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Bacterial Community Dynamics during Start-Up of a Trickle-Bed Bioreactor Degrading Aromatic Compounds

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This study was performed with a laboratory-scale fixed-bed bioreactor degrading a mixture of aromatic compounds (Solvesso100). The starter culture for the bioreactor was prepared in a fermentor with a wastewater sample of a car painting facility as the inoculum and Solvesso100 as the sole carbon source. The bacterial community dynamics in the fermentor and the bioreactor were examined by a conventional isolation procedure and in situ hybridization with fluorescently labeled rRNA-targeted oligonucleotides. Two significant shifts in the bacterial community structure could be demonstrated. The original inoculum from the wastewater of the car factory was rich in proteobacteria of the alpha and beta subclasses, while the final fermentor enrichment was dominated by bacteria closely related to *Pseudomonas putida* or *Pseudomonas mendocina*, which both belong to the gamma subclass of the class *Proteobacteria*. A second significant shift was observed when the fermentor culture was transferred as inoculum to the trickle-bed bioreactor. The community structure in the bioreactor gradually returned to a higher complexity, with the dominance of beta and alpha subclass proteobacteria, whereas the gamma subclass proteobacteria sharply declined. Obviously, the preceded pollutant adaptant did not lead to a significant enrichment of bacteria that finally dominated in the trickle-bed bioreactor. In the course of experiments, three new 16S as well as 23S rRNA-targeted probes for beta subclass proteobacteria were designed, probe SUBU1237 for the genera *Burkholderia* and *Sutterella*, probe ALBO34a for the genera *Alcaligenes* and *Bordetella*, and probe Bcv13b for *Burkholderia cepacia* and *Burkholderia vietnamiensis*. Bacteria hybridizing with the probe Bcv13b represented the main Solvesso100-degrading population in the reactor.

Many branches of industry produce waste gases which contain odorous organic and inorganic components. Apart from the conventional thermal and physicochemical techniques for removal of pollutants from exhaust air, biological waste gas treatment is becoming more and more important. This kind of treatment is advantageous in cases in which the recovery of the components (e.g., absorption in liquids and adsorption in solids) or the utilization of a thermal process (thermal or catalytic combustion) is not economical. Today three different process variations for biological waste gas treatment are used: biofilters, bioscrubbers, and trickle-bed bioreactors. In biofilters and trickle-bed reactors, the pollutant-degrading microorganisms are immobilized on a carrier material, whereas in bioscrubbers the microorganisms are dispersed in the liquid phase. Biofilters and bioscrubbers are preferred in industry, while biofilters are common in compost production and sewage plants (10).

Biological waste gas treatment has a long tradition. Already in 1953, a soil system was employed for the treatment of odorous sewer exhaust gases in Long Beach, Calif. (25), and although up to now a lot of efforts have been funneled into process engineering (14, 17, 18, 24), current knowledge of the involved microorganisms is still very limited. Diversity of the microbial communities in the bioreactors for the exhaust gas purification have mostly been analyzed by culture-dependent methods (9, 12, 28, 31). However, there is a large discrepancy between the total (direct) microscopic cell counts and viable plate counts in many ecosystems and every cultivation medium selects for certain microorganisms. Therefore, cultivation-based studies of bacterial populations may give wrong impressions of the actual community structure in an ecosystem (35). A possible means of avoiding qualitative and quantitative errors in the analysis of microbial community structure in complex ecosystems is the use of fluorescently labeled, rRNA-targeted oligonucleotides (5) for the in situ identification and enumeration of bacteria. This method has already been used successfully in complex microbial communities, such as multispecies biofilms (6, 22, 26), trickling filters (27), and activated sludge (37).

The current study was performed with a laboratory-scale trickle-bed bioreactor degrading a mixture of aromatic compounds (Solvesso100). The starter culture for the inoculation of the bioreactor was an enrichment prepared in a fermentor which was itself started with a wastewater sample from a car painting factory as the inoculum and Solvesso100 as the sole carbon source. The goal of our study was to use for the first time fluorescent in situ hybridization (FISH) to investigate the microbial community structure and dynamics in the fermentor and the bioreactor during start-up. One of the open questions was whether the fermentor enrichment, which is done in suspension, indeed selects for those bacteria that later are immobilized in the bioreactor. In the course of this study, new 16S as well as 23S rRNA-targeted probes for phylogenetic groups within the beta subclass of the class *Proteobacteria* have been developed and applied in order to obtain a higher taxonomic resolution of the molecular techniques. The molecular data were compared to those obtained by traditional cultivation-dependent techniques.

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TABLE 1. Composition of the model pollutant Solvesso100 (7)

Component	Amount (%)
C_8 aromatics	
Ethylbenzene	0.1
p-Xylol	0.6
m-Xylol	0.7
o-Xylol	1.0
Total	2.4
C_9 aromatics	
Isopropylbenzene	0.7
n-Propylbenzene	4.6
1-Methyl-3-ethylbenzene	18.8
1-Methyl-4-ethylbenzene	8.7
1-Methyl-2-ethylbenzene	7.3
1,3,5-Trimethylbenzene	9.2
1,2,4-Trimethylbenzene	35.0
1,2,3-Trimethylbenzene	6.7
Total	91.0
C_{10} aromatics	
t-Butylbenzene	0.1
i-Butylbenzene	0.3
n-Butylbenzene	0.1
1-Methyl-2-isopropylbenzene	0.1
1-Methyl-3-isopropylbenzene	0.2
1-Methyl-4-isopropylbenzene	0.1
1-Methyl-3-*n*-Propylbenzene	0.9
1-Methyl-2-*n*-Propylbenzene	0.2
1-Methyl-4-*n*-Propylbenzene	0.1
1,3-Diethylbenzene	0.1
1,4-Diethylbenzene	0.3
1,2-Diethylbenzene	0.1
1,4-Dimethyl-2-ethylbenzene	0.3
1,3-Dimethyl-4-ethylbenzene	0.3
1,2-Dimethyl-4-ethylbenzene	0.4
1,3-Dimethyl-2-ethylbenzene	0.1
1,2-Dimethyl-3-ethylbenzene	0.5
1,2,4,5-Tetramethylbenzene	0.1
1,2,3,5-Tetramethylbenzene	0.1
Indan	0.5
Total	4.9
C_{11} aromatics	
1,3-Diethyl-5-methylbenzene	0.3
1-Methyl-3-*t*-butylbenzene	
t-Pentylbenzene	
Other C_{11} alkylbenzenes (not identified)	0.1
Total	0.8
Total amount of aromatics	99.1
Aliphates	0.9

MATERIALS AND METHODS

Model pollutant. Solvesso100 (Exxon Chemical GmbH, Cologne, Germany), a mixture of polyalkylated aromatic compounds, was used as a model of a solvent with limited solubility in water. Table 1 shows the typical composition of Solvesso100. It is produced on a large-scale basis from crude oil by catalytic reforming and is used as a solvent in many types of industrial paints, adhesives, and other products.

Starter culture. A sample of wastewater was collected from a car painting facility (BMW AG, Munich, Germany) to prepare a starter culture for the trickle-bed bioreactor. An aliquot of 100 ml (inoculum I) was used to inoculate a 12-liter laboratory fermentor (model L1523; Bioengineering AG, Wald, Switzerland), filled with mineral medium (17). Solvesso100 was delivered as the sole carbon source continuously with a peristaltic pump. For repeated batch fermentations, the following conditions were used: temperature, 30°C; stirring rate, 500 U/min; gas flow rate, 2.5 liters/min; Solvesso100 feed, 5 ml/h. The fermentor was operated under nonsterile conditions and without pH regulation. After 48 days, 3.5 liters of the exponentially growing, Solvesso100-adapted culture (inoculum II) was transferred to the trickle-bed bioreactor.

Trickle-bed bioreactor. For biological waste gas treatment, a laboratory-scale trickle-bed bioreactor was used. It was packed with hydrophilized polypropylene Ralu rings (Raschig AG, Ludwigshafen, Germany). These Ralu rings were perforated hollow cylinders with a length of 18 mm and a diameter of 18 mm. The specific surface area was 320 m^2/m^3; the porosity was 0.94. The experimental set-up is presented in Fig. 1. The volume of the column was 10.8 liters, and the height was 0.7 m. The synthetic waste gas was produced by evaporating a controlled amount of liquid into a stream of purified water-saturated air. The air flow rates were adjusted by electronic mass flow controllers (Brooks Instrument B.V., Veenendaal, The Netherlands). The gas entered the column continuously from the top. The recirculating mineral salt solution (17) was distributed continuously at the top by a simple liquid distributor and flowed concurrently with the gas downward through the column. The recirculating fluid served both as absorbent and as nutrient medium. The temperature in the bioreactor was kept constant at 30°C. All experiments were performed under nonsterile conditions. During start-up the reactor was operated with a Solvesso100 inlet concentration of 600 mg/m^3 and a gas flow rate of 100 liters/min, resulting in a specific pollutant load of 164 g/m^3h.

Analytics for Solvesso100. The degradation of the model pollutant Solvesso100 in the bioreactor was analyzed with a gas chromatograph (Fractovap model 4200; Carlo Erba Strumentazione, Milan, Italy) equipped with a flame ionization detector. Gas probes were drawn from the center line of the column at four different positions as indicated in Fig. 1 and measured successively. Automated data acquisition was performed by utilizing a multitasking real-time microcomputer system based on a Motorola 68000 microprocessor. The operating system RTOS-UH/PEARL, developed at the Institut für Regelungstechnik of the University of Hannover, Hannover, Germany, was used.

Sampling. Samples were collected from the wastewater of the car factory, from the fermentor after 6, 29, and 48 days, and from the bioreactor after 127 and 227 days. Ralu rings were also taken from the bioreactor. The biofilm from the Ralu rings taken at days 127 and 227 was scratched off the carrier under sterile conditions and resuspended in 35 ml of 0.9 M NaCl solution. Glass coverslips, which had been placed in the bottom of the bioreactor for microscopic observation of biofilm development, were fixed as described previously (6). For in situ hybridization, the samples were fixed for 3 h with paraformaldehyde as described before (1). The samples were stored in a 1:1 mixture of phosphate-buffered saline (130 mM sodium chloride, 10 mM sodium phosphate buffer, pH 7.2) and 96% ethanol at −20°C. The samples were also fixed by the addition of ethanol to a final concentration of 50%. In situ hybridizations with probes EUB338 and HGC68a were performed on such ethanol-fixed samples, whereas paraformaldehyde-fixed samples were used for probing gram-negative bacteria.

Membrane filtration and staining with DAPI. Total cell counts were determined by membrane filtration and staining with 4′,6-diamidino-2-phenylindole (DAPI) as described before (35).

Oligonucleotide probes. All probe sequences, hybridization conditions, and references for this study are given in Table 2. Labeling of amino-linked oligonucleotides with carboxytetramethylrhodamine-5-isothiocyanate (Molecular Probes, Eugene, Oreg.) or 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide-ester (Boehringer GmbH, Mannheim, Germany) and purification of the oligonucleotide-dye conjugates were performed as described before (2). Three new probes specific for phylogenetic groups within the beta subclass of proteobacteria were designed by comparative sequence analysis. In situ hybridization conditions for the new oligonucleotide probes were optimized by gradually increasing the formamide concentration in the formamide buffer as previously described (20).

Dot blot hybridization. The specificities of 23S rRNA-targeted oligonucleotide probes were evaluated by dot blot hybridization of reference nucleic acids extracted from 96 pure cultures of bacteria (representing a diverse collection of taxa) with radioactively labeled probes. The extraction of the nucleic acids and their immobilization on nylon membranes were carried out as described previously (29). The membranes were prehybridized in a solution containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 7% sodium dodecyl sulfate, 10× Denhardt solution (0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone, 0.2% Ficoll), and 20 mM NaH_2PO_4 and incubated for 5 h at the hybridization temperature. Hybridization was performed in prehybridization solution containing 5 pmol of the ^{32}P-labelled probe for 3 to 16 h. The membranes were washed twice with 2× SSC–0.1% SDS at hybridization temperature for 10 min. When the hybridization did not show the expected specificity, the washing procedure was repeated at a higher temperature.

Organisms and culture conditions. The organisms used in this study are listed in Table 3. They were grown as indicated in the respective catalogs of strains.

In situ hybridization and probe-specific cell counts. Fixed samples were immobilized on glass slides by air drying, and in situ hybridizations were performed as described by Snaidr et al. (32). Probes BET42a, GAM42a, and BONE23a were used with competitor oligonucleotides as described earlier (23). Slides were examined with an Axioplan microscope (Zeiss, Oberkochen, Germany) using filter sets 01 (for DAPI staining), 09, and 15. For each probe, more than 5,000 cells stained with the probe EUB338 were enumerated. Color photomicrographs were taken with Kodak Panther 1600X films, whereas black-and-white photomicrographs were done with Tmax400 films. Exposure times were 0.01 to 0.06 s for phase-contrast micrographs and 8 to 30 s for epifluorescence micrographs.

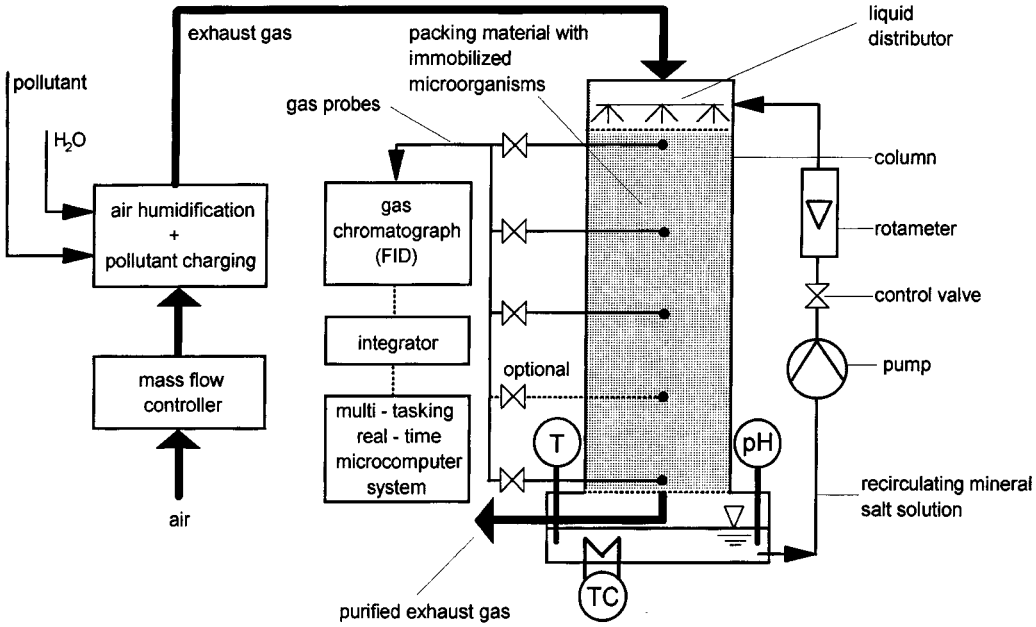

FIG. 1. Schematic diagram of the experimental set-up of the trickle-bed bioreactor. T, temperature sensor; TC, temperature control; FID, flame ionization detector.

Plate counts and cultivation. Serial dilutions in the range of 10^{-1} to 10^{-10} were plated in duplicate on yeast dextrose (YD) agar (tryptose [10 g/liter], yeast extract [2.5 g/liter], dextrose [1 g/liter], NaCl [7 g/liter], agarose [15 g/liter]; pH 7.4), mineral medium (17) with succinate (1 g/liter) as the sole carbon source, and malt extract agar (malt extract [30 g/liter], soy peptone [3 g/liter]; pH 5.6) to determine the total viable counts. The plates were scored after incubation at 30°C for 7 days. Ninety randomly chosen colonies were further cultivated for identification by classical methods and in situ hybridization. Fifty-four isolates were from the biofilm sample, and 36 isolates were from the liquid phase of the trickle-bed bioreactor.

Characterization of isolates. The isolates were subjected to the Gram staining procedure described by Eikelboom and van Buijsen (15) and catalase and oxidase tests. Their morphology and mobility were determined by phase-contrast microscopy. For in situ hybridization, the isolates were cultured in YD agar and cells growing in the logarithmic phase at an optical density at 600 nm (OD_{600}) of 0.5 to 0.8 were harvested, washed, and fixed as previously described (36). The oligonucleotide probes listed in Table 2 were used for identification of the isolates.

The ability of the pure culture isolates to grow with Solvesso100 as the sole carbon source was tested on mineral salt agar. A filter paper soaked with Solvesso100 was placed at the bottom of a glass petri dish. Then, the dishes were incubated in an air-tight metal box at 25°C for 7 days. Solvesso100 is a highly volatile solvent and is almost insoluble in water, so it was not possible to use it as the carbon source in mineral medium as usual. Additionally, it was necessary to use glass plates since plastic plates were completely destroyed by Solvesso100.

RESULTS

Enrichment for Solvesso100-degrading bacteria in a fermentor. The pH of the fermentor inoculum was 7.2, and the dry weight was 265 mg/liter. The total cell count was 6.9×10^7 cells/ml, whereas on YD agar only 1.9×10^4 CFU/ml could be obtained. Solvesso100 was delivered as the sole carbon source continuously to the mineral salt solution in the fermentor. Bacterial growth in the fermentor was observed after an adaption phase of 16 h. The color of the fermentor culture changed during repeated batch fermentation from colorless to yellow, pink, brown, and finally dirty beige. The generation time of the fermentor culture as determined spectrophotometrically during exponential growth was 7 to 10 h. The maximum

TABLE 2. Oligonucleotide probes used in this study

Probe	Specificity	Probe sequence (5′ to 3′)	Target site[a] (rRNA positions)	% FA in situ[b]	Reference
EUB338	Bacteria	GCTGCCTCCCGTAGGAGT	16S (338–355)	0	1
ALF1b	Alpha subclass and several members of delta subclass of *Proteobacteria*, most spirochetes	CGTTCGYTCTGAGCCAG	16S (19–35)	20	21
BET42a	Beta subclass of *Proteobacteria*	GCCTTCCCACTTCGTTT	23S (1027–1043)	35	21
GAM42a	Gamma subclass of *Proteobacteria*	GCCTTCCCACATCGTTT	23S (1027–1043)	35	21
HGC69a	Gram-positive bacteria with high G+C DNA content	TATAGTTACCACCGCCGT	23S (1901–1918)	35	30
ALBO34a	*Bordetella* spp., *Alcaligenes* spp. (sensu stricto)	CGTGCCTTCAACCTGGCC	23S (699–716)	60	This study
Bcv13b	*Burkholderia vietnamiensis*, *Burkholderia cepacia*	GCTCATCCCATTTCGCTC	23S (255–277)	20	This study
SUBU1237	*Burkholderia* spp. and *Sutterella* spp.	CCCTCTGTTCCGACCATT	16S (1237–1254)	35	This study
BONE23a	Beta1 subgroup of *Proteobacteria*	GAATTCCATCCCCCTCT	16S (663–679)	35	4
PS56a	Most true *Pseudomonas* spp.	GCTGGCCTAGCCTTC	23S (1432–1446)	0	31
Ppu56a	*P. putida*, *P. mendocina*	GCTGGCCTAACCTTC	23S (1432–1446)	0	31
BTWO23a	Competitor for BONE23a	GAATTCCACCCCCCTCT	16S (663–679)	35	4

[a] *Escherichia coli* numbering (13).
[b] Percent formamide (FA) in in situ hybridization buffer.

TABLE 3. List of studied strains and results of dot blot hybridizations and FISH with oligonucleotide probes

Organism	Strain[a]	Hybridization with probe[b]				
		Bcv14b		ALBO34a		SUBU1237
		DBH	FISH	DBH	FISH	FISH
Beta subclass of *Proteobacteria*						
Acidovorax avenae avenae	LMG 2117^T	−	−	−	−	−
Acidovorax delafieldii	LMG 5943^T	−	−	−	−	−
Acidovorax konjacii	LMG 5691^T	−	−	−	−	−
Acidovorax temperans	LMG 7169^T	−	−	−	−	−
Alcaligenes denitrificans subsp. *xylosoxidans*	WS 2166^T	−	−	+	+	−
Ralstonia eutrophus	LMG 1199^T	−	−	−	−	−
Alcaligenes faecalis faecalis	LMG 1229^T	−	−	+	+	−
Aquaspirillum metamorphum	DSM 1837^T	−	−	−	−	−
Bordetella avium	LMG 1852^T	−	−	+	+	−
Bordetella parapertussis	LMG 1831^T	−	−	+	+	−
Burkholderia andropogonis	LMG 2129^T	−	−	−	−	+
Burkholderia caryophylli	LMG 2155^T	−	−	−	−	+
Burkholderia cepacia	LMG 1222^T	+	+	−	−	+
Burkholderia cepacia	DSM 50180	+	+	−	−	+
Burkholderia cepacia	DSM 50181	+	+	−	−	+
Burkholderia cepacia	LMG 6859	+	+	−	−	+
Burkholderia cepacia	LMG 6888	+	+	−	−	+
Burkholderia cepacia	LMG 6980	+	+	−	−	+
Burkholderia cepacia	LMG 6988	+	+	−	−	+
Burkholderia cepacia	LMG 10824	+	+	−	−	+
Burkholderia cepacia	LMG 6860 t1	+	+	−	−	+
Burkholderia cepacia	LMG 6860 t2	+	+	−	−	+
Burkholderia gladioli pv. gladiolii	LMG 2216	−	−	−	−	+
Burkholderia glumae	LMG 2196^T	−	−	−	−	+
Ralstonia pickettii	LMG 5942^T	−	−	−	−	+
Burkholderia plantarii	LMG 9035	−	−	−	−	+
Burkholderia solanacearum	LMG 2299^T	−	−	−	−	+
Burkholderia vietnamiensis	LMG 10929	+	+	−	−	+
Chromobacterium violaceum	LMG 1267^T	−	−	−	−	−
Comamonas terrigena	LMG 1253^T	−	−	−	−	−
Comamonas terrigena	LMG 2370	−	−	−	−	−
Comamonas acidovorans	LMG 1226^T	−	−	−	−	−
Comamonas testosteroni	LMG 1800^T	−	−	−	−	−
Hydrogenophaga palleronii	LMG 2366^T	−	−	−	−	−
Hydrogenophaga pseudoflava	LMG 5945^T	−	−	−	−	−
Hydrogenophaga taeniospiralis	LMG 7170^T	−	−	−	−	−
Iodobacter fluviatile	LMG 6630^T	−	−	−	−	−
Leptothrix discophora	LMG 8141	−	−	−	−	−
Neisseria canis	LMG 8383^T	−	−	−	−	−
Neisseria sicca	LMG 5290^T	−	−	−	−	−
Nitrosomonas europaea	ATCC 25978^T	−	ND	−	ND	ND
Rhodocyclus tenuis	LMG 4367^T	−	−	−	−	−
Rubrivivax gelatinosus	LMG 4311^T	−	ND	−	ND	ND
Simonsiella muelleri	LMG 7828^T	−	ND	−	ND	ND
Thiobacillus perometabolis	LMG 8564^T	−	ND	−	ND	ND
Variovorax paradoxus	LMG 1797^T	−	−	−	−	−
Vitreoscilla stercoraria	LMG 7756^T	−	−	−	−	−
Zoogloea ramigera	ATCC 25935	−	ND	−	ND	ND
Zoogloea ramigera	ATCC 19544	−	ND	−	ND	ND
Gamma subclass of *Proteobacteria*						
Escherichia coli	ATCC 11775^T	−	−	−	−	−
Citrobacter freundii	LMG 3246^T	−	ND	−	ND	ND
Klebsiella terrigena	DSM 2687	−	ND	−	ND	ND
Klebsiella plantarum	DSM 3069	−	ND	−	ND	ND
Proteus vulgaris	LMG 2096^T	−	ND	−	ND	ND
Aeromonas hydrophila	ATCC 7966^T	−	ND	−	ND	ND
Aeromonas schubertii	LMG 90745	−	ND	−	ND	ND
Pseudomonas aeruginosa	DSM 5007^T	−	−	−	−	−
Pseudomonas putida	LMG 2257^T	−	−	−	−	−
Pseudomonas fluorescens	DSM 50124	−	−	−	−	−
Acinetobacter calcoaceticus	ATCC 23055^T	−	−	−	−	ND
Acinetobacter lwoffii	ATCC 15309^T	−	ND	−	ND	ND
Shewanella putrefaciens	LMG 2268T	−	ND	−	ND	ND
Agrobacterium rhizogenes	DSM 30148	−	ND	−	ND	ND

Continued on following page

TABLE 3—*Continued*

Organism	Strain[a]	Hybridization with probe[b]				
		Bcv14b		ALBO34a		SUBU1237
		DBH	FISH	DBH	FISH	FISH
Alpha subclass of *Proteobacteria*						
Zoogloea ramigera	ATCC 19623	−	ND	−	ND	−
Paracoccus denitrificans	DSM 65^T	−	−	−	−	ND
Bradyrhizobium japonicum	LMG 6138^T	−	ND	−	ND	ND
Methylobacterium extorquens	DSM 1337^T	−	ND	−	ND	ND
Methylobacterium organophilum	LMG 6083	−	ND	−	ND	ND
Azorhizobium caulinodans	LMG 6463^T	−	ND	−	ND	ND
Rhizobium leguminosum		−	ND	−	ND	ND
Brevundimonas diminuta	DSM 1635	−	−	−	−	−
Brevundimonas vesicularis	WS 1654	−	ND	−	ND	ND
Sphingomonas paucimoilis	LMG 1227^T	−	ND	−	ND	ND
Sphingomonas yanoikuyae	LMG 11252^T	−	ND	−	ND	ND
Flavobacterium devorans	LMG 4017^T	−	ND	−	ND	ND
Cyanobacteria						
Anabaena sp.	ATCC 29151	−	ND	−	ND	ND
Anabaena variabilis	ATCC 29413	−	ND	−	ND	ND
Anacystis nidulans	ATCC 27144	−	ND	−	ND	ND
Gram-positive bacteria with high G+C DNA content						
Corynebacterium betae	DSM 20141	−	ND	−	ND	ND
Corynebacterium glutamicum	DSM 20300	−	ND	−	ND	ND
Rhodococcus ruber	DSM 43338	−	ND	−	ND	ND
Rhodococcus terrae	DSM 93249	−	ND	−	ND	ND
Micrococcus auranticus	ATCC 11731	−	ND	−	ND	ND
Arthrobacter citreus	DSM 20133	−	ND	−	ND	ND
Arthrobacter globiformis	DSM 20124	−	ND	−	ND	ND
Gram positive bacteria with low G+C DNA content						
Microbacterium imperiale	DSM 20530^T	−	ND	−	ND	ND
Lactobacillus curvatus	LTH 1702	−	ND	−	ND	ND
Lactobacillus fermentum	WS 1024T	−	ND	−	ND	ND
Streptococcus bovis	DSM 20480	−	ND	−	ND	ND
Enterococcus cecorum	LMG 12902^T	−	ND	−	ND	ND
Enterococcus hirae	LMG 6399^T	−	ND	−	ND	ND
Staphylococcus carnosus	DSM 20501^T	−	ND	−	ND	ND
Bacillus firmus	DSM 12^T	−	ND	−	ND	ND
Bacillus sphaericus	DSM 28^T	−	ND	−	ND	ND
Mycoplasma bullata	ATCC 4278	−	ND	−	ND	ND

[a] ATCC, American Type Culture Collection, Rockville, MD, USA; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; LMG, Laboratotium voor Microbiologie, Universiteit Gent, Ghent, Belgium; WS, Bakteriologisches Institut der Süddeutschen Versuchs- und Forschungsanstalt für Milchwirtschaft, TU München, Freising-Weihenstephan, Germany.

[b] +, positive for hybridization; −, negative for hybridization; ND, not determined; DBH, dot blot hybridization.

optical density was approximately 7 without and 12 with added $(NH_4)_2SO_4$.

Start-up of trickle-bed bioreactor. The trickle-bed bioreactor was inoculated with 3.5 liters of an exponentially growing fermentor culture (4.2×10^{12} cells) adapted to Solvesso100 for 48 days. The bacteria were allowed to immobilize on the packing material of the bioreactor by recirculating the liquid phase (liquid circulation rate, 300 liters/h) through the trickling filter system. During the first 15 min after inoculation, a rapid decrease in the optical density of the circulation fluid, from an OD_{600} of 3.5 to 2.9, could be observed and then the OD_{600} stabilized at about 2.5 for the next hours. In parallel, dry mass and total cell counts of the circulation fluid decreased by 24%. Five hours after the inoculation of the bioreactor, the OD_{600} started to rise again and reached 4.7 after 2 days before it decreased to low values again (0.5). This oscillation continued for the remainder of the experiment.

In correlation with the decrease during the liquid phase, a rapid increase of dry mass on the packing material could be observed right after inoculation of the bioreactor. After 1, 5, and 24 h it was 0.4, 0.9, and 3.7 mg/Ralu ring. During the next months the dry mass reached and maintained relatively constant values of about 15 mg/Ralu ring.

One hour after start-up of the trickle-bed bioreactor, 76% of biomass transferred to the reactor (dry mass 3.74 g) was still in the liquid phase while 17% was already immobilized on the packing material. The remaining 7% was no longer detectable. At 5 and 24 h after the inoculation of the reactor, the total biomass in the reactor had increased by 14 and 250%, respectively, assuming that the biomass on the packing material was evenly distributed.

Glass coverslips brought into the reactor before the start of the experiment were used to monitor the colonization of surfaces. Phase-contrast microscopy confirmed the rapid development of a biofilm. Already 1 h after the fermentor culture was transferred to the trickle-bed bioreactor, attachment of cells was apparent on the glass surfaces. Individual bacteria with a quite uniform morphology were evenly distributed over the slides. After 5 h single cells, dividing cells, and groups of cells could be observed. A monolayer which continued to grow and became multilayered within the following days was established after 1 day. Finally, a higher morphological heterogeneity and

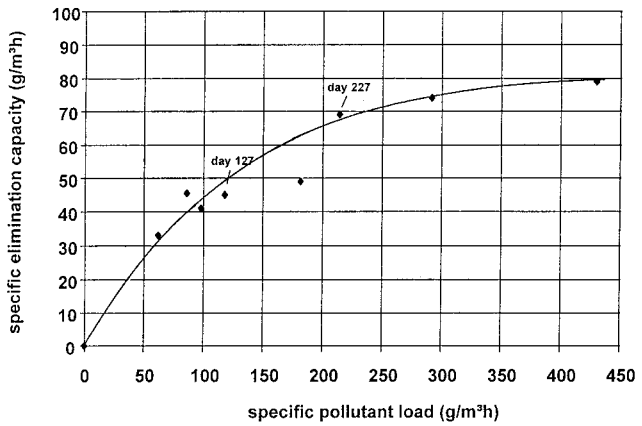

FIG. 2. Measured specific elimination capacity (defined as the difference of gas inlet and outlet concentrations divided by the mean gas residence time) versus specific pollutant load (defined as the gas inlet concentration divided by the mean gas residence time). The days when the samples for the microbial examinations were taken (days 127 and 227) are indicated.

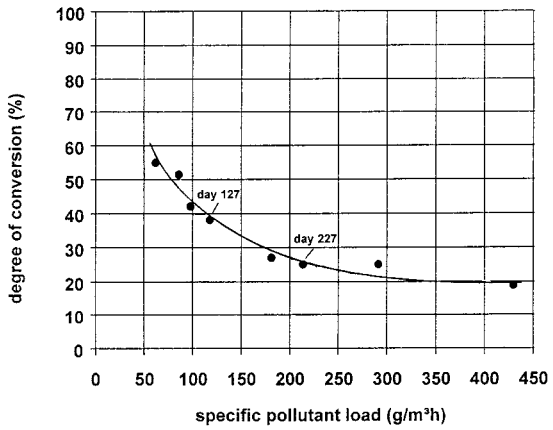

FIG. 3. Measured degree of conversion (defined as the difference of gas inlet and outlet concentrations divided by the gas inlet concentration) versus specific pollutant load. The days when the samples for the microbial examinations were taken (days 127 and 227) are indicated.

production of exopolymers was observed and the biofilm became clearly visible without a microscope.

Trickle-bed bioreactor performance. The trickle-bed bioreactor was monitored over a total period of 6 months. During this time, operation conditions, e.g., the gas inlet concentration and the gas flow rates, were changed several times in order to investigate the stability of the biological system and to evaluate the effects of various process parameters. The liquid circulating rate (300 liters/h) and the temperature (30°C) were, however, kept constant throughout the experiment. Already a few days after the start-up of the trickle-bed bioreactor, efficient degradation of Solvesso100 was achieved. The measured specific elimination capacity (defined as the difference of gas inlet and outlet concentrations divided by the mean gas residence time) is plotted versus the specific pollutant load (defined as the gas inlet concentration divided by the mean gas residence time) in Fig. 2. Figure 3 shows the degree of conversion, defined as the difference of gas inlet and outlet concentrations divided by the gas inlet concentration versus the specific pollutant load. A maximum specific elimination rate of approximately 80 g/m^{3}h was achieved. Depending on the pollutant load, about 20 to 60% of Solvesso100 was degraded. After a specific pollutant load of about 400 g/m^{3}h, the elimination rate did not increase with an increase of the pollutant load. The system maintained its full degradation capacity during variations in pH and discontinuity in the Solvesso100 supply. The pressure drop remained constant at about 20 to 30 Pa during the observed period.

Bacterial community dynamics in the fermentor and the trickle-bed bioreactor as determined by FISH. In the original wastewater sample that was used to inoculate the fermentor (inoculum I), 76% of the microbial cells visualized by DAPI staining were detected by the universal bacterial probe EUB338. Cells detected by probe ALF1b (16%) and probe BET42a (11%) were abundant, whereas only a few cells (<0.1%) were identified as belonging to the gamma subclass of *Proteobacteria* (Fig. 4). In the samples taken from the fermentor after 6, 29, and 48 days, 68, 77, and 90% of the cells, respectively, could be detected with probe EUB338. The hybridization signals became increasingly brighter. Moreover, the fraction of cells hybridizing with the bacterial probe EUB338 which could be identified with the group-specific probes ALF1b, BET42a, and GAM42a increased considerably. In the samples taken after day 6, a significant increase of bacteria belonging to the gamma subclass of *Proteobacteria* could be observed. Cells hybridizing with probe BET42a (30%) and GAM42a (31%) were more frequent than cells hybridizing with probe ALF1b (6%). At days 29 and 48, the fermentor was dominated by bacteria showing a positive hybridization signal with probe GAM42a (72 and 80%, respectively). The amount of cells detected with probe ALF1b decreased to <0.1%. Simultaneous hybridization of the sample taken on day 48 with probes Ppu56a, specific for *Pseudomonas putida* and *Pseudomonas mendocina*, and GAM42a showed that nearly 100% of the cells hybridizing with probe GAM42a also hybridized with probe Ppu56a (Fig. 5A).

Another significant shift in community structure was observed when the fermentor culture was transferred to the trickle-bed bioreactor (Fig. 4). At day 127, cells hybridizing with probe BET42a represented the major part of detectable cells (biofilm and liquid phase, 20 and 13%, respectively, of DAPI cell counts). Cells detected with probe ALF1b (5% in the biofilm, 2% in the liquid phase) were still common, whereas only a few cells were detected with probe GAM42a (0.1% in the biofilm, 0.3% in the liquid phase).

In the sample taken on day 227, the biofilm continued to be rich in bacteria belonging to the beta (16%) and alpha (13%) subclasses of *Proteobacteria*. With probe GAM42a, only 2% of the cells could be detected. In the liquid phase, significantly more cells hybridized with probe ALF1b (15%) than in the sample taken on day 127. Beta subclass proteobacteria (26%) dominate, whereas cells hybridizing with probe GAM42a (0.3%) are still sparse.

Interestingly, the monitoring of the glass coverslips taken out of the reactor 1 h, 5 h, 5 days, and 15 days after the inoculation showed that the changes in the community structure happened very early. One and 5 h after start-up, only bacteria belonging to the gamma subclass of proteobacteria could be detected. In the sample of the liquid phase after 5 h, the number of cells hybridizing with probes GAM42a (87%), BET42a (1.3%), and ALF1b (<0.1%) were in about the same ranges as those in inoculum I. In the 5-day-old biofilm, the community composition had dramatically changed. About 50% of the cells could be detected by probe GAM42a, and about 50% could be detected by probe BET42a. The bacteria belonging to the gamma subclass were significantly bigger than the beta proteobac-

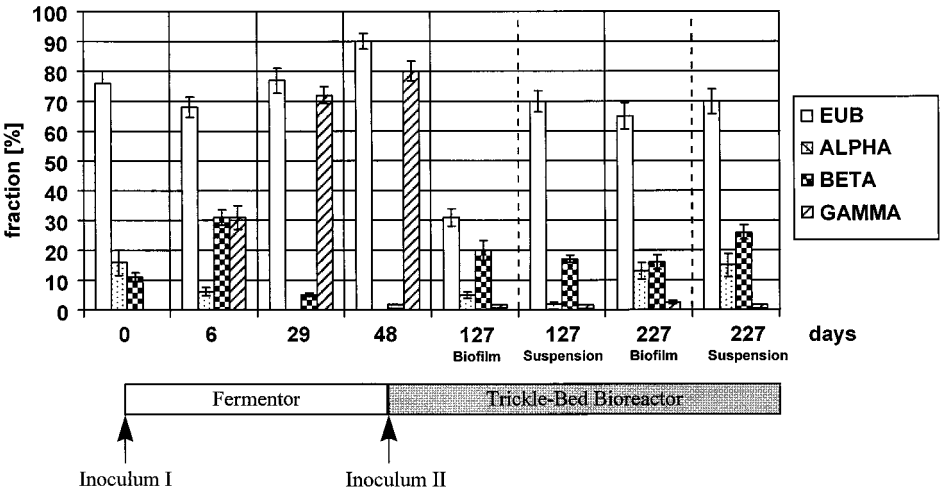

FIG. 4. Comparison of bacterial composition of the samples from the wastewater of a car factory (inoculum I), the fermentor, and the trickle-bed bioreactor (biofilm and liquid phase) as determined by in situ hybridization. Error bars, standard deviations.

teria. After 15 days, almost all bacteria that hybridized with the probe EUB338 also hybridized with probe BET42a. Cells assigned to the gamma subclass were no longer detectable.

Design and optimization of oligonucleotide probes SUBU 1237, Bcv13b, and ALBO34a. Based on the PROBE DESIGN tool of the software package ARB (33), three probes were designed and optimized to increase the taxonomic resolution of FISH. Probe SUBU1237 was targeted to a site on the 16S rRNA typical for the genera *Burkholderia* and *Sutterella* within the beta subclass of *Proteobacteria*. The probe sequence together with the exact target position is given in Table 2. A recent database check (October 1997) showed that all nontarget sequences had at least one mismatch with probe SUBU 1237. The difference in alignment in Fig. 6 displays the locations of mismatches in the 16S rRNA sequences of phylogenetically closely related bacteria. Binding of probe SUBU1237 to these bacteria could be completely prevented by addition of at least 60% formamide to the hybridization buffer (Table 3). Probe SUBU1237 overlaps partially with the already existing probe PPC (11), which was originally designed for *Burkholderia cepacia*. The modified version is specific for the genera *Burkholderia* and *Sutterella.*

Probes Bcv13b for *Burkholderia vietnamiensis* and *Burkholderia cepacia* and ALBO34a for the genera *Bordetella* and *Alcaligenes* (sensu stricto) are both targeted to the 23S rRNA. The oligonucleotide probe Bcv13b is complementary to a sequence in helix 13b/14a of *Burkholderia vietnamiensis* and *Burkholderia cepacia*, whereas probe ALBO is part of helix 34a of *Bordetella pertussis*, *Bordetella parapertussis*, *Bordetella bronchiseptica*, *Bordetella avium*, and *Alcaligenes faecalis*. Probe sequences and exact target positions are given in Table 2. Since the number of 23S rRNA sequences available from databases is still very limited, the specificity of the two 23S rRNA-targeted probes was checked by dot blot hybridization with nucleic acids isolated from 96 reference strains (Table 3). Under stringent hybridization conditions (hybridization temperature, 56°C; washing temperature, 60°C), probe Bcv hybridized only to *Burkholderia vietnamiensis* and *Burkholderia cepacia* strains and therefore allows the detection of a subgroup of those bacteria detected by probe SUBU1237. Probe ALBO34 showed the expected specificity at a hybridization temperature of 62°C and a washing temperature of 65°C. *Ralstonia eutrophus*, formerly *Alcaligenes eutrophus*, and the other *Ralstonia* spp. were not detected with this probe. The results of dot blot hybridization agreed well with the results of in situ hybridization with the fluorescently labeled probes. Sufficient specificity and sensitivity was obtained by applying formamide at a concentration of 20% in the hybridization buffer for the probe Bcv13b and at a concentration of 60% for probe ALBO34a.

In situ hybridization of the sample taken on day 127 with probes SUBU1237, ALBO34a, and Bcv13b. The sample taken on day 127 was dominated by members of the beta subclass of *Proteobacteria*. To further specify this group, biofilm and the liquid phase were hybridized with the three newly designed probes. With probe SUBU1237, specific for *Burkholderia* spp. and *Sutterella* spp., 92% of the cells belonging to the beta subclass of *Proteobacteria* showed a positive hybridization signal (Fig. 5B). Simultaneous hybridization with differently labeled probes SUBU1237 and Bcv13b demonstrated that the majority (83%) of the cells hybridizing with probe SUBU1237 also hybridized with probe Bcv13b, which is specific for *Burkholderia cepacia* and *Burkholderia vietnamiensis*. Probe ALBO34a hybridized to 3% of the bacteria belonging to the beta subclass. Together probes SUBU1237 and ALBO34a detected 96% of the beta subclass proteobacteria. The results in the liquid phase were similar: nearly all cells (98%) belonging to the beta subclass of *Proteobacteria* hybridized with probe SUBU1237 (91%) or probe ALBO34a (7%). With probe Bcv13b, 58% of the cells that hybridized with probe SUBU could be identified.

Cultivation and identification of isolates. Samples from day 127 were also plated on YD agar succinate agar, and malt extract agar. Plating efficiencies of the biofilm samples were surprisingly high: 91% on succinate-agar (3.2×10^8 CFU/ml compared to 3.5×10^8 cells/ml as visualized by DAPI) and 68% on YD agar. The plating efficiency in the liquid phase was lower: 36% on YD agar and 15% on succinate agar. Eighty-six randomly chosen colonies (51 isolates from the biofilm sample, 35 isolates from the liquid phase) from the three different cultivation media were identified by classical methods and by whole-cell hybridizations. All isolates, except for one coccus, were rods varying in size and width. They were all oxidase and catalase positive. Gram staining carried out with the isolates showed that 88% (45 out of 51) of the biofilm isolates and 63% (22 out of 35) of the isolates from the liquid phase were gram negative. The identification of the isolates retrieved from the biofilm by whole-cell hy-

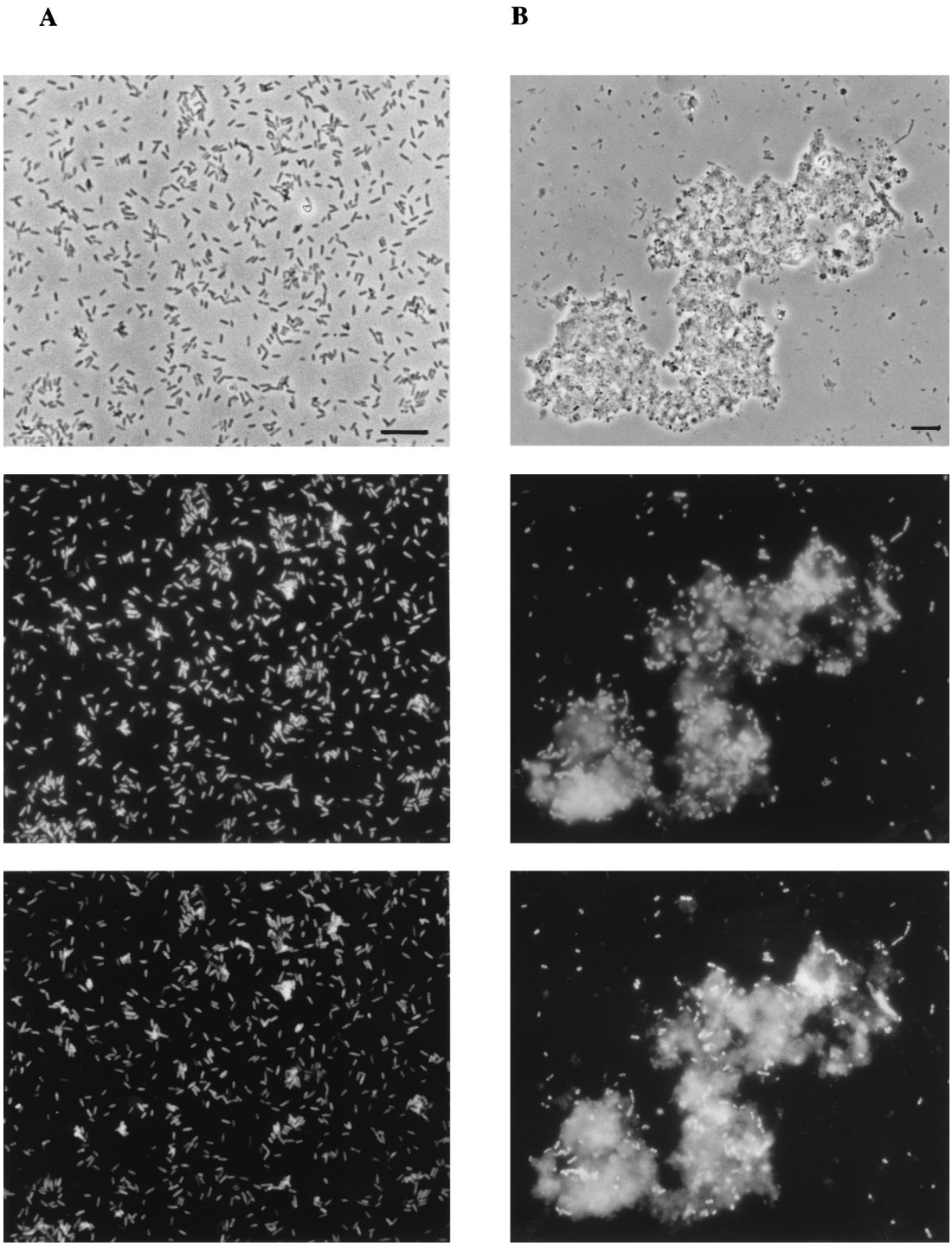

FIG. 5. In situ hybridization with fluorescein- and tetramethylrhodamine-labeled probes. Phase-contrast (upper panels) and epifluorescence micrographs (middle and lower panels) are shown for identical microscopic fields. Bar, 5 μm (all photomicrographs). (A) A sample from the fermentor at day 48 (inoculum II) was hybridized with fluorescein-labeled probe GAM42a (middle panel) and tetramethylrhodamine-labeled probe Ppu56a (lower panel). (B) A sample of the trickle-bed bioreactor biofilm at day 127 was hybridized with tetramethylrhodamine-labeled probe BET42a (middle panel) and fluorescein-labeled probe Bcv13b (lower panel).

bridization showed low complexity. All gram-negative isolates hybridized with probe BET42a, and 5 of the 6 gram-positive isolates hybridized with probe HGC69a. Hybridization of the colonies obtained from the liquid phase showed differences: still, most (20 of 22) of the gram-negative colonies could be detected with probe BET42a, but more colonies, 11 out of 35 (31%), were identified as members of the gram-positive bacteria with high DNA G+C content. One colony hybridized with probes ALF1b, and another hybridized with GAM42a. All 65 isolates from the biofilm and the liquid phase

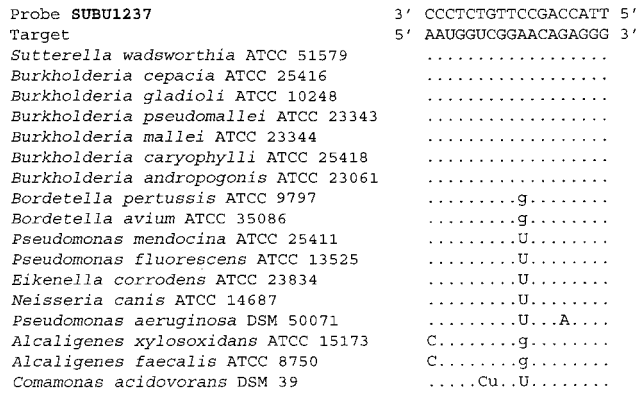

FIG. 6. 16S rRNA sequence alignment showing target regions of probe SUBU1237 for a selection of reference strains. Nucleotides are only identified for mispairings; pairings are indicated by dots. Lowercase letters indicate weakly destabilizing mispairing. Uppercase letters indicate strongly destabilizing mispairings.

belonging to the beta subclass of *Proteobacteria* also hybridized with probe SUBU1237, and 64 hybridized with probe Bcv13b.

The ability of the isolates to grow on Solvesso100 as the sole carbon source was tested to obtain some information about the distribution of pollutant-degrading and saphrophytic members of the biofilm and the liquid phase. From the biofilm isolates, 33 out of 51 (65%) readily grew with Solvesso100 as the sole carbon source, 9 out of 51 (18%) grew slowly, and the rest showed no visible growth. From the isolates of the liquid phase, 16 out of 34 (47%) grew well, 5 (15%) grew weakly, and 13 (38%) did not use Solvesso100 as the sole carbon source. Growth on Solvesso agar also varied among the different bacterial groups. Nearly all (95%) of the tested isolates that hybridized with probe Bcv13b were able to form large, smooth colonies on Solvesso agar. Among the isolates of the other phylogenetic groups growth on Solvesso100 was less frequent. Only 25% of the isolates detected with probe HGC69a and none of the colonies hybridizing with probes ALF1b and GAM42a could utilize Solvesso100.

DISCUSSION

The key finding of this study was that in our experiment the fermentor and the trickle-bed bioreactor selected for different bacteria capable of degrading aromatics. Whereas batch enrichment in the fermentor starting from a complex wastewater sample resulted in an almost pure culture of gamma subclass proteobacteria closely related to *P. putida* or *P. mendocina*, the community structure of the trickle-bed bioreactor inoculated with the fermentor enrichment returned to high diversity, with dominance of members of the beta and alpha subclass *Proteobacteria*—a situation, at least on the group level, similar to the one in the wastewater sample used to inoculate the fermentor. That the gamma subclass proteobacteria dominant in the fermentor were rapidly lost in the trickle-bed bioreactor was quite unexpected, since the bacteria in the starter culture were already adapted to the pollutant and immediately started the immobilization when they were transferred to the reactor, as shown by in situ hybridization. Furthermore, it is well known that *P. putida* and related bacteria readily form biofilms. Banks and Bryers (8) were able to show that a *P. putida* culture deposited on a developed biofilm of *Hyphomicrobium* sp. outgrew the hyphomicrobia and became dominant. Recently, *P. putida* was also visualized as a representative member of the toluene-degrading population in a biofilter for waste gas treatment by scanning confocal laser microscopy using a 16S rRNA-targeted probe (21). In the present study, the dynamics of the bacterial communities were analyzed by FISH and the plate count technique. Even though the results are somehow different, which is not surprising considering the different methods (35), molecular and classical data show the same clear-cut shift from *Pseudomonas* sp. in the fermentor to a mixture of mainly beta and alpha proteobacteria in the trickle-bed bioreactor.

Obviously, even though the start-up of the trickle-bed bioreactor was eventually successful, the pollutant adaptation in the fermentor did not enrich for high numbers of bacteria with an ability for long-term colonization in this particular trickle-bed bioreactor. This is an important result, since the inoculation of bioreactors with adapted fermentor enrichments is a common practice (14, 16, 19, 23). The growth conditions within the biofilm and the liquid phase of the trickle-bed bioreactor are significantly different from those of the fermentor. A fermentor might therefore in certain cases support growth of fast-growing, planktonic cells, whereas a trickle-bed bioreactor would select for a multispecies microbial community characterized by good immobilization and long-term stability.

The three new oligonucleotide probes for the beta subclass of *Proteobacteria* supplement a set of 16S rRNA-targeted probes for genera within the beta1 group of *Proteobacteria* (4). The combination of the nested probes BET42a, SUBU1237, and Bcv13b allowed a rapid and highly reliable identification and enumeration of *Burkholderia cepacia* and *Burkholderia vietnamiensis* in the samples of the trickle-bed bioreactor. This application is an example of the successful combination of 16S and 23S rRNA-targeted probes for the nested identification of bacteria in a complex ecosystem (3) and enabled us to identify the dominant groups of the beta subclass proteobacteria. Among the representatives of the beta subclass of *Proteobacteria*, complexity was quite low. Apparently, the conditions in the trickle-bed bioreactor were so demanding that only a very limited number of bacteria are able to survive.

As already shown in earlier studies (34, 35), the results obtained after cultivation were different from the direct in situ counts. Every cultivation medium is selective; even still, in this case the main Solvesso100 degraders seem to be readily cultivable. The dominance of bacteria belonging to the beta subclass of *Proteobacteria* and the abundance of significant numbers of bacteria belonging to the gamma subclass of *Proteobacteria* still are reflected by the colonies presented on the plates. The dominance of bacteria detected by probes SUBU 1237 and Bcv13b among the isolates is significantly higher than the in situ counts, while no isolate could be detected with the probe ALBO. One more obvious discrepancy between the two methods is shown in the results obtained with probe ALF. Even though three different media were chosen for the cultivation, no members of the alpha subgroup could be isolated from the biofilm. However, alpha subclass *Proteobacteria* from the liquid phase could be cultivated in numbers similar to those detected by in situ hybridization.

In this study, it was demonstrated that pollutant adaptation in fermentors might not enrich for those bacteria that establish in trickle-bed bioreactors. Future studies with the molecular and classical techniques used here should examine whether other inocula are more suitable for start-up and how the methods of pollutant adaptation can be modified to select for the right bacteria.

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