

# Antibiotic Resistance in Bacteria Isolated from the Deep Terrestrial Subsurface

Mindy G. Brown · David L. Balkwill

Received: 21 April 2008 / Accepted: 8 July 2008 / Published online: 2 August 2008  
© Springer Science + Business Media, LLC 2008

**Abstract** Various natural environments have been examined for the presence of antibiotic-resistant bacteria and/or novel resistance mechanisms, but little is known about resistance in the terrestrial deep subsurface. This study examined two deep environments that differ in their known period of isolation from surface environments and the bacteria therein. One hundred fifty-four strains of bacteria were isolated from sediments located 170–259 m below land surface at the US Department of Energy Savannah River Site (SRS) in South Carolina and Hanford Site (HS) in Washington. Analyses of 16S rRNA gene sequences showed that both sets of strains were phylogenetically diverse and could be assigned to several genera in three to four phyla. All of the strains were screened for resistance to 13 antibiotics by plating on selective media and 90% were resistant to at least one antibiotic. Eighty-six percent of the SRS and 62% of the HS strains were resistant to more than one antibiotic. Resistance to nalidixic acid, mupirocin, or ampicillin was noted most frequently. The results indicate that antibiotic resistance is common among subsurface bacteria. The somewhat higher frequencies of resistance and multiple resistance at the SRS may, in part, be due to recent surface influence, such as exposure to antibiotics used in agriculture. However, the HS strains have never been exposed to anthropogenic antibiotics but still had a reasonably high frequency of resistance. Given their long period of isolation from surface influences, it is possible

that they possess some novel antibiotic resistance genes and/or resistance mechanisms.

## Introduction

In nature, bacteria are thought to produce antibiotics that kill or inhibit neighboring bacteria as a strategy for preserving resources [16, 66]. When resources such as nutrients are limited, a bacterium can produce an antibiotic to destroy or inhibit neighboring bacteria, thereby limiting competition for the scarce resources. In order for this strategy to be effective, the bacteria producing the antibiotic must be able to survive by possessing mechanisms of resistance to the antibiotic they produce. These mechanisms can be transferred to other bacteria, and this has led to an ever-increasing threat to global public health by confounding treatment of infections caused by virtually all major pathogens [37, 38, 69]. Given the clinical significance of this problem, it is not surprising that, for many years, research on antibiotic-resistant bacteria and mechanisms of resistance focused almost exclusively on pathogens in clinical settings. More recently, however, it has become apparent that antibiotic resistance is common not only among commensal bacteria of humans and animals [11, 38, 58] but also among environmental bacteria (e.g., [11, 16, 17]). The latter observation is important because bacteria in natural environments likely serve as a reservoir of resistance genes that eventually can be transmitted to pathogenic species [1, 16, 60]. As a result, it is important to develop a more comprehensive understanding of the prevalence and diversity of antibiotic resistance in a broad range of environments worldwide [16] because this information could provide an early warning for future clinically relevant resistance mechanisms [17] and facilitate the design of effective new drugs [53, 68, 69].

**Electronic supplementary material** The online version of this article (doi:10.1007/s00248-008-9431-6) contains supplementary material, which is available to authorized users.

M. G. Brown (✉) · D. L. Balkwill  
Department of Biomedical Sciences,  
Florida State University College of Medicine,  
1115 West Call Street,  
Tallahassee, FL 32306-4300, USA  
e-mail: mindy.brown@med.fsu.edu

Several terrestrial and aquatic environments have been examined for the presence of antibiotic-resistant bacteria, and the resistance mechanisms of these bacteria have been characterized in some cases (see “[Discussion](#)”). However, very little is known about antibiotic resistance in deep terrestrial subsurface environments, which have only in the past several decades been found to contain microbes. These environments were widely believed to be devoid of microbial life until the mid-1980s, when several research groups found substantial numbers of bacteria in shallow aquifers less than 30 m below land surface [29]. Since then, microbes have been shown to occupy a variety of chemically and physically distinct environments, to depths of at least 3.6 km [4, 10, 24, 25, 28].

The first indication that subsurface bacteria possess resistance to antibiotics was a study by Fredrickson et al. [27] that involved plasmid characterization. These investigators were interested in determining the ability of subsurface bacteria to resist metals and antibiotics and wanted to know if this ability corresponded with the presence of plasmids. The bacteria were obtained from several deep and shallow boreholes at the Savannah River Site (SRS) and screened for resistance to six antibiotics, revealing a uniform pattern of resistance for the strains from all of the boreholes. In the late 1990s, 1,800 bacterial strains from a variety of subsurface environments, including two shallow (<30 m below land surface) and three deep (>200 m) examples, were screened for resistance to eight antibiotics [21]. Varying frequencies of resistance and multiple resistances were detected among the strains from all of these environments.

It is not always clear whether a particular subsurface environment has been exposed to commercial antibiotics or if the bacteria in it have been transported there from the surface in recent times. For example, widely studied Atlantic coastal plain sediments at the SRS in South Carolina have been buried for up to 100 million years, but the groundwater that moves through them could be less than 4,000 years old [59]. Similarly, relatively shallow environments, such as aquifers in Oyster, VA, could easily have been exposed to antibiotics used in agriculture, allowing for selection of antibiotic resistant bacteria [32]. In contrast, Ringold formation sediments studied at the DOE Hanford Site (HS) near Richland, Washington, in 1992 have been isolated from any surface influence for at least 3 million years [67]. Clearly, bacteria from these sediments were never exposed to commercial antibiotics or to genes that may have evolved in response to the use of commercial antibiotics. Given their long isolation from the influence of surface bacteria and environments, there is a possibility that the HS strains have evolved mechanisms of resistance distinct from those found in surface bacteria. Although it is becoming clear that subsurface bacteria are resistant to antibiotics, nothing is known about the actual resistance mechanisms utilized by these bacteria.

The purpose of the present study was to confirm that high frequencies of antibiotic resistance are present among deep subsurface bacteria at the SRS and HS and to more fully characterize the types of resistance in aerobic and facultatively anaerobic chemoheterotrophic strains isolated from these two environments. We report in this paper the patterns of resistance to 13 different antibiotics detected in the two groups of subsurface bacteria and provide phylogenetic characterization data relating the strains to established bacterial taxa. Our findings demonstrate that resistance and multiple resistances to antibiotics, as well as a large number of distinct resistance patterns, are present among a phylogenetically diverse range of bacteria from both environments. Future studies will focus on the characterization of selected antibiotic resistance genes, mechanisms, and gene mutations in the strains from the HS.

## Materials and Methods

### Origin and Isolation of Bacterial Strains

The SRS sediment samples were obtained from borehole P24 located within the Upper Atlantic Coastal Plain on the Aiken Plateau, adjacent to the Savannah River, using specialized drilling methods described previously [51, 54]. The strains investigated in this study were isolated from sediment cores extracted from 244 and 259 m below land surface (within the Middendorf Formation) [59]. The HS sediment samples were obtained from the Yakima Barricade borehole located at the western edge of the US DOE Hanford Site, and strains were isolated from Ringold Formation paleosols, fluvial sands, and lacustrine sediments extracted from 173 to 185 m below land surface [34, 45]. Selected physical, chemical, and microbiological characteristics of the sediment samples are given in Supplemental Table 3.

This study focused specifically on aerobic and facultatively anaerobic chemoheterotrophic bacteria at each site. Strains of these bacteria were isolated for further study in a three-part process: Serial dilutions of blended sediment samples were spread on PTYG (peptone 5.0 g/l, tryptone 5.0 g/l, yeast extract 10.0 g/l, glucose 10.0 g/l,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.6 g/l,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  0.07 g/l, agar 15.0 g/l) and 1% PTYG agar plates; morphologically distinct colony types were isolated by streaking and re-streaking on fresh plates; and the isolated strains were grown in liquid PTYG or 1% PTYG and frozen in cryoprotectant [6, 15]. PTYG and 1% PTYG media have been found to yield relatively high numbers and a broad variety of chemoheterotrophic bacteria from various types of subsurface sediment samples [4, 6, 7, 28]. The frozen strains were accessioned into the DOE Subsurface Microbial Culture Collection (SMCC) at Florida State University [7] and have been stored in liquid

nitrogen since that time. For both the phylogenetic and antibiotic resistance analyses carried out in this study, the SRS and HS strains were grown on 5% PTYG agar from the frozen stocks. Recovery rates for the frozen strains in the SMCC have consistently remained above 99.5%.

### Phylogenetic Analysis

The isolated strains were cultured in 5 ml of 5% PTYG broth, and bacterial pellets were collected during early log-phase growth. Chromosomal DNA was isolated from each strain with the DNeasy Tissue Kit (Qiagen, Valencia, CA, USA). Twenty to 100 ng of genomic DNA was used as a template for polymerase chain reaction (PCR) amplification of an approximately 1,500-base segment of the 16S rRNA gene. The PCR primers were 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3') [36], and the resulting PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions, except that the PCR product was eluted in 30  $\mu$ l instead of 50  $\mu$ l. The purified PCR products were sequenced on an ABI 3130xl Genetic Analyzer with the Big Dye Terminator version 3.1 sequencing method. The sequencing primer used was primer G (5'-CCAGGGTATC-TAATCCTGTT-3') [7]. The sequences were then assembled with Sequencer version 4.5 (Gene Codes Corporation, Ann Arbor, MI, USA).

The 16S rRNA sequences inferred from the 16S rDNA sequences determined as described above were aligned with 16S rRNA sequences from selected species from several related genera. Sequences of the reference strains were obtained from the Ribosomal Database Project-II [13] and were chosen based on a high similarity rank with the strains in this study. Representative type strains from the Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes phyla were also included, along with a sequence to serve as an outgroup. Approximately 700 bases were included in the phylogenetic analysis, which was performed with the PAUP computer program [65], using a GTR+G+I model with the following parameter estimates: [A-C]=0.655, [A-G]=1.522, [A-T]=1.314, [C-G]=0.769, [C-T]=3.337, [G-T]=1.000, gamma parameter [G]=0.764, proportion of invariant sites [I]=0.239.

### Antibiotic Resistance Screening

All SRS and HS strains were screened for resistance to 13 antibiotics: vancomycin (32  $\mu$ g/ml), erythromycin (8  $\mu$ g/ml), tetracycline (16  $\mu$ g/ml), chloramphenicol (32  $\mu$ g/ml), gentamicin (16  $\mu$ g/ml), kanamycin (64  $\mu$ g/ml), streptomycin (16  $\mu$ g/ml), neomycin (100  $\mu$ g/ml), ciprofloxacin (4  $\mu$ g/ml), rifampin (4  $\mu$ g/ml), ampicillin (32  $\mu$ g/ml), nalidixic acid (32  $\mu$ g/ml), and mupirocin (30  $\mu$ g/ml). The antibiotics chosen cover a range of classes of antibiotics, and the concentrations

used are the highest inhibitory concentration listed in the CLSI [12]. The strains were plated in triplicate on selective media and checked for growth after 18, 42, and 66 h. Results were recorded as sensitive or resistant; intermediate growth was not considered. Selective media were prepared by incorporating the antimicrobial agent into the 5% PTYG agar medium. The strains were maintained on 5% PTYG until transferred to the selective media. The concentrations of antibiotics (listed with the antibiotics above) used for screening were obtained from the Performance Standards for Antimicrobial Susceptibility Testing [12]. The highest breakpoint minimum inhibitory concentration (MIC) for all bacteria listed in the performance standard was used. The breakpoint MIC for mupirocin and neomycin was not listed in the CLSI. The concentration used for mupirocin was double that (1%) used in Bactroban, according to the GlaxoSmithKline prescribing information [30]. The concentration of neomycin used in this study was the same as that of the other aminoglycosides.

### Accession Numbers for 16S rDNA Sequences

The GenBank accession numbers for the 16S rDNA sequences determined in this study are EU442210-EU442265 for the SRS strains and EU446126-EU446222 for the HS strains.

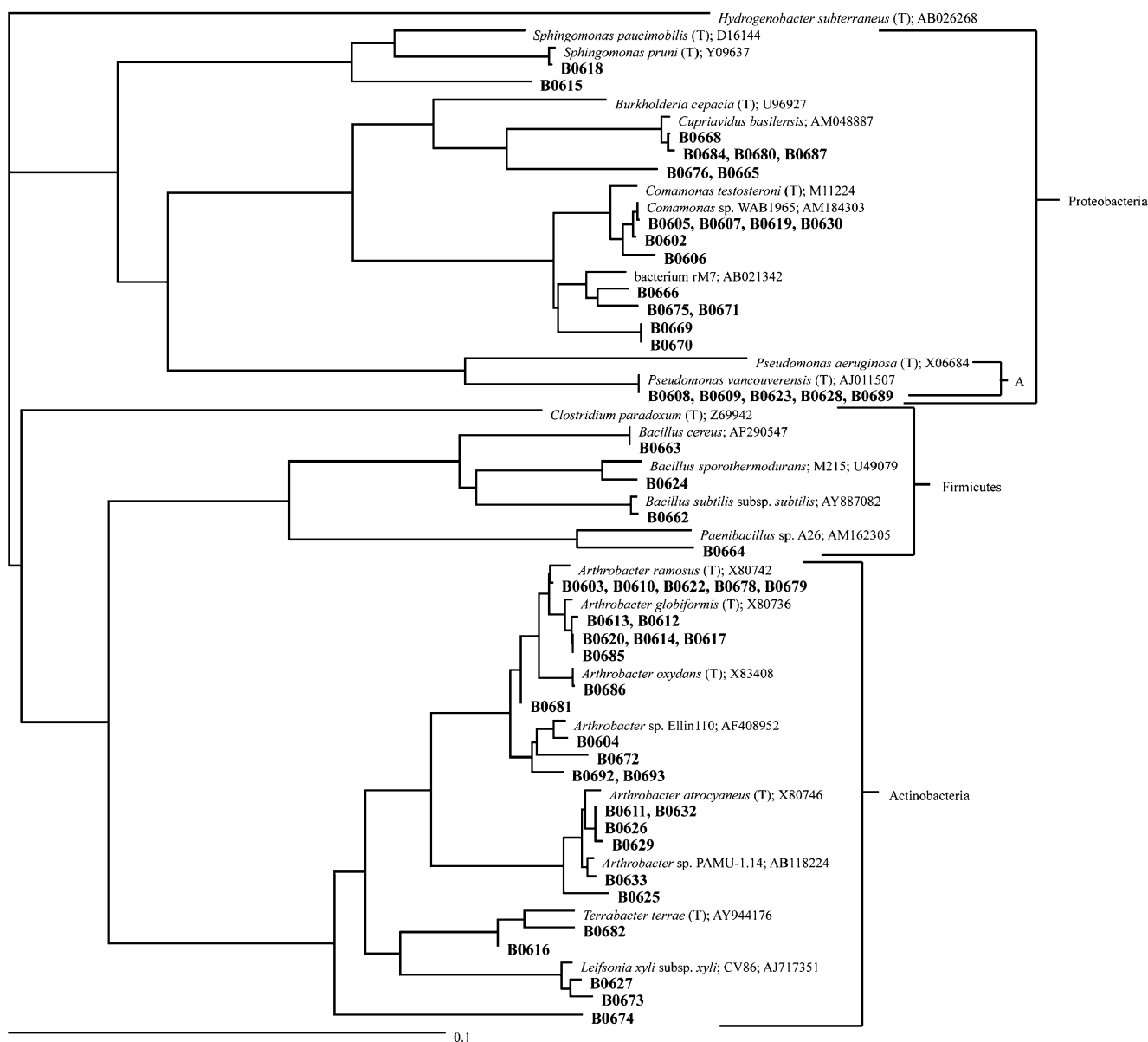
## Results

### Phylogenetic Analysis of Savannah River Site Strains

16S rRNA genes were PCR-amplified from extracted chromosomal DNA and sequenced for phylogenetic characterization of 56 strains from the SRS (Fig. 1). The strains were most closely related to at least nine genera in three phyla of bacteria when compared to sequences from previously described bacterial species. Twenty-four (43%) of the SRS strains grouped with species of *Proteobacteria* and fell into three classes within this phylum. These strains were most closely related to *Sphingomonas* (*Alphaproteobacteria*), *Cupriavidus* or *Comamonas* (*Betaproteobacteria*), or *Pseudomonas* (*Gammaproteobacteria*). Only four (7%) of the strains grouped with low-G+C Gram-positive bacteria in the *Firmicutes* and were most closely related to *Bacillus* or *Paenibacillus*. The largest group of isolates, with 28 strains (50%), clustered with high-G+C Gram-positive genera in the *Actinobacteria* and were most closely related to *Arthrobacter*, *Terrabacter*, or *Leifsonia*.

### Phylogenetic Analysis of Hanford Site Strains

16S rRNA genes were PCR-amplified from extracted chromosomal DNA and sequenced for phylogenetic anal-



**Figure 1** Distance matrix phylogenetic tree showing relatedness of SRS strains to established taxa of bacteria and selected comparison strains and environmental clones. Scale bar represents ten substitutions per 100 bases. Subsurface strains listed on a single branch of the

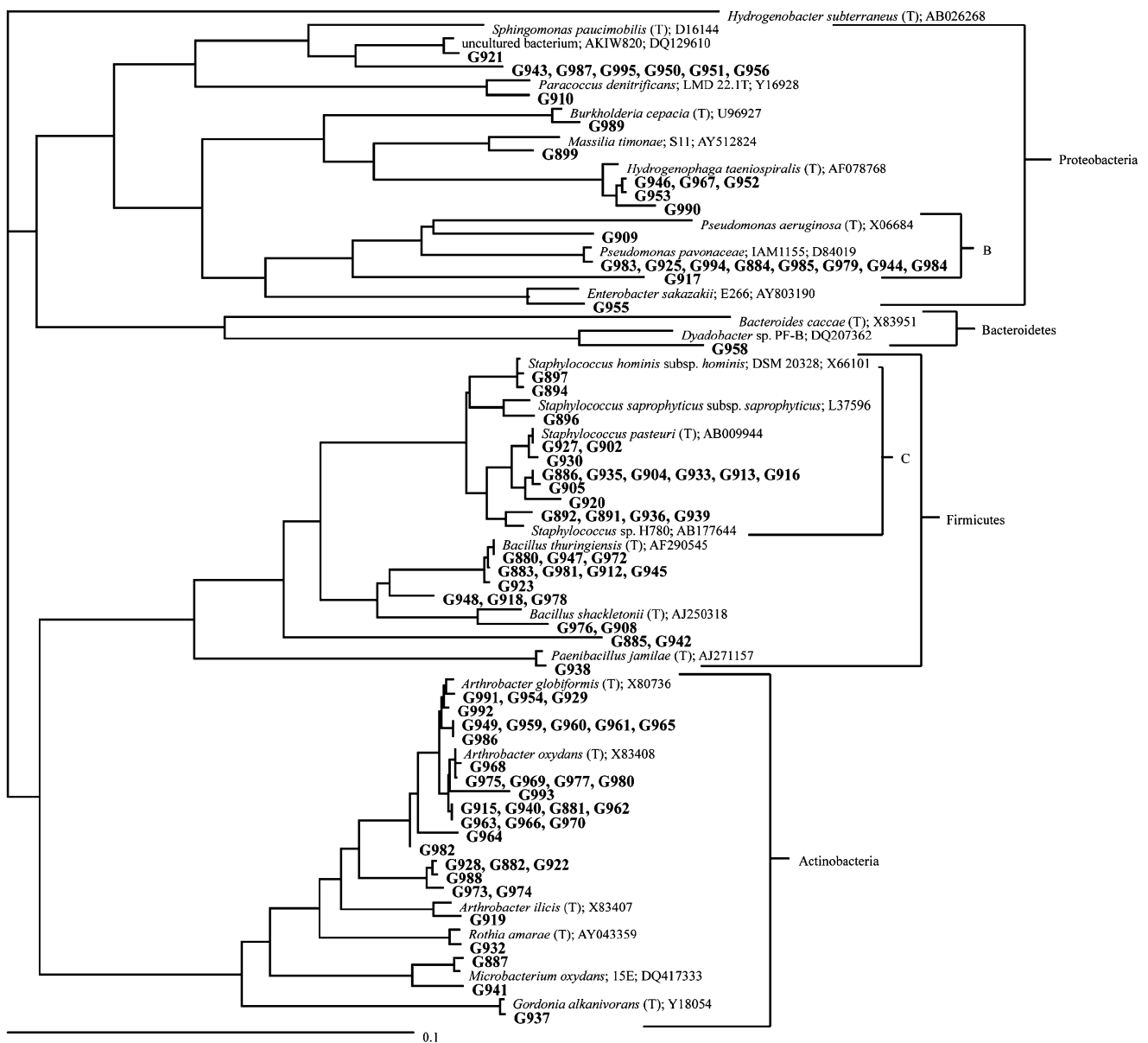
tree had identical 16S rRNA gene sequences. *Hydrogenobacter subterraneus* was used as the outgroup (see “Materials and Methods” for detailed analytical parameters)

ysis of a total of 97 HS strains selected from the SMCC. This analysis (Fig. 2) indicated that, like the SRS strains, the HS strains (with just one exception) fell into genera of the *Proteobacteria*, *Firmicutes*, and *Actinobacteria*. Twenty-six (27%) of the HS strains grouped with the *Proteobacteria* and were most closely related to *Sphingomonas* or *Paracoccus* (*Alphaproteobacteria*), *Hydrogenophaga* or *Burkholderia* (*Betaproteobacteria*), or *Pseudomonas* or *Enterobacter* (*Gammaproteobacteria*). Thirty-four HS strains (34%) grouped with low-G+C Gram-positive bacteria in the *Firmicutes* and were most closely related to *Staphylococcus*, *Bacillus*, or *Paenibacillus*. The largest group of isolates, with

36 strains (36%), clustered with high-G+C Gram-positive genera in the *Actinobacteria* and were most closely related to *Arthrobacter*, *Rothia*, *Microbacterium*, or *Gordonia*. A single HS strain (G958) grouped with the *Bacteroidetes* and was most closely related to *Dyadobacter*.

#### Antibiotic Resistance Screening of SRS Strains

All of the subsurface strains were screened, through plating on selective media, for resistance to 13 antibiotics. Fifty-four of the 56 SRS strains were resistant to at least one antibiotic, and 86% were resistant to more than one antibiotic (Supplemental



**Figure 2** Distance matrix phylogenetic tree showing relatedness of HS strains to established taxa of bacteria and selected comparison strains and environmental clones. Scale bar represents ten substitutions per 100 bases. Subsurface strains listed on a single branch of the

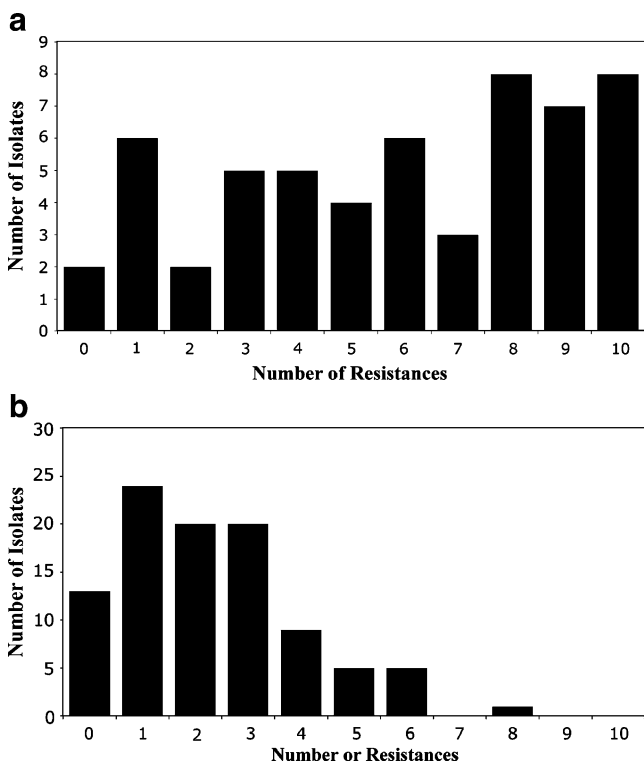
tree had identical 16S rRNA gene sequences. *Hydrogenobacter subterraneus* was used as the outgroup (see “Materials and Methods” for detailed analytical parameters)

Table 1; Fig. 3a). Over half (64%) of the strains were resistant to five or more antibiotics, and 14% were resistant to ten antibiotics. Of the 36 strains resistant to more than five antibiotics, 23 (64%) were resistant to either eight, nine, or ten drugs (Fig. 3a). The highest frequency of resistance was to ampicillin (79% of the strains; Table 1). The antibiotic with the highest efficacy was erythromycin, with only 5% of strains showing resistance.

The *Actinobacteria* (high-G+C Gram-positive) group contained the highest frequency (93%) of strains with five or more resistances. The *Actinobacteria* group contains all but one of the eight strains with ten resistances.

#### Antibiotic Resistance Screening of HS Strains

Overall, the frequency of resistance in the HS strains was lower than in the SRS strains. Eighty-six percent of the HS strains were resistant to at least one antibiotic, but only 62% of the strains were resistant to more than one antibiotic (Supplemental Table 2). One strain was resistant to eight antibiotics (the maximum number of resistances detected in any one strain). The majority of the strains (66%) were resistant to either 1, 2, or 3 antibiotics (Fig. 3b), and the highest frequencies of resistance among these strains were to nalidixic acid (65%) or mupirocin (57%; Table 1). None



**Figure 3** Frequency and levels of multiple antibiotic resistance (MAR) among SRS strains (a) and HS strains (b) examined in this study

of the HS strains were resistant to gentamicin, and only four strains were resistant to kanamycin or rifampin.

The *Proteobacteria* group contained the highest frequency of HS strains (22%) with five or more resistances. The *Actinobacteria* (high-G+C Gram-positive) group contains the lowest frequency of sensitive strains, with only one strain sensitive to all the antibiotics.

**Analysis of Distinct Resistance Profiles**

The subsurface strains from both sample sites were found to possess a broad range of distinct resistance profiles. Forty-seven distinct profiles were detected among the 54 resistant SRS strains, with 43 strains having unique profiles that were not detected in any other strains. Five SRS strains

were resistant to ampicillin, but no other distinct profiles were shared by more than two strains. Forty distinct profiles were detected among the 84 HS strains that were resistant to at least one antibiotic, and 29 of these strains had unique profiles that were not detected in any other strains. Thirteen HS strains were resistant to nalidixic acid and mupirocin, 12 were resistant to nalidixic acid only, seven were resistant to mupirocin only, and six were resistant to vancomycin, nalidixic acid, and mupirocin. No other distinct profile was shared by more than four HS strains. If the two groups of strains are considered as a single group, the 138 strains that were resistant to at least one antibiotic possessed 83 distinct resistance profiles, only 16 of which were found in more than one strain (Supplemental Tables 1 and 2).

Distinct resistance profiles were frequently detected among strains that were closely related to one another. Among the SRS strains, for example, five phylogenetically identical strains closely related to *Arthrobacter ramosus* (B0603, etc.; Fig. 1) all had different resistance profiles (Supplemental Table 1). Similarly, seven phylogenetically identical resistant HS strains related to *Pseudomonas pavonaceae* (G983, etc.; Fig. 2) had six distinct resistance profiles (Supplemental Table 2).

**Discussion**

The purpose of the present study was to examine the frequency and diversity of antibiotic resistance among bacteria in an environment that, to our knowledge, has not been studied systematically in this regard—the deep terrestrial subsurface. We have confirmed that a high frequency of antibiotic resistance is present among culturable heterotrophic bacteria from two subsurface environments that differ considerably in chemical (e.g., pH and  $E_h$ ) and physical (e.g., hydraulic conductivity and particle size distribution) characteristics, as well as microbial population density (for detailed information, see Supplemental Table 3). One of these environments (the HS) apparently has not been exposed to anthropogenic antibiotics or to microbes that

**Table 1** Total resistances to screened antibiotics for SRS and HS strains

	Antibiotic												
	VAN	ERY	TET	CHL	GEN	KAN	STR	NEO	CIP	RIF	AMP	NAL	MUP
<b>SRS strains</b>													
Total strains resistant to antibiotic	12	3	26	22	11	19	22	26	39	24	44	42	39
% of strains resistant to antibiotic	21	5	46	39	20	34	39	46	70	43	79	75	70
<b>HS strains</b>													
Total strains resistant to antibiotic	13	8	9	12	0	4	7	16	22	4	10	63	55
% of strains resistant to antibiotic	13	8	9	12	0	4	7	16	23	4	10	65	57

have been influenced by them. Ninety-six percent of the strains from SRS sediments and 86% of those from the HS sediments were resistant to at least one antibiotic. This finding is generally consistent with those of other studies in which varying frequencies of antibiotic resistance were detected among bacteria in natural environments including soils [17, 50, 60], seawater and marine sediments [19, 47, 55], and freshwater environments [5, 39, 44]. Therefore, our results lend support to a growing body of evidence that antibiotic resistance is extremely widespread in natural environments, including relatively pristine habitats that have not been exposed to antibiotic-resistant human pathogens or commercial sources of antibiotics.

Our results also show that a high frequency of multiple antibiotic resistance (MAR; resistance to two or more antibiotics) is present in subsurface bacteria. All but eight of the strains from the SRS were resistant to two or more of the 13 antibiotics tested, with 64% of them resistant to five or more and 41% resistant to eight or more antibiotics. MAR was also detected, at a somewhat lower frequency, among the HS strains (62% resistant to two or more and 21% resistant to four or more drugs). Varying frequencies of MAR have been found in other environments, including river and stream water [5, 39], rural groundwater [44], soils [17], seawater [19, 47], marine sediments [47], and beach sands [46], so MAR may be common among environmental bacterial populations.

Analysis of resistance profiles indicated that subsurface bacteria vary widely in terms of the specific antibiotics to which they are resistant. The 138 resistant strains screened in this study possessed 83 distinct resistance profiles, only 16 of which were found in more than one strain. The few other studies that have included this type of analysis have also detected a wide range of specific resistance patterns. D'Costa et al. [17] found nearly 200 distinct resistant profiles among 480 soil *Streptomyces* strains, while Martins da Costa et al. [43] reported 69 distinct profiles among 537 *Enterococcus* isolates from poultry slaughterhouse wastewater and sludge. Both of these studies detected considerable diversity within a single genus. We did not screen large numbers of strains from any single genus, but our results imply that a similar pattern occurs among subsurface bacteria. For example, the 32 *Arthrobacter*-related HS strains had 13 distinct resistance profiles, and the 23 SRS *Arthrobacter*-related strains had 22 distinct profiles.

Resistance and MAR were detected in a diverse range of chemoheterotrophic bacteria in both environments examined in this study. Phylogenetic analyses assigned the SRS strains into three phyla and at least nine genera of bacteria and the HS strains into four phyla and at least 15 genera. Only limited comparisons can be made with other environments in terms of the diversity of resistant bacteria because most recent studies have been limited to specific types or groups of bacteria [17, 20, 22, 33, 39, 47]. However,

McKeon et al. [44] examined a comparatively broad range of Gram-negative strains from groundwater and found resistance among eight genera and 14 species, while De Souza et al. [19] found resistance among three phyla and at least nine genera of psychrotrophic Antarctic seawater bacteria. In general, it appears that antibiotic resistance typically occurs in a taxonomically wide range of environmental bacteria. Moreover, it should be noted that the actual diversity of resistant types in the environment might be higher, given that most studies have been limited to the culturable organisms that represent only a small fraction of the total microflora thought to reside in natural environments.

The SRS strains were found to have a somewhat higher frequency of resistance and MAR than the HS strains in this study. The higher frequency of resistance at the SRS may well be due to recent surface influence. Whereas the sediments at the HS are thought to have been entirely isolated from the surface for over 3 million years [15], the groundwater flowing through the sediments at the SRS may be <4,000 years old [59] and originates in recharge zones subject to possible exposure to antibiotics and antibiotic-resistant bacteria or resistance genes from agricultural sources. In addition, many monitoring wells have been drilled into the SRS sediments with drilling methods involving drilling muds that contain large numbers of surface-soil bacteria. Although considerable precautions were taken to prevent contamination of the sediment samples from the SRS with materials or microbes from the surface [51, 54], the possibility of recent exposure of the environments to surface influence prior to sampling cannot be fully eliminated. Therefore, the frequencies of resistance and MAR at the SRS could, in part, have been influenced by the agricultural use of antibiotics in animal feeds, a practice that has now been banned in the European Union because it is believed to increase resistance among bacteria in surface soils and other environments (e.g., see [9, 37, 49, 50]). Given that the SRS sediments are situated in a regime with flowing groundwater that contains a diverse microflora, it is also possible that the frequency of resistance at this site has been influenced by communication between the established subsurface bacterial community and planktonic species moving through the system.

Interestingly, some of the environmental strains examined in this study are closely related to known human pathogens. Several of the strains from both sample sites (Group A, Fig. 1 and Group B, Fig. 2) group with *Pseudomonas* and are resistant to multiple antibiotics. A group of 18 strains from the HS that includes six strains with MAR (Group C, Fig. 2) appears to comprise several species of *Staphylococcus*, a genus known to contain human pathogens and opportunistic pathogens. Another HS strain, G989, is closely related to *Burkholderia cepacia*, an opportunistic pathogen. Opportunistic pathogens such as *B. cepacia* are often intrinsically

resistant to antibiotics [70]. There are other opportunistic human pathogens that are becoming more of a clinical problem because they can infect extremely ill or immunocompromised patients [52], and while these bacteria normally would not pose a threat, they are now becoming increasingly dangerous in hospital settings [38].

As stated previously, both sites have strains with MAR to different classes of antibiotics. While there are only a few different general types of resistance mechanisms (e.g., efflux pumps, target site modification, and inactivation of the antibiotic), bacteria often possess distinct genes for resistance to different classes of antibiotics. However, for the bacteria in this study to be resistant to different classes of antibiotics, they could possess an efflux gene capable of decreasing the concentration of any antibiotic [48]. The possibility of a multi-drug efflux pump seems likely, given that some of the HS strains are resistant to human-altered/synthesized antibiotics that they could not have been exposed to if they were isolated from the surface for such an extended period of time. The SRS strains that are resistant to eight to ten antibiotics would theoretically require numerous antibiotic resistance genes. Yet, maintaining a single or limited number of resistance mechanisms covering all antibiotics would be more efficient, given the low levels of nutrients in the deep subsurface [15, 26]. *P. aeruginosa*, an opportunistic pathogen isolated from soil and fresh water, possesses efflux pumps that provide maximum flexibility for this organism in regard to antibiotic resistance [70].

An alternative to a general efflux pump for MAR would be the acquisition of antibiotic resistance genes in a gene cassette. It is known that the acquisition of antibiotic resistance can be due to horizontal gene transfer (HGT), which is aided by selective pressure due to increased levels of the antibiotic [18] and is a major factor contributing to the adaptation and evolution of bacteria [42, 62]. HGT is known to occur in soils and aquatic environments [49], and there is some evidence that it occurs in the terrestrial subsurface [14, 41, 42, 62], but little is known about the actual mechanisms of transfer in the subsurface. In regard to the specific environments examined in this study, mechanisms requiring direct contact between cells, such as conjugation, might be more likely in the SRS sediments, where the numbers of bacteria are high ( $10^5$ – $10^7$  culturable bacterial cells per gram;  $10^7$ – $10^8$  total cells per gram) [61]. In contrast, transformation might be a more significant transfer mechanism in the HS sediments, where the bacterial numbers are so low ( $<10^2$  culturable cells per gram; often  $<10^4$  total cells per gram; see Supplemental Table 3) that spatial separation between cells might rule out conjugation. Transformation has been shown to occur often among environmental isolates [40], and the HS strains fall into genera known to have naturally transformable species [40]. Bacteria may also be intrinsically resistant to anti-

biotics, most often due to structure, and do not require the acquisition of a resistance mechanism. This has been taken into consideration for this study and is noted in Supplemental Tables 1 and 2.

The acquisition and retention of resistance genes by the strains examined in this study could imply that the bacteria were being exposed to an antibiotic in the subsurface. The selective pressure of an antibiotic will allow bacteria that have acquired a novel mutation or characteristic for resistance to proliferate [8] and continue to transfer the beneficial characteristic. Antibiotic-producing bacteria could well be present in the subsurface, given the limited resources available in these environments. Bacteria will produce an antibiotic to attack neighboring bacteria to preserve their environmental resources [1, 66]. Another reason for retaining the resistance genes might be that they perform other functions related to survival, such as removal of heavy metals or other toxins from the cell [18, 35, 64] or serving a metabolic function [2]. Given the location of the sediments the strains were isolated from (i.e., DOE production sites), the possibility of maintaining resistance genes due to heavy metals or toxins seems more likely [62].

The long period of isolation from the surface indicates two possibilities for such a high frequency of antibiotic resistance among the HS strains: Antibiotic resistance mechanisms were shared traits when these bacteria were isolated from the surface 3 million years ago and/or they have evolved different mechanisms during this period. Several studies have shown that  $\beta$ -lactamase antibiotic resistance genes predate the antibiotic era [23, 31, 35, 56, 63] and were only detected once the clinical use of the antibiotic started. Song et al. [63] demonstrated the presence of antibiotic resistance genes in sediments isolated for about 10,000 years. It also seems likely that, over such a long time period, these bacteria have developed new resistance genes, mechanisms, or gene mutations to provide resistance. There may be a reservoir of uncharacterized antibiotic resistance mechanisms in the environment [1, 3, 16, 53, 57], especially in the deep terrestrial subsurface (a possibility that is now under investigation in our laboratory), and information on these mechanisms could potentially aid in antibiotic development and prevention of future resistance problems [17, 53, 68]. Studying the origin and evolution of antibiotic resistance genes also provides the potential ability to predict the future evolution of antibiotic resistance genes [31].

**Acknowledgments** Part of this research was funded by a Leaders Educated to Make a Difference (LEAD) Grant to MGB from the Student Council on Research and Creativity (SCRC) at Florida State University. Other portions of this research were supported by the US Department of Energy Office of Science, Office of Biological and Environmental Research. We thank Jim Wilgenbusch in the Florida State University School of Computational Science for assistance with the phylogenetic analysis and modeling.



## References

- Alonso A, Sanchez P, Martinez JL (2001) Environmental selection of antibiotic resistance genes. *Environ Microbiol* 3:1–9
- Aminov RI, Garrigues-Jeanjean N, Mackie RI (2001) Molecular ecology of tetracycline resistance: development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. *Appl Environ Microbiol* 67:22–32
- Aminov RI, Mackie RI (2007) Evolution and ecology of antibiotic resistance genes. *FEMS Microbiol Lett* 271:147–161
- Amy PS, Haldeman DL (1997) The microbiology of the terrestrial deep subsurface. Lewis, New York, NY
- Ash RJ, Mauck B, Morgan M (2002) Antibiotic resistance of Gram-negative bacteria in rivers, United States. *Emerg Infect Dis* 8:713–716
- Balkwill DL, Fredrickson JK, Thomas JM (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
- Balkwill DL, Reeves RH, Drake GR, Reeves JY, Crocker FH, King MB, Boone DR (1997) Phylogenetic characterization of bacteria in the Subsurface Microbial Culture Collection. *FEMS Microbiol Rev* 20:201–216
- Baquero F, Negri MC, Morosini MI, Blazquez J (1998) Antibiotic-selective environments. *Clin Infect Dis* 27(Suppl 1): S5–11
- Boxall AB, Kolpin DW, Halling-Sorensen B, Tolls J (2003) Are veterinary medicines causing environmental risks? *Environ Sci Technol* 37:286–294
- Chapelle F (1993) Ground-water microbiology and geochemistry. Wiley, New York, NY
- Chopra I, Roberts M (2001) Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol Mol Biol Rev* 65:232–260
- CLSI (2005) Performance standards for antimicrobial susceptibility testing. Fifteenth informational supplement, CLSI document M100-S15 ed. Clinical and Laboratory Standards Institute, Wayne, PA
- Cole JR, Chai B, Farris RJ, Wang Q, Kulam-Syed-Mohideen AS, McGarrell DM, Bandela AM, Cardenas E, Garrity GM, Tiedje JM (2007) The ribosomal database project (RDP-II): introducing myRDP space and quality controlled public data. *Nucleic Acids Res* 35(Database issue):D169–D172
- Coombs JM, Barkay T (2004) Molecular evidence for the evolution of metal homeostasis genes by lateral gene transfer in bacteria from the deep terrestrial subsurface. *Appl Environ Microbiol* 70:1698–1707
- Crocker FH, Fredrickson JK, White DC, Ringelberg DB, Balkwill DL (2000) Phylogenetic and physiological diversity of *Arthrobacter* strains isolated from unconsolidated subsurface sediments. *Microbiology* 146:1295–1310
- D'Costa VM, Griffiths E, Wright GD (2007) Expanding the soil antibiotic resistome: exploring environmental diversity. *Curr Opin Microbiol* 10:481–489
- D'Costa VM, McGrann KM, Hughes DW, Wright GD (2006) Sampling the antibiotic resistome. *Science* 311:374–377
- Davison J (1999) Genetic exchange between bacteria in the environment. *Plasmid* 42:73–91
- De Souza MJ, Nair S, Loka Bharathi PA, Chandramohan D (2006) Metal and antibiotic-resistance in psychrotrophic bacteria from antarctic marine waters. *Ecotoxicology* 15:379–384
- Dhakephalkar PK, Chopade BA (1994) High levels of multiple metal resistance and its correlation to antibiotic resistance in environmental isolates of *Acinetobacter*. *Biometals* 7:67–74
- Drake GR, DeFlaun MF, Streger S, Levy SB, Bueker CL, Balkwill DL (1999) Widespread antibiotic resistance among bacteria isolated from subsurface environments, Abstr. 4th International Symposium on Subsurface Microbiology, Vail, CO, USA
- Ferreira da Silva M, Vaz-Moreira I, Gonzalez-Pajuelo M, Nunes OC, Manaia CM (2007) Antimicrobial resistance patterns in Enterobacteriaceae isolated from an urban wastewater treatment plant. *FEMS Microbiol Ecol* 60:166–176
- Fevre C, Jbel M, Passet V, Weill FX, Grimont PA, Brisse S (2005) Six groups of the OXY beta-Lactamase evolved over millions of years in *Klebsiella oxytoca*. *Antimicrob Agents Chemother* 49:3453–3462
- Fredrickson JK, Balkwill DL (2006) Geomicrobial processes and biodiversity in the deep terrestrial subsurface. *Geomicrobiol J* 23:345–356
- Fredrickson JK, Fletcher M (2001) Subsurface microbiology. Wiley, New York, NY
- Fredrickson JK, Garland TR, Hicks JM, Thomas JM, Li SW, McFadden KM (1989) Lithotrophic and heterotrophic bacteria in deep subsurface sediments and their relation to sediment properties. *Geomicrobiol J* 7:53–65
- Fredrickson JK, Hicks RJ, Li SW, Brockman FJ (1988) Plasmid incidence in bacteria from deep subsurface sediments. *Appl Environ Microbiol* 54:2916–2923
- Ghiorse WC (1989) Special issue on deep subsurface microbiology. *Geomicrobiol J* 7:1–136
- Ghiorse WC, Wilson JT (1988) Microbial ecology of the terrestrial subsurface. *Adv Appl Microbiol* 33:107–172
- GlaxoSmithKline (2004) Bactroban Cream: Prescribing Information. Research Triangle Park, NC.
- Hall BG, Barlow M (2004) Evolution of the serine beta-lactamases: past, present and future. *Drug Resist Updat* 7:111–123
- Hall JA, Mailloux BJ, Onstott TC, Scheibe TD, Fuller ME, Dong H, DeFlaun MF (2005) Physical versus chemical effects on bacterial and bromide transport as determined from on site sediment column pulse experiments. *J Contam Hydrol* 76:295–314
- Jacobs L, Chenia HY (2007) Characterization of integrons and tetracycline resistance determinants in *Aeromonas* spp. isolated from South African aquaculture systems. *Int J Food Microbiol* 114:295–306
- Kieft TL, Fredrickson JK, McKinley JP, Bjornstad BN, Rawson SA, Phelps TJ, Brockman FJ, Piffner SM (1995) Microbiological comparisons within and across contiguous lacustrine, paleosol, and fluvial subsurface sediments. *Appl Environ Microbiol* 61:749–757
- Kobayashi T, Nonaka L, Maruyama F, Suzuki S (2007) Molecular evidence for the ancient origin of the ribosomal protection protein that mediates tetracycline resistance in bacteria. *J Mol Evol* 65:228–235
- Lane D (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) *Nucleic acid techniques in bacterial systematics*. Wiley, New York, NY, pp 131–175
- Levy SB (2002) The 2000 Garrod lecture. Factors impacting on the problem of antibiotic resistance. *J Antimicrob Chemother* 49:25–30
- Levy SB, Marshall B (2004) Antibacterial resistance worldwide: causes, challenges and responses. *Nat Med* 10:S122–S129
- Lima-Bittencourt CI, Cursino L, Goncalves-Dornelas H, Pontes DS, Nardi RM, Callisto M, Chartone-Souza E, Nascimento AM (2007) Multiple antimicrobial resistance in Enterobacteriaceae isolates from pristine freshwater. *Genet Mol Res* 6:510–521
- Lorenz MG, Wackernagel W (1994) Bacterial gene transfer by natural genetic transformation in the environment. *Microbiol Rev* 58:563–602
- Maiden MC (1998) Horizontal genetic exchange, evolution, and spread of antibiotic resistance in bacteria. *Clin Infect Dis* 27(Suppl 1):S12–S20
- Martinez RJ, Wang Y, Raimondo MA, Coombs JM, Barkay T, Sobecky PA (2006) Horizontal gene transfer of PIB-type ATPases

- among bacteria isolated from radionuclide- and metal-contaminated subsurface soils. *Appl Environ Microbiol* 72:3111–3118
43. Martins da Costa PM, Vaz-Pires PM, Bernardo FM (2006) Antibiotic resistance of *Enterococcus* spp. isolated from wastewater and sludge of poultry slaughterhouses. *J Environ Sci Heal B* 41:1393–1403
  44. McKeon DM, Calabrese JP, Bissonnette GK (1995) Antibiotic resistant Gram-negative bacteria in rural groundwater supplies. *Water Res* 29:1902–1908
  45. McKinley IG, Hagenlocher I, Alexander WR, Schwyn B (1997) Microbiology in nuclear waste disposal: interfaces and reaction fronts. *FEMS Microbiol Rev* 20:545–556
  46. Mudryk ZJ (2005) Occurrence and distribution antibiotic resistance of heterotrophic bacteria isolated from a marine beach. *Mar Pollut Bull* 50:80–86
  47. Neela FA, Nonaka L, Suzuki S (2007) The diversity of multi-drug resistance profiles in tetracycline-resistant *Vibrio* species isolated from coastal sediments and seawater. *J Microbiol* 45:64–68
  48. Nikaido H (1998) Antibiotic resistance caused by gram-negative multidrug efflux pumps. *Clin Infect Dis* 27(Suppl 1):S32–S41
  49. Nwosu VC (2001) Antibiotic resistance with particular reference to soil microorganisms. *Res Microbiol* 152:421–430
  50. Patterson AJ, Colangeli R, Spigaglia P, Scott KP (2007) Distribution of specific tetracycline and erythromycin resistance genes in environmental samples assessed by macroarray detection. *Environ Microbiol* 9:703–715
  51. Phelps TJ, Fliermans CB, Garland TR, Piffner SM, White DC (1989) Methods for recovery of deep terrestrial subsurface sediments for microbiological studies. *J Microbiol Meth* 9:267–279
  52. Quinn JP (1998) Clinical problems posed by multiresistant nonfermenting Gram-negative pathogens. *Clin Infect Dis* 27 (Suppl 1):S117–S124
  53. Riesenfeld CS, Schloss PD, Handelsman J (2004) Metagenomics: genomic analysis of microbial communities. *Annu Rev Genet* 38:525–552
  54. Russell BF, Phelps TJ, Griffin WT, Sargent KA (1992) Procedures for sampling deep subsurface microbial communities in unconsolidated sediments. *Ground Water Monit R* 12:96–104
  55. Sabry SA, Ghozlan HA, Abou-Zeid DM (1997) Metal tolerance and antibiotic resistance patterns of a bacterial population isolated from sea water. *J Appl Microbiol* 82:245–252
  56. Saier MH Jr, Paulsen IT, Sliwinski MK, Pao SS, Skurray RA, Nikaido H (1998) Evolutionary origins of multidrug and drug-specific efflux pumps in bacteria. *Faseb J* 12:265–274
  57. Salyers A, Shoemaker NB (2006) Reservoirs of antibiotic resistance genes. *Anim Biotechnol* 17:137–146
  58. Salyers AA, Gupta A, Wang Y (2004) Human intestinal bacteria as reservoirs for antibiotic resistance genes. *Trends Microbiol* 12:412–416
  59. Sargent KA, Fliermans CB (1989) Geology and hydrology of the deep subsurface microbiology sampling sites at the Savannah River Plant, South Carolina. *Geomicrobiol J* 7:3–13
  60. Schmitt H, Stooß K, Hamscher G, Smit E, Seinen W (2006) Tetracyclines and tetracycline resistance in agricultural soils: microcosm and field studies. *Microb Ecol* 51:267–276
  61. Sinclair JL, Ghiorse WC (1989) Distribution of aerobic bacteria, protozoa, algae, and fungi in deep subsurface sediments. *Geomicrobiol J* 7:15–31
  62. Smets BF, Morrow JB, Arango Pinedo C (2003) Plasmid introduction in metal-stressed, subsurface-derived microcosms: plasmid fate and community response. *Appl Environ Microbiol* 69:4087–4097
  63. Song JS, Jeon JH, Lee JH, Jeong SH, Jeong BC, Kim SJ, Lee JH, Lee SH (2005) Molecular characterization of TEM-type beta-lactamases identified in cold-seep sediments of Edison Seamount (south of Lihir Island, Papua New Guinea). *J Microbiol* 43: 172–178
  64. Summers AO (2006) Genetic linkage and horizontal gene transfer, the roots of the antibiotic multi-resistance problem. *Anim Biotechnol* 17:125–135
  65. Swofford D (1989–2002) PAUP, Phylogenetic Analysis Using Parsimony, 4.0 Beta edn. Sinauer, Sunderland, MA
  66. Séveno NA, Kallifidas D, Smalla K, Dirk van Elsas J, Collard JM, Karagouni AD, Wellington EMH (2002) Occurrence and reservoirs of antibiotic resistance genes in the environment. *Rev Med Microbiol* 13:15–27
  67. van Waasbergen LG, Balkwill DL, Crocker FH, Bjornstad BN, Miller RV (2000) Genetic diversity among *Arthrobacter* species collected across a heterogeneous series of terrestrial deep-subsurface sediments as determined on the basis of 16S rRNA and recA gene sequences. *Appl Environ Microbiol* 66:3454–3463
  68. Waters B, Davies J (1997) Amino acid variation in the GyrA subunit of bacteria potentially associated with natural resistance to fluoroquinolone antibiotics. *Antimicrob Agents Chemother* 41:2766–2769
  69. Wise R (2004) The 2003 Garrod Lecture: The relentless rise of resistance? *J Antimicrob Chemother* 54:306–310
  70. Wright GD (2007) The antibiotic resistome: the nexus of chemical and genetic diversity. *Nat Rev Microbiol* 5:175–186