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In Situ Analysis of Denitrifying Toluene- and *m*-Xylene-Degrading Bacteria in a Diesel Fuel-Contaminated Laboratory Aquifer Column

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A diesel fuel-contaminated aquifer was bioremediated in situ by the injection of oxidants (O_2 and NO_3^-) and nutrients in order to stimulate microbial activity. After 3.5 years of remediation, an aquifer sample was excavated and the material was used (i) to isolate bacterial strains able to grow on selected hydrocarbons under denitrifying conditions and (ii) to construct a laboratory aquifer column in order to simulate the aerobic and denitrifying remediation processes. Five bacterial strains isolated from the aquifer sample were able to grow on toluene (strains T_2 to T_4, T_6, and T_{10}), and nine bacterial strains grew on toluene and *m*-xylene (strains M_3 to M_7 and M_9 to M_{12}). Strains T_2 to T_4, T_6, and T_{10} were cocci, and strains M_3 to M_7 and M_9 to M_{12} were rods. The morphological and physiological differences were also reflected in small sequence variabilities in domain III of the 23S rRNA and in the 16S rRNA. Comparative sequence analyses of the 16S rRNA of one isolate (T_3 and M_3) of each group revealed a close phylogenetic relationship for both groups of isolates to organisms of the genus *Azoarcus*. Two 16S rRNA-targeted oligonucleotide probes (Azo644 and Azo1251) targeting the experimental isolates, bacteria of the *Azoarcus tolulyticus* group, and *Azoarcus evansii* were used to investigate the significance of hydrocarbon-degrading *Azoarcus* spp. in the laboratory aquifer column. The number of bacteria in the column determined after DAPI (4′,6-diamidino-2-phenylindole) staining was 5.8×10^8 to 1.1×10^9 cells g of aquifer material^{-1}. About 1% (in the anaerobic zone of the column) to 2% (in the aerobic zone of the column) of these bacteria were detectable by using a combination of probes Azo644 and Azo1251, demonstrating that hydrocarbon-degrading *Azoarcus* spp. are significant members of the indigenous microbiota. More than 90% of the total number of bacteria were detectable by using probes targeting higher phylogenetic groups. Approximately 80% of these bacteria belonged to the β subdivision of the class *Proteobacteria* (β-*Proteobacteria*), and 10 to 16% belonged to the γ-*Proteobacteria*. Bacteria of the α-*Proteobacteria* were present in high numbers (10%) only in the aerobic zone of the column.

Diesel fuel-contaminated soils and aquifers can be partially remediated by pumping hydrocarbons occurring in free phase back to the soil surface or by stripping the subsurface with air (7). Residual hydrocarbons, however, are often trapped in cracks and pores of the subsurface, and they may be removed by in situ bioremediation. This technique is usually based on the infiltration of water supplemented with oxidants (e.g., O_2 and NO_3^-) and/or nutrients (e.g., NH_4^+ and PO_4^{3-}) to stimulate the catabolic activity of microorganisms in the subsurface and thereby the biodegradation of the hydrocarbons (18, 23–25, 32).

An in situ bioremediation process was applied in a diesel fuel-contaminated aquifer in Menziken, Switzerland (23). Groundwater supplemented with O_2 (329 μM) and NO_3^- (1,020 μM) as electron acceptors and $NH_4H_2PO_4$ (75 to 125 μM) as the nutrient was infiltrated into the contaminated zone. At a monitoring well 20 m downstream of the infiltration point, the oxidants were found to be almost completely depleted (23). However, under field conditions the hydrocarbon mineralization and oxidant consumption could be analyzed only with poor spatial resolution, and the carbon mass, nitrogen, and electron balances could not be established accurately. Therefore, after the in situ bioremediation had been carried out for 3.5 years it was decided to excavate some of the contaminated aquifer material and simulate the processes in laboratory aquifer columns (20). These columns had lengths of 40.5 cm, and they were operated under continuous-flow conditions with artificial groundwater containing O_2 (4.4 mg liter^{-1}) and NO_3^- (1,610 μM). After a few days of operation, distinct redox zones were established. Within 24 cm, the concentration of O_2 decreased from 4.4 mg liter^{-1} to a value below the detection limit of 0.1 mg liter^{-1}. Denitrification was found to occur over the entire length of the column, regardless of the presence of O_2. Data on the evolution of dissolved inorganic carbon and the ratio of *n*-alkanes to *i*-alkanes suggested that biodegradation of hydrocarbons (initial hydrocarbon concentration, 1,100 μg g^{-1}) occurred over the entire column length (20).

Diesel fuel consists of a large variety of hydrocarbons that can be degraded under aerobic conditions and partially degraded under anaerobic conditions (8). As groundwater pollutants, aromatic hydrocarbons such as benzene, toluene, ethylbenzene, and xylene are of major concern since they are highly soluble in water and rather toxic. Diesel fuel contains on average 1.35 mg of toluene g^{-1} and 1.43 mg of *m*- plus *p*-xylene g^{-1} (30). During the last few years, many pure cultures of bacteria that are able to degrade toluene under aerobic and anaerobic—that is, denitrifying (12, 14, 17, 35, 40), iron-reducing (26), and sulfate-reducing (34)—conditions have been obtained. However, the significance of these isolates in their natural habitat, i.e., contaminated soil and aquifer material, is not known.

The aim of our study was to investigate the significance of the bacterial isolates in the diesel fuel-contaminated laboratory

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aquifer column. These isolates were obtained from the excavated aquifer material from Menziken and had the capacity to degrade toluene and/or *m*-xylene under denitrifying conditions. The studies included (i) the molecular characterization of the isolates by comparative sequence analysis of the 16S rRNA, and (ii) the use of specific rRNA-targeted oligonucleotide probes to enumerate the isolates by in situ hybridization.

MATERIALS AND METHODS

Isolation and growth conditions. Toluene- and *m*-xylene-degrading isolates were obtained from denitrifying enrichment cultures inoculated with excavated material from a diesel fuel-contaminated aquifer (Menziken, Switzerland) (23). Samples of the aquifer material (10 g) were added to 50 ml of oxygen-free basal medium (46) supplemented with 1.4 mM Na_2SO_4, 5 mM KNO_3, 1 ml of nonchelated trace element mixture SL10 (46) liter^{-1}, 1 ml of selenite-tungstate liter^{-1}, 0.5 ml of vitamin solution (41) liter^{-1}, 15 ml of $NaHCO_3$ solution liter^{-1}, and either 0.19 mM toluene or 0.16 mM *m*-xylene at pH 7.2. The cultures were incubated at 28°C for 10 weeks. Isolates were obtained after aerobic incubation of serial dilutions of the enrichment cultures on agar plates containing basal medium supplemented with 0.1% pyruvate and 0.05% KH_2PO_4. They were subsequently grown and maintained in liquid culture under denitrifying conditions with either 0.19 mM toluene or 0.16 mM *m*-xylene. The cultures were incubated at 25°C on a rotary shaker (100 rpm). Growth of all isolates on both substrates was also tested under aerobic conditions.

Molecular characterization of isolates. Cells of well-grown cultures of the isolates (3 ml) were harvested by centrifugation at 14,000 × *g* for 1 min. Cells were resuspended in 100 μl of distilled water and lysed by the addition of 50 μl of proteinase K (6 mg ml^{-1}; Appligene, Basel, Switzerland) and 1.5 μl of 10% sodium dodecyl sulfate (SDS) and incubation for 30 min at 37°C. Nucleic acids were extracted with phenol-chloroform, precipitated with ethanol, dried, and resuspended in distilled water (39).

Phylogenetic characterization was based on sequence comparison of approximately 1,450 bp of the 16S rRNA of two isolates, T_3 and M_3, and of a 240-bp fragment of the 23S rRNA of all 14 strains obtained. The 16S rRNA was amplified by PCR with oligonucleotide primer FGPS6 (5′GGAGAGTTAGAT CTTGGCTCAG), containing an internal *Bgl*II site (6), and primer 1510 (5′GT GCTGCAGGGTTACCTTGTTACGACT), extended with a *Pst*I site (13). The 23S rRNA fragment was amplified with primers 23InsV (5′CACAGATCTMA DGCGTAGNCGAWGG), extended with a *Bgl*II site (37), and 23InsR (5′CAC AAGCTTGTGWCGGTTTNBGGTA), extended with a *Hin*dIII site (37). Amplification by PCR was performed in a total volume of 100 μl containing 10 μl of 10× PCR buffer (500 mM KCl, 25 mM $MgCl_2$, 200 mM Tris-HCl [pH 8.4], 0.1% Triton X-100), 2 μl of deoxynucleoside triphosphates (each at 10 mM in 10 mM Tris-HCl, pH 7.5), 0.2 μl of *Taq* polymerase (5 U μl^{-1}; Appligene), 3 μl of both primers (100 ng each), and DNA (approximately 100 ng). Thirty rounds of temperature cycling (Crocodile II Thermocycler; Appligene) at 95°C, 50°C, and 70°C for 30 s each were followed by a final 7-min incubation at 70°C.

The amplification products were treated with proteinase K (final concentration, 60 ng per 100 μl; Appligene) at 37°C for 15 min (11), extracted with phenol-chloroform, and precipitated with ethanol (39). Subsequently, the DNA was cleaved with the restriction enzymes *Pst*I (Appligene) and *Bgl*II (Catalys; Promega, Wallisellen, Switzerland) for 16S rRNA and *Bgl*II and *Hin*dIII (Promega) for 23S rRNA fragments and cloned into pGEM-3zf (Promega) by standard methods (39). The DNA was transformed into *E. coli* DH5α, and plasmid preparation was performed by using the alkaline lysis method (39).

Cloned amplification products were sequenced with the Thermo Sequenase fluorescence-labeled primer cycle sequencing kit (Amersham, Zurich, Switzerland) according to the manufacturer's instructions. The sequences of the isolates were compared to published 16S rRNA sequences in the EMBL Nucleotide Sequence Database by FASTA analysis of the GCG sequence analysis package (17a).

Probes and stains. Oligonucleotide probes targeting rRNA of *Bacteria* (Eub338) (3), the α-, β-, and γ-*Proteobacteria* (Alf1b, Bet42a, and Gam42a) (27), gram-positive bacteria with high G+C DNA contents (HGC69a) (38), and probes Azo644 (5′GCCGTACTCTAGCCGTGC; positions 644 to 661 on the 16S rRNA according to *Escherichia coli* numbering [9]) and Azo1251 (5′CGCG CTTTGGCAGCCCT; positions 1251 to 1267 on the 16S rRNA according to *E. coli* numbering) were synthesized with a primary amino group at the 5′ end (MWG Biotech, Ebersberg, Germany). The fluorescent Cy3 Reactive Dye (Amersham) was covalently bound to the amino group of the oligonucleotide probe, and the dye-oligonucleotide conjugate (1:1) was purified from unreacted components and stored at −20°C at concentrations of 25 to 30 ng μl^{-1} (2).

The DNA intercalating dye 4′,6-diamidino-2-phenylindole (DAPI; Sigma, Buchs, Switzerland) was stored in a 1-mg ml^{-1} solution at −20°C (33). Staining was performed by the addition of DAPI to the hybridization reaction mixture (final concentration, 20 ng μl^{-1}) and was always used as a control stain to detect all bacteria present in the preparation.

Column operation and analytical procedures. A detailed description of the column operation and analytic protocols is given in reference 20. After 96 days of operation the column was disconnected and the aquifer material was frozen and stored at −20°C until further use.

Cell fixation and pretreatment. For whole-cell hybridization, the frozen aquifer core was cut into pieces 2.5 or 5 cm in length (see Fig. 3). This material and cells of pure cultures were fixed in 3 volumes of 4% paraformaldehyde in phosphate-buffered saline (PBS; 0.13 M NaCl, 7 mM Na_2HPO_4 and 3 mM NaH_2PO_4 [pH 7.2] in water) (19) for 3 h at 4°C. Fixed samples were washed with 50% ethanol in PBS and stored in 50% ethanol in PBS at −20°C (15).

Before being applied to slides, 40 μl of the aquifer homogenate was mixed with 960 μl of 0.1% sodium pyrophosphate and the material was dispersed with a 2-mm-diameter ultrasonic treatment probe (Sonifier B-12; Branson, Danbury, Conn.) at 20% for 1 min. Twenty microliters was subsequently applied to each well (8 mm in diameter) on gelatin-coated slides [0.1% gelatin, 0.01% $KCr(SO_4)_2$] and allowed to air dry (2). After dehydration in 50, 80, and 96% ethanol for 3 min each, the preparations were treated with lysozyme (Fluka, Buchs, Switzerland) (1 mg, corresponding to 37,320 U dissolved in 1 ml of distilled water) at 37°C for 10 min (16, 22), followed by a final dehydration in 50, 80, and 96% ethanol.

Whole-cell hybridization. Hybridizations were carried out in 8 μl of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl, 10 mM EDTA, 0.01% SDS [pH 7.2]) in the presence of 10 to 30% formamide (for Alf1b, 10%; for HGC69a, Azo644, and Azo1251, 20%; for Eub338, Bet42a, and Gam42a, 30%), 1 μl of the probe (25 to 30 ng μl^{-1}), and 1 μl of the DAPI solution (200 ng μl^{-1}) at 40°C for 2 h. After hybridization, the slides were washed in buffer containing 20 mM Tris-HCl, 10 mM EDTA, 0.01% SDS, and either 440, 308, or 102 mM NaCl, depending on the formamide concentration during hybridization (10, 20, and 30%, respectively), for 15 min at 48°C and subsequently rinsed with distilled water and air dried.

Samples were mounted with Citifluor (Canterbury, United Kingdom) solution and examined with a Zeiss (Oberkochen, Germany) Axiophot microscope fitted for epifluorescence detection with a high-pressure mercury bulb and filter sets 02 (DAPI) and HQ Cy3 (AHF Analysen Technik, Tübingen, Germany) (Cy3). Microorganisms were counted at a magnification of ×1,000 in 20 fields covering an area of 0.01 mm^2 each (15).

RESULTS AND DISCUSSION

Characterization of isolates. Five toluene-degrading isolates (T_2 to T_4, T_6, and T_{10}) and nine *m*-xylene-degrading isolates (M_3 to M_7 and M_9 to M_{12}) were obtained under denitrifying conditions from the excavated aquifer material from Menziken. Under denitrifying conditions, isolates M_3 to M_7 and M_9 to M_{12} grew on both toluene and *m*-xylene, while isolates T_2 to T_4, T_6, and T_{10} were able to grow only on toluene. Under aerobic conditions, all isolates were able to grow on toluene, but none of the isolates grew on *m*-xylene. The toluene-degrading isolates grew as cocci, while the *m*-xylene-degrading isolates grew as rods. The morphological and physiological differentiation between the two types of isolates was also reflected at the rRNA level. Sequence comparisons of domain III of the 23S rRNA of all isolates revealed identical sequences within the respective groups, which may indicate multiple isolations of the same organisms. Between the isolates of each group, however, similarity values of only 93% were found (Fig. 1). Large differences were also obtained by comparative sequence analyses of the complete 16S rRNA of representative isolates (T_3 and M_3) of each group, for which a similarity value of only 96.2% was obtained (Table 1).

Comparative 16S rRNA analysis revealed a close phylogenetic relationship of isolates T_3 and M_3 to organisms of the genus *Azoarcus* (Table 1). The sequences of the 16S rRNA showed 97 to 98% similarity to sequences of the *Azoarcus tolulyticus* group, which had been isolated from a petroleum-contaminated site and which had the capacity to degrade toluene under aerobic and denitrifying conditions (10, 17, 47). One of these organisms, *A. tolulyticus* Td-15, was also able to grow on *m*-xylene under denitrifying conditions. High levels of similarity to the toluene-, ethylbenzene-, and propylbenzene-degrading isolates ToN1, EbN1, and PbN1, respectively, which were phylogenetically classified as members of the β-*Proteobacteria* (35), were also obtained. Similarity values of about 98%, however, were also obtained with the 16S rRNA sequences of *Azoarcus evansii* KB740, which can use benzoate

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    1                                   30
T   GGATCTCAGG CGTAGCCGAT GGGAAACGGG
M   GGATCTCAGG CGTAGCCGAA GGGAAACAGG

    31                                  60
T   TCAATATTCC CGTACCGGTT CTGGATGCGA
M   TCAATATTCC TGTACCGATT TTAGATGCGA

    61                                  90
T   TGGGGGGACG GAGAAGGTTA GGCCAGCCGG
M   TGGGGGGACG GAGAAGGTTA GGTCAGCCGG

    91                                  120
T   GTGTTGGACG TCCCGGTTTA AGCGTGTAGG
M   GTGTTGGACG TCCCGGTTTA AGCGTGTAGG

    121                                 150
T   CGTGCCCCGT AGGCAAATCC GCGGGGATTA
M   CGTGCACCGT AGGCAAATCC GCGGAG.CTA

    151                                 180
T   AGCTGAGGCG TGATGACGAG GGCTCTTTGA
M   AGCTGAGGCG TGATGACGAG GTCTCTTTGA

    181                                 210
T   GCCCGAAGTG GTTGATACCA TGCTTCCAGG
M   GACCGAAGTG ACTAATACCA TGCTTCCAGG

    211                                 240
T   AAAAGCCTCT AAGCTTGAGT ATTCTATAGT
M   AAAAGCCTCT AAGCTTGAGT ATTCTATAGT
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FIG. 1. Sequence comparison of domain III, beginning at primer 23InsV, of the 23S rRNA of isolates T_2 to T_4, T_6, and T_{10} (*T*) and M_3 to M_7 and M_9 to M_{12} (*M*).

and 4-hydroxybenzoate but not toluene as a carbon source under denitrifying conditions (5). Another *Azoarcus* species, *Azoarcus indigens* VB32 (36), a nitrogen-fixing bacterium living in close association with Kallar grass (*Leptochloa fusca* (L.) Kunth), showed about 95% similarity to isolate T_3 and 98% similarity to isolate M_3. Though these results support an assignment of isolates T_3 and M_3 to the genus *Azoarcus*, a more detailed characterization of both isolates is required in order to resolve their definite taxonomic position within this genus.

Probe design. Based on the 16S rRNA sequences of isolates T_3 and M_3, 16S rRNA-targeted oligonucleotide probes (Azo644 and Azo1251, respectively) were designed with a database containing the 16S rRNA sequences of about 4,000 bacteria and the ARB probe design program (kindly provided by W. Ludwig, Technische Universität, Munich, Germany). Probe Azo644 was designed to detect both toluene-degrading and toluene- and *m*-xylene-degrading isolates, whereas probe Azo1251 was designed to differentiate between the isolates. A search for target sequences for both probes in the EMBL database showed identical sequences in the 16S rRNA of *A. tolulyticus* (strains Tol-4, Td-1, Td-15, Td-17, Td-19, and Td-21), *A. evansii* KB740, and *Azoarcus* sp. strain pF6. A sequence identical to the target sequence of probe Azo644 was also detected in the 16S rRNA of β-*Proteobacteria* strain PbN1, while the identical target sequence of probe 1251 was found in the 16S rRNA of β-*Proteobacteria* strain ToN1. Several strains showed target sequences with one mismatch to those of the probes. For *A. tolulyticus* Td-2 and Td-3, as well as for *Azoarcus denitrificans*, this mismatch represented a weak change which allowed detection of a sequence similar to the target sequences without a mismatch. In contrast, the mismatches in *Azoarcus* sp. strain BH72, *Thauera aromatica* K172, and β-*Proteobacteria* strains EbN1 and mXylN1 represented strong changes allowing a differentiation between these sequences and the target sequence under high-stringency hybridization conditions (31).

The applicability of both probes to whole-cell hybridization and their specificity were demonstrated by whole-cell hybridization on fixed cells of the isolates, *A. tolulyticus* Td-15, *A.*

TABLE 1. Percent similarity between the 16S rRNAs of toluene- and *m*-xylene-degrading isolates T_3 and M_3 and closely related organisms[a]

Organism (GenBank accession no.)	% Similarity to organism:																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1. T_3 (Y11041)	100																				
2. M_3 (Y11040)	96.2	100																			
3. *A. tolulyticus* Tol-4 (L33694)	97.7	97.9	100																		
4. *A. evansii* KB740 (X77679)	97.9	98.4	98.8	100																	
5. *A. tolulyticus* Td-1 (L33687)	98.1	97.9	99.9	98.8	100																
6. *A. tolulyticus* Td-2 (L33691)	97.9	97.7	99.7	98.6	99.7	100															
7. *A. tolulyticus* Td-15 (L33688)	97.4	98.1	98.8	99.6	98.8	98.6	100														
8. *A. tolulyticus* Td-17 (L33689)	97.7	98.2	98.4	99.2	98.4	98.2	99.4	100													
9. *A. tolulyticus* Td-19 (L33690)	97.2	97.7	98.1	99.2	98.1	97.9	98.9	98.4	100												
10. *A. denitrificans* (U82665)	97.4	97.2	98.8	97.8	98.9	98.5	97.6	97.6	97.2	100											
11. *Azoarcus* sp. strain pF6 (U44853)	97.9	98.4	98.8	100	98.8	98.6	99.6	99.2	99.2	97.8	100										
12. *Azoarcus* sp. strain BH72 (L15530)	95.9	95.4	96.4	95.6	96.4	96.2	96.1	95.9	95.5	95.7	95.9	100									
13. *A. indigens* VB32 (L15531)	94.9	98.1	95.5	94.9	95.3	95.2	95.1	94.4	94.8	94.2	95.3	97.5	100								
14. *Azoarcus* sp. strain S5B2 (L15532)	93.2	91.8	92.7	91.9	92.7	92.5	92.4	92.2	91.6	92.1	92.4	93.6	93.6	100							
15. *T. aromatica* K172 (X77118)	93.8	93.1	94.0	94.5	94.0	93.8	94.1	94.3	93.5	92.4	94.4	94.6	94.5	93.2	100						
16. β-*Proteobacteria* strain ToN1 (X83534)	97.8	98.5	98.8	99.8	98.8	98.6	99.4	99.0	99.1	98.1	99.8	96.0	95.3	92.1	94.4	100					
17. β-*Proteobacteria* strain PbN1 (X83532)	97.9	95.8	97.2	96.4	97.2	97.0	96.6	96.5	96.2	96.4	96.8	95.6	95.2	93.2	93.8	96.7	100				
18. β-*Proteobacteria* strain EbN1 (X83531)	97.5	95.4	97.1	96.2	96.4	96.9	96.5	96.4	95.8	96.3	96.6	95.6	95.2	93.2	94.5	96.3	98.3	100			
19. β-*Proteobacteria* strain mXyN1 (X83533)	94.1	93.4	94.3	94.7	94.3	94.1	94.4	94.6	93.9	92.7	94.7	95.0	94.7	93.3	99.7	94.6	94.0	94.7	100		
20. *Zoogloea ramigera* (X74913)	92.3	92.2	92.7	92.8	93.2	92.6	92.9	93.2	92.3	92.0	93.0	93.4	92.9	91.1	92.8	93.2	92.7	92.5	92.9	100	
21. *Rhodocyclus tenuis* (D16208)	90.8	90.1	90.8	90.6	90.7	90.5	90.7	90.7	90.2	90.9	90.7	91.3	91.3	93.1	90.8	90.8	90.5	90.5	90.9	91.3	100

[a] Calculated from data obtained from the EMBL nucleotide sequence database by using the GAP program with creation penality 5.0 and extension penality 0.3.

TABLE 2. Whole-cell hybridization of isolates used in this study and some reference bacteria with probes Azo644 and Azo1251

Organism(s)	Azo644[a]		Azo1251[b]	
	Hybridization	No. of mismatches	Hybridization	No. of mismatches
T_2 to T_4, T_6, and T_{10}	+	0	−	3
M_3 to M_7 and M_9 to M_{12}	+	0	+	0
A. tolulyticus Td-15	+	0	+	0
A. indigens VB32	−	3	−	3
T. aromatica K172	−	1	−	3
C. testosteroni	−	2	−	3
Alcaligenes sp.	−	3	−	3
N. europaea	−	4	−	3
B. cepacia	−	4	−	4

[a] 18 bp.
[b] 17 bp.

indigens VB32, and *T. aromatica* K172, and several reference organisms which were shown by the ARB program to contain target sequences similar to those of the isolates (*Alcaligenes* sp. [GenBank accession no. L31650], *Burkholderia cepacia* DSM 50181 [L28675], *Comamonas testosteroni* DSM 50251 [M11224], and *Nitrosomonas europaea* ATCC 25978 [M96399]). Under highly stringent hybridization conditions with 30% formamide, isolates T_2 to T_4, T_6, T_{10}, M_3 to M_7, and M_9 to M_{12}, as well as *A. tolulyticus* Td-15, were detected by whole-cell hybridization with Cy3-labeled probe Azo644 (Table 2). Probe Azo1251, however, hybridized only to isolates M_3 to M_7, M_9 to M_{12}, and *A. tolulyticus* Td-15 (Table 2; Fig. 2). Neither the reference organisms nor *A. indigens* VB32 or *T. aromatica* K172 showed hybridization signals to either of the probes under these conditions. These results demonstrate the applicability of whole-cell hybridization with probes Azo644 and Azo1251 for the detection of isolates T_2 to T_4, T_6, T_{10}, M_3 to M_7, and M_9 to M_{12}. The specificity of the probes for these isolates, however, is restricted because hybridization results obtained with bacteria of the *A. tolulyticus* group as well as *A. evansii* suggest a more general specificity for hydrocarbon-degrading *Azoarcus* spp.

Characterization of microbial populations in the column. Whole-cell hybridization with a combination of probes Azo644 and Azo1251 was subsequently used to study the significance of populations of isolates T_2 to T_4, T_6, T_{10}, M_3 to M_7, and M_9 to M_{12}, as well as the closely related bacteria of the *A. tolulyticus* group and *A. evansii*, in the laboratory aquifer column simulating the processes at Menziken. Data on the O_2, NO_3^-, and NO_2^- concentrations in this column were reported in detail by Hess et al. (20). The first 12 cm of the column was aerobic, with an O_2 concentration of >0.1 mg liter^{-1}, while the zone from 24 to 40.5 cm was anaerobic, with an O_2 concentration below the detection limit of 0.1 mg liter^{-1}. The zone between 12 and 24 cm had an O_2 concentration of approximately 0.1 mg liter^{-1} and was termed microaerobic. Total bacterial numbers determined after DAPI staining were highest in the aerobic (1.1×10^9 cells g of aquifer material^{-1} at 1.5 cm of column depth) and anaerobic (9.1×10^8 cells g of aquifer material^{-1} at 32 cm of column depth) zones of the column (Fig. 3c). In the aerobic zone, the large numbers of cells may have been due to favorable environmental conditions, since O_2 and NO_3^- consumption and hydrocarbon mineralization were high (Fig. 3b). In the anaerobic zone, the increase in cell numbers could be correlated to the increase in NO_3^- consumption. In the microaerobic zone of the column, bacterial numbers were low (5.8×10^8 cells g of aquifer material^{-1}). This result is also in

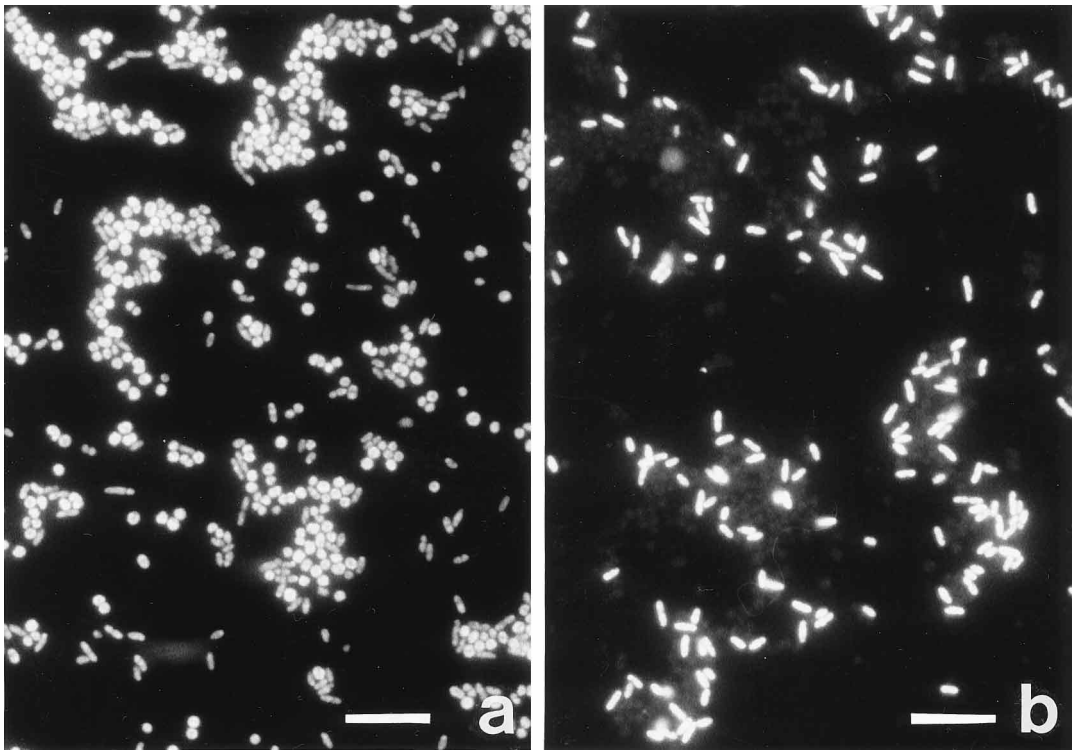

FIG. 2. Epifluorescence micrographs of mixtures of isolates T_3 (cocci) and M_3 (rods) after whole-cell hybridization with Cy3-labeled probes Azo644 (a) and Azo1251 (b). Bar, 5 μm.

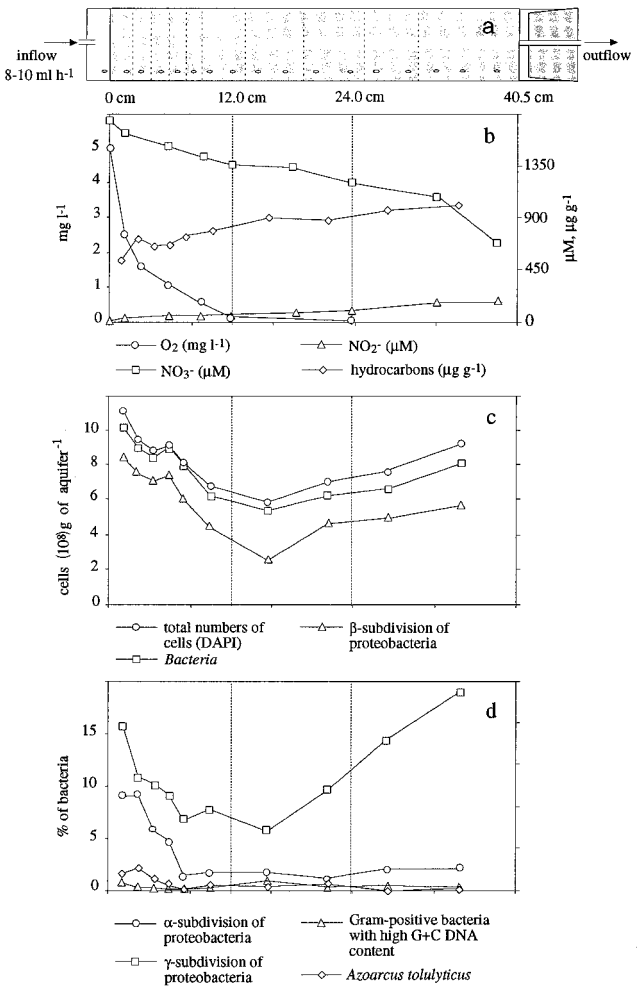

FIG. 3. (a) Column set-up; (b) concentrations of O_2, NO_3^-, and NO_2^- in groundwater, averaged over 96 days, and concentration of residual hydrocarbons after 96 days in the column (initial concentration, 1,100 μg of hydrocarbons g^{-1}); (c and d) results of in situ hybridization.

agreement with the chemical data, which showed that O_2 was almost depleted and that NO_3^- consumption was low (Fig. 3b).

Whole-cell hybridization with probe Eub338, targeting all bacteria, revealed that between 86 and 99% of DAPI-stained bacteria were detected throughout the column (Fig. 3c). The highest percentage (99%) was obtained in the aerobic zone; the lowest percentage (86%) was obtained in the anaerobic zone. The large percentage of cells detectable by whole-cell hybridization revealed the presence of metabolically active cells containing sufficient amounts of rRNA for detection and, at the same time, sufficient permeability for in situ detection with oligonucleotide probes (4). Comparably high percentages of cells detected by whole-cell hybridization have been reported for nutrient-rich environments such as activated sludge, with up to 89% detection (42–44), or lake snow, with 55 to 100% detection (45). In environments supplying less-eutrophic conditions, such as drinking water, 70% of surface-associated and 40% of planktonic cells were detected (28). In the winter cover and pelagic zones of a high mountain lake, the percentage of cells detected by whole-cell hybridization was 40 to 81% (1); in bacterioplankton, it was 35 to 67% (21); and in earthworm casts, it was up to 37% (15). Therefore, the large percentage of cells detected in our study by whole-cell hybridization also suggests that the aquifer column offered favorable growth conditions.

In this laboratory aquifer column, bacteria of the β-*Proteobacteria* were numerically dominant (Fig. 3c), accounting for between 80 and 87% of the bacteria detected by whole-cell hybridization in the aerobic zone. These numbers decreased to 42% in the middle of the column and then increased to 66% in the anaerobic zone (Fig. 3c). The remaining bacteria belonged mainly to the γ-*Proteobacteria*, with numbers between 10% in the aerobic zone and 16% in the anaerobic zone of the column, while bacteria of the α-*Proteobacteria* were detected in large amounts (10%) only in the aerobic zone (Fig. 3d). Bacteria with high G+C DNA contents were detected only in low numbers.

It was reported previously that the microbial communities in aquatic environments were also dominated by bacteria of the β-*Proteobacteria*, e.g., 27 to 42% of the cells in lake snow (45) or 6.5 to 116% in the winter cover and pelagic zones of a mountain lake (1). In earthworm casts, numbers of bacteria of the α- and γ-*Proteobacteria* exceeded numbers of bacteria of the β-*Proteobacteria* by far (15).

Our target organisms, i.e., the toluene- and *m*-xylene-degrading isolates, bacteria of the *A. tolulyticus* group, and *A. evansii*, were detected throughout the column (Fig. 3d) by whole-cell hybridization with a combination of probes Azo644 and Azo1251. Their populations were higher in the aerobic zone of the column close to the inlet (2%) than in the rest of the column (1%). Compared to genus-specific detection of, e.g., *Acinetobacter*, which is present in numbers that constitute less than 8% of the total bacterial community in activated sludge with enhanced biological phosphate removal (42, 44), the detection of hydrocarbon-degrading *Azoarcus* spp. in numbers between 1 and 2% suggests that these *Azoarcus* spp. may play an important role during bioremediation in diesel fuel-contaminated aquifer material.

Because of the extreme differences between column and field conditions, an extrapolation of the population data obtained in our laboratory aquifer column (scale, 40 cm, with very limited heterogeneity) to the field (scale, larger than 20 m, with considerable spatial heterogeneity) is difficult. More information on the catabolic activity of the hydrocarbon-degrading *Azoarcus* population in columns and the field will probably be obtained when recent advances in studies on the anaerobic toluene mineralization pathway (11, 29) lead to relevant genetic information. These sequences, we hope, will allow the design of probes directed against the mRNA of crucial catabolic enzymes and hence the determination of the in situ activity of microbial populations in the subsurface.

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