Microbial Ecology

FISH Shows That *Desulfotomaculum* spp. Are the Dominating Sulfate-Reducing Bacteria in a Pristine Aquifer

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Abstract

The hydrochemistry and the microbial diversity of a pristine aquifer system near Garzweiler, Germany, were characterized. Hydrogeochemical and isotopic data indicate a recent activity of sulfate-reducing bacteria in the Tertiary marine sands. The community structure in the aquifer was studied by fluorescence *in situ* hybridization (FISH). Up to 7.3×10^5 cells/mL were detected by DAPI-staining. Bacteria (identified by the probe EUB338) were dominant, representing 51.9% of the total cell number (DAPI). Another 25.7% of total cell were affiliated with the domain Archaea as identified by the probe ARCH915. Within the domain Bacteria, the β-Proteobacteria were most abundant (21.0% of total cell counts). Using genus-specific probes for sulfate-reducing bacteria (SRB), 2.5% of the total cells were identified as members of the genus *Desulfotomaculum*. This reflects the predominant role these microorganisms have been found to play in sulfate-reducing zones of aquifers at other sites. Previously, all SRB cultured from this site were from the spore-forming genera *Desulfotomaculum* and *Desulfosporosinus*.

Introduction

Cultivation-based techniques for assessing microbial diversity in natural environments are limited in their ability to identify all contributing microorganisms. Only a small proportion of the microbial population is revealed by the isolation of pure cultures, no matter whether they have been obtained from enrichment cultures or dilution series. Since molecular tools became available, a much higher microbial diversity has been detected, reflecting most of the natural microbial communities in various environments including hot springs, soils, sewage sludge, intestines of higher organisms, marine habitats, freshwater systems, and contaminated groundwater systems [11, 20, 25, 34, 39, 49, 51]. Compared to these habitats, knowledge about structure and function of the microbial community in non-contaminated subsurface environments is rather limited (for review, see [26]). Until recently the microbial communities in subsurface environments were mostly described by cultivation-based methods or by cloning of 16S rRNA genes [9, 11, 30, 36, 37, 48]. Nowadays FISH represents a powerful tool for the identification and quantification of different phylogenetic groups in environmental samples [2, 3, 35].

The investigated groundwater system, situated near Cologne, western Germany, is geologically well characterized [8, 42, 43]. Marine sands of Tertiary age with intercalated lignite seams confine a multiple aquifer system. Information obtained before this investigation, on the groundwater chemistry, pointed strongly toward recent metabolic activity of microorganisms. A strong isotopic discrimination signature of dissolved sulfate and the occurrence of methane already indicated complete anoxic conditions and the presence of sulfate-reducing bacteria (SRB) and methanogenic microorganisms [14]. Therefore, we used group- and genus-specific oligonucleotide probes to gain an insight into the microbial diversity of this ecosystem.

Materials and Methods

Study Site and Sampling Procedure. The study site is situated in the Lower Rhine Embayment, Germany next to the open-pit lignite mine Garzweiler I. The general

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Table 1. Hydrochemical and microbiological properties of groundwater from pristine aquifer sampled at four depths

Well	Sampling depth (m)	pH	Temperature (°C)	O_2 (mg/L)	NO_3^- (mg/L)	SO_4^{2-} (mg/L)	$\delta^{34}S$ SO_4^{2-} (‰)	DOC (mg/L)	$\delta^{13}C$ DIC (‰)	Total cell number (10^5 cells/mL)	Eub 338 (% DAPI)	Arc 915 (% DAPI)
1	120	7.74	13.0	0.0	0.04	17.1	0.23	26.30	−15.79	0.53	10.1	0.0
2	125	6.94	13.2	1.1	0.10	2.8	37.23	1.31	−16.55	0.89	0.0	0.0
3	123	7.06	11.8	0.0	0.04	5.3	21.93	2.54	−17.72	7.26	51.9	25.7
4	121	7.59	11.3	0.0	0.04	7.1	28.97	2.12	−15.94	1.30	9.6	18.9

lithostratigraphic column comprises a succession of up to 250 m of marine sands with a Tertiary age covered by up to 100 m of Quaternary fluvial sediments. A clay horizon (Reuver Ton) and up to three lignite seams (Morken, Frimmersdorf, Garzweiler) are intercalated, resulting in up to five different aquifers. The uppermost aquifer is unconfined while all lower aquifers are confined.

Sampling of groundwater monitoring wells was performed using a standard method as described in detail elsewhere [16]. Samples were taken after pumping for ≥40 min and after parameters such as temperature, pH, redox potential, oxygen and conductivity of the groundwater had remained stable for ≥15 min due to recharge of aquifer water. For chemical analysis samples were stored under anoxic conditions at +4°C in the dark.

Chemical and Isotopic Measurements. Basic hydrochemical information about the groundwater in the study area was obtained using standard procedures as outlined elsewhere [8, 42, 43]. The main objective of this study was a characterization of the isotopic compositions of carbon and sulfur components within the aquifer system with special emphasis on evidence for biologically driven processes. Analytical procedures and data were outlined previously [14].

Isolation of Sulfate-Reducing Bacteria. The isolates used as references in this study have been isolated from the same habitat using media and protocols described previously [14]. The sequences of their 16S rRNA encoding genes were obtained using standard protocols [14]. The nearly complete sequences were loaded into the 16S rRNA sequence database of the Technical University of Munich using the program package ARB [47]. The tool ARB_Align was used for sequence alignment. The alignment was visually inspected and corrected manually. Sequences of *Desulfotomaculum* or other closely related genera of relevance not included in the database were obtained from the NCBI database.

Fluorescence in Situ Hybridization (FISH). Samples for FISH were fixed with 4% (w/v) paraformaldehyde solution on polycarbonate filters and stored as described in detail by Glöckner et al. [18]. Cy3-labeled oligonucleotides were purchased from Interactiva (Ulm, Germany). Hybridization and microscopy counts of hybridized and 4′,6′-diamidino-2-phenylindole (DAPI)-stained cells were performed as described previously [45]. Means were calculated from 10 to 20 randomly chosen fields on each filter section, corresponding to 800–1000 DAPI stained cells. Counting results were always corrected by subtracting signals observed with the probe NON338. Formamide concentrations and oligonucleotide probes used are given in Table 2. Probes BET42a, GAM42a, PLA886, CREN499R, EURY498R, and BONE23a were used with competitor oligonucleotides as described previously [3, 10, 28, 33].

Results

Groundwater. The hydrochemical and microbiological parameters of the investigated groundwater samples are summarized in Table 1. Water samples were retrieved from four groundwater monitoring wells at a depth of 120–125 m and had a temperature between 11.3 and 13.2°C. Samples of well 2 and well 3 displayed circumneutral pH. Wells 1 and 4 had pH values of 7.6 to 7.7. All water samples except for those from well 2 were anoxic and contained only minor concentrations of nitrate. In contrast to water samples of well 1, all other samples showed comparatively low concentrations of sulfate, paralleled by strongly positive $\delta^{34}S$ values, indicative of a recent activity of SRB.

All samples contained dissolved organic carbon (DOC). Negative $\delta^{13}C$-values, indicative of microbial degradation of organic matter, were lowest in water samples of well 3. This groundwater contained the highest cell numbers (7.3×10^5 cells/mL) and additionally showed the highest percentage of FISH-detected cells with domain-specific probes EUB338 and ARCH915 (77.6% vs 28.5% in groundwater well 4). This indicates that well 3 harbored the most active microbial population of all groundwater wells sampled. Groundwater samples from well 3 were therefore chosen for further characterization of the microbial diversity in this aquifer.

Microbial Diversity. FISH results are summarized in Fig. 1. Of the total cell counts indicated by DAPI-

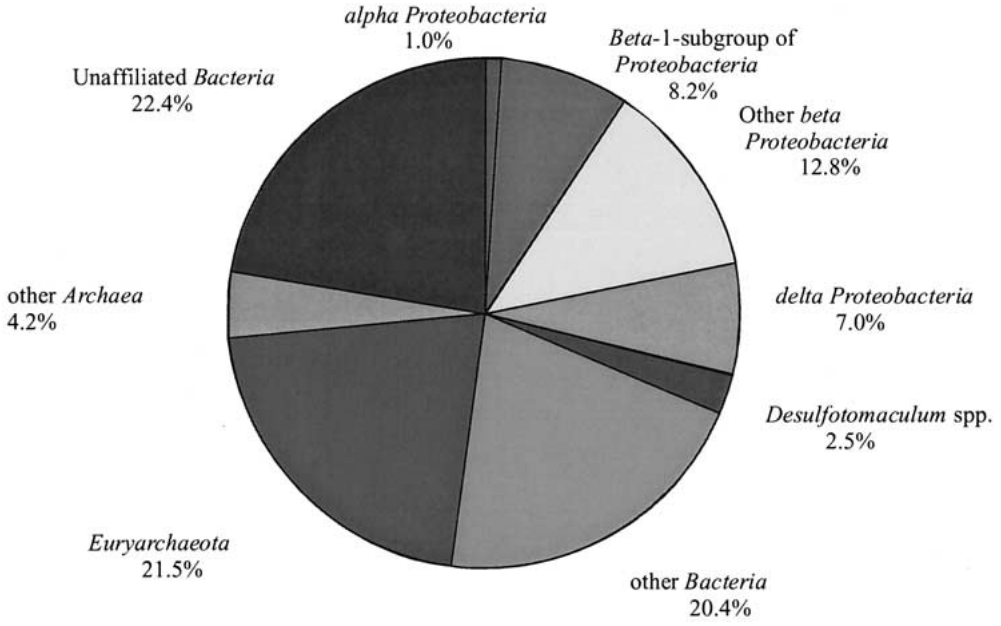

Figure 1. Microbial diversity revealed by FISH in groundwater well 3.

staining, 77.6% hybridized with probes specific for the domains Bacteria and Archaea. The Bacteria were more abundant than the Archaea (51.9% of total cells, Table 1). Among the cells detected with the bacterial probe EUB338, Proteobacteria were most abundant (29.0% of total cells). Within the Proteobacteria, members of the β-group were dominant (21.0% of total cells), whereas the α-group played a minor role (1.0% of total cells). A significant fraction of the β-Proteobacteria was affiliated with the β-1-subgroup (8.2% of total cells). Members of the γ- and ε-Proteobacteria, the Cytophaga–Flavobacterium cluster, Planctomycetales, and Gram-positives with high G+C DNA (Table 2) were below the detection limit for FISH. The majority of cells detected with the archaeal probe ARCH915 were affiliated with Euryarchaeota (21.5% of total cells).

Sulfate-Reducing Microorganisms. The general probe SRB385 hybridized with 7.0% of total cells in samples from groundwater well 3. This probe covers most bacteria of the δ-Proteobacteria, but also targets cells from other taxa (e.g., *Clostridia* and other Gram-positive bacteria) and might therefore cause an overestimation of this group. More specific probes for certain genera of Gram-negative SRB (see Table 2) showed no hybridization with cells in the samples. Members of the genera *Desulfobotulus*, *Desulfobulbus*, *Desulfomicrobium*, *Desulfomonile*, *Desulfovibrio*, and *Syntrophus*, which have been frequently isolated from freshwater habitats [29, 35], were not found. The only positive result for SRB was with probe DTM229, which targets members of the genus *Desulfotomaculum*. Only *Desulfotomaculum acetoxidans*, *Desulfotomaculum alcaliphilum*, and *Desulfosporosinus orientis* (formerly *Desulfotomaculum orientis*-cluster) are not targeted by this probe [19].

Discussion

Microbial Activity. In this communication we describe the community composition of the groundwater-associated microbial population. Hydrochemical data of the characterized groundwater monitoring wells indicated anaerobic processes in three of the four sampled wells (wells 2, 3, and 4). Positive $\delta^{34}S$-values paralleled by low concentrations of dissolved sulfate in wells 3 and 4 are pointing strongly toward recent bacterial sulfate reduction [42, 43]. Nevertheless, sulfate might not be used exclusively as terminal electron acceptor in this system. The presence of high microbial activity is supported by the finding of the highest cell numbers and highest percentage of FISH-detected cells in these samples. Some cells may be undetectable due to spore-formation, starvation, dormancy, or cell death and are therefore considered to be an indicator for low *in-situ* activity [6]. Only groundwater samples from well 3 contained a microbial population with >75% hybridizable cells. In samples from other wells, <30% of DAPI-stained cells hybridized with EUB 338 and ARCH915 probes even though the other samples were retrieved from a similar depth (Table 1). Obviously, aquifer systems display a significant heterogeneity with respect to their microbial activity.

Microbial Diversity. FISH can be a suitable method for the quantification of phylogenetic groups in environmental samples. Nevertheless, this method has

Table 2. Oligonucleotide probes used in this study

Probe	*Specificity*	*Sequence (5′ → 3′)*	*Target site[a] (rRNA position)*	*FISH [FA][b]*	*Reference*
EUB338	Bacteria	GCTGCCTCCCGTAGGAGT	16S (338–355)	10	[5]
NON338		ACTCCTACGGGAGGCAGC	16S (338–355)	10	[50]
ARCH915	Archaea	GTGCTCCCCCGCCAATTCCT	16S (915–935)	35	[46]
CREN499R	Crenarchaeota	CCAGRCTTGCCCCCCGCT	16S (499–515)	0	[10]
EURY498R	Euryarchaeota	CTTGCCCRGCCCTT	16S (498–510)	0	[10]
ALF968	Alpha subclass of Proteobacteria	GGTAAGGTTCTGCGCGTT	16S (968–986)	35	[32]
BET42a	Beta subclass of Proteobacteria	GCCTTCCCACTTCGTTT	23S (1027–1043)	35	[28]
BONE23a	β1 group of Proteobacteria	GAATTCCATCCCCCTCT	16S (663–679)	35	[4]
GAM42a	Gamma subclass of Proteobacteria	GCCTTCCCACATCGTTT	23S (1027–1043)	35	[28]
CF319a	*Cytophaga–Flavobacterium* cluster	TGGTCCGTGTCTCAGTAC	16S (319–336)	35	[27]
PLA886	Planctomycetales	GCCTTGCGACCATACTCCC	16S (886–904)	35	[33]
HGC69a	Gram–positive with high G+C DNA content	TATAGTTACCACCGCCGT	23S (1901–1918)	20	[40]
LGC354a	Gram–positive with low G+C DNA	TGGAAGATTCCCTACTGC	16S (354–372)	35	[31]
LGC354b	Content	CGGAAGATTCCCTACTGC			
LGC354c		CCGAAGATTCCCTACTGC			
ARC94	*Arcobacter* spp.	TGCGCCACTTAGCTGACA	16S (94–111)	20	[45]
ARC1430	*Arcobacter* spp.	TTAGCATCCCCGCTTCGA	16S (1430–1447)	20	[45]
SRB385	SRB of the delta Proteobacteria plus several gram–positive bacteria (e.g., *Clostridium*)	CGGCGTCGCTGCGTCAGG	16S (385–402)	20	[5]
DSR651	*Desulforhopalus*	CCCCCTCCAGTACTCAAG	16S (651–668)	35	[29]
DSS658	*Desulfosarcina, Desulfococcus*, *Desulfofaba*, *Desulfofrigus*	TCCACTTCCCTCTCCCAT	16S (658–685)	60	[29]
DSV698	*Desulfovibrio*	GTTCCTCCAGATATCTACGG	16S (698–717)	35	[29]
DSV214	*Desulfomicrobium*	CATCCTCGGACGAATGC	16S (214–230)	10	[29]
DSV407	*Desulfovibrio*	CCGAAGGCCTTCTTCCCT	16S (407–424)	50	[29]
DSV1292	*Desulfovibrio*	CAATCCGGACTGGGACGC	16S (1292–1309)	35	[29]
DSD131	*Desulfovibrio*	CCCGATCGTCTGGGCAGG	16S (131–148)	20	[29]
DSMA488	*Desulfovibrio/Desulfomonile/Syntrophus*	GCCGGTGCTTCCTTTGGCGG	16S (488–507)	60	[29]
660	*Desulfobulbus*	GAATTCCACTTTCCCCTCTG	16S (660–679)	60	[15]
221	*Desulfobacterium*	TGCGCGGACTCATCTTCAAA	16S (221–240)	35	[15]
DSB985	*Desulfobacter/Desulfobacula*	CACAGGATGTCAAACCCAG	16S (985–1003)	20	[29]
DRM432	*Desulfuromonas/Pelobacter*	CTTCCCCTCTGACAGAGC	16S (432–449)	40	[38]
DTM	*Desulfotomaculum*				[19]
DSBO224	*Desulfobotulus*	GGGACGCGGACTCATCCTC	16S (224–240)	60	[29]

[a]Position in the 16S/23S rRNA of *E. coli*.
[b]Formamide concentrations in the hybridization buffer.

limitations when cell walls are impermeable, when target sites are poorly accessible, or when the cellular rRNA content is too low. This makes it unsuitable for samples where cellular activity is low. However, it would seem reasonable to use FISH for well 3 as metabolic activity is obviously high. By using FISH, 77.6% of the total cell number in samples from well 3 could be affiliated with specific domains, giving an overview of structural and functional aspects of the microbial population in this aquifer.

As in other freshwater systems, a large proportion of the abundant microorganisms belonged to the β-Proteobacteria [13, 17, 37]. The *Comamonas–Variovorax* group (β-1 subgroup) seems to be of particular importance. Microorganisms belonging to these taxa were frequently isolated from subsurface environments [7, 11, 22, 36, 37, 52]. Obviously β-Proteobacteria are generally well adapted to freshwater conditions and might be important for the oxidation of dissolved organic carbon.

The abundance of Euryarchaeota can most probably be attributed to methanogens. This finding correlates well with the detection of biogenic methane as identified by typical $\delta^{13}C$ signatures [42, 44]. The probe EURY498R also hybridizes with members of the genus *Archaeoglobus*, but the presence of these thermophilic sulfate-reducing archaea (temperature range for growth between 60 and 95°C) at this low temperature is very unlikely. Since sulfate concentrations are very low, carbon dioxide is important as an electron acceptor in this aquifer system. Fe^{3+} as electron acceptor was important only at lower depths, whereas Mn^{4+} was not relevant in this system [8]. In contrast to hydrogen-dependent methanogenesis, which would be outcompeted by sulfate reduction, the acetate-dependent methanogenesis would probably not be affected, because of the low ability of sulfate-reducing bacteria to compete for acetate as electron donor in nonmarine habitats [1]. The coexistence of acetoclastic methanogenic archaea, homoacetogenic bacteria, and

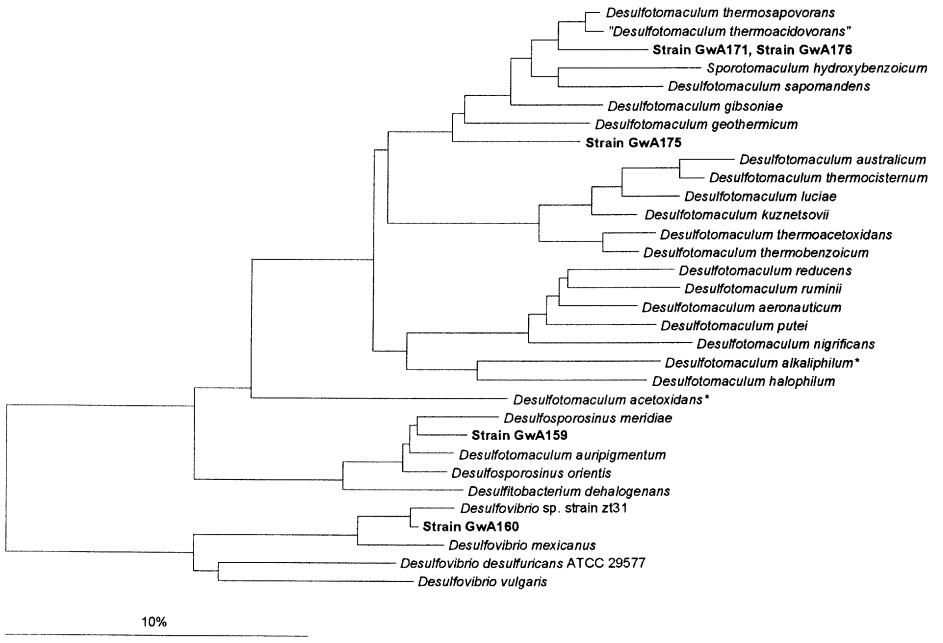

Figure 2. Phylogenetic position of sulfate-reducing isolates (in bold type) from a pristine aquifer. *Desulfotomaculum* spp., which are not detected with DFM229, are indicated with an asterisk. The tree was constructed using 1255 unambiguously aligned nucleotides using the maximum-likelihood method. All sequences belong to the Gram-positive bacteria except the *Desulfovibrios*, which are members of the δ-Proteobacteria and function as an outgroup.

sulfate-reducing bacteria has been demonstrated in deep granitic rock groundwater [23, 31].

In the investigated aquifer system SRB are present and active as indicated by the isotopic signature of the residual sulfate, depletion of sulfate and formation of sulfide in the groundwater [14]. Although many of the oligonucleotide probes which have been used seem to be restricted to marine SRB, the absence of *Desulfovibrio* spp. and *Desulfomicrobium* spp. in the groundwater was surprising. The positive results with SRB385 but negative results using more specific probes for SRB could be explained by the presence of members of the *Geobacteraceae.* These microorganisms use sulfur or ferric iron as electron acceptor or are growing by fermentation. The dominant population of SRB in our aquifer system are spore-forming members of the genus *Desulfotomaculum* (Fig. 1). These results perfectly reflect the isolates we have obtained from this aquifer system which were spore-forming SRB affiliated with *Desulfotomaculum* spp. and *Desulfosporosinus* spp. as indicated by comparative analysis of the 16S rRNA sequences (Fig. 2; [14]). All of these isolates are able to use a large variety of organic compounds as electron donors and show similar physiological properties as has been demonstrated for other members of this taxa (e.g., [24]). In contrast to *Desulfovibrio* and *Desulfomicrobium*, they are not restricted to a few organic compounds and hydrogen as electron donors. *Desulfotomaculum* spp. seem to be widespread in subsurface environments [7, 9, 12, 21]. Many *Desulfotomaculum* spp. can cope with low levels or complete absence of sulfate, because they can grow by fermentation of organic compounds or by homoacetogenesis using hydrogen and carbon dioxide [24]. It is possible that hydrogen, the favored electron donor for *Desulfovibrio* spp. and *Desulfomicrobium* spp., is not present together with sulfate at sufficient levels in the aquifer, or hydrogen is effectively removed by homoacetogenesis as is the case in similar environments [1, 23, 35]. Additionally, the formation of spores by *Desulfotomaculum* spp. allows survival under varying redox conditions. Generally Gram-positive bacteria play an important role in the subsurface [21, 52]. Members of the genera *Desulfosarcina* and *Desulfococcus* are widespread in marine environments [38, 41]. However, because of their universalistic physiological traits *Desulfotomaculum* spp. seem to be the counterpart in this niche in the subsurface.

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