Microbial Ecology

Mineralogy Influences Structure and Diversity of Bacterial Communities Associated with Geological Substrata in a Pristine Aquifer

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Abstract

Our understanding of mineralogical influences on subsurface microbial community structure and diversity has been difficult to assess due to difficulties in isolating this variable from others in the subsurface environment. In this study, biofilm coupons were used to isolate specific geological substrata from the surrounding geological matrix during colonization by microorganisms suspended in the surrounding groundwater for an 8-week period. Upon retrieval, the structure and diversity of the microbial community associated with each type of substratum was evaluated using 16S rDNA-based terminal-restriction fragment length polymorphism (T-RFLP). Phylogenetic affiliations of the populations associated with each type of substratum were established based on sequence analysis of near full-length 16S rDNA obtained through construction of a clone library. Hematite, quartz, and saprolite each harbored a community dominated by members of the division *Proteobacteria* (>67% of community). However, the different substrata selected for different subdivisions of bacteria within the *Proteobacteria*. After accounting for the influence exerted by substratum type on recovery of DNA from the attached populations, both phylogenetic data and Jaccard and Bray–Curtis similarity indices derived from terminal-restriction fragment (T-RF) profiles suggested a strong mineralogical influence on the structure and composition of the solid phase-associated community. The results suggest that mineralogical heterogeneity influences microbial community structure and diversity in pristine aquifers.

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Introduction

It has recently been estimated that microbial biomass in subsurface environments greatly exceeds the combined biomass of all other microbial reservoirs [32, 45]. According to the literature, most of the microbial biomass in the subsurface is associated with surfaces of minerals and other solid-phase constituents that make up the geological substrata [1, 4, 20]. Our current knowledge of the structure and diversity of the microbial community associated with the solid phase is based largely on analysis of bulk core material, which is often geologically heterogeneous [36]. It is unclear whether geological heterogeneity at this scale exerts an influence on the structure and diversity of the solid phase-associated microbial community because it has been difficult to isolate substratum effects from other variables present in the system.

Using phospholipid fatty acid signatures as an indicator of microbial diversity, Peacock *et al.* [33] demonstrated that the microbial community associated with Bio-Sep beads (a composite of aramid polymer and powdered activated carbon) was more diverse than that associated with glass wool incubated in a nuclear fuel-contaminated zone of a subsurface aquifer. While the artificial substrata used in the aforementioned study permitted characterization of the composition and activities of microbial communities in the subsurface, the communities selected by these substrata may not resemble or behave like those that develop on the geological substrata comprising the aquifer.

Recently, Reardon *et al.* [38] exposed different geological substrata to the groundwater microbial com-

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munity in the saturated zone of a pristine aquifer in a manner that suggested a mineralogical influence on the structure and diversity of the solid-phase-associated microbial community. Analysis of terminal-restriction fragments (T-RFs) of PCR-amplified community 16S rRNA genes using Jaccard similarity coefficients and Euclidean distance revealed differences in the communities that developed on the different geological substrata after an 8-week exposure period. However, Reardon *et al.* [38] used a different DNA extraction method to recover DNA from the communities associated with hematite than that used to recover DNA from communities associated with the other geological substrata under investigation, making comparisons difficult.

The purpose of this study was to determine the influence of different types of geological substrata on the structure and diversity of substrata-associated subsurface microbial communities using a common DNA extraction technique. A secondary goal of the study was to determine the extent to which the structure and diversity of a substratum-associated community varied with different DNA extraction techniques. Analysis of T-RF profiles of communities associated with hematite, quartz, and saprolite substrata, the same types of geological substrata used in the aforementioned study, yielded robust, new evidence of a geological substratum influence on the structure and diversity of microbial communities at the particle scale. Furthermore, the use of different methods to extract DNA from a substrata-associated community resulted in a different community T-RF profile.

Methods

Site Description. Well FW303 at the Field Research Center (FRC), Oak Ridge Reservation, Oak Ridge, TN was used as the subsurface environment for evaluating the influence of geological substrata on microbial community structure and diversity. Well FW303 (latitude 35.94113923; longitude 84.33627935) penetrates a pristine aquifer in a weathered shale composed of unconsolidated clay-rich saprolite supported by a bedrock of interbedded shales, siltstones, and limestones residing 5–10 m below the land surface. Overlying the saprolite is a thin veneer of organic- and clay-rich soil ranging from 0.5 to 3 m in thickness. The water table varies from <1 to 3 m below the land surface. The predominant minerals in the shales include illite, quartz, kaolinite, chlorite, calcite, and plagioclase feldspar. Calcite, goethite, and kaolinite occur as coatings on fractures (Schreiber, ME, 1995, University of Wisconsin PhD thesis) while low-Mg calcite, dolomite, and ferroan dolomite are contained within the carbonates [19]. Groundwater flux in the saturated zone occurs primarily within an interval defined by the interface between the competent bedrock and overlying highly weathered saprolite (porosity of 30–50%) [22–24, 46]. It is believed that bacteria reside only in the high-permeability fractures due to size exclusion from the matrix (http://public.ornl.gov/nabirfrc/sitenarrative.cfm). Aquifer geochemistry has been described elsewhere (Reardon et al. [38]; URL: http://public.ornl.gov/nabirfrc/sitenarrative.cfm#Anchor1).

Biofilm Coupon Preparation and Incubation. Biofilm coupons, composed of capped stainless steel mesh cylinders (25.4 × 1.27 cm; 1-mm mesh size) (Fig. 1), were prepared with approximately 10 g of the following minerals: quartz, specular hematite, and saprolite. Quartz sand (98–99% SiO_2; 1.0–1.4 mm particle size) was obtained from Ricci Bros. Sand Co. Inc., Port Norris, NJ, USA. Specular hematite particles, obtained from Minas Gerais, Brazil (kindly provided by K. Rosso, Pacific Northwest National Laboratory, Richland, WA, USA) and saprolite from the FRC subsurface (a gift from P. Jardine, Oak Ridge National Laboratory) were crushed and sieved. Only particles in the 1–3 mm diameter size range were used in an attempt to minimize differences in particle surface area, porosity, and permeability. All substrata were separated within the coupon by a plug of glass wool and mineral-loaded coupons were sterilized by combustion (550°C, 6 h). Coupons were lowered by fishing line into the saturated zone of the well and incubated for an 8-week period to promote colonization of substrata by those microorganisms with a propensity for attachment. The large coupon mesh size allowed access of the particle surfaces to bacteria suspended in the surrounding groundwater. After incubation, coupons were retrieved, frozen, and shipped on dry ice to the Idaho National Laboratory (Idaho Falls, ID), and stored at −80°C for molecular analyses.

Community DNA Extraction and Amplification of 16S rRNA Genes From Colonized Mineral Substrata. Total community DNA extraction was performed using the Bio101 FastDNA SPIN Kit for Soil (Q-Biogene, Irvine, CA, USA). To account for heterogeneity in community diversity and structure as well as variability in DNA extraction efficiency, each type of geological substratum from the biofilm coupon was sampled in triplicate (0.5 g) and extracted along with positive and negative controls according to the manufacturer's instructions with the following exceptions: silica matrix-bound DNA was allowed to settle for 30 min and the SPIN Filter was allowed to air-dry in a sterile PCR hood for 30 min after the salt–ethanol wash. Equal parts of each triplicate extraction

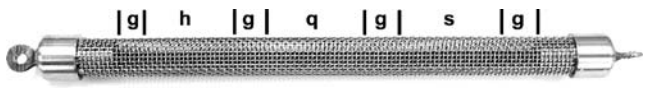

Figure 1. Biofilm coupon (25.4 × 1.27 cm) used to retain geologic substrata during incubation in monitoring wells. Glass wool spacer (g), hematite (h), quartz (q), saprolite (s).

from each substratum type were combined and quantified using the High DNA Mass Ladder (Invitrogen, Carlsbad, CA, USA). Approximately 1.5–2.0 ng of DNA was used as template under the following cycling conditions: initial denaturation at 94°C (4 min) followed by 30 cycles of denaturation at 94°C (1 min), annealing at 55°C (1 min), primer extension at 72°C (1.5 min), and a final extension step at 72°C (20 min). The final reaction mixture (50 μL) contained 2 mM $MgCl_2$ (Invitrogen), 200 μM of each deoxynucleotide triphosphate (Eppendorf, Hamburg, Germany), 0.5 μM of each forward and reverse primer, 0.4 μg μL^{-1} molecular-grade bovine serum albumin (Roche, Indianapolis, IN, USA), and 0.25 U Taq DNA Polymerase (Invitrogen) in 1× PCR buffer (Invitrogen). Conserved regions of the 16S rRNA gene were targeted with bacterial forward primer 8F-FAM (5-AGAGTTTGAT CCTGGCTCAG-3′) modified with phosphoramidite fluorochrome 5-carboxyfluorescein (FAM) at the 5′ end (Invitrogen) and the universal reverse primer 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Integrated DNA Technologies, Coralville, IA, USA). After electrophoresis of amplicons in a matrix of 1.2% agarose, it was decided that a seminested PCR reaction was necessary to generate a higher yield of DNA product for T-RFLP analysis. PCR products were quantified using the Low DNA Mass Ladder (Invitrogen) and 10–20 ng of DNA was used as template for the seminested amplification using the bacterial forward primer 8F-FAM and bacterial reverse primer 907R (5′-CCGTCAATTCMTTTRAGTTT-3′ where M = A or C and R = A or G). The following conditions were used for seminested PCR: initial denaturation 94°C (4 min) followed by 25 cycles of denaturation at 94°C (1 min), annealing at 55°C (1 min), and primer extension at 72°C (1.5 min) with a final extension step at 72°C (20 min). Each primary reaction was subjected to seminested PCR amplification once, including three individual negative controls, yielding triplicate seminested PCR reactions of each. Successful PCR was verified after the seminested reaction on a 1.2% agarose gel prior to preparation for T-RFLP.

Intact Biofilm Polymerase Chain Reaction From Hematite and Quartz Surfaces. Intact biofilm-polymerase chain reaction (IB-PCR) was used to identify microbial populations through direct amplification (i.e., no extraction step) of DNA from the communities associated with hematite and quartz substrata. IB-PCR was performed in triplicate following previously published protocols [38]. Briefly, colonized particles of hematite or quartz were added directly to PCR tubes containing nuclease-free water and 1× PCR buffer (Invitrogen) yielding a 25 μL total "premix" aqueous volume. The tubes and their contents were heated at 99°C for 15 min to lyse cells. The temperature was lowered to 80°C, before subjecting the DNA to the same PCR conditions as used to amplify DNA extracted by bead-beating to maintain the same PCR bias for DNA extracted by the different methods. After electrophoresis of amplicons in a matrix of 1.2% agarose, it was determined that a seminested PCR was necessary to generate sufficient DNA product for T-RFLP analysis. Seminested IB-PCR was performed using the same conditions as those used for seminested PCR of DNA extracted by the bead-beating method.

T-RFLP Analysis of PCR-amplified 16S rRNA Genes. T-RFLP was used to compare the subsurface microbial communities colonizing the different geological substrata in the biofilm coupon. Forty microliters of each triplicate seminested PCR reaction were pooled, yielding a total volume of 120 μL. Pooled PCR amplicons were purified using the Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA) and DNA concentration was determined using the Low DNA Mass Ladder (Invitrogen) in a matrix of 1.2% agarose.

Approximately 200 ng of PCR-amplified DNA from each type of substratum was subjected to digestion at 37°C for 12 h in an Eppendorf Mastercycler Gradient thermocycler using the 4-base-recognition restriction enzyme MspI (C^CGG) (New England BioLabs, Ipswich, MA). The digestion mixture (50 μL total volume) contained 10 U of MspI (20 U/μL), 5 μL 10X NEB2 Buffer (New England BioLabs), 200 ng DNA, and nuclease-free water (Sigma, St. Louis, MO, USA). Digestion was performed in triplicate for each substratum type. Restriction digests were purified with 3 μL of sodium acetate (3 M, pH 5.2) and 66 μL ethanol (70%), air-dried, and resuspended in 10 μL nuclease-free water (Sigma). Nucleic acids were denatured by heating to 95°C for 3 min followed by submersion in an ice bath. The denatured DNA (2 μL), along with the internal Rox 1000 standard (Applied Biosystems, Foster City, CA, USA), was loaded onto a model 377 DNA sequencer (Applied Biosystems) employing a Cambrex Long Ranger XL 5% polyacrylamide denaturing gel. Terminal restriction fragments (T-RF) were separated by electrophoresis (51°C, 3 kV) for 4.5 h.

T-RFs generated by gel electrophoresis were analyzed using Genescan version 2.1 (Applied Biosystems). T-RFs that migrated to within 0.5 bp of each other were, for the purposes of this study, considered to have originated from the same phylotype. Only T-RFs present in all three of the triplicate digests were retained for community characterization. T-RFs greater than 900 bp were discarded in order to reduce the number of errors associated with fragment drift due to increasing T-RF length [6, 26]. Total fluorescence (TF) contributed by the sum of all T-RFs in each digest was standardized to a value of 10,000 units [6]. Composite community profiles were generated from the triplicate digests by averaging peak migration distances and the percent TF. The same

screening criteria used for substrata-derived community structure analysis was applied to electropherograms produced by nested negative T-RFLP reactions. T-RFs present in the composite profiles of both the seminested negative T-RFLP reaction and seminested substrata-associated reactions were discarded without further consideration and all remaining T-RFs were again standardized to 10,000 units.

Construction of Bacterial 16S rDNA Clone Library. To identify individual phylotypes that contributed T-RFs, a clone library was constructed from IB-PCR-amplified 16S rDNA from hematite mineral surfaces. Primary PCR, nested PCR, amplicon purification, and DNA concentration determination were carried out as described above for the IB-PCR amplification of hematite-associated microbial communities. However, for the clone library, the forward primers used in primary and nested PCR were not conjugated with FAM. Amplicons and pGEM-T Easy Vector (Promega) were ligated at room temperature for 1 h followed by ligation overnight at 4°C according to the manufacturer's directions. Competent *Escherichia coli* JM109 cells were transformed with the vector construct as recommended by the manufacturer. Transformed cells were plated on S-GAL agar (Sigma) using 100 μg mL^{-1} ampicillin sodium salt (Fluka, Seelze, Germany) as the selection agent. Plates were incubated at 37°C for 18 h and a total of 100 clones were selected for sequencing and T-RF analysis.

Transformants were grown in 3 mL LB (100 μg mL^{-1} ampicillin sodium salt) at 37°C for 18 h. Cells were harvested by centrifugation and the plasmids were purified (QIAprep Spin Miniprep Kit, Qiagen, Valencia, CA, USA). Primers used in sequencing were 8F, 515F (5′-GTGCCAGCMGCCGCGGTAA-3′, where M = A + C), 519R (5′-ATTACCGCGGCTGCTGG-3′), and 907R. Sequencing reactions were performed using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and an ABI 3700 automated DNA sequencer (Applied Biosystems). Sequences were assembled using the BioEdit sequence alignment editor freeware (version 7.0.1, http://www.mbio.ncsu.edu/BioEdit/bioedit.html) [17]. Sequences were examined for chimeric artifacts using the CHIMERA_CHECK function of the Ribosomal Database project II (RDP) (http://rdp8.cme.msu.edu/cgis/chimera.cgi?su=SSU) and were aligned using ClustalW (http://clustalw.genome.jp/). Aligned sequences were grouped using a 97% similarity threshold [41] using the Sequence Grouper program (Andrew Shewmaker, Idaho National Laboratory, Idaho Falls, ID, USA). A representative of each group was subjected to nucleotide–nucleotide Basic Local Alignment Search Tool (BLASTn) analysis [2] against the "nr" database provided by the National Center for Biotechnology Information (NCBI).

Nucleotide Sequence Accession Numbers. All clone sequences have been deposited in the GenBank, DDBJ, and EMBL databases under the accession numbers DQ003152–DQ003205 as well as DQ004244 and DQ004245 (Supplemental Table 1).

Clone T-RFLP. A clone from each group was subjected to T-RF analysis to assign a phylotypic identity to T-RFs generated from the microbial community associated with each type of geological substrata. Purified plasmids from a representative clone from each group were subjected to PCR amplification as described above using the primers 8F-FAM and 907R using 20 ng of purified plasmid as template. Reaction conditions were: initial denaturation at 94°C (4 min) followed by 30 cycles of denaturation at 94°C (1 min), annealing at 55°C (1 min), primer extension at 72°C (1.5 min), and a final extension step at 72°C (20 min). Successful PCR was determined on a 1.2% agarose gel. Reaction products were purified, quantified, and subjected to digestion as described above except that 25 ng of DNA was digested. Clone T-RF lengths were determined as described above and corresponding peaks from the clone T-RF and community T-RF electropherograms were considered to be the same if they migrated to within 0.5 bp of each other.

Table 1. Relative abundance[a] of T-RFs revealed by bead beating DNA extraction from various substrata

T-RF[b]	*Hematite*	*Quartz*	*Saprolite*	*Glass wool*
1			7.7	
3		9.6	5.0	5.0
4			38.4	
5	10.8	13.5	8.4	9.1
6	5.9	5.5		6.4
7			25.5	
10	3.9			
12	3.9	5.9		
13		5.2		
14	5.1			
16		6.8		7.6
17				3.7
20	15.6	46.4		24.4
21	27.6			30.4
22	4.7			
23	4.1	7.0		3.2
24			4.2	
25			7.0	
26				4.9
27	3.8			
28			3.8	
30	11.0			
32				5.2
33	3.7			
Total	100.0	100.0	100.0	100.0

[a]Percent of total fluorescence (TF) of phylotypes representing greater than 3.0% TF.
[b]Terminal-restriction fragment (T-RF) reference numbers here refer to the same fragments in Table 3 and Supplemental Table.

Statistical Analysis of T-RFLP Electropherograms. For the purposes of this study, distinct T-RFs were considered to be unique operational taxonomic units (OTU) and were the unit by which individual phylotypes were demarcated. Richness (R) was calculated as the total number of phylotypes. Shannon–Weaver diversity indices (H) were calculated using the formula $H = -\sum P_i(\ln P_i)$ where P_i represents the proportional abundance of individuals or phylotypes (i) and an increase in H indicates an increase in diversity. Shannon equitability (J) was calculated according to the formula $J = -H(\ln S)$ resulting in values of 0–1.0, with 1.0 indicating maximal diversity [5]. Simpson Index (dominance) values were calculated using the formula $D = \sum P_i^2$ which results in values from zero (infinite diversity) to 1.0 (no diversity), and an increase in the inverse $\left(1/\sum P_i^2\right)$ indicates an increase in diversity [5]. Cluster analysis and calculations of Jaccard and Bray–Curtis (Odum) similarity coefficients were completed using PAST freeware software [18].

Results

The influence of mineralogy on the structure and diversity of microbial communities that associate with different mineral phases in subsurface environments was evaluated using T-RFLP of PCR-amplified community 16S rDNA extracted from different geological substrata after 8-week incubation in a subsurface aquifer. Before conducting this evaluation, it was first necessary to (1) establish an appropriate minimum fluorescence for T-RFs representing populations comprising a community and (2) determine whether the nature of the substratum influenced the efficiency of extraction of DNA from the different substratum-associated populations.

Criteria for Including a Population in a Substratum-Associated Community. A range of baseline values by which a T-RF is considered real or background noise are reported in the literature including 0.5% TF [38] and 1.0% [10, 27]. In the present study, the similarity of the microbial communities associated with different substrata was found to be influenced by the threshold fluorescence value used to select T-RF peaks representing the populations that comprise each community. The highest unweighted Jaccard similarity coefficient obtained for communities associated with hematite and quartz using DNA extracted by either IB-PCR or bead-beating methods was achieved when a minimum fluorescence value of 3% of total profile fluorescence (TF) was used to filter out background fluorescence and populations in low abundance. Of the 18 phylotypes recovered from hematite and quartz substrata using the IB-PCR nucleic acid extraction, seven were shared, resulting in a Jaccard similarity coefficient of 0.389 (Table 1). Of the 15 phylotypes recovered from hematite and quartz substrata using bead-beating nucleic acid extraction, five were shared (Table 1), resulting in a Jaccard similarity coefficient of 0.333 (Table 2). When threshold fluorescence values were set above or below 3.0%, a smaller fraction of shared phylotypes were recovered from the hematite and quartz substrata, yielding lower Jaccard similarity coefficients (data not shown). The minimum fluorescence value that maximizes the similarity of the communities associated with different substrata should facilitate detection of only the most significant substratum influences on community structure. Consequently, all subsequent community comparisons were conducted using communities defined by a 3% TF threshold value.

Table 2. T-RFLP-based Jaccard and Bray–Curtis similarity coefficients for bacterial communities associated with different geological substrata subjected to IB-PCR or bead-beating nucleic acid extraction

		IB-PCR		*Bead-Beating Extraction*			
		Hematite	*Quartz*	*Hematite*	*Quartz*	*Saprolite*	*Glass Wool*
Jaccard similarity coefficients							
IB-PCR	Hematite	1.000	0.389	0.294	0.385	0.059	0.429
	Quartz		1.000	0.174	0.211	0.095	0.190
Bead-beating extraction	Hematite			1.000	0.333	0.053	0.294
	Quartz				1.000	0.143	0.500
	Saprolite					1.000	0.125
	Glass wool						1.000
Bray–Curtis similarity coefficients							
IB-PCR	Hematite	1.000	0.460	0.391	0.576	0.084	0.512
	Quartz		1.000	0.212	0.250	0.124	0.214
Bead-beating extraction	Hematite			1.000	0.399	0.084	0.614
	Quartz				1.000	0.134	0.541
	Saprolite					1.000	0.134
	Glass wool						1.000

Bias of Different Methods of DNA Recovery from Substratum-associated Microbial Populations. The influence of the substratum on recovery of DNA from the substratum-associated populations was examined by first identifying two DNA extraction methods that exhibit unique biases for a specific substratum-associated microbial community and then evaluating whether the uniqueness of the bias varies with the type of substratum. Bias uniqueness was determined by comparing T-RF profiles of the same substratum-associated community whose DNA was extracted by IB-PCR and bead-beating methods. Profile similarity was evaluated using both unweighted Jaccard and weighted Bray–Curtis similarity coefficients. The Jaccard similarity algorithm accounts only for the presence/absence of a phylotype, whereas the Bray–Curtis similarity algorithm accounts not only for presence/absence but also relative abundance of a phylotype. If the two extraction methods exhibited no bias or the same bias in extracting DNA from members of the same substratum-associated community, both the Jaccard and Bray–Curtis similarity coefficients should be 1.00 for a given substratum. A value less than 1.00 describes the uniqueness of the bias.

Bead-beating and IB-PCR nucleic acid extraction methods exhibited unique biases for recovery of DNA from the hematite- and quartz-associated microbial communities. IB-PCR and bead-beating together yielded 17 unique phylotypes from the hematite-associated community, only five of which were recovered by both extraction methods (Table 3), yielding an unweighted Jaccard similarity coefficient of 0.294 and a weighted Bray–Curtis similarity coefficient of 0.391 (Table 2). For the quartz-associated community, the two extraction methods together yielded 19 unique phylotypes, only four of which were shared (Table 3), yielding a Jaccard similarity coefficient of 0.211 and a Bray–Curtis similarity coefficient of 0.250 (Table 2). Thus, the IB-PCR and bead-beating methods of DNA extraction methods met the criteria for their subsequent use in assessing the influence of substratum properties on the efficiency of recovery of DNA from the different phylotypes present.

Similarity of Communities Associated With Different Geologic Substrata Using a Common DNA Extraction Method. In this study, the influence of substratum type on the efficiency of extracting DNA from microbial communities associated with different substrata was investigated by comparing the similarity of the T-RF profiles of communities associated with hematite and quartz whose DNA was extracted by IB-PCR to the similarity of the profiles of communities associated with the same two substrata whose DNA was extracted by bead-beating. T-RF profiles of hematite- and quartz-associated communities whose DNA was extracted using IB-PCR yielded a Jaccard similarity coefficient of 0.389 (Table 2). A similar Jaccard coefficient (0.333) was obtained from T-RF profiles of the same two communities when their DNA was extracted by bead beating (Table 2). The similarity of the profiles from the two communities based on the Bray–Curtis algorithm was 0.460 and 0.399 when their DNA was extracted by IB-PCR and bead beating, respectively (Table 2). The difference in the Bray–Curtis similarity values (0.460–0.399 = 0.061) was similar to the difference in the Jaccard similarity values (0.389–0.333 = 0.056).

Because not all populations gave rise to a T-RF of unique size (Supplemental Table 1), the similarity of T-RF profiles generated from DNA extracted by the two methods could be a result of different populations within the community contributing the same T-RF. However, it is unlikely that the different extraction methods recovered different populations from the same substratum that not only produced a T-RF of the same size, but that were also present in approximately the same relative abundance as indicated by the Bray–Curtis similarity

Table 3. Relative abundance[a] of T-RFs revealed by IB-PCR or bead-beating DNA extraction from hematite- and quartz-associated communities

	Extraction method yielding fragment			
	Relative abundance of fragment on hematite		*Relative abundance of fragment on quartz*	
T-RF[b]	IB-PCR[c]	BB[c]	IB-PCR[c]	BB[c]
2			10.7	
3			6.5	9.6
5	11.2	10.8	7.4	13.6
6		5.9		5.5
8			4.1	
9	8.3		5.0	
10		3.9		
11			5.0	
12	11.1	3.9	7.6	5.9
13				5.2
14		5.1		
15			7.1	
16	5.6			6.8
17	7.6		6.2	
18	4.9		8.7	
19			5.6	
20	28.3	15.6	5.2	46.4
21		27.6		
22	9.8	4.7	12.4	
23	6.7	4.1		7.0
27		3.8		
29			4.1	
30		11.0		
31			4.6	
32	6.7			
33		3.7		
Total	100.0	100.0	100.0	100.0

[a]Percent of total fluorescence (TF) of phylotypes representing greater than 3.0% TF

[b]Terminal-restriction fragment (T-RF) reference number corresponding to clone library (see supplemental information)

[c]Intact biofilm-PCR (IB-PCR) or bead beating (BB)

coefficient. Because it was demonstrated above that each extraction method has unique biases for the same populations, the most reasonable interpretation of these results is that hematite and quartz exert a similar, yet minimal influence on the efficiency in which the two extraction methods recover DNA from the populations associated with these substrata.

Substratum Influence on Community Diversity and Structure. Assuming that other geological substrata exert no more of an influence on community DNA extraction efficiency than hematite and quartz, then any substrata exerting a greater influence on their community's structure and diversity than that imposed by these two minerals should produce Jaccard or Bray–Curtis similarity coefficients of less than 0.333 or 0.399, respectively, when the bead-beating method is used to extract DNA from the communities. The communities associated with all substrata evaluated, with the exception of quartz and glass wool, when subjected to the bead-beating method of DNA extraction, yielded Jaccard similarity coefficients lower than those of the communities associated with hematite and quartz (Table 2). The high similarity of the communities associated with quartz and glass wool is not unexpected because both are silica-based substrata, whereas, hematite is an iron oxide mineral. The communities associated with hematite and saprolite shared a single phylotype (Table 1) and therefore exhibited the lowest Jaccard similarity coefficient (0.056) (Table 2).

When phylotype abundance was factored into the determination of community similarity using the Bray–Curtis algorithm, trends similar to those produced by the unweighted Jaccard algorithm were preserved (Table 2). The exception was a higher similarity coefficient for the communities associated with hematite and glass wool (0.614) than for the communities associated with quartz and glass wool (0.541). This reflected the fact that the communities associated with hematite and glass wool shared two dominant phylotypes (>15% TF), whereas, the quartz- and glass wool-associated communities shared only one dominant phylotype (Table 1). This resulted in a different branch order in dendograms constructed with Jaccard and Bray–Curtis similarity coefficients (Fig. 2). Except for the communities associated with saprolite and quartz, Bray–Curtis similarity coefficients for communities associated with different substrata were higher than Jaccard similarity coefficients for the corresponding communities (Table 2). In spite of the differences in community similarity when phylotype abundance was taken into account, the results strongly suggest that the structure and diversity of solid phase-associated microbial communities are influenced by the nature of the solid-phase substratum.

The influence of geologic substrata on the structure and diversity of the microbial communities whose DNA

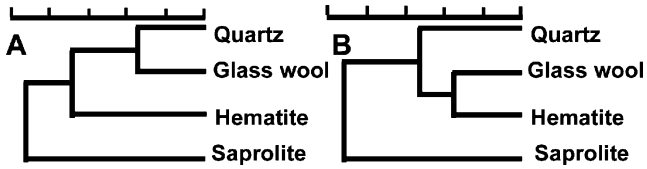

Figure 2. Cluster analysis based on Jaccard (A) or Bray–Curtis (B) similarity analysis of bacterial communities associated with different substrata revealed by bead-beating DNA extraction. Tick marks represent similarity units of 0.2 over a range of 0–1.0.

was extracted by bead-beating was further investigated using various mathematical algorithms that describe richness, dominance, equitability, and diversity. Community richness (S), a measure of the total number of individual phylotypes, was found to depend on the substratum on which the community developed (Tables 1 and 4). The hematite-associated community contained 50% more phylotypes than the community associated with saprolite, indicating a substratum influence on community structure. The ability to deduce substratum effects can be obscured by the richness index because this index does not take into account phylotype abundance or community evenness. Shannon equitability (J), a measure of the relative proportion of individuals among a given set of phylotypes (i.e., phylotype abundance) is a standardized numerical representation of community evenness. An equitability value of close to 0 indicates low species evenness or a high degree of dominance by a single phylotype. Equitability values approaching 1 indicate equal abundance of all phylotypes or maximal evenness. The equitability value was higher for the phylotypes associated with hematite and saprolite than for the phylotypes associated with quartz and glass wool, indicating that the phylotypes comprising the hematite- and saprolite-associated communities were more evenly distributed than the phylotypes associated with quartz and glass wool communities (Table 4). Dominance (Simpson index) describes the probability that any two members of a community chosen at random will belong to the same phylotype. Higher dominance values indicate an uneven distribution among individual phylotypes. Dominance indices ranged from 0.257 for the community associated with quartz to 0.135 for the community associated with hematite (Table 4). No one index offers a comprehensive measure of community structure and diversity. The Shannon–Weaver index of diversity (H), however, sums phylotypes weighted by their abundance and therefore takes into account both community richness and equitability. Shannon–Weaver indices varied from 2.194 for the hematite-associated community to 1.695 for the quartz-associated community indicating that the former was more diverse than the latter (Table 4). The high Shannon–Weaver index associated with the hematite community is

Table 4. T-RFLP-based diversity statistics[a] of bacterial communities associated with different geological substrata subjected to bead-beating DNA extraction

	Diversity statistics			
Geologic substratum	*S*	*J*	*D*	*H*
Hematite	12	0.883	0.135	2.194
Glass Wool	10	0.825	0.236	1.991
Quartz	8	0.815	0.257	1.695
Saprolite	8	0.865	0.184	1.715

[a]Calculated using peaks >3.0% total community fluorescence
S Richness, *J* Shannon equitability, *D* dominance, *H* Shannon–Weaver index

consistent with the high phylotype richness and the high equitability (evenness) of the community. Correspondingly, the quartz-associated community had the lowest Shannon–Weaver index, phylotype richness, and equitability. Similar trends between Shannon–Weaver indices, richness, and equitability were also observed when the glass wool- and saprolite-associated communities were analyzed (Table 4). In summary, the differences in richness, evenness, and Shannon–Weaver index values obtained for the communities associated with the different substrata support the results of the Jaccard and Bray–Curtis similarity analyses indicating that both community structure and diversity are strongly influenced by the nature of the geological substratum with which the community is associated.

Phylogenetic Affiliations of Substratum-associated Community Members. A 16S rDNA clone library was constructed from the hematite-associated microbial community whose DNA was extracted using IB-PCR to establish the identity of phylotypes contributing T-RFs to the community profiles. DNA extracted from the hematite-associated community by IB-PCR was chosen for library construction because T-RFLP analysis indicated that it provided the greatest Shannon–Weaver index of diversity, thereby offering the greatest opportunity to relate the maximum number of phylotypes associated with this and other geological substrata to phylotypes in the ribosomal RNA database. Phylogenetic analysis of 16S rDNA clones indicates that very few of the clones exhibited close affiliation with any cultured *Bacteria* (Supplemental Table 1). Furthermore, phylogenetic and T-RF analysis of rDNA clones indicate that distinct populations that belonged to the same class did not always yield unique T-RFs. For these reasons, only class-level taxonomic data are reported here. Sixty seven to 96% of the TF of T-RF profiles generated by bead-beating extraction of the different substrata were phylogenetically identified by T-RF analysis of 16S rDNA clones (Fig. 3). A smaller fraction of the TF of community T-RF profiles generated by IB-PCR extraction of hematite and quartz were identified by individual clone T-RF analysis than for communities generated by bead-beating extraction (Fig. 3). This difference was attributed to four unidentified phylotypes (T-RFs) associated with hematite and six unidentified phylotypes associated with quartz that each contributed greater than 4%TF (data not shown).

Members of the *Proteobacteria* dominated the communities associated with all geologic substrata evaluated in this study regardless of whether T-RF profiles were based on DNA extracted by bead-beating or IB-PCR. The different geologic substrata each selected for a microbial community dominated by a different subdivision of *Proteobacteria* when bead beating was used to extract DNA from the substrata-associated communities. Hematite-associated communities were dominated by phylotypes aligning with the γ-*Proteobacteria* (40% TF) and the α-*Proteobacteria* (26% TF) (Fig. 3). β-*Proteobacteria* (56% TF) and α-*Proteobacteria* (19% TF) dominated the community associated with the quartz substratum (Fig. 3). The glass wool-associated community was dominated by γ- and β-*Proteobacteria* (45 and 30% TF, respectively) (Fig. 3). The phylogenetic structure of the saprolite-associated community was markedly different from that of the communities associated with the other geologic substrata with 51% of the phylotypes falling within α-*Proteobacteria* (Fig. 3). These results indicate that not only do different geological substrata select for different phylotypes but that the majority of phylotypes associated with each substratum align within different classes of the *Proteobacteria*.

It is interesting to note that the bias introduced by DNA extraction method on the composition of the communities associated with the different substrata was evident at the subdivision-level. The hematite-associated

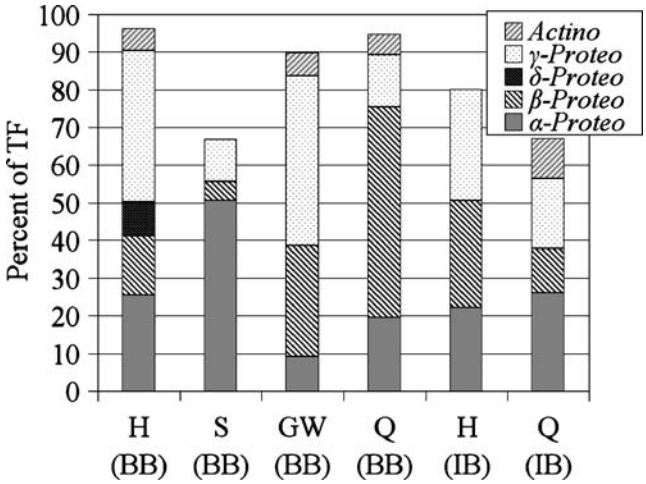

Figure 3. 16S rRNA-based phylogenetic composition of bacterial communities associated with hematite (H), saprolite (S), quartz (Q), and glass wool, (GW) subjected to bead beating (BB) or IB-PCR (IB) nucleic acid extraction. Abbreviations: TF, total fluorescence; *Actino*, *Actinobacteria*; *Proteo*, *Proteobacteria*.

bacterial community whose DNA was extracted using IB-PCR contained a larger fraction of β-*Proteobacteria* than the same community whose DNA was extracted by bead beating (Fig. 3). Like the hematite-associated community, the quartz-associated bacterial community whose DNA was extracted using IB-PCR contained a greater fraction of non-*Proteobacteria* and γ-*Proteobacteria* than the same community whose DNA was extracted by bead beating (Fig. 3). Unlike the hematite-associated community, the quartz-associated bacterial community whose DNA was extracted using IB-PCR contained a smaller fraction of β-*Proteobacteria* than the same community whose DNA was extracted by bead beating (Fig. 3). The results support similarity coefficient-based data indicating that different DNA extraction methods have different biases for recovering DNA from *Proteobacteria* and non-*Proteobacteria* as well as from the different subdivisions of *Proteobacteria* associated with a substratum-associated bacterial community. These results also support the similarity coefficient-based data suggesting that geological substrata influence bacterial community structure.

Discussion

Subsurface sediments often contain a heterogeneous mixture of minerals suitable for colonization by groundwater microbial populations [36]. The present study employed T-RF analysis of PCR-amplified 16S rRNA genes extracted from communities that developed on different minerals during subsurface incubation to demonstrate a substratum influence on community structure and diversity. The unweighted Jaccard coefficient provided a quantitative assessment of the similarity of the microbial communities associated with different minerals based on the presence or absence of distinguishable populations. As expected, communities associated with substrata with similar properties such as quartz and glass wool yielded a higher similarity coefficient than communities associated with substrata with different properties such as hematite and quartz. Using an experimental approach and methodology similar to those used in the present study, Reardon *et al.* [38] obtained Jaccard coefficients for the communities associated with quartz and glass wool (0.464–0.600) and with quartz and hematite (0.345) similar to those reported in the present study (0.550 and 0.333, respectively). A similar coefficient was anticipated from the two studies for the communities associated with quartz and glass wool because both studies used the same bead-beating DNA extraction protocol after incubation in the same well at the same depth for equivalent periods of time, but in different years.

The similarity of the coefficients reported in this study and by Reardon *et al.* [38] for the communities associated with hematite and quartz was not expected because Reardon *et al.* [38] used IB-PCR and bead-beating to obtain DNA from the hematite- and quartz-associated communities, respectively, while the present study used bead-beating to extract DNA from both communities. Different extraction techniques are known to have their own unique biases for recovering DNA from different populations [13, 28, 29]. The results of the present study suggest that any bias experienced by Reardon *et al.* [38] from the use of different DNA extraction methods to recover DNA from hematite- and quartz-associated communities was masked by the substratum influence on the Jaccard-based measure of community similarity. The similar Jaccard coefficients (0.389 and 0.33) obtained in the present study for communities associated with these two substrata when DNA was extracted using either IB-PCR or bead-beating protocols, respectively support this conclusion. While seasonal variations in community composition may have occurred during the 1-year period that elapsed between the two studies, the results suggest that conditions in the well were at least sufficiently similar during the respective incubations to preserve structure and diversity of the microbial communities associated with each mineral phase.

Jaccard similarity coefficients for communities associated with saprolite and the other substrata evaluated in this study were consistently lower than those reported by Reardon *et al.* [38]. For example, the similarity coefficient of 0.143 obtained in the present study for the communities associated with saprolite and quartz was lower than the 0.419 value obtained by Reardon *et al.* [38]. The difference in this case is difficult to explain because the same bead-beating protocol was used in both studies to extract DNA from the communities associated with these two substrata.

The low similarity of the saprolite-associated community and other substratum-associated communities in the present study compared to those reported by Reardon *et al.* [38] may be due, in part, to the different stringencies employed by the two studies for including a T-RF in a community profile. Whereas Reardon *et al.* [38] included a T-RF if it contributed greater than 0.5% TF and appeared in two of three replicate T-RFLP digestions, the present study included a T-RF only if it contributed greater than 3.0% TF and occurred in all three replicate digestions. The higher stringency employed in the present study tends to decrease the probability that a particular T-RF is shared by two communities, which likely yields a lower Jaccard similarity coefficient than when lower stringencies are used. Whereas 17 phylotypes qualified for inclusion in the hematite-associated community using the criteria applied in the present study, 28 phylotypes qualified when the criteria of Reardon *et al.* [38] were used (data not shown). Of the 39 total phylotypes associated with the saprolite and hematite communities

compiled using the criteria of Reardon *et al.* [38], seven were shared, resulting in a Jaccard similarity of 0.179. This value is only modestly larger than the value obtained using the 3.0%TF threshold used to establish a community profile in the present study (0.143), and is much lower than the value of 0.370 reported by Reardon *et al.* [38]. In spite of the Jaccard-based differences in similarity between the saprolite-associated community and the communities associated with other substrata used in the two studies, Jaccard coefficient-based cluster analysis yielded similar dendrogram topologies for the microbial communities associated with hematite, quartz, saprolite, and glass wool spacer material (Fig. 2 this study; Reardon *et al.* [38]).

Factors such as relative abundance of phylotypes are not accounted for by the Jaccard similarity algorithm. A similarity index such as Bray–Curtis, which factors in relative abundance along with presence/absence of a phylotype should produce a lower coefficient value than that produced by the Jaccard index if there is a significant difference in the relative abundance of the phylotypes shared between two communities. Bray–Curtis similarity coefficients were higher than the corresponding Jaccard coefficient for communities associated with different substrata, the exception being those that described the similarity between the saprolite-associated community and the communities associated with other substrata. The higher similarity coefficient between substrata-associated communities when abundance is taken into account can be attributed to the sharing of dominant phylotypes between communities. For example, the quartz and glass wool communities, which exhibited a significantly higher coefficient when abundance was accounted for in the similarity algorithm, shared 5 T-RFs that each contributed >5% TF (Table 1). Together, these five phylotypes accounted for 81.8% of the quartz-associated community and 52.5% of the glass wool-associated community, which is reflected in the similarly high dominance indices for the two substrates (Table 4). Furthermore, the hematite and glass wool-associated communities which also showed a marked increase in community similarity when abundance was accounted for, shared a total of four T-RFs that each contributed >5% TF (Table 1). Together, these four phylotypes comprised 59.9% of the hematite-associated community and 70.3% of the glass wool-associated community. Consequently, the similarity indices for the quartz- and the glass wool- and the hematite- and the glass wool-associated communities increased by 0.041 and 0.320, respectively, when phylotype abundance (%TF) was included in community similarity calculations.

The reason for the higher similarity achieved between the hematite- and glass wool-associated communities than between the quartz- and glass wool-associated communities using the Bray–Curtis algorithm is not readily apparent. Because this study focused on the effect of geologically relevant substrata on microbial community structure and the purpose of the glass wool was primarily to isolate these substrata during colonization by the groundwater populations, this anomaly was not investigated further.

A potentially important difference between the hematite and quartz substrata that could have contributed to the development of different microbial communities on these minerals is that hematite can serve as a terminal electron acceptor for respiration by some subsurface microorganisms during anaerobic conditions, whereas quartz cannot [31]. Therefore, if anaerobic conditions had developed within the coupons during the incubation, hematite but not quartz should have selected for subsurface microbial populations capable of Fe transformations. However, no 16S rDNA sequences were recovered from either the hematite-associated community or the communities associated with the other substrata that aligned with those of known Fe-reducers or Fe-oxidizers deposited in the Ribosomal RNA Databases. Instead, phylotypes were recovered from the various substrata whose 16S rDNA aligned most closely with *Sphingomonas* spp., *Pseudomonas* spp., *Caulobacter* spp., and *Novasphingobium* spp. These genera are known to be physiologically diverse and have a propensity to colonize surfaces [3, 25, 37, 43]. These traits, however, provided little indication of the basis of the observed substratum influence on community structure and diversity.

The dependence of bacterial community composition on the nature of the geological substratum that was observed in the present study is consistent with results of previous studies, which used artificial instead of geological substrata to capture subsurface microbial communities [19]. *Proteobacteria* comprised >67% of the total microbial community associated with the three geologic substrata (hematite, quartz, and saprolite) evaluated in the present study. *Proteobacteria* were also the primary phylotypes recovered from Bio-Sep beads incubated in a different part of the same aquifer by Peacock *et al.* [33]. α-*Proteobacteria* with 16S rDNA sequences that most closely aligned with *Sphingomonas* (93–96% similarity) accounted for >10% TF of the communities associated with all three geological substrata evaluated in the present study. α-*Proteobacteria* (*Sphingomonas* spp. and *Rhodopseudomonas* sp.), β-*Proteobacteria*, and δ-*Proteobacteria* aligning with the genus *Frateuria* were also detected on Bio-Sep beads by Peacock *et al.* [33]. The results reported in this study are also consistent with previous results indicating a highly diverse groundwater community comprised exclusively of α-, β-, γ-, and δ-*Proteobacteria* as well as high and low G+C gram-positive bacteria in the pristine portion of the FRC aquifer [12].

Proteobacteria also dominated the 16S rDNA clone library (96% of total clones) constructed from the hematite-associated community by Reardon *et al.* [38] using the IB-PCR nucleic acid extraction method. At the subdivision level, however, composition of the IB-PCR-extracted, hematite-associated community obtained in

the present study differed from that reported by Reardon *et al.* [38]. Whereas, the β-*Proteobacteria*, γ-*Proteobacteria*, and α-*Proteobacteria* contributed 69, 21, and 3.5% of the clones, respectively, in the Reardon *et al.* [38] study, they contributed 28, 30, and 22% of the total community T-RF fluorescence, respectively, in the present study. DNA used for construction of clone libraries in both studies was extracted using IB-PCR from the same types of substrata incubated in the same well for identical (8-week) periods of time, but in different years. However, similar Jaccard coefficients generated from T-RF community profiles in the two studies for quartz- and glass wool-associated communities and for quartz- and hematite-associated communities suggested that the structure and diversity of the communities associated with these substrata had not changed during the intervening 1-year period in which the two studies were conducted.

The community composition differences noted above likely resulted from the different approaches used by the two studies to assemble community structure information. The T-RFLP method used in the present study is independent of sample size and thus offers the opportunity to achieve greater coverage of the true community structure than the clone library subsampling ($n = 95$ clones) approach used by Reardon *et al.* [38]. However, T-RFLP is limited by the fact that only those members divergent enough in the 16S rRNA gene yield different length T-RFs. The use of different or multiple restriction enzymes could enhance diversity of a community T-RF profile. However, to achieve this result, the recognition and cut site for each enzyme cannot be conserved among all members of a community. Furthermore, T-RFLP does not provide phylogenetic information for those T-RFs that are resolved unless complemented with a 16S rDNA clone library that yields a unique clone for each T-RF.

Denaturing gradient gel electrophoresis (DGGE) is an alternative approach that has been widely used to describe microbial community structure and diversity [11, 30, 33]. This approach has the advantage of analyzing all members of a community 16S rDNA pool rather than only those members whose 16S rDNA gene yields T-RFs of unique size. However, those phylotypes whose 16 rRNA gene sequence share similar melting characteristics may not be resolved, leading to an underestimation of community richness [21]. Thus, it may be necessary to employ multiple approaches to achieve a higher degree of certainty of the community diversity associated with the geological media utilized in the present study. Nevertheless, the approaches employed in this study permitted detection, quantification, and characterization of the influence of mineralogy on the structure and diversity of the microbial communities that develop on subsurface geological media.

The results of the present study also serve as a reminder that community structure and diversity data obtained using different DNA extraction methods may not be directly comparable due to the unique biases of each method [6, 13, 28, 29]. The different DNA extraction techniques used in this study clearly influenced subdivision-level community composition (Fig. 3). For example, IB-PCR recovered proportionally fewer γ-*Proteobacteria* of the hematite-associated community and proportionally fewer β-*Proteobacteria* in the quartz-associated community than did the bead-beating extraction method. The results of the current study suggest that the type of substratum which the community is associated influences the detection of certain microbial populations by the PCR-based T-RFLP method because other biases such as those associated with the PCR [34, 42] should be the same for both bead beating and IB-PCR extracts. The results also suggest that the substratum-derived bias is quantifiable and distinguishable from an actual substratum influence on community structure and diversity.

A potentially important consideration not investigated in the present study is how efficiency of extraction of DNA from the different substratum-associated populations is influenced by the amount of biomass present [13, 35, 44]. Unfortunately, the efficiency of chemically based methods used to quantify microbial biomass in subsurface environments such as phospholipid fatty acid analysis may also be influenced by substratum properties, precluding acquisition of absolute biomass values [40]. Direct microscopic methods of biomass determination are hampered by particle masking of cells and uncertainties associated with assigning carbon content to populations of cells that vary in size and physiological state [7, 8, 15].

In summary, 16S rDNA-based T-RFLP and clone library analysis provided new evidence supporting previous studies that different geological media are colonized by communities of microorganisms with unique structure and diversity. The substratum influence on community structure and diversity was isolated from the substratum influence on efficiency of extraction of DNA from the substratum-associated community. To our knowledge, this is the first study that has determined the influence of both the type of substratum and extraction method on the diversity and phylogenetic composition of communities associated with subsurface geological media. The present study described the structure and diversity of microbial communities associated with different geological media at the particle scale. In the subsurface, however, geological heterogeneity occurs over a wide range of scales [9, 14, 16, 36]. We presently have little understanding of how microbial community structure and diversity at the particle scale relates to that which exists at the aquifer scale where these community parameters likely control important processes such as contaminant fate and transport. The results presented in this study represent a first step toward achieving this goal.

Acknowledgments

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