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Bacterial Community Composition in Central European Running Waters Examined by Temperature Gradient Gel Electrophoresis and Sequence Analysis of 16S rRNA Genes▽†

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The bacterial community composition in small streams and a river in central Germany was examined by temperature gradient gel electrophoresis (TGGE) with PCR products of 16S rRNA gene fragments and sequence analysis. Complex TGGE band patterns suggested high levels of diversity of bacterial species in all habitats of these environments. Cluster analyses demonstrated distinct differences among the communities in stream and spring water, sandy sediments, biofilms on stones, degrading leaves, and soil. The differences between stream water and sediment were more significant than those between sites within the same habitat along the stretch from the stream source to the mouth. TGGE data from an entire stream course suggest that, in the upper reach of the stream, a special suspended bacterial community is already established and changes only slightly downstream. The bacterial communities in water and sediment in an acidic headwater with a pH below 5 were highly similar to each other but deviated distinctly from the communities at the other sites. As ascertained by nucleotide sequence analysis, stream water communities were dominated by *Betaproteobacteria* (one-third of the total bacteria), whereas sediment communities were composed mainly of *Betaproteobacteria* and members of the *Fibrobacteres/Acidobacteria* group (each accounting for about 25% of bacteria). Sequences obtained from bacteria from water samples indicated the presence of typical cosmopolitan freshwater organisms. TGGE bands shared between stream and soil samples, as well as sequences found in bacteria from stream samples that were related to those of soil bacteria, demonstrated the occurrence of some species in both stream and soil habitats. Changes in bacterial community composition were correlated with geographic distance along a stream, but in comparisons of different streams and rivers, community composition was correlated only with environmental conditions.

Bacterial biomass is an important component of natural stream systems. Due to low autochthonous primary production and a high ratio of stream bank length to water volume, allochthonous organic material is usually the main source of organic matter for the food web in such environments. Heterotrophic bacteria connect organic matter with protozoa and metazoa at the base of the microbial food chain. Thus, not only are these bacteria decomposers, as assumed in classical ecological concepts (55), but their biomass is a major food resource for higher trophic levels in streams (40, 44, 45, 51).

The compositions of bacterial communities in streams have been studied using classical methods based on cultivation techniques (e.g., references 4, 25, and 34), including numerical taxonomy analysis and/or microtechniques (27, 28, 44). These methods suffer from a number of artifacts, mainly because only a small, and probably not representative, number of bacteria are cultivable and identifiable (2). Nevertheless, differences in the community composition between different stream habitats, especially between water and sediment, have been observed using these techniques (27, 44).

The same cosmopolitan clusters have been found in different freshwater environments all over the world (23, 70, 74), suggesting the existence of typical global bacterial populations. Nucleotide sequences of these bacteria have been detected in lakes and rivers but, so far, not in headwater streams. While substantial information on the compositions of bacterial communities in rivers and estuaries has been accumulated using fluorescence in situ hybridization (FISH) (e.g., references 7, 8, 32, 34, and 65), as well as denaturing gradient gel electrophoresis and sequence analysis (e.g., references 6, 14, 19, 33, 64, 65, and 72), fewer reports of studies of headwaters have been published, and these studies have used mainly FISH (5, 12, 18, 22, 30, 49, 57, 56).

In this study, the structure and diversity of bacterial communities in different streams, as well as in different habitats of the same stream, were determined by temperature gradient gel electrophoresis (TGGE) analysis of PCR-amplified 16S rRNA genes. This genetic fingerprinting technique has been described to compare temporal and spatial population dynamics (54). TGGE yields geometric patterns that allow comparisons between samples and estimates of the complexity of their population structures. However, it does not generate information on community composition and the phylogenetic relationships of the organisms. Therefore, this study was complemented by sequence analyses of cloned PCR products from 16S rRNA genes.

The study focuses on whether different habitats of the examined streams harbor different bacterial communities and

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whether the bacterial communities within habitats vary with downstream distance. We compared the 16S rRNA genes in samples from (i) water and sediment in three streams (Breitenbach, Rohrwiesenbach, and Jossa) and one river (Fulda), (ii) several habitats (water, sediment, leaf litter, biofilms, and soil adjacent to the stream) around one stream (Breitenbach), (iii) different-depth layers of the sediment in one stream (Breitenbach), and (iv) the water and sediment along one stream (Breitenbach) from the source to the mouth. The data were further considered for their adequacy to evaluate general concepts in microbial biogeography.

MATERIALS AND METHODS

Study sites. The investigations were performed with samples from three unpolluted first-order upland streams and one river, located in central Germany about 100 km northeast of Frankfurt am Main. The Breitenbach stream (50°40′N, 9°37′E), about 4.2 km in length, has been the focus study area for most investigations from the Limnological River Station in Schlitz for many years (45). The source is at an altitude of about 350 m above sea level, and the confluence with the river Fulda is at about 220 m above sea level. About 2,000 m downstream from the source, water from two alternative springs enters the stream, providing most of the water to the middle and lower reaches. Along most of its length, the stream is flanked by grassland with some scattered alder trees (*Alnus glutinosa*). The catchment area is covered mainly by forest, which extends to a distance of about 50 m from the stream bank. Thus, the stream has higher autochthonous primary production than a typical woodland stream. However, allochthonous organic matter is by far the most important carbon and energy source for the stream community (46). The geological composition of this area consists mainly of Bunter sandstone, resulting in low ion concentrations in the stream water and a circumneutral pH yielding low buffering capacity (45). Further chemical characteristics are given in Table 1.

The upper reach of the Rohrwiesenbach (50°41′N, 9°30′E) is surrounded by woodland dominated by beech (*Fagus sylvatica*). The stream water is calcareous, with pHs around 8 (40) due to chalk deposits in the upper part of the catchment area.

The Jossa headwaters (50°40′N, 9°28′E) are located in a completely forested catchment area dominated by spruce (*Picea abies*) and composed of Bunter sandstone, causing low ion concentrations and pH values below 5 (21). However, due to liming activities in the catchment area some years ago, the pH has slightly increased since the previous report (Table 1).

The river Fulda is the main water course in this area, draining the whole region. Its source is located in the Rhön Mountains. At the sampling region, about 60 km below the source, the river is slightly polluted (Table 1), mainly due to municipal and agricultural influences.

Sample collection. Water and sediment samples (designated with the prefixes W and S, respectively) were collected on 10 June 2002 from seven study sites along the Breitenbach (designated Btb1 to Btb7) and one site each along the Rohrwiesenbach, Jossa, and Fulda (designated Rwb, Jos, and Ful). A Breitenbach spring (two sites) was sampled 1 week later. The locations of the sampling sites are illustrated in Fig. 1. The sites Btb1 and Btb2 were located in the upper reach, above the point of inflow of the two additional springs. One of these springs was sampled directly at its outlet from the rocks (site BQ1) and in the center of the spring pool (BQ2), which was about 2 m in diameter. The sites Btb3 to Btb7 were positioned along the middle and lower reaches. The sampling sites at the Rohrwiesenbach and the Jossa were located close to the sources of these streams. The Fulda sampling site (50°39′N, 9°36′E) was located about 1 km upstream from the point of inflow of the Breitenbach.

Sediment cores (0 to 8 cm) were taken from Btb4 and Btb5 on 11 June 2002. The cores were divided into four layers of 2 cm each, and three replicates from each layer were examined. The same day, leaves from oak (*Quercus robur)*, beech (*Fagus sylvatica*), and alder (*Alnus glutinosa*) trees, as well as two stones with biofilms, were collected from the Breitenbach area at Btb4. Soil samples (Ah horizon) were taken about 2 m from the Breitenbach bank, one at Btb4 and one at Btb5.

Bacterial cell counts. Water and sediment samples were fixed immediately after collection with 5% formaldehyde solution (final concentration), which had been buffered to obtain a pH of 7 and filtered through a 0.2-μm-pore-size filter, and were stored at 4°C. Bacteria from selected water and sediment samples were stained with SYBR green I and counted as described previously (9).

TABLE 1. Physical and chemical characteristics of the streams Breitenbach, Rohrwiesenbach, and Jossa and the river Fulda on the day of sampling[a]

Site	Temp (°C)	Discharge rate (liter s^{-1})	pH	Conductivity ($\mu S_{25°C}$ cm^{-1})	% O_2 saturation	Concn of:			
						DOC (mg $liter^{-1}$ of C)	Orthophosphate (μg $liter^{-1}$ of P)	Ammonia (μg $liter^{-1}$ of N)	Nitrate (μg $liter^{-1}$ of N)
Btb1	10.3	ND	6.7	142	103	2.5	33	20	750
Btb2	13.0	4.1	6.8	142	104	2.5	34	11	720
BQ1	8.4	ND	6.1	162	93	1.8	53	3	1,010
BQ2	8.7	2.6	6.0	162	91	1.9	55	4	1,020
Btb3	10.6	ND	6.4	155	103	2.5	48	9	760
Btb4	11.4	10.0 (3.8–111)	7.0	155	105	2.2	43	9	710
Btb5	11.9	ND	6.9 (6.4–7.2)	154 (138–154)	103 (96–105)	2.3 (2.2–4.4)	42 (33–46)	12 (7–20)	840 (610–1,030)
Btb6	12.3	ND	7.0	160	102	2.5	44	29	710
Btb7	12.5	13.4 (7.5–131)	7.1	161	102	2.7	41	17	870
Rohrwiesenbach	14.0	0.82 (0.36–23.7)	8.2 (7.2–8.3)	533 (156–618)	95 (70–104)	3.4 (3.3–14.4)	22 (4–27)	22 (7–54)	290 (160–500)
Jossa	14.1	ND	4.9 (4.4–5.1)	196 (149–197)	87 (85–103)	10.0 (8.2–13.5)	15 (1–65)	21 (11–55)	810 (690–1,040)
Fulda	18.5	7.9×10^3[b]	7.6	551	81	4.2	195	110	2,350

[a] When available, the range of data collected during the whole year of 2002 is given in parentheses. Btb1 and Btb2, Breitenbach upper reach; Btb3 and Btb4, Breitenbach middle reach; Btb5 to Btb7, Breitenbach lower reach; BQ1 and BQ2, Breitenbach spring; ND, not determined.

[b] Measured about 13 km above the sampling site.

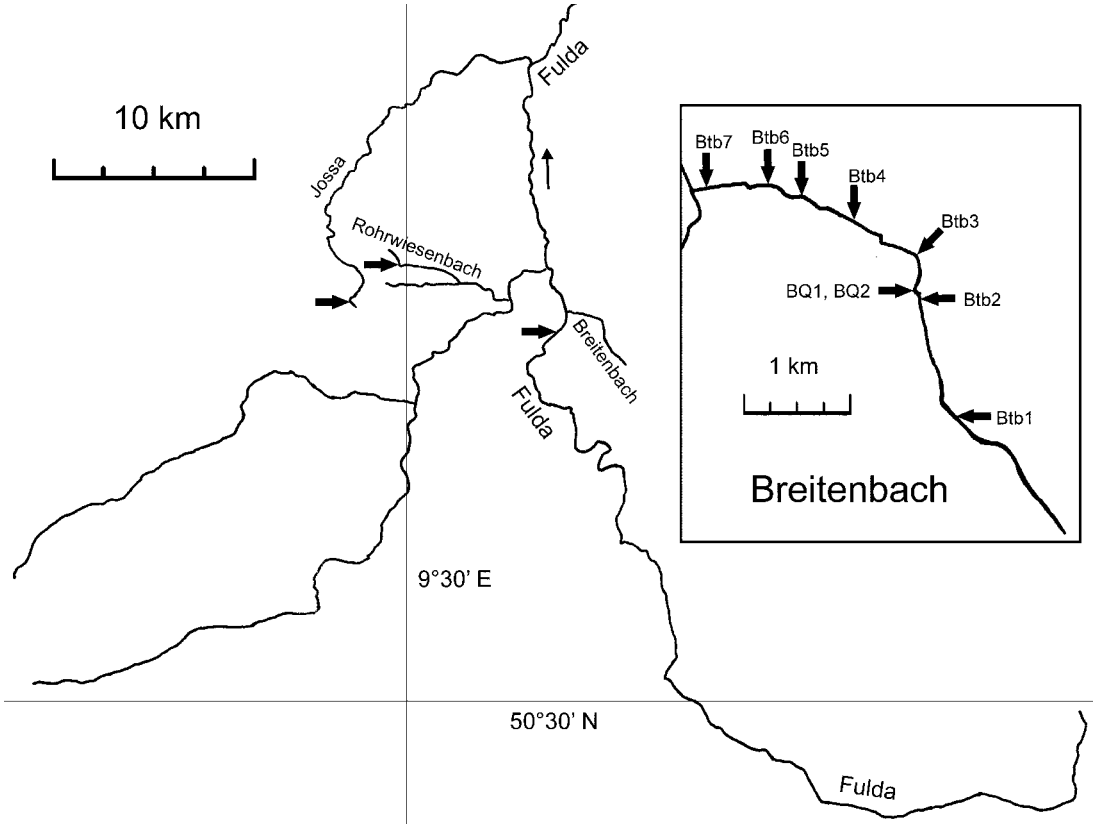

FIG. 1. Map of the upper Fulda River catchment in central Germany showing the locations of the sampling sites (indicated by arrows).

DNA extraction. DNA was extracted with the UltraClean soil DNA kit (Mo Bio) directly from samples of about 0.25 g (wet weight), either from sediment, leaves (cut into pieces), or soil. DNA from stream water (500 to 1,000 ml) or biofilm suspensions (100 to 250 ml) was extracted after filtration through polyethersulfone filters (0.2-μm pore size; Pall Corporation). Biofilm (ca. 12 cm^2) from stones was released by ultrasonication with a Piezon Master 400 (Electro Medical Systems) according to the method of Werneke (71) and suspended in sterile Milli-Q water.

PCR and cloning. Eubacterial 16S rRNA genes were amplified with primers EUB 9-27 and EUB 1542 (5′-GAGTTTGATCCTGGCTCAG-3′ and 5′-AGAAAGGAGGTGATCCAGCC-3′) (36). In a second nested PCR, we used the primer pair 341f and 534r (5′-CCTACGGGAGGCAGCAG-3′ and 5′-ATTACCGCGGCTGCTGG-3′) with a GC clamp (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3′) (53) attached to the forward primer. Hot-start PCR was carried out in a 50-μl reaction mixture containing 5 μl of 10× buffer provided by the manufacturer (Sigma) with 15 mmol of $MgCl_2$/liter, 1 μmol of each primer/liter, 200 μmol of deoxynucleoside triphosphates/liter, 1 U of *Taq* DNA polymerase (Sigma), and 0.2 to 1.0 μl of DNA extract. The touchdown temperature program consisted of 6 min at 94°C; 30 cycles of 15 s at 94°C, 30 s at the annealing temperature, and 2 min and 30 s at 72°C; and a final extension at 72°C for 3 min. During the first 20 cycles, the annealing temperature was decreased by 0.5°C in each cycle from 50 to 40°C. For nested PCR with the primer pair 341f and 534r, the temperature program consisted of 2 min at 94°C and 30 cycles of 15 s at 94°C, 1 min at the annealing temperature, and 1 min 30 s at 72°C. The annealing temperature was decreased during the first 20 cycles by 0.5°C in each cycle from 65 to 55°C, and a final extension of 3 min at 72°C was added. PCR products were checked for concentration, purity, and appropriate size by agarose gel electrophoresis and ethidium bromide staining.

A clone library was constructed with PCR products generated with the eubacterial primers EUB 9-27 and EUB 1542 and the proofreading DNA polymerase *Pfu* (Promega). The 50-μl reaction mixture contained 5 μl of 10× buffer provided by the manufacturer (Promega) with 20 mmol of $MgSO_4$/liter, 0.5 μmol of each primer/liter, 0.2 mmol of each deoxynucleoside triphosphate/liter, 1.5 U of *Pfu* DNA polymerase, and 1 μl of DNA extract. The temperature program consisted of 2 min at 95°C and a hot start at 80°C, followed by 25 cycles of 30 s at 95°C, 30 s at 40°C, and 3 min at 73°C and a final extension for 5 min at 73°C. In order to reduce the risk of chimeric artifacts, the number of cycles was reduced to the minimum necessary to obtain sufficient amplification products.

PCR products from the sediment samples S-BQ2, S-Btb7, S-Jos, and S-Rwb, as well as the water sample W-Btb7, were used for cloning with the Zero Blunt PCR cloning kit as described in the instructions of the manufacturer (Invitrogen). From each sample, 96 clones were selected and checked for inserts of the appropriate size by PCR with *Taq* DNA polymerase (Roche) and M13f and M13r primers.

TGGE. Products obtained from the nested PCR were used for TGGE (54, 60) with a TGGE Maxi system (Biometra). Gels (1 mm thick) were composed of 20% formamide, 6% acrylamide (37.5:1), 10% 10× Tris-acetate-EDTA buffer (pH 8.3), 5% glycerol, and 8 mol of urea/liter. Polymerization was catalyzed by 110 μl of *N,N,N′*,N′-tetramethylethylenediamine and 80 μl of 10% ammonium persulfate solution added to 50 ml of the gel solution. The gel was loaded with 5 μl of 341f-534r-GC clamp products and 1 μl of dye solution and run for 12 h at 195 V with a parallel temperature gradient ranging from 36 to 52°C. After electrophoresis, the gel was silver stained (62) and dried.

Gel images were scanned with a flat-bed scanner (CanoScan N670U) at 400 dots per inch. The relative heights of the peaks in a densitometric curve that had been calculated from the gel images with the software package Gene Tools (version 3.00.22; Syn Gene) were used to establish banding patterns for each sample. Peaks with similar positions in the gel were considered identical and were identified visually. Cluster analysis was performed (using SPSS for Windows, version 11.5) with squared Euclidean distances in order to compare banding patterns of samples on the same gel. Dendrograms were calculated by using the Ward linkage.

The application of quantitative calculation tools to the results from PCR amplification must be considered with care, because biases from PCR, variation in 16S rRNA gene copy numbers, or DNA extraction may cause variations in the numbers and intensities of bands (17, 52, 68). In this study, a strictly qualitative evaluation of banding patterns was impossible due to the high number of weak bands that became undetectable after the digitalization of the gel images. The

TABLE 2. Numbers of bacterial cells from selected sampling sites (± 95% confidence limits)[a]

Sampling site (depth)	No. of cells ml^{-1} of:	
	Water (10^6)	Sediment (10^9)
Btb1	0.95 (±0.05)	6.3 (±1.7)
BQ2	0.039 (±0.011)	1.5 (±0.4)
Btb4	ND	2.3 (±0.5)
Btb7	2.2 (±0.2)	3.1 (±0.7)
Rohrwiesenbach	0.94 (±0.13)	4.0 (±0.9)
Jossa	3.0 (±0.3)	4.0 (±0.5)
Fulda	8.9 (±2.6)	4.2 (±0.4)
Btb4 (0–2 cm)		2.3 (±0.5)
Btb4 (2–4 cm)		3.3 (±0.4)
Btb4 (4–6 cm)		2.2 (±0.3)
Btb4 (6–8 cm)		1.9 (±0.1)

[a] Sampling sites are as defined in Table 1, footnote *a*. ND, not determined.

squared-Euclidean-distance method was used for examining similarities of banding patterns because this method is influenced only slightly by the removal of weak bands, unlike binary cluster analysis (63).

To analyze differences in bacterial community composition between sampling sites and the relationships of these differences to environmental variables, a principal-component analysis (PCA) was performed with TGGE banding patterns as active variables and environmental variables as passive variables. To verify the statistical significance of these relationships, a multivariate general linear model (GLM) was run with species data derived from TGGE profiles (bands were operational taxonomic units considered as surrogates of bacterial species) as dependent variables and the environmental variables as continuous predictor variables by using forward stepwise selection to retain only significant predictors in the model. The PCA and GLM analysis were conducted using the Statistica 7.1 software package (StatSoft Inc., Tulsa, OK).

To test the potential influence of spatial separation on genetic dissimilarity, Mantel tests (XLSTAT, version 2007.4) with 10,000 permutations were performed on the dissimilarity matrices derived from TGGE data and the geographic-distance matrix. Geographic distances between Breitenbach samples were derived from measurements along the stream including bending; all other distances were calculated as linear distances between geographic coordinates.

Sequencing and phylogenetic analysis. After cleaning with a MultiScreen plate (Milli-Q), M13f-M13r PCR products were used for cycle sequencing with the BigDye Terminator kit, version 3.1 (ABI Prism), and analyzed with the ABI 3100 automatic capillary sequencer (ABI Prism) according to the manufacturer's recommendations. To obtain the full-length sequence of the insert (forward and reverse with overlap), partial sequences obtained with the primers M13f, 341f, 926f, M13r, 907r, and 534r were edited and assembled with the AutoAssembler program (ABI Biosystems). The consensus sequences were checked for chimeras by using the CheckChimera function of the Ribosomal Database Project (13) and the Bellerophon chimera checking tool (29). Two sequences of 45 were identified as chimeras and omitted from further analysis. The remaining sequences, as well as related sequences found via BLAST searching, were imported into the 1400ssu database (ssu_jan04_corr_opt) supplied on the ARB home page (http://www2.mikro.biologie.tu-muenchen.de/arb/about.html) by using the ARB program package (38). The automatic alignment of the sequences was corrected manually. A rooted phylogenetic tree was constructed by maximum-likelihood analysis with consensus sequences of >1,400 bp from which highly variable regions were excluded by filters. Sequences of <1,400 bp were included by parsimony criteria into an existing tree. Good's coverage index (24), calculated by the following equation, was used to estimate if the clone library reflected the actual bacterial diversity in the samples: $C = 1 - (n_1/N)$, where C is the coverage index, n_1 is the number of phylotypes appearing only once, and N is the number of all sequences in the library. Coverage was indicated on a scale from 0 to 1, with 0 being no coverage and 1 being complete coverage. Sequences were regarded as representing the same phylotype if their similarity was 97% or higher (66).

Nucleotide sequence accession numbers. The sequences determined in this study have been deposited in GenBank under accession no. DQ017910 to DQ017952.

RESULTS

Bacterial cell numbers. Cell densities in sediment samples exceeded those in water samples by up to 4 orders of magnitude. The lowest numbers in both water and sediment samples were found in samples from the spring site, and the highest numbers were found in Fulda water and Breitenbach upper reach (Btb1) sediment samples. The relative fluctuations among water samples were distinctly larger than those among sediment samples. The maximum cell density was detected in sediment from depths between 2 and 4 cm (Table 2).

TGGE analyses. Between 4 and 22 bands were identified by densitometry, though visual inspection of the gels had showed more bands for almost all samples. Two samples (S-Btb2 and the sediment core sample designated Btb4-6b) were excluded from further analysis because only one or two bands were detected. Pictures of TGGE gels are provided in the supplemental material.

Comparison of different Breitenbach habitats. Bacterial communities in the habitats analyzed by TGGE band patterns were clearly different and separated into distinct clusters (Fig. 2). Differences between habitats were more pronounced than those between samples from the same habitat. The most significant differences occurred between soil, sediment, and biofilm communities and water and leaf communities. Soil communities clearly differed from those in sediment and biofilm, as did leaf communities from water communities. Unrelated samples of sediment and biofilm were more similar than unrelated samples of water and soil. The similarity between sediment and biofilm samples was in the same range as that between unrelated samples collected within the other habitats. Communities from *Quercus* and *Fagus* leaves were more similar to each other than to those from *Alnus* leaves. Apart from the distinct clustering of the examined habitats, in all cases, shared bands were observed in comparisons of two different habitats.

Comparison of water and sediment samples from different locations. Communities from water and surface sediment samples from all streams, including samples taken along the entire Breitenbach, as well as single-site samples from the Rohrwiesenbach, the Jossa, and the river Fulda, were compared using cluster analysis (Fig. 3). This analysis revealed two main groups. Cluster

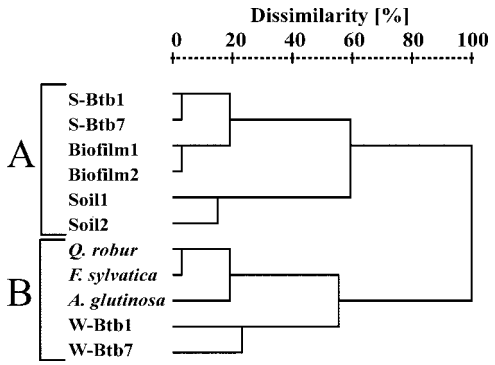

FIG. 2. Comparison of the bacterial community compositions in different habitats of the Breitenbach by cluster analysis of TGGE profiles prepared with 16S rRNA gene fragments. The dissimilarity matrix for each sample was calculated using squared Euclidean distances, and clustering was performed by the Ward method. The highest dissimilarity value between samples was set at 100%.

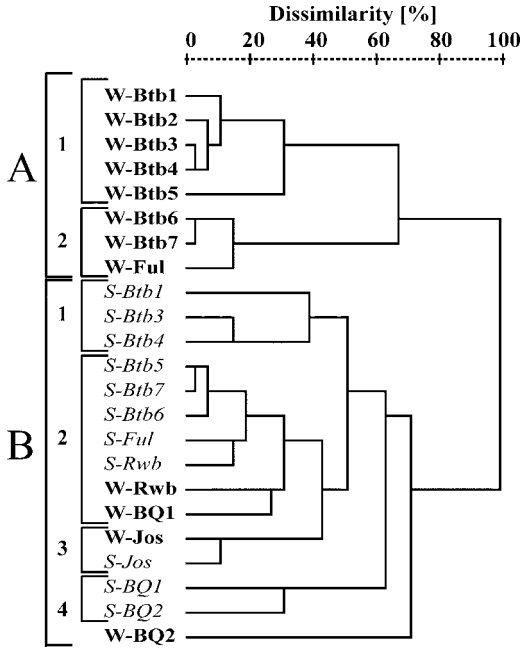

FIG. 3. Comparison of the bacterial community compositions in water and sediment samples from the Breitenbach (sites Btb1 to Btb7), the Breitenbach spring (BQ1 and BQ2), the Rohrwiesenbach and Jossa streams, and the river Fulda. The dendrogram was prepared as described in the legend to Fig. 2.

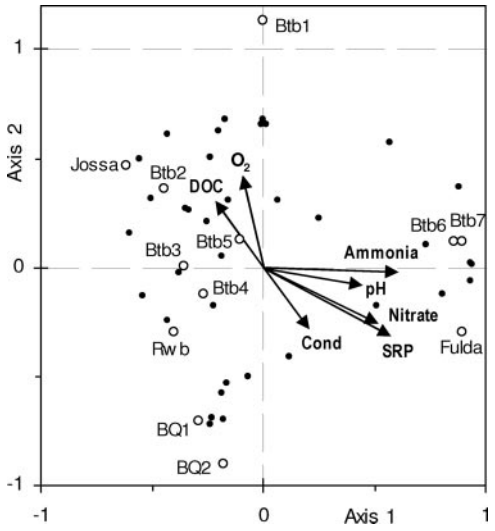

FIG. 4. PCA biplot of the TGGE band patterns (active variables) and environmental data (passive variables) from water samples from all studied streams and rivers. Axes 1 and 2 explain 40% of the variance in bacterial community composition. Small points symbolize operational taxonomic units (TGGE bands of identical positions), and large open circles indicate the sampling sites. Environmental variables are as follows: DOC concentration, soluble reactive phosphorus (SRP) concentration, and conductivity (cond).

A contained only communities from water samples from the Breitenbach and Fulda. Cluster B included communities from all the sediment and water samples from the Jossa, the Rohrwiesenbach, and the Breitenbach spring. The water samples from the Breitenbach were clearly divided into those from the upper and middle reaches (Btb1 to Btb4) versus those from the lower reach (Btb6 and Btb7). The bacterial community from Btb5, the site located at the transition from the middle to the lower reach, below the point of outflow of a fishpond, had an intermediate position between the groups from the middle and lower reaches. The community from Fulda water was most similar to those from water of the lower Breitenbach (group A2).

Community patterns and the relationships of these patterns to environmental variables are summarized in a PCA biplot (Fig. 4). Axes 1 and 2 explained 40% of the bacterial community variation. Concentrations of ammonia, inorganic phosphorus, and nitrate and the pH were most strongly associated with axis 1 (loadings of 0.59, 0.56, 0.52, and 0.43, respectively). The oxygen concentration was most strongly associated with axis 2 (0.41), and the dissolved organic carbon (DOC) content (−0.21, 0.29) and conductivity (0.21, −0.27) had lower loadings than the other factors on both axes. The importance of ammonia and DOC was confirmed in the GLM test (Table 3), which demonstrated the significance of these two variables for the TGGE banding patterns.

Dissimilarity between water and sediment communities was more pronounced than dissimilarity between communities from different sites of the same habitat. Sediment from the Fulda and Rohrwiesenbach was grouped with sediment from the lower Breitenbach (group B2). For all sites along the Breitenbach, communities in sediment and water samples (clusters A and B) were significantly different, while the communities in samples from the Jossa were very similar (group B3). The Jossa site was the only site where sediment and water communities were so closely related. Communities from Breitenbach spring samples were distributed among different groups within cluster B.

A dependence of genetic similarity between bacterial communities on geographic distance, as estimated by the Mantel test, existed only for sediment communities of the Breitenbach ($P = 0.030$). Water communities of the Breitenbach exhibited no significant relationship with distance ($P = 0.265$). However, if site Btb5 (the site directly below the point of outflow of a fishpond) was removed from the matrix, genetic similarity and geographic distance proved to be correlated ($P = 0.003$). A comparison of all streams and sites gave no significant correlation between community similarity and geographic distance ($P = 0.311$ and 0.797 for water and sediment communities,

TABLE 3. Multivariate GLM of the effects of environmental variables on the occurrence of operational taxonomic units (bands of identical gel positions) generated by TGGE of 16S rRNA gene fragments[a]

Variable	Wilks' lambda	*F*	Effect (df)	Error (df)	*P*
Intercept	0.000105	1,187	8	1	0.0224
pH					
Conductivity					
Concn of:					
O_2					
DOC	0.000009	13,394	8	1	0.0067
SRP					
NH_4	0.000066	1,884	8	1	0.0178
NO_3	0.008546	14.5	8	1	0.2005

[a] Empty cells indicate variables excluded from the model by forward stepwise selection. SRP, soluble reactive phosphorus.

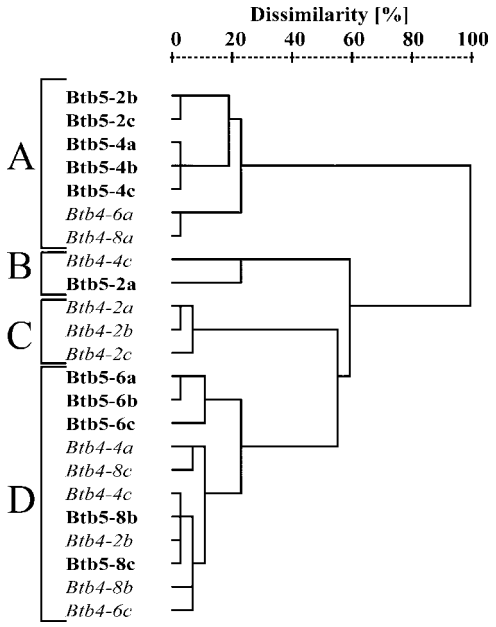

FIG. 5. Comparison of the bacterial community compositions in depth profiles from two sediment cores from sampling sites Btb4 and Btb5 in the middle reach of the Breitenbach. The dendrogram was prepared as described in the legend to Fig. 2. Depths are indicated as follows: 2, 0 to 2 cm; 4, 2 to 4 cm; 6, 4 to 6 cm; and 8, 6 to 8 cm. Suffixes *a* to *c* denote subsamples.

respectively). The genetic variation between streams appeared to be independent from their geographic distance. But the variation within the Breitenbach samples ranged from very close similarity to about half of the maximal values for dissimilarity between streams (data not shown).

Breitenbach sediment depth profiles. Two sediment cores, taken from Breitenbach sites 4 and 5, were studied for depth-related differences in the bacterial communities. A comparison between the surface layer samples S-Btb4-2 and S-Btb5-2 (Fig. 5) confirmed differences between the bacterial communities in these sediment samples that were also observed in the independent sample set taken along the whole stream (Fig. 3). The core from Btb5 exhibited clear stratification of the communities across depths. The three subsamples from each depth (except Btb5-2a) were grouped into the same cluster. The most significant differences observed were between the upper part (0 to 4 cm; cluster A) and the lower part (4 to 8 cm; cluster D) of this core. Comparing the bacterial communities at the different depths of the core from Btb4 did not reveal distinct stratification; only the surface layer (Btb4-2) was clearly separated from the deeper ones.

Phylogenetic analysis. Nine of the 43 sequences analyzed in this study originated from organisms from Breitenbach water (W-Btb7). All other sequences were from bacteria isolated from sediments: 3 from organisms from the Breitenbach spring (S-BQ2), 10 from bacteria from the Breitenbach lower reach (S-Btb7), 12 from isolates from the Rohrwiesenbach (S-Rwb), and 9 from organisms from the Jossa (S-Jos).

In total, 37 different phylotypes were sampled, and their sequences were widely distributed across bacterial divisions (Fig. 6 and 7). Four phylotypes were represented by more than one sequence: those of clones (designated according to sample origin) W-Btb7_46, S-Rwb_22, S-Rwb_26, and S-Rwb_36 within the *Betaproteobacteria*, S-Btb7_57 and S-Btb7_59 within the *Deltaproteobacteria*, and S-Rwb_44 and S-Jos_44, as well as S-Btb7_17 and S-Btb7_22, within the *Fibrobacteres/Acidobacteria* group. Good's coverage index was 0.14. We observed no clear clustering of isolated sequences dependent on their origin from different locations. Three of nine sequences from stream water were affiliated with the *Betaproteobacteria* (Fig. 6). Two sequences were related to those of the *Gammaproteobacteria*, and one sequence each was related to those of the *Alphaproteobacteria*, the *Cytophaga-Flavobacterium-Bacteroidetes* (CFB) group, the *Actinobacteria*, and the *Fibrobacteres/Acidobacteria* group. Among sequences from bacteria from sediment samples (Fig. 7), both the *Betaproteobacteria* and the *Fibrobacteres/Acidobacteria* group were represented by about 25% (8 and 10 sequences, respectively). Four clones each were related to the *Alphaproteobacteria* and the *Deltaproteobacteria*, three to the *Verrucomicrobia*, and two to the *Actinobacteria*, and one clone each was related to the *Spirochaetes*, the *Planctomycetes*, and the *Chloroflexi*. Considering all sequences from bacteria from sediment and water, most belonged to the *Betaproteobacteria* and the *Fibrobacteres/Acidobacteria* groups.

The majority of GenBank entries that were related to the sequences from this study were derived from organisms from freshwater habitats, soil, or sludge. For eight of nine sequences from organisms from Breitenbach water, the closest relatives listed in GenBank were from bacteria isolated from freshwater habitats. Three of these sequences were grouped into pure-freshwater clusters: those of clones W-Btb7_24 and W-Btb7_56 into cluster betaII of the *Betaproteobacteria*, containing *Polynucleobacter necessarius* as a cultured organism (23), and that of clone W-Btb7_91 into acII, one of four lineages suggested for the *Actinobacteria* (70). Further sequences grouped together with sequences from bacteria from freshwater or habitats that are in contact with freshwater. These were the sequences of the betaI cluster of the *Betaproteobacteria* (23), to which five sequences (those of clones W-Btb7_46, S-Rwb_36, S-Rwb_22, S-Jos_09, and S-Rwb_26) were closely related, the alphaIV cluster, to which one sequence (that of clone S-Btb7_18) was most closely related, and the further lineage acIV of the *Actinobacteria* (70), with which one sequence (that of clone S-Btb7-38) was affiliated. Sequences isolated from bacteria from soil or sludge samples were often the nearest relatives of sequences affiliated with other phylogenetic groups, especially the *Fibrobacteres/Acidobacteria* group.

DISCUSSION

Abundance and diversity. All cell numbers for water and sediment samples (Table 2) were in a range typical for these environments (43, 44). The complex TGGE patterns, with up to 40 bands per sample observed visually in the TGGE gels, suggest that a large number of bacterial species occurs at most of the sites investigated, although the number of bands may not necessarily represent the exact number of phylotypes (11, 20, 69). These findings confirm the results from an earlier study of the Breitenbach using a numerical taxonomy analysis of the bacteria isolated from stream and spring water, sediment, and soil adjacent to the stream (44). The results also match with findings from other

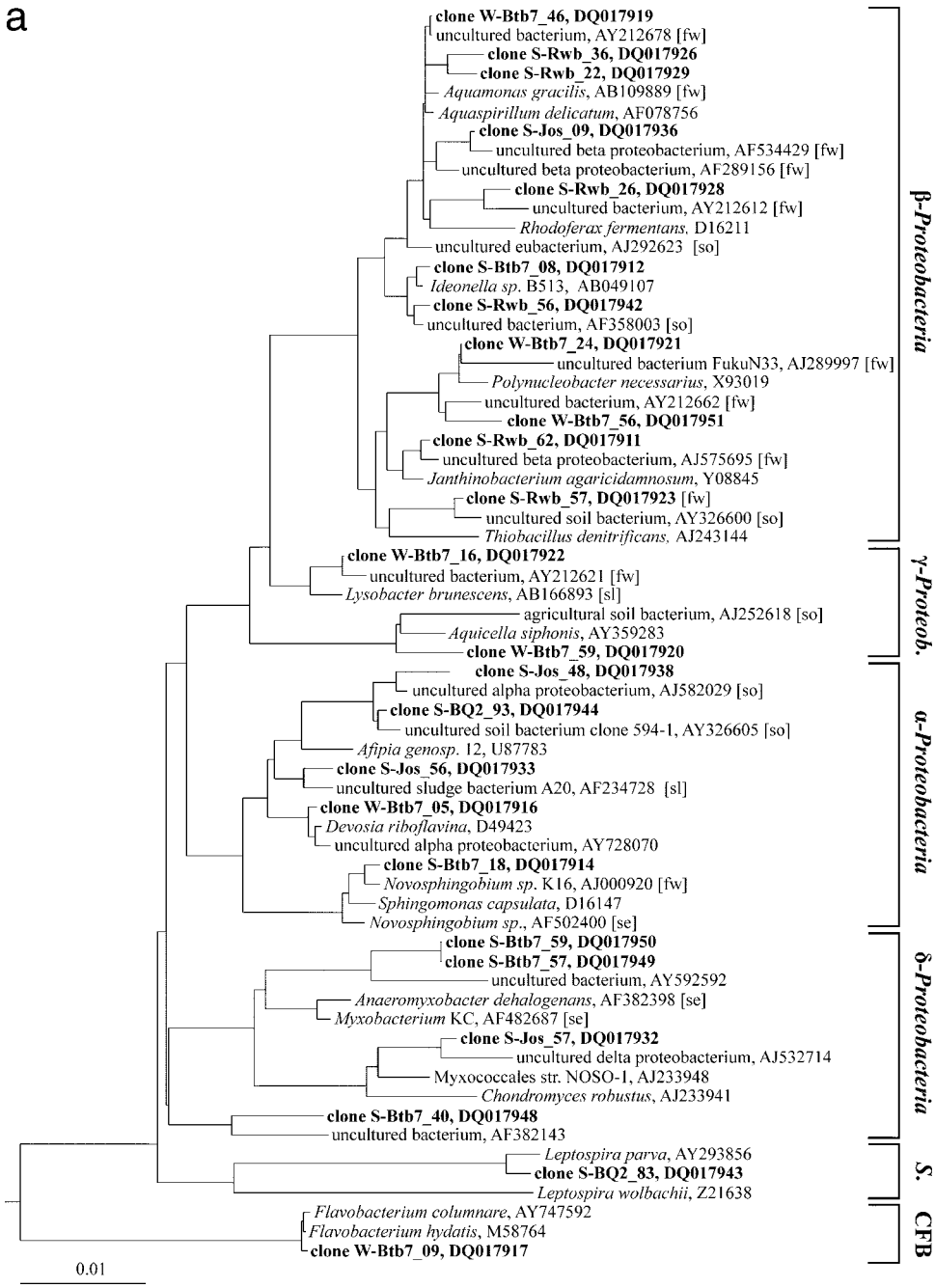

FIG. 6. Phylogenetic trees based on partial 16S rRNA gene sequences obtained from bacteria from water and sediment samples (in boldface) and their nearest relatives from GenBank. Sequences are coded by sample designation, clone number, and GenBank accession number. If known, the habitat of the nearest relative is given in brackets (fw, freshwater; se, sediment, freshwater, or estuary; so, soil; and sl, activated sludge). The trees were generated by using the ARB software package with the maximum-likelihood algorithm. (a) *Proteobacteria* (γ-*Proteob*., *Gammaproteobacteria*), *Spirochaetes* (*S*.), and the *Bacteroidetes*/*Chlorobi* group (CFB). The scale bar represents 1 nucleotide substitution per 100 nucleotides. (b) The *Fibrobacteres*/*Acidobacteria* group, *Verrucomicrobia* (*Verruco*.), and *Planctomycetes* (*Plancto*.). The scale bar represents 1 nucleotide substitution per 20 nucleotides. (c) *Actinobacteria* and *Chloroflexi* (*Chl*.). The scale bar represents 1 nucleotide substitution per 20 nucleotides.

studies that suggested high levels of bacterial diversity in many environments, including streams (30) and rivers (16).

Comparison of different habitats. The TGGE patterns for samples from identical habitats in one stream, the Breitenbach, were similar to each other but indicated clear differences in bacterial community composition between the habitats (Fig. 2). The communities in sediment, biofilm, and soil were distinctly different from those in stream water or on leaves. This finding agrees with those from earlier studies of streams and rivers showing marked differences between communities in stream water and sediment (27) but also clear differences between both stream water and sediment communities and those

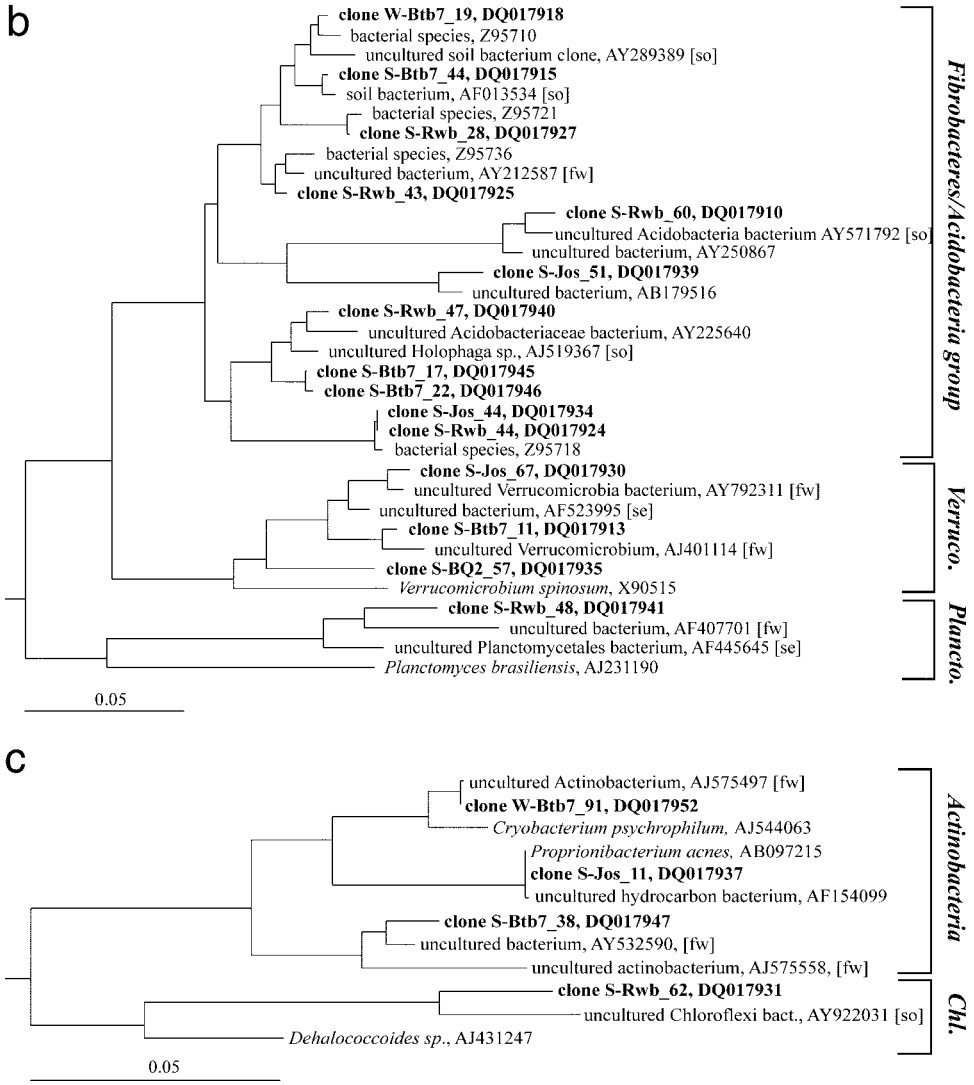

FIG. 6—*Continued*.

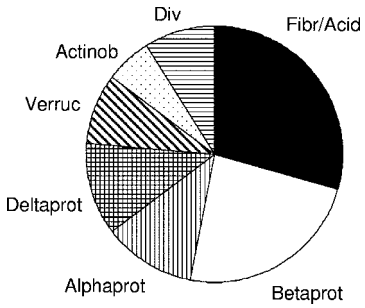

FIG. 7. Taxonomic distribution of the clonal 16S rRNA gene sequences from bacteria from stream sediments in eastern Hesse (Germany). Fibr/Acid, *Fibrobacteres/Acidobacteria* group; Betaprot, *Betaproteobacteria*; Alphaprot, *Alphaproteobacteria*; Deltaprot, *Deltaproteobacteria*; Verruc, *Verrucomicrobia*; Actinob, *Actinobacteria*. The category Div contains *Spirochaetes*, *Planctomycetes*, and *Chloroflexi*.

in soil and spring water (44). Even if cluster analyses suggest distinct bacterial communities in water and sediment samples, the appearance of shared bands in TGGE gels indicates that identical bacterial phylotypes can occur in sediment and water.

Similarities between habitats were highest when bacterial communities in biofilms on stones and on sandy surface sediments were compared. In both these habitats, communities develop on hard, inorganic substrata. Soil communities had the closest relationships to stream sediment communities. Among the five types of habitats investigated, soil and sediment were most similar with respect to spatial structure and organic matter supply but were very different in water content. The occurrence of similar bacteria of several taxonomic groups in soil and freshwater has been reported previously (23, 70).

Along-stream variation. Differences in bacterial community composition in stream water and sandy sediment at different sampling sites along the Breitenbach were not as significant as those between these two habitats (Fig. 3). The distribution of the communities in both habitats, as detected by cluster anal-

ysis, indicated a continuous change of bacterial community composition from the uppermost site (Btb1) to the mouth (Btb7) of the Breitenbach. This observation was confirmed by the results of the Mantel test, which showed increasing differences in bacterial community composition with increasing distance between sampling sites. A similar relationship between the genetic dissimilarity of bacteria and geographic distance was found previously in a study of a South Carolina stream (48). The entering of spring water (from sites BQ1 and BQ2) did not change the community structure noticeably. This finding is in agreement with the low number of bacteria in spring water (4% of that in the Btb1 sample) and a somewhat lower rate of water discharge from the spring than from the Btb2 site (2.6 versus 4.1 liters/s) (Table 1).

Communities in the sediment samples from the Breitenbach spring (S-BQ1 and S-BQ2) formed a group of their own (cluster B4) (Fig. 3), separated from all others. This result is similar to that for the water sample from the spring pool (W-BQ2). The differentiation may be due to the facts that the springs are fed by groundwater of deep origin, the sediments are well perfused, the water residence time is very short (a few minutes), and the temperature is low (annual variation, 7.7 to 8.4°C). Thus, the existence of a community that is different from those in the main stream channel seems quite reasonable.

Mantel tests showed correlation between the genetic divergence of bacterial communities and geographic distance for Breitenbach sediment and water communities, but the application of a multivariate GLM resulted in no significant environmental predictor variables for the TGGE banding patterns (data not shown). This result indicates that transportation events are more important for the variation in bacterial communities detected along the Breitenbach than environmental parameters and is in agreement with the concept of the spiraling movement of bacteria along a stream (35).

Between-stream variations. The similarity between bacterial communities in the river Fulda and the lower reach of the Breitenbach (Fig. 3), as shown for sediment and water samples, is surprising. The environments are very different, e.g., the river Fulda has a 1,000-fold-higher discharge rate at a lower flow velocity and significantly higher nutrient concentrations than the Breitenbach (Table 1).

Bacterial community structures in sediment and water samples from the Jossa (S-Jos and W-Jos) were very similar to each other but differed from those in all other environments. High levels of similarity between communities in water and sediment samples were observed only at the Jossa site (cluster B3) (Fig. 3). The discharge rate was very low at the time of investigation, resulting in almost lentic conditions, thus facilitating the exchange of bacteria between both habitats without downstream transport of suspended cells, as in typical lotic situations. The low pH and the high DOC concentration in the Jossa (Table 1) result in extreme habitat conditions that may favor the development of similar populations in water and sediment. PCA (Fig. 4) suggested the influence of DOC concentrations and pH on TGGE patterns of water bacterial communities, although both variables were not determined as the main drivers. However, other studies identified pH and DOC concentrations to be among the major factors influencing bacterial community composition in freshwater habitats (22, 37, 58). Multivariate tests of significance (GLM) performed for water bacterial communities from this study revealed the importance of only the DOC concentration, besides the ammonia concentration, for bacterial community composition (Table 3).

The numbers of bacteria (Table 2) and the community compositions in the Rohrwiesenbach sediment (S-Rwb), the Fulda sediment (S-Ful), and the sediments of the Breitenbach lower reach (S-Btb5 to S-Btb7) were similar (Fig. 3), supporting the argument for the existence of typical sediment communities that are similar in different running waters.

An influence of geographic distance on the relationship of bacterial community structures in different environments was not observed in this study. Although streams from three catchments were considered, Mantel tests showed that dissimilarities between different streams and rivers were independent from their geographic distance. Levels of intrastream dissimilarities among the Breitenbach samples appeared to be in the lower range or were even lower than those among samples from different streams. This finding corresponds with results for North American streams, where limited differences in bacterial communities within a stream, but much larger differences in communities in different streams, have been observed (22).

The effects of the chemical and physical environments seemed to be of greater importance than geographic distance. PCA (Fig. 4) suggested a general influence of nutrients (N components and inorganic P), loading mainly on axis 1, but also of oxygen and DOC concentrations on the bacterial community compositions in stream and river water. Other studies have shown that further environmental factors can be important drivers, like the phosphorus concentration and temperature (61). Also, for soil bacteria, genetic divergence was found to be related to the degree of environmental divergence (47). Future, more-detailed analyses than were possible with this snapshot study may help to determine the drivers for bacterial community composition in streams and rivers with more reliability.

Sediment depth profiles. The two profiles taken from the sandy sediments (0 to 8 cm) of the Breitenbach showed contradictory results (Fig. 5). In the profile from Btb5, bacterial communities in subsamples from the same layer were similar but communities differed among layers. At this site, the stratification of the sediments remained undisturbed for some months, thus allowing separate communities to be established at different sediment depths. These conditions differed from those at Btb4, where coarser deposits, exposed to more turbulent conditions, were sampled. These differences may explain the very heterogeneous distribution of subsamples. The dissimilarities between subsamples from the same layer were in the same range as those between samples from different layers. Only the subsamples from the top layer (0 to 2 cm) had discernible within-layer similarities and were clearly separated from those from the deeper layers. This part of the sediment is subjected to surface influences, like the penetration of stream water and light and the deposition of particles available as a substrate, and is thus under different conditions than the deeper layers.

Phylogenetic analysis. The large number of phylotypes appearing only once in our clone libraries indicated, like the TGGE patterns, the high level of bacterial diversity in the systems studied. The low Good's coverage index (0.14) showed

that the number of clones did not yet describe the total phylotype richness.

Considering the few sequences available (9 from bacteria from water samples and 34 from bacteria from sediment samples), a comparison between different systems needs to be preliminary. However, a rough evaluation of differences between water and sediment communities, as well as a general comparison of the compositions of running-water bacterial communities from the environments in this study with those from other environments, may be meaningful.

The high abundance of *Betaproteobacteria* (accounting for one-third and one-quarter of the sequences from water and sediment samples, respectively) agrees with results from Hullar et al. (30) for small Pennsylvania piedmont streams. Nevertheless, sequences from the *Fibrobacteres/Acidobacteria* group were found only at much lower proportions in the Pennsylvania samples than in the samples from the German streams. In the present study, this group was even slightly more common (frequency, 29%) than the *Betaproteobacteria* (frequency, 24%). FISH analysis of bacterial community composition in the samples investigated in our study revealed frequencies of *Betaproteobacteria* of about 30 to 40% in water samples and about 8 to 20% in sediment samples (J. Marxsen, unpublished data). These observations agree with results of studies in which the FISH technique was applied to samples from headwaters in Europe and North America (5, 18, 22). In these habitats, high percentages of *Betaproteobacteria* were found, but sometimes similar amounts of *Alphaproteobacteria* and *Gammaproteobacteria* or members of the CFB group were also found. There is no possibility of evaluating the occurrence of representatives of the *Fibrobacteres/Acidobacteria* group because they have not been considered in any FISH study of streams and rivers so far. High abundances of *Betaproteobacteria* have also been detected in large rivers and estuaries (3, 8, 15), in lakes (50), and in drinking water (31). In contrast, marine ecosystems usually contain only very few *Betaproteobacteria* (59, 67). Also, in soil the proportion of *Betaproteobacteria* is much lower than that of *Alphaproteobacteria* and especially than that of members of the *Fibrobacteres/Acidobacteria* group (10, 73).

The results of this study differ significantly from those of an earlier study (44) of the composition of bacterial communities in different Breitenbach habitats by numerical taxonomy analysis of the isolates. This earlier study suggested a clear dominance of *Flavobacteria* and *Cytophaga*-like organisms (the CFB group) in stream and spring water (79 and 62% of bacteria, respectively). The rest were mainly strains from groups now affiliated with the *Betaproteobacteria* and *Gammaproteobacteria*. These last two groups, however, dominated the populations isolated from sediment (together accounting for about 70%), whereas CFB strains occurred at a frequency of only about 10%. Gram-positive bacteria (*Actinobacteria* and *Firmicutes*) were represented at a level below 10% in all samples.

In this study, the sequences from different bacterial divisions resembled those of the cosmopolitan freshwater clusters (23, 70, 74). However, we also observed a considerable number of sequences related to those from soil communities, confirming the results of an earlier study (30) of small Pennsylvania piedmont streams. It is typical of small streams for soil and stream sediment habitats to be closely connected (1). Thus, this finding is not surprising. The low number of clones related to sequences in the database from organisms isolated from freshwater sediments may be due to the fact that the number of available sequences originating from bacteria from freshwater sediments is much lower than the number of sequences from bacteria isolated from soil.

Water versus sediment bacterial communities. Sequences that were arranged into pure-freshwater-habitat clusters were from bacteria isolated from water samples only, confirming the findings of former studies (23, 70, 74) that these organisms are indigenous freshwater species. The observation of these organisms in a small first-order stream with a low temperature (8 to 13°C in the middle reach and 6 to 12°C in the lower reach during the 2 weeks before sampling [H.-H. Schmidt, personal communication]), a high flow rate, and a very short residence time (4 to 10 h from site Btb1 to Btb7, as calculated from the discharge rate during this study) is surprising and raises the question of their origin. Compared to the TGGE patterns for samples from the upper reach of the Breitenbach, those for samples from site Btb7 in the lower reach, where the bacteria with these sequences were isolated, changed only slightly. It is unlikely that a special community has a chance to develop in the water under these conditions. The TGGE data even suggest that in the upper reach of the Breitenbach a special suspended bacterial community is already established and changes only slightly, but continuously, downstream (Fig. 3). Such differences have been observed in other studies, not only in the Breitenbach (44), but also in other streams (27). As was suggested previously (44), there may be a selective, continuous replenishment of bacterial cells from the surrounding habitats, especially from sediment and biofilm and even from soil, establishing the typical bacterial community observed in the water. It has been shown previously (26) that organisms from the pure-freshwater clusters, for example, *Polynucleobacter*-like organisms, are ultramicrobacteria with a cell size of $<0.1\ \mu m^3$. This volume facilitates selective seeding and can cause an enrichment of the water body with ultramicrobacteria. This assumption is supported by the very small cell volume (on average, about $0.1\ \mu m^3$) of bacteria in the water of the streams under investigation (42, 43, 41). Cell seeding is particularly important under high-level-water conditions but should also be recognizable during periods with low water levels, as occurred during sampling for this study.

Conclusions. The results of this study into the diversity of bacterial running-water communities by molecular biology techniques confirmed some fundamental results of earlier studies based on cultivation techniques, especially the finding of distinct community compositions in different habitats. But the taxonomic affiliations of the strains derived by cultivation were different from the affiliations of the sequences obtained by PCR amplification of 16S rRNA genes. The data obtained with the new techniques can be expected to describe the bacterial communities more truly because the new techniques are less selective.

TGGE patterns, as well as data from sequence analyses, confirm results from former studies suggesting that freshwater habitats harbor typical bacterial communities. However, the occurrence in stream water of special bacterial communities that are markedly distinct from those in other habitats remains an unexplained phenomenon.

Environmental effects have been repeatedly shown to influ-

ence community composition on scales of a few kilometers when distance effects have been found to be negligible (39). Significant distance effects were observed on larger scales of more than 10 km. The present study of Central European streams was able to confirm that the distribution of microorganisms reflects the influence of environmental variation. But the role of historical contingencies, reflected by distance effects, cannot be considered from the data because the sampling locations were separated by too-short distances of only a few kilometers. However, there was a clear intrastream distance effect on bacterial genetic diversity. This effect may be due to the special situations of running-water systems, which are characterized by unidirectional flow and transport, sedimentation versus suspension effects, and colonization versus the release of organisms, resulting in the spiraling of matter, organisms, and genetic information (35).

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