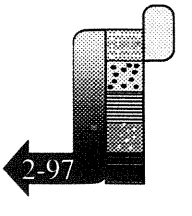
Molecular Analysis of Deep Subsurface Bacteria

Luis Jiménez,[1] Geralyne López de Victoria,[2] John Wear,[3]
Carl B. Fliermans,[3] and Terry C. Hazen[3]
[1]Center for Environmental Biotechnology,
University of Tennessee, Knoxville, TN;
[2]Department of Biology, University of South Carolina, Columbia, SC,
[3]Environmental Sciences Section, Savannah River Laboratory,
Westinghouse Savannah River Company, Aiken, SC.

Abstract

Bacterial isolates from deep sediment samples from three sites, P24, 28, and 29 at the Savannah River Site (SRS), near Aiken, SC were studied to determine their microbial community composition and genetic structure by total DNA hybridization and mol %G+C. Standard phenotypic identification underestimated the bacterial diversity at the three sites, since bacteria with the same phenotype have different genetic composition. The mol %G+C of deep subsurface bacteria ranged from 20 to 75%, with more than 60% and 12% of the isolates tested showing values similar to the Pseudomonas sp. and Acinetobacter sp., respectively. No significant difference was found between the average mol %G+C content of isolates from different sites, which suggests that the same bacterial genus was the most abundant in all sites. Total DNA hybridization and mol %G+C analysis of deep sediment bacterial isolates showed that each formation was comprised of different microbial species. No isolates from deeper formations showed the same genetic structure of isolates from upper formations, although geological age of the sediment appeared to limit the diversity of bacterial genera. The Pseudomonaceae and Acinetobacteriaceae were the only bacterial families found in deeper formations, suggesting a long period of adaptation to the environmental conditions of the deep subsurface does exist.



Introduction

Deep subsurface microbial communities have been found at the Savannah River Site, near Aiken, SC, that have shown higher bacterial densities and diversity than previous studies done at shallow depths.[1,7] A diverse microbial community was found across the geological profile, including very different physiological groups, (i.e., methanogens, sulfate and nitrate reducers, and heterotrophic bacterial populations).[9] Moreover, the diversity of the heterotrophic bacterial populations did not decrease with depth.[1] A large number of different plasmids were found that were encoded for antibiotic and metal resistance. Plasmids larger than 200 kb were most frequently found at deeper aquifers.[9] Plasmid frequency increased with greater depth suggesting a different bacterial composition across the depth profile. A great majority of these communities were aerobic or facultative chemoheterotrophic bacteria, and most of them were oxidative (82%) rather than fermentative (4%).[1] Considerable bacterial diversity was observed even within defined geological formations based upon the Rapid API-NFT test.[1]

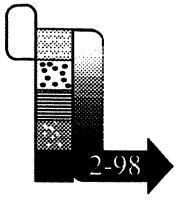
The identification of deep subsurface bacteria using standard biochemical assays had brought out a number of unidentified strains from the three sites already sampled.[1,5] However, several studies have shown that phenotypic tests alone are not an accurate way to identify and separate bacterial populations in natural environments (i.e., those bacteria that are identified by these assays may not be identified correctly).[6,23] Accurate identification, detection, and discrimination of bacterial communities in nature have been done by molecular techniques such as nucleic hybridization and the mol %G+C.[2,10,19] Microbial ecologists and bacterial taxonomists have used the mol %G+C and DNA hybridization for the classification of bacterial isolates from clinical and natural environments.[3,13,22]

Since significant microbial populations occur in deep subsurface sites, it is very important to know the composition and genetic structure of these microorganisms. Moreover, they will play a significant role in the transformation and mobilization of different pollutants in the deep subsurface and in the formation of the geological layers.

A number of studies have been reported regarding the phenotypic diversity of subsurface bacteria, but none have presented information about the genetic structure and diversity of these communities. No effort has been given to assessing genetic changes across a depth profile as well. Using molecular analysis (e.g., mol %G+C and total DNA homology), the genetic structure and diversity of deep subsurface bacteria were studied across a geological profile, and the phenotypic identification and genetic content of these communities were compared in order to understand the community structure of deep subsurface bacterial communities.

Materials and Methods

Study site. Subsurface sediments were obtained at three different sites, (P24, P28, and P29) at the Savannah River Site, near Aiken, SC. For a more complete description of the site, sampling procedures, and geological profiles, see Balkwill et al.[1] and Fliermans and Balkwill.[7]



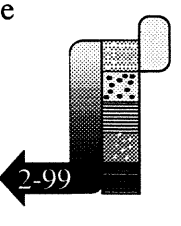
Bacteriology. Deep subsurface bacterial isolates used in the genetic studies were provided by Dr. David Balkwill from Florida State University, Tallahassee, Florida, from the Subsurface Microbiology Culture Collection (SMCC). For further details on the isolation of deep subsurface bacteria and media used, see Balkwill et al.[1] After receiving all of the isolates, they were analyzed again using the API-NFT Rapid tests[1] to corroborate previous identification.

DNA melting point. More than 65 bacterial isolates were randomly chosen from all three sites across the geological profile and their DNA was isolated using a variation of the Marmur technique[16] as described by Bermudez and Hazen.[2] A preparation was considered pure when it had an optical density at 260 nm/optical density at a 280 nm ratio of 1:8.[15] The thermal denaturation method[12] was used for measuring DNA base composition. Samples were prepared as described before.[2] Standard DNA's (e.g., Escherichia coli (ATCC 11773) and Pseudomonas aeruginosa (ATCC 9721)) were used in each run as a reference standard. The melting profiles were determined with a 300 DMS Varian UV spectrophotometer (Varian Instrument Co., Texas) and connected to a thermal program from an Apple IIe computer. The mol %G+C of each bacterial isolate was determined by using the Marmur and Doty equation.[17]

DNA homology. Whole chromosomal bacterial DNA was labeled *in vitro* using a nick translation kit (New England Nuclear, Boston, MA) with ^{3}H-thymidine (specific activity: 75 Ci/ m mol). The DNA probe was precipitated as described by Crouse and Amorese.[4] Specific activity of the nucleic acid probe was measured by the method of Maniatis et al.[15] The probe was fragmented by sonication, denatured at 100°C for five minutes, and rapidly chilled on ice.

Denatured DNA from bacterial isolates was immobilized as previously described.[2] DNA hybridization and washings were done as described before by Bermudez and Hazen.[2] After being washed, individual membranes were removed from the wells in the filtration plate, air dried, and counted in a liquid scintillation analyzer model 2000 CA (United Technologies Packard, Virginia). Percentage of homology was calculated as described elsewhere.[2] Similar mol %G+C values and $\geq$70% DNA homology were used to discriminate among different bacterial species.

Vertical distribution of bacterial genotypes across the geological profile. To assess the distribution of bacterial genotypes across the geological and depth profile, more than 65 bacterial isolates from the three sites were selected and their mol %G+C values were determined as described above. This allowed for preliminary genetic comparisons of the different bacterial species present in each formation at the three sites. In addition, whole chromosomal probes of four bacterial isolates were prepared as described before to measure the DNA relatedness of bacteria from different sites and geological formations and to assess the genetic relatedness across the depth profile. Pseudomonas aeruginosa (ATCC 9721) DNA was extracted, labeled by nick translation, and hybridized against deep subsurface isolates identified as P. aeruginosa to determine the reliability of the API system and to determine known bacterial species.



Data analysis. Programs developed for a Macintosh computer were used for all statistical analyses. Analysis of variance was used to determine differences between sites and formations in the DNA homology and base composition analysis. Data were subjected to the appropriate transformation before statistical analysis as described by Zar (1987). Any statistical probability less than or equal to 0.05 was considered significant.

Results

Total DNA hybridizations and DNA base content of isolates from deep sediments. The identification of deep sediment isolates randomly chosen for the DNA tests from the three sites by the Rapid API-NFT identification system showed that 28% were unknown species. Based on these physiological tests, 40% of the isolates were identified as Pseudomonas sp. and 25% as Acinetobacter sp. (Table 1). The species Pseudomonas luteola was found across the geological profile at all of the sites, while Acinetobacter sp. was found in all formations at sites P24 and P28, with the exception of the Tobacco Road. Unknown species were found throughout the geological profile at all sites.

The guanine and cytosine content of deep subsurface bacterial isolates that were randomly chosen from the three sites ranged from 20 to 76% (Table 2). More than 60% of the isolates have their values between 58 and 75%. Of the isolates not identified by the phenotypic tests, 55% showed mol %G+C values between 57 and 70% while 27% of the isolates had values between 38 to 49% (Tables 1 and 2). The mol %G+C of bacterial isolates from site P28 ranged from 32 to 72% while sites P24 and P29 showed ranges of 36-60% and 43-74%, respectively. The average mol %G+C of bacteria from site P28 was 58.67 $\pm$ 3.1%, whereas a 53.4 $\pm$ 16.2% was found in site P24. On the other hand, site P29 showed an average of 59.3 $\pm$ 3.1%. There was no significant difference between sites in the total mol %G+C values found.

Three whole chromosomal probes were used to study the molecular relatedness between and within site isolates A0481 (site P28), B0703 (site P24), and C0397 (site P29). Isolates A0481 and B0703 had the same phenotype and were identified as Pseudomonas luteola using the API-NFT code (Table 1); however, their mol %G+C was different (66.0 and 64.0) and the DNA homology between the two was less than 50% (Table 3). Thus, they could not be the same species and were misidentified as P. luteola. Probe CO397 had a mol %G+C of 61.0 and was identified as P. acidovorans by the API-NFT code (Tables 1 and 2). Since all three probes were different species, they were used as diversity probes against isolates from different sites and formations.

The average DNA homology of probe A0481 (P28) against isolates from site P24, P28 and P29 were 32.5 $\pm$ 10.3, 19.5 $\pm$ 7.3, and 19.7 $\pm$ 14.2, respectively. Probe B0703 showed DNA homologies against isolates from the same three sites of 20.1 $\pm$ 6.2, 26.6 $\pm$ 10.9, and 20.2 $\pm$ 14.4. Probe C0397 showed DNA homologies of 29.0 $\pm$ 6.7, 37.6 $\pm$ 15.9, and 29.2 $\pm$ 23.5. Overall there was no significant difference in DNA relatedness between isolates from different sites and formations. DNA

relatedness between the same formations and sites were not significant at the three sites. Nevertheless, no bacterial isolate from upper formations or different sites had the same genetic structure of isolates from deeper stratas, since none of them showed $\geq$ 70.0 DNA homology under stringent conditions or similar mol %G+C values (Tables 2 and 3).

Bacteria with the same phenotype from different geological formations did not show the same genetic composition. Therefore, since phenotypic tests did not suggest the genetic differences between deep subsurface bacteria, several isolates from the same geological formation and phenotype were analyzed using total DNA hybridizations and base composition to see whether this was a common pattern in these communities. All isolates were hybridized against a phenotypically identical probe (A0481) from site P28 with a mol %G+C of 66.0%, and none showed more than 52.0% DNA homology. Their DNA base composition was significantly different ($F = 217.9, dF = 1$ and 6, $P < 0.05$). Isolates from site P28 showed an average of 71.4% of G+C, while isolates from site P29 showed an average of 31.6% (Table 4). The same reaction was found with isolates from site P24. Although they come from the same site and formation and have the same phenotype, their DNA homology against isolate B0703 was not equal to or higher than 60% (Table 4).

The accuracy of the phenotypic tests to describe known bacterial species was determined by using a whole chromosomal DNA probe from Pseudomonas aeruginosa type strain ATCC 9721. The mol %G+C of the type strain was 67.9%. However, when the mol %G+C of deep subsurface bacteria identified as P. aeruginosa (by the API-NFT) was done, they showed values from 46.8 to 64.7%. None of the isolates showed similar values with the type strain. Total DNA hybridization of the type strain against these isolates showed DNA homologies lower than 50% (Table 5).

To study the effect of depth on the distribution of bacterial genotypes in deep subsurface environments, at least three randomly chosen bacteria from each formation were tested across the depth profile at site P24 (Table 6). These bacteria were hybridized against one isolate each from the Middendorf and Tobacco Road formations. In addition, the mol %G+C of each bacterial DNA was determined. In that way, the genetic distance between isolates from upper and deeper aquifers was measured. Isolates from the Tobacco Road formation showed a broader range of mol %G+C (29-73%), whereas the Middendorf isolates had a narrower range (64 - 71.6%). No isolate showed a significant hybridization (e.g., $\geq$70%) against the two probes tested.

Discussions

Three of the four probes used were in the genus Pseudomonas, since they all were Gram-negative, glucose nonfermentative rods with mol %G+C of 61-66%.[24] Although two of these probes were phenotypically identical, they did not share the same genotype (i.e., less than 50% DNA homology and different mol %G+C). The mol %G+C of deep subsurface bacteria ranged from 20 to 75%, with more than 60% of the isolates between 57-75%. According to Palleroni,[24] this is the range of the family Pseudomonaceae. Moreover, of the 28% unknown bacteria not identified by

the API-NFT system, 55% showed mol %G+C values within this range; therefore, since they were all Gram-negatives rods, oxidase positive, glucose nonfermentors, they probably should be classified as Pseudomonas sp. Based on the physiological tests and the mol %G+C analysis, it can be concluded that the Pseudomonas spp. were the most abundant heterotrophic bacteria in the isolates tested.

Nevertheless, a great majority of the isolates that were identified by the API-NFT system as Pseudomonas luteola did not show the reported mol %G+C values for this species.[11] Holmes et al.[11] divided the Pseudomonas luteola species into two groups based on their differences in DNA base composition, Flavimonas spp. with a mol %G+C of 63 ± 1.6% and Chryseomonas spp. at 56.8%. Bacterial isolates B0428, B0703, and B0725 showed similar mol %G+C values with the Flavimonas spp. These two new species were created to separate this genetically heterogeneous group in different genera. Since deep subsurface bacterial communities showed a number of unknown phenotypic types, it is possible that a number of new species are present in the deep subsurface. An extensive number of phenotypic tests and electron microscopy studies have to be done to gather enough information about it. Also, different rRNA probes are currently available that can be used to identify some of these isolates to the species level.[20]

Deep subsurface isolates from the three sites identified as P. aeruginosa did not have the same mol %G+C value of the type strain, and their homology was less than 50% against a type strain. Thus, they were misidentified as P. aeruginosa. Bacterial isolates A0474, A0735, B0344, B0103, B0121, B0444, and B0457, were identified as Acinetobacter spp. by the API-NFT test. Since they all had mol %G+C values that ranged from 38 to 49%, they could be Acinetobacter spp.[14] Overall, DNA homology studies did not show any positive correlation with phenotypic identification for the identification of bacterial species, because bacteria with the same phenotype from the same or different formations not only showed less than 50% of DNA homology but different mol %G+C as well. Thus, phenotypic tests alone underestimated the bacterial diversity at the three sites and did not provide an accurate assessment of the structure and composition of heterotrophic bacteria from the deep subsurface. Busse et al.[3] found that the API-NFT system may not yield valuable results even with isolates characterized as true Pseudomonas spp. due to the limited number of reference strains and because of the ill-defined status of this genus. Indeed, some of the Pseudomonas species listed in the API code book have been placed in separate genus and did not reflect the actual status of the family Pseudomonaceae.

When the Middendorf (deepest) and Tobacco Road (upper) geological formations were compared, they did not show any mol %G+C content in common. Moreover, no isolate from different geological formations and sites belonged to the same species (i.e., showed more than 70% DNA homology and same mol %G+C value). Therefore, each geological formation seems to have a distinct microbial community. Nevertheless, bacterial isolates tested from the Middendorf formation showed narrower mol %G+C values than Tobacco Road isolates, suggesting that a single bacterial genus, probably Pseudomonas sp., was dominant in the deepest

stratas. It seems that the deeper the formation, the narrower the range of mol %G+C values found. This suggests that greater distances from the recharge zones and older depositional sediments diminish the diversity of bacterial genera, since only mol %G+C values similar to the Pseudomonas and Acinetobacter sp. were found in deeper aquifers. Therefore, it seems that based on these genetic tests and those reported by Balwkwill et al.,[1] each geological formation has its own microbial community that was laid down during the deposition of the sediments, and that specific bacterial species were selected by particular conditions such as stratigraphy, geochemistry, and hydrology in each formation.

Deep subsurface bacterial species probably reflect phenotypic and genetic adaptations to the prevailing climatic and abiotic conditions of the deep subsurface. Similar results were reported by Yayanos et al.[26] when deep sea bacteria were closely related to, but distinct from, members of the genus Vibrio. Olsen et al.[20] reported that Thiobacillus spp. 5S rRNA sequences are the most predominant species living in symbiosis inside worms in deep hydrothermal vents. They stated that these new species are related to, but different from other Thiobacillus species based on the divergence in the RNA sequences.

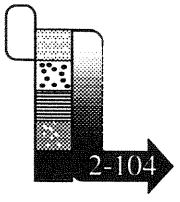
The family Pseudomonaceae has been found as part of the predominant heterotrophic flora from the Schirmacher Oasis in the Antarctica based on phenotypic characteristics and mol %G+C of bacterial isolates.[24] However, the differentiation of the Pseudomonaceae and Acinetobactereaceae at the species level in ecological studies as shown in this paper is difficult due to their phenotypic and genetic heterogeneity. Woese[25] has shown that both families are comprised of phylogenetically distant strains and that they are spread over more than five different rRNA homology groups. The metabolic versatility of the genus Pseudomonaceae has been well documented and their widespread distribution in the environment is a result of this characteristic.[18] Since more than 70% of deep subsurface bacteria tested are in those two families the potential uses of these communities for subsurface bioremediation strategies are excellent. This is due to the reported metabolic capacity of these two genera to degrade a wide range of xenobiotics.

Conclusion

In a single Pseudomonas species, there may be several different pathways by means of which dissimilar organic compounds are broken down. Deep subsurface bacteria are an ideal choice to be used for environmental detoxification studies due to their metabolic and genetic capability, and they may offer new strategies for *in situ* bioremediation of deep aquifers and unsaturated vadose zone sediments. This capability can be increased by gene amplification and increased expression of the degradative gene.[22] Further genetic studies on deep subsurface bacteria will provide additional evidence to understand the variability, evolution, establishment, and survival of these communities in deep subsurface environments.

Acknowledgements

The author was supported by a predoctoral fellowship in bioengineering from Oak Ridge Associated Universities Program. I want to acknowledge the Environmental Sciences Section at the Savannah River Laboratory for their help and cooperation during the development of this work. Terry C. Hazen made some helpful comments and reviews of the manuscript. The information contained in this article was developed during the course of work under Contract No. DE-AC09-76SR00001 with the U. S. Department of Energy.



References

1. Balkwill, D. L., J. K. Fredrickson, and J. M. Thomas, 1989. "Vertical and Horizontal Variations in the Physiological Diversity of the Aerobic Chemoheterotrophic Bacterial Microflora in Deep Southeast Coastal Plain Subsurface Sediments". *Appl. Environ. Microbiol.* **55**:1058-1065.

2. Bermúdez, M. and T. C. Hazen, 1988. "Phenotypic and Genotypic Comparison of Escherichia coli from Pristine Tropical Waters". *Appl. Environ. Microbiol.* **54**:979-983.

3. Busse, H. J., T. El-Banna, and G. Auling, 1989. "Evaluation of Different Approaches for Identification of Xenobiotic-Degrading Pseudomonads". *Appl. Environ. Microbiol.* **55**:1578-1583.

4. Crouse, J. and D. Amorese, 1987. "Ethanol Precipitation: Ammonium Acetate as an Alternative to Sodium Acetate". *Focus (Bull. Bethesda Res. Lab.)* **9**(2): 3-5.

5. Department of Energy. "Microbiology of Subsurface Environments. *Proceedings of the Second Investigators' Meeting-Savannah River Exploratory Deep Probe.* US DOE Report DOE/ER-0312, Office of Energy Research, Washington, DC, 1986.

6. Desmarchelier, P. M. and W. G. Zumf, 1987. "A Phenotypic and Genetic Study of Sucrose Nonfermenting Strains of Vibrio mimicus and Vibrio chlolerae". *Curr. Microbiol.* **10**:41-48.

7. Fliermans, C. B. and D. Balkwill, 1989. "Microbial Life in Deep Terrestrial Subsurfaces". *Bioscience* **39**:370-377.

8. Frederickson, J. K. and R. J. Hicks, 1987. "Probing Reveals Many Microbes Beneath Earth's Surface". *ASM News* **53**:78-79.

9. Fredrickson, J. K., R. J. Hicks, S. W. Li, and F. J. Brockman, 1988. "Plasmid Incidence from Deep Subsurface Sediments". *Appl. Environ. Microbiol.* **54**: 2916-2923.

10. Hazen, T. C. and L. Jimenez, 1988. "Enumeration and Identification of Bacteria from Environmental Samples Using Nucleic Acid Probes". *Microbiol. Sci.* **5**:340-343.

11. Holmes, B., A. G. Steigerwalt, R. E. Weaver, and D. J. Brenner, 1987. "Chryseomonas luteola Comb. Nov. and Flavimonas oryzihabitans Gen. Nov., Pseudomonas-Like Species from Human Clinical Specimens and Formerly Known Respectively as Groups Ve-1 and Ve-2". *Int. J. Syst. Bacteriol.* **37**: 245-250.

12. Johnson, J. L. "Determination of DNA Base Composition". G. Gottschalk (ed.), *Methods in Microbiology* (vol. 18). Academic Press, Inc., London. pp 1-31, 1985.

13. Johnson, R. C., W. Burgdorfer, R. S. Lane, A. G. Barbour, S. F. Hayes, and F. W. Hyde, 1987. "Borrelia coriaceae Sp. Nov: Putative Agent of Epizootic Bovine Abortion". *Int. J. Syst. Bacteriol.* **37**:72-74.

14. Krieg, N. R. and J. G. Holt. *Bergey's Manual of Systematic Bacteriology* (vol. 1). The Williams and Wilkins Co., Baltimore, 1984.

15. Maniatis, T., E. F. Fritsch, and J. Sambrook. *Molecular Cloning*. Cold Spring Harbor Laboratory Publication, Cold Spring Harbor, New York, 1987.

16. Marmur, J, 1961. "A Procedure for the Isolation of Deoxyribonucleic Acid from Microorganisms". *J. Mol. Biol.* **3**:208-218.

17. Marmur, J. and P. Doty, 1962. "Determination of the Base Composition of Deoxyribonucleic Acid from Its Thermal Denaturation Temperature". *J. Mol. Biol.* **5**:109-118.

18. McCormick, D, 1985. "One Bug's Meat...". *Bio/Technology* **3**:429-435.

19. Ogram, A. and G. S. Sayler, 1988. "The Use of Gene Probes in the Rapid Analysis of Natural Microbial Communities". *J. Ind. Microbiol.* **3**:281-292.

20. Olsen, G. J., D. J. Lane, S. J. Giovannoni, and N. R. Pace, 1986. "Microbial Ecology and Evolution: A Ribosomal RNA Approach". *Ann. Rev. Microbiol.* **40**:337-365.

21. Olson, B. H. and R. A. Goldstein, 1988. "Applying Genetic Ecology to Environmental Management". *Environ. Sci. Technol.* **22**:370-372.

22. Palleroni, N. J., R. Kunisawa, R. Contopoulou, and M. Doudorofoff, 1973. "Nucleic Acid Homologies in the Genus Pseudomonas". *Int. J. Syst. Bacteriol.* **23**:333-339.

23. Palleroni, N. J. "Genus I. Pseudomonas Migula 1894". N. R. Krieg and J. G. Holt (eds.), *Bergeys Manual of Systematic Bacteriology* (vol. 1). The Williams and Wilkins Co., Baltimore, 1984.

24. Shivaji, S., N. Shyamalarao, L. Saisree, V. Sheth, G. S. N. Reddy, and P. M. Bhargava, 1989. "Isolation and Identification of Pseudomonas spp. from Schirmacher Oasis, Antarctica". *Appl. Environ. Microbiol.* **55**:767-770.

25. Woese, C. R, 1987. "Bacterial Evolution". *Microbiol. Rev.* **51**:221-271.

26. Yayanos, A., A. S. Dietz, and R. Van Baxtel, 1979. "Isolation of A Deep-Sea Bacterium and Some of Its Growth Characteristics". *Science* ***205***:808-812.

27. Zar, J. H. *Biostatistical Analysis*. Prentice-Hall, Inc., Englewood Cliffs, NJ, 1984.

Tables

Table 1. API Identification of deep subsurface bacterial isolates used in the genetic tests.

Isolate	Species ID (API-NFT)	API Code	Formation
A1037	Pseudomonas aeruginosa	1-144-455	Congaree
A0098	Pseudomonas aeruginosa	1-144-455	Pee Dee
A0099	Moraxella phenylpyruvica	0-200-004	Pee Dee
A0101	Unknown	1-154-655	Pee Dee
A0231	Pseudomonas lutheola	0-476-643	Pee Dee
A0232	Unknown	1-154-655	Pee Dee
A0234	Pseudomonas aeruginosa	1-144-455	Pee Dee
A0474	Acinetobacter calcoaceticus lwoffi	0-000-051	Middendorf
A0481	Pseudomonas lutheola	0-476-643	Middendorf
A0619	Unknown	1-677-764	Middendorf
A0735	Acinetobacter calcoaceticus lwoffi	0-000-051	Middendorf
A0747	Pseudomonas aeruginosa	1-144-455	Middendorf
A1270	Unknown	1-154-655	Middendorf
A1271	Unknown	1-154-655	Middendorf
A0371	Acinetobacter calcoaceticus lwoffi	0-000-051	Middendorf
A0376	Acinetobacter calcoaceticus lwoffi	0-200-000	Middendorf
B0088	Pseudomonas luteola	0-476-643	Tobacco Road
B0096	Unknown	0-677-667	Tobacco Road
B0344	Acinetobacter calcoaceticus lwoffi	0-200-000	Dry Branch
B0348	Unknown	0-050-224	Dry Branch
B0259	Unknown	1-154-655	Congaree
B0267	Acinetobacter calcoaceticus lwoffi	0-000-053	Congaree
B0103	Acinetobacter calcoaceticus lwoffi	0-000-051	Ellenton
B0121	Acinetobacter calcoaceticus anitratus	0-041-473	Ellenton
B0428	Pseudomonas luteola	0-467-667	Pee Dee
B0444	Acinetobacter calcoaceticus lwoffi	0-000-051	Pee Dee
B0457	Acinetobacter calcoaceticus lwoffi	0-000-051	Pee Dee
B0550	Unknown	0-677-557	Black Creek
B0617	Pseudomonas luteola	0-476-643	Middendorf
B0703	Pseudomonas luteola	0-476-643	Middendorf
B0725	Pseudomonas luteola	0-476-643	Middendorf
C0081	Pseudomonas luteola	0-476-643	Tobacco Road
C0101	Pseudomonas cepacia	4-477-777	Dry Branch
C0128	Pseudomonas luteola	0-476-643	Dry Branch
C0198	Unknown	1-154-655	Congaree
C0200	Moraxella lacunata	1-010-053	Congaree
C0397	Pseudomonas acidovorans	1-000-456	Middendorf
C0464	Pseudomonas luteola	0-476-643	Middendorf
C0593	Pseudomonas luteola	0-476-643	Middendorf
C0679	Unknown	1-154-655	Middendorf

Table 2. DNA base composition as determined by DNA thermal melting point of deep subsurface bacteria.

Isolate	Mol %G+C	Formation	Site
A1037	65.9	Congaree	28
A0098	67.4	Pee Dee	28
A0099	70.5	Pee Dee	28
A0111	66.2	Pee Dee	28
A0231	59.5	Pee Dee	28
A0232	64.7	Pee Dee	28
A0234	61.5	Pee Dee	28
A0474	39.5	Middendorf	28
A0481	66.0	Middendorf	28
A0619	45.4	Middendorf	28
A0735	32.7	Middendorf	28
A0747	N.D.	Middendorf	28
A1270	54.4	Middendorf	28
A1271	46.8	Middendorf	28
A0371	67.4	Middendorf	28
A0376	72.2	Middendorf	28
B0088	51.2	Tobacco Road	24
B0096	46.8	Tobacco Road	24
B0344	36.1	Dry Branch	24
B0348	57.0	Dry Branch	24
B0259	64.4	Congaree	24
B0267	55.1	Congaree	24
B0103	49.8	Ellenton	24
B0121	36.2	Ellenton	24
B0428	62.0	Pee Dee	24
B0448	46.8	Pee Dee	24
B0457	49.8	Pee Dee	24
B0619	45.4	Middendorf	24
B0703	64.0	Middendorf	24
B0725	63.5	Middendorf	24
C0081	47.8	Tobacco Road	29
C0101	62.5	Dry Branch	29
C0128	43.5	Dry Branch	29
C0200	74.0	Congaree	29
C0198	54.7	Congaree	29
C0397	61.0	Middendorf	29
C0464	69.0	Middendorf	29
C0593	65.8	Middendorf	29
C0679	62.5	Middendorf	29
E. coli	51.7	NA	NA

Table 3. DNA homologies between deep subsurface bacterial isolates.

Site Isolate	Probe (P28) A0481	Probe (P24) B0703	Probe (P29) C0397	Formation	Site
A1037	0.0	35.0	6.80	Congaree	P28
A0098	8.7	58.4	2.5	Pee Dee	P28
A0099	1.2	35.8	0.0	Pee Dee	P28
A0111	33.0	1.0	ND	Pee Dee	P28
A0231	38.0	59.9	0.0	Pee Dee	P28
A0232	45.0	11.0	ND	Pee Dee	P28
A0234	3.0	45.6	55.1	Pee Dee	P28
A0474	0.0	15.3	18.9	Middendorf	P28
A0481	100	62.8	46.3	Middendorf	P28
A0619	ND	47.0	36.3	Middendorf	P28
A0735	0.0	20.2	24.1	Middendorf	P28
A0747	0.0	40.0	9.5	Middendorf	P28
A1270	44.0	0.0	ND	Middendorf	P28
A1271	13.0	4.0	ND	Middendorf	P28
A0371	0.0	54.0	45.3	Middendorf	P28
A0376	0.0	23.0	12.5	Middendorf	P28
B0088	0.0	0.0	0.0	Tobacco Road	P24
B0096	0.0	0.0	0.0	Tobacco Road	P24
B0344	0.0	10.0	25.6	Dry Branch	P24
B0348	34.2	39.0	ND	Dry Branch	P24
B0259	39.0	0.0	ND	Congaree	P24
B0267	0.0	13.4	36.7	Congaree	P24
B0103	0.0	49.9	17.1	Ellenton	P24
B0121	14.3	22.8	15.3	Ellenton	P24
B0428	21.3	31.1	77.4	Pee Dee	P24
B0448	12.2	39.1	0.0	Pee Dee	P24
B0457	5.5	61.9	14.5	Pee Dee	P24
B0550	0.0	0.0	0.0	Black Creek	P24
B0619	21.8	13.9	41.3	Middendorf	P24
B0703	61.0	100	21.6	Middendorf	P24
B0725	0.0	11.6	60.4	Middendorf	P24
C0081	0.0	0.0	0.0	Tobacco Road	P29
C0101	46.7	18.2	0.0	Dry Branch	P29
C0128	ND	79.5	ND	Dry Branch	P29
C0200	55.0	59.6	27.9	Congaree	P29
C0198	10.0	17.0	ND	Congaree	P29
C0397	18.0	25.9	100	Middendorf	P29
C0464	30.0	50.9	43.7	Middendorf	P29
C0593	40.2	80.0	15.0	Middendorf	P29
C0679	46.0	0.0	ND	Middendorf	P29
E. coli	0.0	2.0	0.0	NA	NA

NA = Not applicable.
ND = Not determined.

Table 4. DNA homologies and base composition of bacteria with identical phenotypic profile classified as Pseudomonas luteola, API-NFT Code 0-476-642.

PROBE A0481

Bacteria	%DNA Homology	%G+C	Site
A0481	100	66.0	P28
A01366	24.8	71.3	P28
A1379	48.2	71.2	P28
A1373	18.6	71.6	P28
C0520	33.8	35.2	P29
C0543	50.3	32.1	P29
C0574	32.6	33.8	P29
C0564	30.2	25.1	P29

PROBE B0703

Bacteria	%DNA Homology
B0703	100
B0612	0.0
B0617	0.0
B0620	0.0
B0603	10.0
B0719	29.0
B0694	57.0
B0622	13.0
B0679	13.0
B0671	35.0

Table 5. Total DNA hybridizations of Pseudomonas aeruginosa and isolates phenotypically identified by API-NFT as P. aeruginosa.

Isolate	%G+C	%DNA Homology	Site
P. aeruginosa	67.0	100	—
A01270	54.4	52.3	P28
C0198	54.7	50.9	P29
B0259	64.4	8.3	P24
A0111	66.2	0.0	P28
A0232	64.7	29.4	P28
A1271	46.8	45.1	P28
C0679	62.5	41.4	P29

Table 6. DNA base composition and homology of bacterial isolates across the geological profile in site P24.

Geological Formation (Depth)	Mol %G+C	DNA Homology BO703	BO169
MIDDENDORF (235-257 m)			
B0703	64.0	100	0.0
B0694	71.6	57.0	0.0
B0622	66.7	13.0	0.0
B0671	66.5	35.0	0.0
BLACK CREEK (199-202 m)			
B0550	69.5	10.0	0.0
B0549	71.4	11.0	0.0
B0560	72.9	1.0	0.0
B0547	64.1	45.0	0.0
PEE DEE (145-180 m)			
B0477	20.0	0.0	0.0
B0476	50.8	0.0	0.0
B0474	75.2	0.0	0.0
B0470	77.0	0.0	0.0
ELLENTON (117-139 m)			
B0379	72.0	8.2	0.0
B0390	71.2	5.5	0.0
B0380	73.0	0.0	0.0
CONGAREE (91 m)			
B0277	75.0	0.0	0.0
B0278	70.7	1.3	0.0
B0306	66.8	21.2	0.0
DRY BRANCH (45-65 m)			
B0180	72.0	1.2	0.0
B0185	71.8	2.3	0.0
B0179	71.8	0.0	0.0
TOBACCO ROAD (34 m)			
B0163	29.4	0.0	10.0
B0165	51.2	0.0	0.0
B0167	73.0	3.6	0.0
B0169	33.4	0.0	100