobacter*, *Bacillus*, and *Stenotrophomonas*. The frequency of plasmid-bearing isolates cultivated from contaminated FRC soils ranged from (20%) to (60%) while the lowest frequency of plasmid-bearing bacteria (10%) was observed in bacteria cultivated from the background (control) site. The occurrence of a considerable lower frequency of (culturable) plasmid-bearing bacteria in uncontaminated soils and sediments has also been reported for other environments including marine systems (Sobecky et al. 1997, Reyes et al. 1999). However, the high frequency of plasmid-bearing isolates in contaminated FRC soils (i.e., 60%) is among one of the highest plasmid incidence we have detected in sediments (Sobecky, unpublished). Four different media types (varying in the concentration of organic nutrients and in the absence of heavy metal selection) were used to obtain as much culturable bacterial diversity as possible. However, there was no difference in the frequency of plasmids observed when either low or high nutrient conditions were initially used to cultivate subsurface bacteria (data not shown).

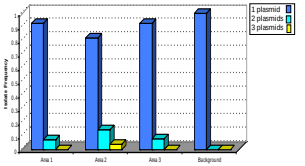


**Figure 6.** Incidence of single and multiple plasmids detected in plasmid-bearing subsurface bacterial isolates. Although the vast majority of the isolates contained only one plasmid, numerous isolates contained multiple plasmid bands. Given the relative positions of the bands in the agarose gels, in most cases it is unlikely that the bands represent open circular or multimer forms of the same plasmid.

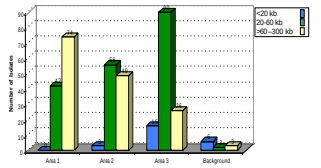


**Figure 7.** Comparison of known plasmid size standards to the plasmid-bearing isolates to obtain an estimate of plasmid mass indicated a broad range of plasmid sizes. The majority of plasmids in the FRC bacterial isolates, particularly those obtained from contaminated soils, were generally large and ranged from 20 to 300-kb.

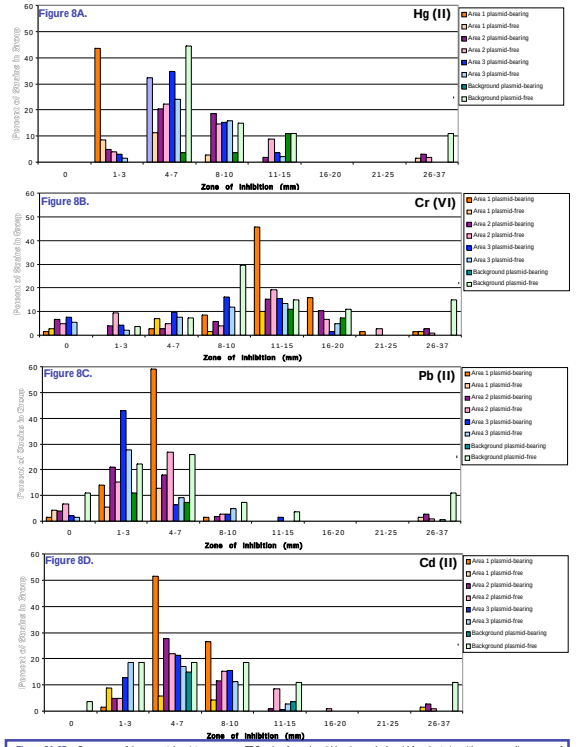


**Figure 8A-8D.** Occurrence of heavy metal resistance among FRC subsurface plasmid-bearing and plasmid-free bacteria with corresponding zones of inhibition. Four metals: cadmium [Cd(II); 500 nmol), chromium [Cr(VI); 2 µmol], mercury [Hg(II); 50 nmol] and lead [Pb(II); 500 nmol] were tested as described in Benyehuda et al. (2003). Strains are considered resistant if inhibition zones are ≤ 5 mm (Hg); ≤ 3 mm (Cr); ≤ 2 mm (Pb, Cd). The metal concentrations and assay conditions were chosen so that a direct comparison between subsurface strains, previously isolated from DOE's Hanford and Savannah River Site (SRS) sites [Balkwill et al., 1997; Kieft et al., 1995], and characterized by Barkay and co-workers could be conducted (Benyehuda et al. 2003). Plasmid-bearing and (putatively) plasmid-free FRC strains exhibited a higher metal tolerance for Hg(II) and Cr(VI) [Fig. 8A and 8B]. A similar trend was observed for Cd(II) and Pb(II), although in contrast to Hg(II) and Cr(VI) data, a number of (plasmid-free and plasmid-bearing) strains from the background reference site exhibited metal tolerance. A greater number of FRC isolates displayed a higher tolerance to Hg(II) when compared to SRS and Hanford strains while a greater tolerance to Cr(V I) was reported for the SRS and Hanford strains (Benyehuda et al. 2003). A comparable percentage of Hanford, SRS and FRC isolates tolerant to Pb(II) was detected. Resistance to Cd(II) was not determined for Hanford and SRS strains. With the possible exception of resistance to Hg(II) [Fig. 8A], a comparable percentage of plasmid-bearing and plasmid-free FRC isolates were resistant to cadmium, chromium, and lead. However, direct testing for plasmid-encoded resistance determinants is underway to confirm the occurrence of metal resistance determinants on mobile genetic plasmid elements.

## Discussion

Conjugation, transduction and transformation are mechanisms that facilitate the rapid evolution of microbial phenotypes by the mobilization of segments of DNA resulting in the inheritance of entire gene systems during a single transfer event (Levy and Miller, 1992; Bushman, 2002). Although point mutations contribute to microbial adaptation, lateral gene transfer is more critical to promoting rapid genomic flexibility and microbial evolution (Davison, 1999). In contrast to transduction and transformation, conjugation may mediate very broad host range transfers (i.e., between unrelated species). The rapid and widespread occurrence of antibiotic resistance genes throughout the *Enterobacteriaceae* is oft-cited evidence for the importance of conjugal transfer in natural populations. Gene recruitment elements (e.g., insertion sequences, transposons, integrons) carried on plasmids provide a mechanism of adaptation by promoting recombination in bacterial genomes. Analyses of complete genome sequences have indicated that a significant portion of laterally transferred genes can be attributed to plasmids and their associated "hitchhiking" transposable elements (Ochman *et al.*, 2000). Together, these gene recruitment elements comprise a portion of the horizontal gene pool that provides microbial communities with a means by which to respond to changing environmental conditions and exploit new ecological niches. Considering the magnitude and importance of these processes, surprisingly little is known regarding the evolutionary relationships, phenotypes, and ecological roles of plasmids from diverse habitats, particularly those with broad-host-range (BHR) capabilities (del Solar *et al.*, 1996) that can be shared by many different species.

The little information that is available for subsurface systems indicates the commonality of plasmids in subsurface populations. For example, Fredrickson *et al* (1988) showed that 33% of approximately 200 strains obtained from deep sediments of the SRS site had plasmids, the majority of which were large (> 150-kb), and some carried genes with homology to the TOL plasmid. Aromatic hydrocarbon degradation genes were subsequently found to be located on plasmids and chromosomes in isolates from the SRS (Kim *et al.*, 1996). A complete sequence of pNL1, a 184-kb conjugative catabolic plasmid from *Sphingomonas aromaticivorans* F199, has been reported (Romine *et al.*, 1999). The putative *rep* proteins encoded on pNL1 are dissimilar to existing *rep* origins supporting our hypothesis regarding new plasmid lineages among subsurface strains. The frequent occurrence of plasmids (Fredrickson *et al.*, 1988) and plasmid-carriage of degradative genes (Kim *et al.*, 1996; Romine *et al.*, 1999) clearly suggest an ecological role for plasmids in promoting genetic diversity and evolution of traits important for bioremediation among subsurface microbial communities. Our ongoing identification and characterization of plasmid replicons in subsurface bacteria will add a critical element to our proposed molecular studies on the significance of lateral gene transfer in the evolution of metal and radionuclide resistance and transformations in subsurface microbial communities (Sobecky et al. 1998).

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