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16S rRNA Gene-Based Oligonucleotide Microarray for Environmental Monitoring of the Betaproteobacterial Order "*Rhodocyclales*"

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For simultaneous identification of members of the betaproteobacterial order "*Rhodocyclales*" in environmental samples, a 16S rRNA gene-targeted oligonucleotide microarray (RHC-PhyloChip) consisting of 79 probes was developed. Probe design was based on phylogenetic analysis of available 16S rRNA sequences from all cultured and as yet uncultured members of the "*Rhodocyclales*." The multiple nested probe set was evaluated for microarray hybridization with 16S rRNA gene PCR amplicons from 29 reference organisms. Subsequently, the RHC-PhyloChip was successfully used for cultivation-independent "*Rhodocyclales*" diversity analysis in activated sludge from an industrial wastewater treatment plant. The implementation of a newly designed "*Rhodocyclales*"-selective PCR amplification system prior to microarray hybridization greatly enhanced the sensitivity of the RHC-PhyloChip and thus enabled the detection of "*Rhodocyclales*" populations with relative abundances of less than 1% of all bacteria (as determined by fluorescence in situ hybridization) in the activated sludge. The presence of as yet uncultured *Zoogloea*-, *Ferribacterium*/*Dechloromonas*-, and *Sterolibacterium*-related bacteria in the industrial activated sludge, as indicated by the RHC-PhyloChip analysis, was confirmed by retrieval of their 16S rRNA gene sequences and subsequent phylogenetic analysis, demonstrating the suitability of the RHC-PhyloChip as a novel monitoring tool for environmental microbiology.

Members of the provisional betaproteobacterial order "*Rhodocyclales*" comprise a physiologically versatile assemblage of bacteria, many of them responsible for the removal of anthropogenic compounds in the environment or in biotechnological systems. While most members of the genera *Azoarcus* and *Thauera* can couple the anaerobic reduction of nitrate with the degradation of aromatic hydrocarbons (7, 40) or halogenated compounds (50), other *Azoarcus* species are associated with grass roots, where they fix nitrogen (17, 44). Furthermore, it has been recognized only recently that the "*Rhodocyclales*" genera *Dechloromonas* and *Azospira* harbor the dominant (per)chlorate-reducing bacteria in the environment (1, 11). Another important bioremediation process which exploits bacteria of the order "*Rhodocyclales*" to ameliorate anthropogenic damage is sewage treatment. For example, an uncultured bacterium provisionally named *Candidatus* "Accumulibacter phosphatis" catalyzes enhanced biological phosphorous removal in wastewater treatment plants (WWTPs) (13, 25, 59). Other "*Rhodocyclales*" to date also recalcitrant to cultivation were the numerically dominant bacteria in activated sludge from a nitrifying-denitrifying WWTP, where they presumably contributed to denitrification (27).

Due to their importance for bioremediation and agriculture, several approaches for detection of members of the order "*Rhodocyclales*" have been developed. Besides traditional cultivation methods (48), molecular detection of members of this order has been based on taxon- or clone-selective 16S rRNA gene-targeted PCR primers or probes (4, 13, 24–27, 43, 45). While these molecular methods are well suited for the detection of a few selected subgroups or species within the order "*Rhodocyclales*," tools for surveying the diversity of members of this order in parallel are missing. DNA microarrays, which have recently been introduced to microbial ecology (22) and generally fulfill all requirements for high-resolution monitoring of complex microbial communities (9, 10, 16, 32, 35, 41, 49, 55, 60–62), but a dedicated microarray for the order "*Rhodocyclales*" is not yet available.

In this study we have developed and applied a 16S rRNA gene-targeted oligonucleotide microarray consisting of 79 probes for the parallel detection of all bacteria of the order "*Rhodocyclales*" at different hierarchical or identical phylogenetic levels (RHC-PhyloChip). The use of three newly designed primer pairs for selective amplification of "*Rhodocyclales*" 16S rRNA genes prior to microarray hybridization allowed the detection of rare "*Rhodocyclales*" groups in activated sludge from an industrial sewage treatment plant. The microarray results were confirmed and extended by comparative 16S rRNA gene sequence and quantitative fluorescence in situ hybridization analyses.

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MATERIALS AND METHODS

Reference organisms. Tables 1 and 2 list the 12 pure cultures and the 17 16S rRNA gene-containing clones that were used to evaluate the RHC-PhyloChip.

TABLE 1. "*Rhodocyclales*" reference strains

Pure-culture species	Strain[a]	16S rRNA sequence accession no.
Azoarcus anaerobius	DSM 12081^T	Y14701
Azoarcus communis	DSM 12120	AF011343
Azoarcus evansii	DSM 6898^T	X77679
Azoarcus indigens	LMG 9092^T	L15531
Azoarcus sp.	LU1	AJ007007
Azonexus fungiphilus	LMG 19178^T	AF011350
Azospira oryzae (Dechlorosoma suillum)	DSM13638^T	AF170348
Dechloromonas agitata	DSM 13637^T	AF047462
Propionivibrio pelophilus	DSM 12018^T	AF016690
Rhodocyclus tenuis	DSM 109^T	D16210
Thauera mechernichensis	DSM 12266^T	Y17590
Thauera terpenica	DSM 12139^T	AJ005817

[a] Strains were obtained as lyophilized cells or active cultures. DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; LMG, Laboratorium voor Microbiologie, Universitat Gent, Belgium.

Sampling of activated sludge. Activated sludge samples were collected in June 2002 from the intermittently aerated nitrification-denitrification basin of an industrial WWTP. This treatment plant received its sewage from a rendering plant (Tierkörperbeseitigungsanstalt Kraftisried, Kraftisried, Germany). For DNA isolation, aliquots (4 ml) of the samples were pelleted by centrifugation (5,000 rpm for 2 min) at the treatment plant, immediately put on ice, and stored at −20°C upon arrival at the laboratory. For fluorescence in situ hybridization (FISH), an activated sludge sample was fixed at the WWTP with paraformaldehyde as outlined previously (15).

DNA extraction. Genomic DNA was isolated from reference organisms by using the FastDNA kit (Bio101, Vista, Calif.). DNA from Kraftisried activated sludge was extracted by using a modification (35) of a previously described protocol (21).

PCR amplification of 16S rRNA genes. For DNA microarray hybridization, 16S rRNA gene fragments from DNA of "*Rhodocyclales*" reference pure cultures were amplified by using the bacterial primer pair 616V and 630R (Table 3), whereas 16S rRNA gene inserts of reference clones were amplified with cloning vector-specific primers M13F(−20) (5′-GTAAAACGACGGCCAG-3′) and M13R (5′-CAGGAAACAGCTATGAC-3′) (Invitrogen Corp., San Diego, Calif.). Amplification of 16S rRNA gene fragments from DNA of the activated sludge sample was performed by using the bacterial primer pairs 616V and 630R and 616V and 1492R or the newly designed primer pairs A, R, or Z, each targeting different "*Rhodocyclales*" subgroups (Table 3).

Positive controls containing purified DNA from suitable reference organisms were included in all of the PCR amplification experiments along with negative

TABLE 2. "*Rhodocyclales*" reference 16S rRNA gene clones

16S rRNA gene clone	16S rRNA sequence accession no. or source	Insert region (*E. coli* numbering)
Kraftisried WWTP clones		
A13	AF072927	0008–1545
A16	AF234726	0008–1545
A33	AF072925	0008–1545
H7	AF234684	0008–1545
H23	AF072926	0008–1545
S3	AF072918	0008–1545
S21	AF234738	0008–1545
S23	AF072921	0008–1545
KRA34	AY689089	0094–1439
KRR56	AY689085	0175–1306
KRZ64	AY689092	0066–1439
KRZ65	AY689091	0066–1439
WWTP clones		
BNP269	N. Lee, unpublished data	0008–1511
hBPR4	N. Lee, unpublished data	0107–1263
hBPR24	N. Lee, unpublished data	0107–1263
Wadi Gaza clones		
WGAR24	AY687927	0107–1263
WGAR25	AY687928	0107–1263

TABLE 3. 16S rRNA gene-targeted primers

Short name[a]	Full name[b]	Annealing temp (°C)	Sequence 5′-3′	Specificity[c]	Reference
616V	S-D-Bact-0008-a-S-18	52	AGA GTT TGA TYM TGG CTC	Most *Bacteria*	28
630R	S-D-Bact-1529-a-A-17	52	CAK AAA GGA GGT GAT CC	Most *Bacteria*	28
1492R	S-*-Proka-1492-a-A-19	52, 60[c]	GGY TAC CTT GTT ACG ACT T	Most *Bacteria* and *Archaea*	Modified from 29
AT+94F[d]	S-*-AT-0094-a-A-18	60	GCC GGC GAG TGG CGA ACG	Genera *Azoarcus*, *Thauera*, and other bacteria	This study
ATD1420R[d]	S-*AT-1420-a-S-20	60	CCT ACT TCT GGT GAA ACC CA	Genera *Azoarcus*, *Thauera*, and *Denitromonas*	This study
RHC175F[e]	S-*-Rhc-0175-a-A-19	60	CCG CAT ATT CTG TGA GCA G	Genera *Candidatus* "Accumulibacter," *Rhodocyclus*, *Propionivibrio*, *Dechloromonas*, *Azospira*, and *Ferribacterium*	This study
RHC+1289R[e]	S-*-Rhc-1289-a-S-18	60	TCC GGA CTA CGA TCG GCT	Genera *Candidatus* "Accumulibacter," *Rhodocyclus*, *Propionivibrio*, *Dechloromonas*, *Azospira*, *Ferribacterium*, and other bacteria	This study
ZOGLO66F[f]	S-*-Zoglo-0066-a-A-18	60	ACG GTA ACA GGG AGC TTG	Genus *Zoogloea*, not *Z. resiniphila*	This study
ZOGLO1421R[f]	S-*Zoglo-1421-a-S-19	60	CCT ACT TCT GGT AAA CCC C	Genus *Zoogloea*	This study

[a] The short name used in the reference or in this study.
[b] The full name of the 16S rRNA gene-targeted oligonucleotide primer is based on the nomenclature of Alm et al. (2).
[c] Target organisms with a perfectly matched primer target site.
[d] Primer pair A.
[e] Primer pair R.
[f] Primer pair Z.

controls (no template DNA added). For 16S rRNA gene amplification, reaction mixtures (total volume, 50 μl) containing each primer at a concentration of 25 pM were prepared by using 10× *Ex Taq* reaction buffer and 2.5 U of *Ex Taq* polymerase (Takara Biomedicals, Otsu, Shiga, Japan). Additionally, 20 mM tetramethylammonium chloride (Sigma, Deisenhofen, Germany) was added to each amplification mixture to enhance the specificity of the PCR (31). Thermal cycling was carried out by using an initial denaturation step of 94°C for 1 min, followed by 30 cycles of denaturation at 94°C for 40 s, annealing at temperatures ranging from 52°C to 60°C (depending on the primer pair [Table 3]) for 40 s, and elongation at 72°C for 1.5 min. Cycling was completed by a final elongation step of 72°C for 10 min.

The presence and sizes of the amplification products were determined by agarose (1%) gel electrophoresis of the reaction product. Ethidium bromide-stained bands were digitally recorded with a video documentation system (Cybertech, Hamburg, Germany).

DNA microarray hybridization. 16S rRNA-targeted oligonucleotides were designed in silico by using the ARB probe design and probe match tools (37) and obtained from MWG Biotech (Ebersberg, Germany). Table 4 lists the sequence, specificity, and microarray position of all oligonucleotide probes. The theoretical melting temperatures (T_m) of the probes were calculated according to the nearest neighbor method by using the OligoAnalyzer 3.0 software with default settings (http://biotools.idtdna.com/analyzer/oligocalc.asp). For each probe and each reference organism, the free energies, ΔG, of the perfectly matched and the mismatched (up to 4.5 weighted mismatches; as determined by using the ARB probe match tool) probe-target hybrids were calculated. ΔG calculation was performed with the two-state hybridization server (concentration of Na^+ and temperature were set to 0.829 M and 42°C, respectively) at the mfold website (http://www.bioinfo.rpi.edu/applications/mfold/) (63). Additional information on RHC-PhyloChip probes can be viewed at the probeBase website (http://www.microbial-ecology.net/probebase) (34).

Each oligonucleotide probe contained a spacer element consisting of 15 dTTPs at the 5′ end and was aminated at the 5′-terminal nucleotide to allow covalent coupling to aldehyde group-coated CSS-100 glass slides (CEL Associates, Houston, Tex.). Fluorescence labeling of PCR amplicons, manufacturing and processing of microarrays, and reverse hybridization on microarrays were performed as outlined previously (35). The concentration of oligonucleotide probes before printing was adjusted to 50 pmol μl^{-1} in 50% dimethyl sulfoxide to prevent evaporation during the printing procedure. RHC-PhyloChips with triplicate spots for each probe were printed by using a GMS 417 contact arrayer (Affymetrix, Santa Clara, Calif.). Spotted DNA microarrays were dried overnight at room temperature in the dark to allow efficient cross-linking. Free aldehyde groups at the slide surface were reduced with sodium borohydride solution (35). For each reference organism, a separate microarray was hybridized, washed, and scanned under identical conditions and settings.

Scanning of microarrays and image analysis. Fluorescence images of the RHC-PhyloChips were recorded by scanning the slides with a GMS 418 array scanner (Affymetrix). The fluorescence signals were quantified by using the ImaGene 4.0 software (BioDiscovery, Inc., Los Angeles, Calif.). A grid of individual circles defining the location of each spot on the array was superimposed on the image to designate each fluorescent spot to be quantified. The mean signal intensity of each spot and the local background area surrounding each spot was determined. Subsequently, for each probe the signal-to-noise ratio (SNR) was calculated according to the following formula:

$$\mathrm{SNR} = [I_P - (I_N - I_{NLB})] \times {I_{PLB}}^{-1}$$

where I_P is the mean pixel intensity of all replicate probe spots, I_N is the mean pixel intensity of all nonsense probe spots, I_{NLB} is the mean pixel intensity of the local background area around all nonsense probe spots (note that $I_N - I_{NLB}$ must always have a lower value than I_P), and I_{PLB} is the mean pixel intensity of the local background area around all replicate probe spots. Probes for which the SNR was equal to or greater than 2.0 were considered positive (35). Furthermore, in the reference strain evaluation experiments the SNR of each probe was normalized against the SNR of the bacterial EUB338 probe, recorded on the same microarray, according to the following formula:

$$\mathrm{nSNR} = \mathrm{SNR} \times \{[I_{EUB} - (I_N - I_{NLB})] \times {I_{EUBLB}}^{-1}\}^{-1}$$

where nSNR is the normalized SNR of the specific probe, I_{EUB} is the mean pixel intensity of all EUB338 probe spots, and I_{EUBLB} is the mean pixel intensity of the local background area around all EUB338 probe spots.

Cloning and sequencing. Prior to cloning, the PCR amplification products were purified by low-melting-point agarose (1.5%) gel electrophoresis (NuSieve 3:1; FMC Bioproducts, Biozym Diagnostics GmbH, Oldendorf, Germany) and stained in SYBR Green I solution (10 μl of 10,000× SYBR Green I stain in 100 ml of TAE buffer [40 mM Tris, 10 mM sodium acetate, 1 mM EDTA, pH 8.0]; Biozym Diagnostics GmbH) for 45 min. Bands of the expected size were excised from the agarose gel with a glass capillary and melted with 80 μl of double-distilled water for 10 min at 80°C (this procedure was also done for the amplicon obtained with primer pair A, although no band was visible). Four microliters of each solution was ligated as recommended by the manufacturer (Invitrogen Corp.) into the cloning vector pCR2.1 of the TOPO TA cloning kit. Nucleotide sequences were determined by the dideoxynucleotide method (46) following a previously published protocol (42).

Phylogeny inference. All phylogenetic analyses were performed by using the alignment and treeing tools implemented in the ARB program package (37). All almost-full-length 16S rRNA sequences (>1,300 bases) which have been assigned to the order "*Rhodocyclales*" in the preview release of the RDP II database (version September 2003) (12) and all 16S rRNA sequences obtained from the activated sludge samples in this study were added to an ARB alignment of about 20,000 small-subunit rRNA sequences by using the alignment tool ARB_EDIT. Alignments were refined by visual inspection. Chimeric "*Rhodocyclales*" sequences were identified by independently subjecting base positions 1 to 513, 514 to 1026, and 1027 to 1539 (*Escherichia coli* numbering) of the 16S rRNA sequence to phylogenetic analysis. Inconsistent affiliation of the gene fragments in the phylogenetic trees was interpreted as being caused by a chimeric sequence. In addition, the CHECK_CHIMERA program of the RDP II was used for confirmation. Ambiguous base positions were excluded during calculation of 16S rRNA sequence similarities.

16S rRNA phylogenetic analyses were performed by applying distance-matrix, maximum-parsimony, and maximum-likelihood methods (36). A representative assortment of type strain sequences of different orders of the *Beta-* and *Gammaproteobacteria* was used as the outgroup for treeing. Variability of the individual alignment positions was determined by using the ARB_SAI tools and used as a criterion to remove or include variable positions for phylogenetic analyses. The neighbor-joining method combined with a Jukes-Cantor correction was used to infer distance-matrix trees. Maximum-likelihood trees were calculated by Tree-puzzle (54) and by applying the new A(x)ccelerated Maximum-Likelihood (AxML) algorithm (52). Maximum-parsimony analyses (treeing and bootstrapping) were performed with the Phylogeny Inference Package (PHYLIP, version 3.57c, J. Felsenstein, Department of Genetics, University of Washington, Seattle). For parsimony bootstrap analysis, 100 resamplings were used. All phylogenetic consensus trees were drawn according to recommendations outlined previously (36).

FISH. The abundance of selected "*Rhodocyclales*" groups in the activated sludge sample was determined by FISH combined with subsequent image analysis (14, 47). Fluorescently labeled oligonucleotide probes (Table 5) (34) were purchased from Thermo Hybaid (Ulm, Germany). Hybridization under optimal conditions was performed as described previously (27, 38).

Bacterial nomenclature. The names of the bacterial taxa were used in accordance with the prokaryotic nomenclature proposed in the latest taxonomic outline of the second edition of *Bergey's Manual of Systematic Bacteriology* (http://dx.doi.org/10.1007/bergeysoutline200310) (20).

Nucleotide sequence accession numbers. The sequences determined in this study were deposited at GenBank under accession numbers AY689085 to AY689093.

RESULTS AND DISCUSSION

16S rRNA-based phylogeny of "*Rhodocyclales*." The latest taxonomic outline of *Bergey's Manual of Systematic Bacteriology* lists 30 validly published species assigned into the 12 recognized genera of the "*Rhodocyclaceae*" (*Azoarcus*, *Thauera*, *Zoogloea*, *Azovibrio*, *Azospira*, *Rhodocyclus*, *Propionivibrio*, *Dechloromonas*, *Ferribacterium*, *Quadricoccus*, *Azonexus*, and *Sterolibacterium*), the only family within the betaproteobacterial order "*Rhodocyclales*." The order additionally encompasses the species "*Denitromonas aromaticus*" and the as yet uncultured *Candidatus* species "Accumulibacter phosphatis" (25), both of which still await valid description. In addition, 92 isolates and 104 environmentally retrieved 16S rRNA sequences affiliated to this order were included in the analysis.

To establish a robust and detailed phylogenetic backbone

TABLE 4. 16S rRNA-targeted probes used for microarray hybridization.

Original name	Name	Full name[a]	Primer[b]	Microarray position(s)	Sequence (5′-3′)	T_m (°C)	ΔG (kcal mol^{-1})	Specificity	Reference
	CONT-COMP				CTT CCT TCC TTC CTT CCT			Complementary to control oligonucleotide	35
	CONT			A1, A48, B1, B48, C1, C48, D1, D48	AGG AAG GAA GGA AGG AAG	51.3	−18.8	Control oligonucleotide	35
	NONSENSE			A2, A25, A47, C25, D47	AGA GAG AGA GAG AGA GAG	48.5	−17.7	Nonbinding control	35
UNIV1390	UNIV1389a	S-D-Univ-1389-a-A-18	R	A27, C27, D5	ACG GGC GGT GTG TAC AAG	58.3	−22.2	*Bacteria,* not *Epsilonproteobacteria*	Modified from 51
UNIV1390	UNIV1389b	S-D-Univ-1389-b-A-18	R	D6	ACG GGC GGT GTG TAC AAA	58	−21.9	*Eucarya*	Modified from 51
UNIV1390	UNIV1389c	S-D-Univ-1389-c-A-18	R	D7	ACG GGC GGT GTG TGC AAG	62	−23.9	*Archaea*	Modified from 51
EUB338	EUB338	S-D-Bact-0338-a-A-18		A3, A46, C26, D2, D46	GCT GCC TCC CGT AGG AGT	59.5	−22.4	Most *Bacteria*	5
EUB338II	EUB338II	S-*-BactP-0338-a-A-18		D3	GCA GCC ACC CGT AGG TGT	60.7	−23.0	*Planctomycetes*	14
EUB338III	EUB338III	S-*-BactV-0338-a-A-18		D4	GCT GCC ACC CGT AGG TGT	60.7	−23.0	*Verrucomirobia*	14
BTWO23a	BTWO663	S-*-Btwo-0663-a-A-18		A34, D9	GGA ATT CCA CCC CCC TCT	57	−21.0	Most *Rhodocyclales,* but not the *Zoogloea* and the *Azospira* lineages	Modified from 4
	RHC143	S-*-RHC-0143-a-A-18	R	A4, A19, A44, D19	TCG CTA CGT TAT CCC CCA	56	−21.2	Most members of the *Dechloromonas-Ferribacterium-Quadricoccus-Azonexus,* the *Azospira,* and the *Rhodocyclus-Propionivibrio-Accumulibacter* lineages, *Zoogloea* spp., and few members of the *Azoarcus-Thauera-Denitromonas* lineage	This study
S-G-Rhc-0175-a-A-18	RHC175a	S-*-RHC-0175-a-A-18		A42, D17	TGC TCA CAG AAT ATG CGG	53	−20.0	Most members of the *Dechloromonas-Ferribacterium-Quadricoccus-Azonexus,* the *Azospira,* and the *Rhodocyclus-Propionivibrio-Accumulibacter* lineages	25
S-G-Rhc-0439-a-A-18	RHC439	S-*-RHC-0439-a-A-18		A43, D18	CNA TTT CTT CCC CGC CGA	54.9–58.3		*Rhodocyclus* spp., most members of the *Candidatus* "Accumulibacter" cluster, *Azospira* lineage	25
	RHAC855	S-*-RhAc-0855-a-A-18		A7	TCA CGC GTT AGC TAC GGC	58.2	−22.8	*Rhodocyclus* spp., most members of the *Candidatus* "Accumulibacter" cluster	This study
PAO846	ACCBA846	S-G-Accba-0846-a-A-18		A10	AGC TAC GGC ACT AAA AGG	52.6	−19.7	Most members of the *Candidatus* "Accumulibacter" cluster	Modified from 13
PAO651	ACCBA651	S-G-Accba-0651-a-A-18		A6	CCC TCT GCC AAA CTC CAG	55.8	−20.9	Most members of the *Candidatus* "Accumulibacter" cluster	13
	ACCBA443	S-*-Accba-0443-a-A-18		A8	CAA GCA ATT TCT TCC CCG	52.2	−19.6	Some members of the *Candidatus* "Accumulibacter" cluster	This study
S-G-Rhx-0456-a-A-17	ACCBA455	S-*-Accba-0455-a-A-18		A9	AGG GTA TTA ACC CAA GCA	51.4	−18.8	Some members of the *Candidatus* "Accumulibacter" cluster	25
	RHO828	S-G-Rho-0828-a-A-18		A12	TTA ACC CCA CCA ACA CCT	54.4	−20.0	*Rhodocyclus* spp.	This study
	RHO842	S-G-Rho-0842-a-A-18		A13	CGG CAC TAA TGG GTT TAA	50.5	−18.8	*Rhodocyclus* spp.	This study
	RHOTE1280	S-S-Rho.te-1280-a-A-18		A15	CGA TCG GCT TTG CGG GAT	58.9	−22.6	*Rhodocyclus tenuis*	This study
	AZP471	S-G-Azp-0471-a-A-18		A29	GTA CCG TCA TCA ACA ACG	51.4	−19.5	Most *Azospira* spp.	This study
	AZP456	S-G-Azp-0456-a-A-18		A30	ACG GAT ATT AGC CGT TGC	53.1	−20.1	Most *Azospira* spp.	This study

	AZP737	S-G-Azp-0737-a-A-18		A28	GTC AGT ACT AAC CCA GGG	51.6	−19.1	Most *Azospira* spp.	This study
	DAF1030	S-*-D.A.F-1030-a-A-18		A22, A45, D20	TGT GTT CCA GCT CCC TTT	54.7	−20.2	Some bacteria of the *Dechloromonas-Ferribacterium-Quadricoccus-Azonexus* lineage	This study
	DEMFE455	S-*-DemFe-0455-a-A-18		A21	AGG GTA TTA ACC CAT GCG	52.5	−19.5	*Ferribacterium limneticum,* few *Dechloromonas* spp.	This study
	QUACO135	S-*-Quaco-0135-a-A-18		D40	TTA TCC CCC ACT CAA TGG	51.8	−19.0	*Quadricoccus australiensis,* reactor clone PHOS-HE23	This study
	DCMAG455	S-S-Dcm.ag-0455-a-A-18		A20	CAG GTA TTA GCT GAT GCG	50.7	−19.1	*Dechloromonas agitata*	This study
	A08KA458	S-*-A08KA-0459-a-A-18		D44	ACA CCC CGT ATT AGA GAG	50.7	−18.7	Oral strain A08KA lineage	This study
TH14	RHC175b	S-*-RHC-0175-b-A-18		A39, C4, C28, C40, D14	CCC TCA GGA CGT ATG CGG	57.9	−22.1	Some *Thauera, Azoarcus, Zoogloea, Sterolibacterium,* and *Azovibrio* spp.	Modified from 26
TH3	RHC222	S-*-RHC-0222-a-A-18		A36, B2, C2, C41, D11	ACA TCG GCC GCT CCA ATC	58.6	−22.4	Some members of the *Azoarcus-Thauera-Denitromonas, Zoogloea,* and *Azovibrio* lineages	26
	AZV211	S-G-Azv-0211-a-A-18		C42	TCC AAT CGC ACA AGG TCC	55.7	−21.2	*Azovibrio* spp.	This study
	AZVRE847	S-*-Azv.re-0847-a-A-18		C43	TAG CTC CGT TAC TAA TAG	45.1	−17.1	*Azovibrio restrictus*	This study
AT1458	ATD1459	S-*-ATD-1459-a-A-18	A,R,Z	A35, D10	TCT CAC CGT GGT AAG CGC	58	−22.4	Most members of the *Azoarcus-Thauera-Denitromonas* lineage	Modified from 43
	RHC630	S-*-RHC-0630-a-A-18		A40, D15, D25	TGC AGT CAC AAA CGC AGT	56.1	−21.2	Most *Thauera, Zoogloea,* and *Rhodocyclus* spp.	This study
	AZA1006	S-*-Aza-1006-a-A-18		A38, B4, D13	TCC CTG ATC TCT CAA GGA	52.1	−19.5	Most members of the *Azoarcus* cluster	This study
	AZA483	S-*-Aza-0483-a-A-18		B6	CTT CTT CTG ACA GTA CCG	49.7	−18.6	*Azoarcus* cluster	This study
Azo644	AZA645	S-*-Aza-0645-a-A-18		B5, B43	GCC GTA CTC TAG CCG TGC	58.3	−22.5	Most members of the *Azoarcus* cluster	24
Azo1251	AZA1252	S-*-Aza-1252-a-A-18		B7	TCG CGC TTT GGC AGC CCT	64.1	−24.8	*Azoarcus evansii, Azoarcus toluvorans, Azoarcus tolulyticus, Azoarcus toluclasticus*, and related *Azoarcus* spp.	Modified from 24
	AZA444	S-*-Aza-0444-a-A-18		B9	GGA AGC GTT TTC TTT CCG	52.4	−19.9	*Azoarcus evansii, Azoarcus tolulyticus* str. Td-3, *Azoarcus tolulyticus* str.Td-19, *Azoarcus sp.* ToN1	This study
	AZTOLY452	S-S-Az.toly-0452-a-A-18		B10	GTA TTG ACC CAC CCG ATT	52.4	−19.5	*Azoarcus tolulyticus*	This study
	AZANBU228	S-*-Az.an.bu-0228-a-A-18		B12	AAT CCG ACA TCA GCC GCT	57.7	−21.8	*Azoarcus anaerobius, A. buckelli,* and related *Azoarcus* spp.	This study
	AZA844	S-*-Aza-0844-a-A-18		B20	TGC GTC ACT CAG CGC GTT	61.1	−23.7	*Azoarcus* spp. PH002 and CR23	This study
	AZA452	S-*-Aza-0452-a-A-18		B19	CTA TTC ACG CAC CCG ATT	53	−20.0	*Azoarcus* spp. PH002 and CR23	This study
	AZA463	S-*-Aza-0463-a-A-18		B15	ATC CAG GCA CGC TAT TCA	54.6	−20.3	*Azoarcus* spp. PbN1 and HxN1	This study
	AZA835	S-*-Aza-0835-a-A-18		B16	CAG AAA GTT ACC TTC CCG	50.5	−18.8	*Azoarcus* sp. PbN1	This study
	AZAN465	S-S-Az.an-0465-a-A-18		B14	TCA TCC AGG CTC GCT ATT	54	−20.3	*Azoarcus anaerobius*	This study
	AZAN130	S-S-Az.an-0130-a-A-18	R	B13	CCC CTC GAC TGG GTA CGT	59	−22.2	*Azoarcus anaerobius*	This study
S-*-OTU2-0132-a-A-18	ATDe132	S-*-ATDe-0132-a-A-18	R	B24	CCC CCA CAA CAT GGG TAC	55.9	−20.9	Activated sludge clones A33, H25, H30, H35, S3, S10, and S23 of the *Azoarcus-Thauera-Denitromonas* lineage	27

Continued on following page

TABLE 4—*Continued*

Original name	Name	Full name[a]	Primer[b]	Microarray position(s)	Sequence (5′-3′)	T_m (°C)	ΔG (kcal mol^{-1})	Specificity	Reference
	ATDe442	S-*-ATDe-0442-a-A-18		B25	ACC CCG TTT CTT CCC AAC	55.7	−20.8	Activated sludge clones A33, H25, H30, H35, S3, S10, and S23 of the *Azoarcus-Thauera-Denitromonas* lineage	This study
	ATDe830	S-*-ATDe-0830-a-A-18		B26	CGT TAC CGC TCC GAA CAA	55.8	−21.4	Activated sludge clones A33, H25, H30, H35, S3, S10, and S23 of the *Azoarcus-Thauera-Denitromonas* lineage	This study
S-*-OTU2-0467-a-A-20	ATDe467	S-*-ATDe-0467-a-A-18		B27	CGT CAT TAG GAT CCT ATG	46.6	−17.2	Activated sludge clones A33, H25, H30, H35, S3, S10, and S23 of the *Azoarcus-Thauera-Denitromonas* lineage	Modified from 27
	S3-486	S-*-S3-0486-a-A-18		B31	GTG CTT CTT CCG TCG GTA	54.9	−21.4	Activated sludge clone S3	This study
	A33-587	S-*-A33-0587-a-A-18		B34	CAC CTG TCT TAC CAA ACC	50.7	−18.9	Activated sludge clone A33	This study
	DENAR176	S-S-Denar-0176-a-A-18		C16	TCC CTC AGG AAA TAT GCG	52.1	−19.6	*Denitromonas aromaticus*	This study
	DENAR453	S-S-Denar-0453-a-A-18		C17	CGT ATT CGG GGC GAT GAT	55.3	−21.0	*Denitromonas aromaticus*	This study
	DENAR845	S-S-Denar-0845-a-A-18		C18	GCT GCG TTA CCC AGA AAG	54.2	−20.6	*Denitromonas aromaticus*	This study
	AZCOM447	S-S-Az.com-0447-a-A-18		B42	AGC CCA CAC GTT TTC TTC	53.8	−20.2	*Azoarcus communis*	This study
TH5	AZIND1023	S-S-Az.ind-1023-a-A-18		A37, B3, B38, C3, D12	CTG GTT CCC GAA GGC ACC	58.9	−22.3	*Azoarcus indigens, Azoarcus* sp. BH72	Modified from 26
	AZIND433	S-S-Az.ind-0433-a-A-18		B39	CTT TCC GTC CGA AAG AGC	53.8	−20.5	*Azoarcus indigens, Azoarcus* sp. BH72	This study
	AZIND449	S-St-Az.ind-0449-a-A-18		B40	TTA GCC CGC GCG ATT TCT	58.2	−22.2	*Azoarcus* sp. BH72	This study
	AZIND455	S-St-Az.ind-0455-a-A-18		B41	CGG GTA TTG GCC GAA GCG	59.4	−23.0	*Azoarcus indigens* (T)	This study
	THAU832	S-G-Thau-0832-a-A-18		C5	TGC ATT GCT GCT CCG AAC	57	−21.7	*Thauera* spp.	This study
	THAU455a	S-*-Thau-0455-a-A-18		C6	ACT ATG TTA GAG TGC GCG	52.6	−19.9	*Thauera chlorobenzoica, Thauera mechernichensis*	This study
	THAU443	S-*-Thau-0443-a-A-18		C7	AAC ACG ATT TCT TCC CGG	53.2	−20.0	*Thauera selenatis, Thauera phenylacetica,* and related *Thauera* spp.	This study
	THAU580	S-*-Thau-0580-a-A-18		C8	CTT ACA AAA CCG GCC TCG	54.2	−20.6	*Thauera selenatis,* soil clone AX39	This study
	THAU455b	S-*-Thau-0455-b-A-18		C9	ACT ATG TTA GAG TCG CCG	51.6	−19.4	*Thauera* sp. PIV-1, TCB-transforming microbial consortium clone SJA-186	This study
	THAU468	S-*-Thau-0468-a-A-18		C10	CCG TCA TCC AGC GAC TAT	54.5	−20.6	*Thauera* sp. PIV-1, TCB-transforming microbial consortium clone SJA-186	This study
	THATE461	S-S-Tha.te-0461-a-A-18		C11	CCA CAC CCT ATG TTA GAG	49.2	−18.2	*Thauera terpenica*	This study
	ZOGLO828	S-G-Zoglo-0828-a-A-18		C30	TCT CCT CAC CGA ACA ACT	53.6	−20.1	*Zoogloea* spp.	This study
S-*-OTU1-1415-a-A-20	ZOGLO1416	S-G-Zoglo-1416-a-A-18		C29	TCT GGT AAA CCC CAC TCC	54.1	−20.2	*Zoogloea* spp.	Modified from 27
ZRA (ZRA23a)	ZOGLO647	S-*-Zoglo-0647-a-A-18		C31	CTG CCG TAC TCT AGT TAT	48.2	−17.8	Most members of the *Zoogloea* lineage, not *Z. resiniphila*	45

	ZOGLO455	S-*-Zoglo-0455-a-A-18		C32	AGA GTA TTA TCC TGC GCG	52.1	−19.6	Some members of the *Zoogloea* lineage (activated sludge clones H13, H11, H22, H10, H40, H27, and S21), not *Z. ramigera* and *Z. resiniphila*	This study
S-*-H7-1014-a-A-18	H7-1014	S-*-H7-1014-a-A-18		C33	TCG GGC ACC CCT CAA TCT	59.4	−22.3	Activated sludge clone H7	27
	ZORAM211	S-S-Zo.ram-0211-a-A-18		C34	TCG TAT AAC GTG AGG CCT	53.4	−20.2	*Zoogloea ramigera*	This study
	ZORAM441	S-S-Zo.ram-0441-a-A-18		C35	TGC GAT TTC TTT CCA CCT	52.6	−19.5	*Zoogloea ramigera*	This study
S-*-OTU3-1426-a-A-18	STEBA1426	S-*-Steba-1426-a-A-18	A, R, Z	D26	ACT ACC TAC TTC TGG TGG	50.6	−18.5	Some members of the *Sterolibacterium* lineage	27
	STEBA468	S-*-Steba-0468-a-A-18		D28	CCG TCA TTA GTA GCC CGT	54.5	−20.5	Some members of the *Sterolibacterium* lineage (activated sludge clones S28, A13, S40, H12, H23, and H20)	This study
S-*-OTU3-0445-a-A-20	STEBA448	S-*-Steba-0448-a-A-18		D27	TAG GGG CCA CCG TTT CGT	59.8	−23.1	Some members of the *Sterolibacterium* lineage (activated sludge clones S28, A13, S40, H12, H23, and H20)	Modified from 27
	STEBA635	S-*-Steba-0635-a-A-18		D29	AGT CCT ACA GTC ACA AAC	49.3	−18.2	Few members of the *Sterolibacterium* lineage (activated sludge clones S28 and A13)	This study
	STEBA643	S-*-Steba-0643-a-A-18		D30	CAC ACT CGA GTT ATG CAG	50.8	−19.2	Few members of the *Sterolibacterium* lineage (activated sludge clones S40, H12, H23, and H20)	This study
	STEBA214	S-*-Steba-0214-a-A-18		D32	CGC TCC TCT CGC GCG AGG	63.5	−25.1	Few members of the *Sterolibacterium* lineage (clones SBR1001, SBR2080, and GC24)	This study
BONE23a	BONE663[c]	S-*-Bone-0663-a-A-18		A33, D8	GGA ATT CCA TCC CCC TCT	54.1	−19.9	Beta1 group of *Betaproteobacteria*	Modified from 4
	RHOTE206[c]	S-S-Rho.te-0206-a-A-18		A14	AAG CGC AAG GTC CTA AGA	54.4	−20.5	*Rhodocyclus tenuis*	This study
	PPV1239[c]	S-*-Ppv-1239-a-A-18		A11	ACC CTC TGA ACC GAC CAT	56.4	−20.9	*Propionivibrio* spp., few members of the *Candidatus* "Accumulibacter" cluster	This study
	AZA1269[c]	S-*-Aza-1269-a-A-18		B8	AAG GGA TTG GCT CCA GCT	57.2	−21.1	*Azoarcus evansii, Azoarcus toluvorans, Azoarcus tolulyticus, Azoarcus toluclasticus,* and related *Azoarcus* spp.	This study
	AZA829[c]	S-*-Aza-0829-a-A-18		B18	GTT ACC GCA CCG AAC AAC	54.7	−21.0	*Azoarcus* spp. EbN1 and pCyN1	This study
	AZA234[c]	S-*-Aza-0234-a-A-18		B17	CCA GCT AAT CCG ACA TCA	52.1	−19.4	*Azoarcus* spp. EbN1 and pCyN1	This study
	AZA221[c]	S-S-Aza-0221-a-A18		B45	CAT CGG CCA CTC CAA TCA	55.5	−20.9	*Azoarcus* sp. LU1	This study

[a] Name of 16S rRNA gene-targeted oligonucleotide probe based on the nomenclature of Alm et al. (2).
[b] When "*Rhodocyclales*" subgroup-selective primer pair A, R, or Z was used for microarray analysis, this probe has its target site outside the amplified 16S rRNA gene fragments and must be ignored during interpretation of the hybridization pattern.
[c] Probe was removed from the RHC-PhyloChip because it hybridized nonspecifically to many reference organisms that have mismatches in the 16S rRNA gene target site.

TABLE 5. 16S rRNA-targeted probes used for FISH

Name	Sequence, 5′-3′	Formamide concn (%)	Specificity	Reference
EUB338[a]	GCT GCC TCC CGT AGG AGT	0–50	Most *Bacteria*	6
EUB338II[a]	GCA GCC ACC CGT AGG TGT	0–50	*Planctomycetes*	14
EUB338III[a]	GCT GCC ACC CGT AGG TGT	0–50	*Verrucomicrobia*	14
GAM42a	GCC TTC CCA CAT CGT TT	35	*"Gammaproteobacteria"*	38
BET42a	GCC TTC CCA CTT CGT TT	35	"*Betaproteobacteria*"	38
AT1458	GAA TCT CAC CGT GGT AAG CGC	50	Most members of the *Azoarcus-Thauera-Denitromonas* lineage	43
S-*-OTU1-1415-a-A-20	TTC TGG TAA ACC CCA CTC CC	25	*Zoogloea* lineage (including all KRZ Kraftisried clones from this study)	27
S-*-OTU3-0445-a-A-20	TTA GGG GCC ACC GTT TCG TT	30	Kraftisried activated sludge clones of the *Sterolibacterium* lineage	27

[a] EUB338, EUB338II, and EUB338III were applied simultaneously to target most *Bacteria* (14).

for subsequent design of microarray probes and for future taxonomic and environmental studies, an evaluation of the phylogeny of cultivated and yet uncultivated "*Rhodocyclales*" was performed. Initially, 10 sequences from environmental 16S rRNA clones (GenBank accession numbers AJ009452, AF245350, AF280861, AF281119, AY118150, AF529340, AY082472, AB089100, AB089101, and AF204249) were identified as chimeras and omitted from all subsequent analyses. The remaining 218 almost-full-length 16S rRNA sequences of "*Rhodocyclales*" were phylogenetically analyzed by calculating similarities and applying distance-matrix, maximum-parsimony, and maximum-likelihood methods for treeing.

The minimum 16S rRNA sequence similarity of two members of the "*Rhodocyclales*" was 88.1%. This is in the range of minimal similarities previously reported for other bacterial families e.g., 83% each for "*Desulfobacteraceae*" and "*Desulfovibrionaceae*" (33), 89% for "*Nitrosomonadaceae*" (30), and 90% for *Chlamydiaceae* (18), and therefore legitimates subclassification of all "*Rhodocyclales*" into the single family "*Rhodocyclaceae*" (http://dx.doi.org/10.1007/bergeysoutline200310) (20) from an rRNA-based point of view.

Independently of the phylogeny inference method applied, members of the "*Rhodocyclaceae*" could be subdivided into nine different monophyletic lineages (Fig. 1). The phylogenetic positions of these lineages to other betaproteobacterial orders could not be unambiguously determined, as shown by a polytomic tree topology (Fig. 1). With the exception of the anaerobic consortium clone SJA-109 lineage, designated according to an environmental clone sequence from an anaerobic, trichlorobenzene-transforming microbial consortium (58), each lineage was represented by at least one validly described or cultured species, indicating that lineage-level biodiversity of "*Rhodocyclaceae*" is well reflected by available cultured strains. Detailed phylogenetic trees showing the affiliation of all members of each "*Rhodocyclaceae*" lineage can be downloaded at http://www.microbial-ecology.net/supplements.asp (supplemental Fig. S1).

Probe design and microarray format. In general, the same strategies for in silico development and the same technical set-up for fabrication and hybridization of the RHC-PhyloChip were used as for the development of a 16S rRNA-targeted oligonucleotide microarray for detection of all lineages of recognized sulfate-reducing prokaryotes (SRP-PhyloChip) (35).

Initially, the specificities of all previously published 16S rRNA-targeted probes and primers for "*Rhodocyclales*" (13, 24–27, 43, 45) were reevaluated with the updated 16S rRNA database. Eighteen probes were found to target "*Rhodocyclales*" only and were therefore included on the RHC-PhyloChip, although not all of them target monophyletic "*Rhodocyclales*" groups (Table 4 and supplemental Fig. S2). In addition, 60 oligonucleotide probes were designed according to the "multiple probe concept" (3, 8) to specifically target "*Rhodocyclales*" at hierarchical and identical phylogenetic levels (Table 4 and Fig. S2). Because multiple nested probes can at least partly compensate for unspecificities of individual probes (32, 35), this probe design strategy is particularly valuable if microarray formats are used which only allow hybridization or washing at a single stringency. In summary, the RHC-PhyloChip probe set consists of 78 specific probes covering the complete diversity of "*Rhodocyclales*" known so far (see above), two probes targeting betaproteobacterial groups at a broader specificity (BONE663 and BTWO663) (4), six bacterial or universal probes, and two probes as positive and negative hybridization controls (CONT and NONSENSE) (Table 4). All probes were designed to have the same length (18 bases, excluding the T-spacer), but the G+C contents of the probes varied between 38.9 and 77.8%. To attenuate the influence of differing G+C contents of probe-target duplexes on duplex stabilities, 3M tetramethylammonium chloride was added to the washing buffer (8, 35, 39).

RHC-PhyloChip evaluation with reference strains. The specificity of the individual probes was tested under monostringent conditions (i.e., the same hybridization and washing conditions for all probes and microarrays). Cy5-labeled 16S rRNA gene amplicons of each pure culture and each 16S rRNA gene-containing clone ($n = 29$) were hybridized with a separate RHC-PhyloChip. For 60 "*Rhodocyclales*"-specific probes, this set of reference 16S rRNA genes contained at least one sequence with a perfectly matched target site. None of these probes showed false-negative signals. Out of the 18 probes for which no perfectly matched reference sequence was available, 10 yielded positive signals with mismatching reference 16S rRNA gene amplicons, indicating that the respective probe-target sites were accessible for hybridization. A detailed list of the individual hybridization results can be downloaded at http://www.microbial-ecology.net/supplements.asp (supplemental Table S1). Seven of the probes hybridized nonspecifically with many reference organisms not having fully comple-

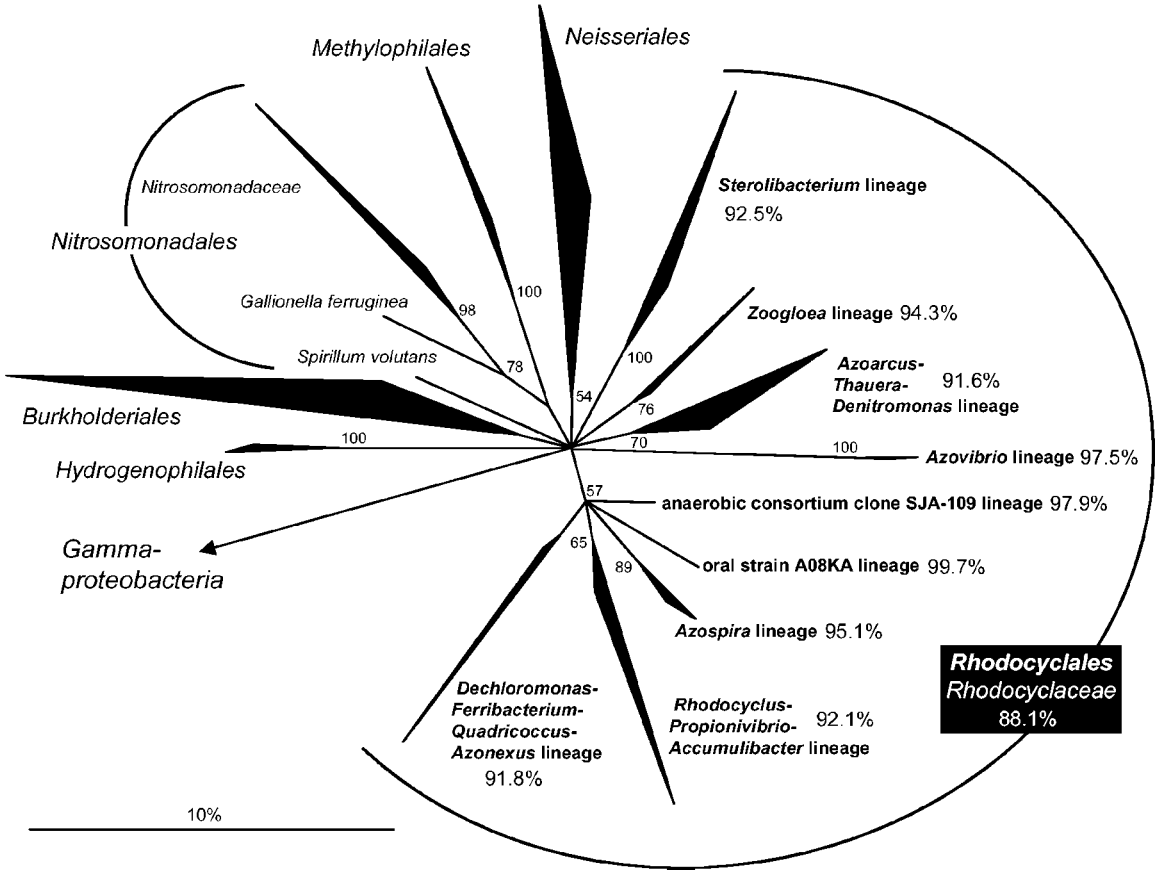

FIG. 1. 16S rRNA-based phylogenetic tree of the "*Rhodocyclales*" and selected type strains of other betaproteobacterial orders. The consensus tree is based on maximum-likelihood analysis (AxML) performed with a 50% conservation filter for the "*Betaproteobacteria*." The bar indicates 10% estimated sequence divergence. Polytomic nodes connect branches for which a relative order could not be determined unambiguously by applying neighbor-joining, maximum-parsimony, and maximum-likelihood treeing methods. Numbers at branches indicate percent parsimony bootstrap values. Branches without numbers had bootstrap values of less than 50%. The minimum 16S rRNA sequence similarity for each "*Rhodocyclales*" lineage is shown.

mentary probe target sites and were therefore excluded from the final version of the RHC-PhyloChip (listed separately in Table 4 and supplemental Table S1).

In order to compare the hybridization efficiency of the different RHC-PhyloChip probes, the signal-to-noise ratios (SNRs) of the probes were normalized against the SNR of the bacterial probe EUB338 recorded on the same microarray. The resulting nSNRs for perfectly matched probe-target duplexes ranged from 0.2 (for probe A33-587 with Kraftisried WWTP clone A33) to 48.0 (for probe BTWO663 with *Azonexus fungiphilus*), demonstrating that the signal intensities of individual probes vary strongly if excess target DNA is added. It has been observed previously that the duplex yield of different rRNA gene-targeted microarray probes can differ considerably (32, 35, 41), and on the RHC-PhyloChip the duplex yield was significantly positively correlated with the theoretical T_m of the probe (Spearman nonparametric correlation test: $R = 0.342$, $P = 0.013$). The latter point demonstrated that the addition of tetramethylammonium chloride did not completely abolish the influence of the G+C content on the duplex yield of different probes.

Of the 2,291 different hybridizations (each reference DNA

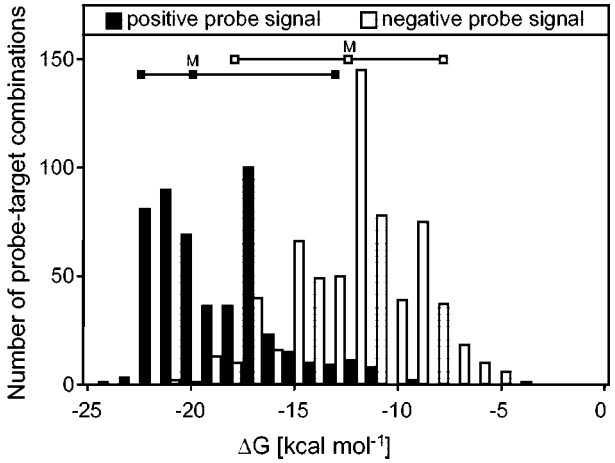

FIG. 2. Frequency distribution of ΔG values for positive (black bars) and negative (white bars) probe-target combinations having up to five mismatches. The horizontal lines indicate the 5th and 95th percentiles and the median value (M). The difference between the ΔG values of positive and negative probe-target combinations was highly significant (analysis of variance, $P < 0.001$).

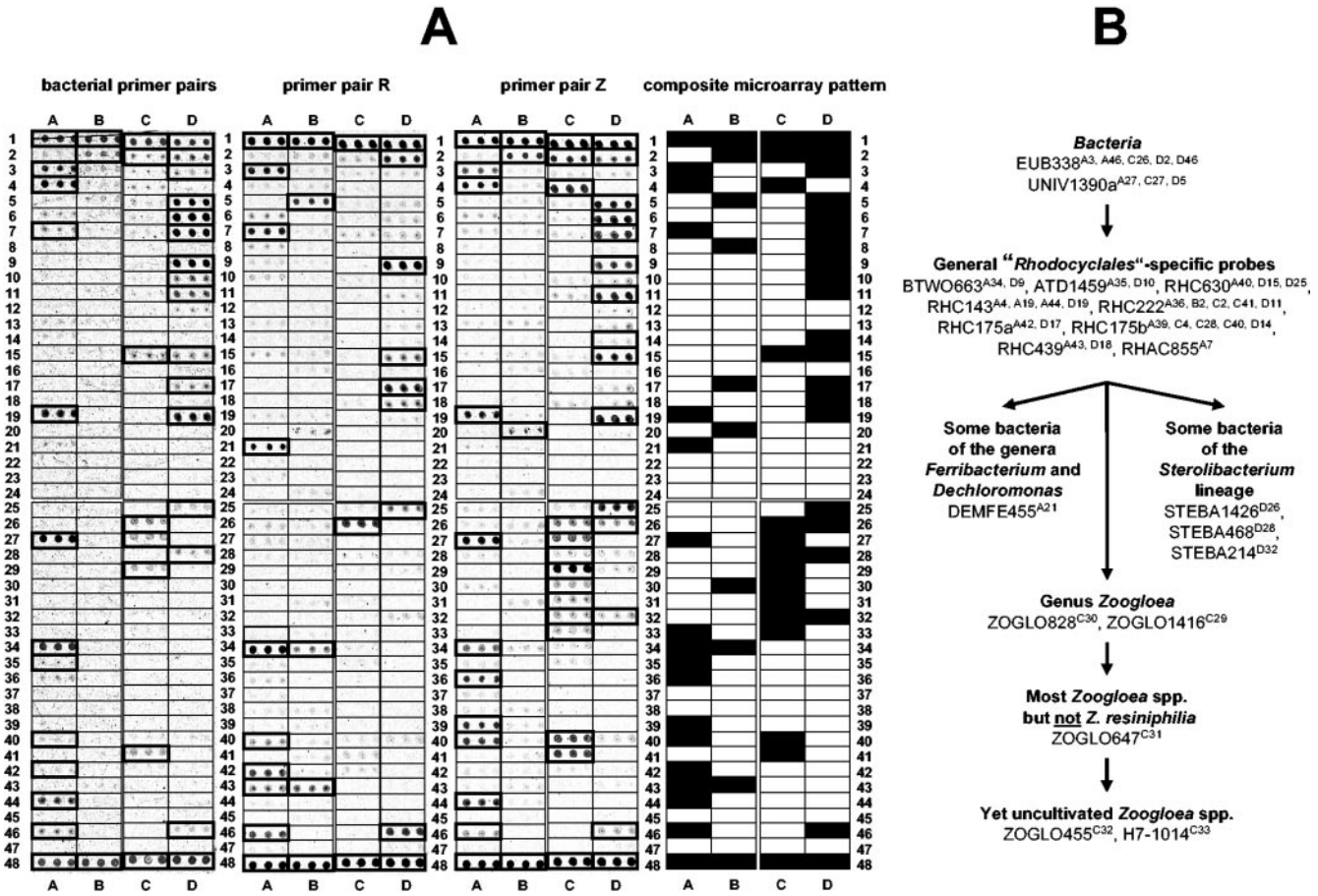

FIG. 3. (A) DNA microarray diversity analysis of "*Rhodocyclales*" in activated sludge from the industrial WWTP Kraftisried. Three RHC-PhyloChips were hybridized separately with fluorescently labeled 16S rRNA gene PCR amplicons that were retrieved from the activated sludge sample by using either bacterial or "*Rhodocyclales*" subgroup-selective (R or Z) primer pairs (Table 3). Each probe was spotted in triplicate. For each microarray position, the probe sequence and specificity are depicted in Table 4. Probe spots having a signal-to-noise ratio (SNR) equal to or greater than 2.0 are indicated by boldface boxes and were considered positive. In the composite microarray pattern, probes which were positive in any of the three individual RHC-PhyloChip hybridizations are indicated by black boxes. (B) Flow chart illustrating the presence of distinct "*Rhodocyclales*" groups in the activated sludge from Kraftisried as inferred from the composite microarray pattern. For each probe, the position on the microarray is indicated in the superscript text.

with each probe) which were performed in total, 208 (9.1%) were false-positive (positive probe signal with a nontarget organism having one or more mismatches in the probe target site), while no hybridizations were false-negatives (supplemental Table S1). The occurrence of some false-positive results is almost impossible to avoid with a monostringent microarray hybridization approach, because the stability of mismatched probe-target hybrids is difficult to predict in silico and influenced by many factors, such as (i) the number of mismatches, (ii) the nature of the mismatching nucleotides, (iii) the position of the mismatches in the probe target site, and (iv) possible stacking interactions of nucleotides adjacent to the mismatches (19, 53, 56, 57). However, specific identification of target organisms is still possible with the RHC-PhyloChip because of the multiple probe concept (the theoretical specificities of the nested probes are depicted in supplemental Fig. S2). Nevertheless, future microarray probe design would be further improved if oligonucleotide probe parameters were available which allow estimation of the hybridization behavior of each probe in silico (53).

One hybridization parameter that might be a suitable candidate is the free energy, ΔG, of a given perfectly matching or mismatching probe-target hybrid (23, 55). On the RHC-PhyloChip, the ΔG values of most (88%) of the false-positive probe-target hybrids with one or two mismatches were in a similar range (-22 to -16 kcal mol^{-1}) to ΔG values of all perfectly matched probe-target pairs (-25 to -17 kcal mol^{-1}), providing an explanation of why discrimination was not successful under monostringent hybridization conditions. As can be inferred from Fig. 2, most of the positive probe-target hybrids (84%) (including false-positive signals) had a ΔG below -16 kcal mol^{-1}. Additionally, only 3% of all probe-target combinations that yielded no hybridization signal had also a ΔG below -16 kcal mol^{-1}. Therefore, a ΔG threshold of -16 kcal mol^{-1} could provide useful guidance for future preselection of appropriate probes in silico (Fig. 2) but does not abolish

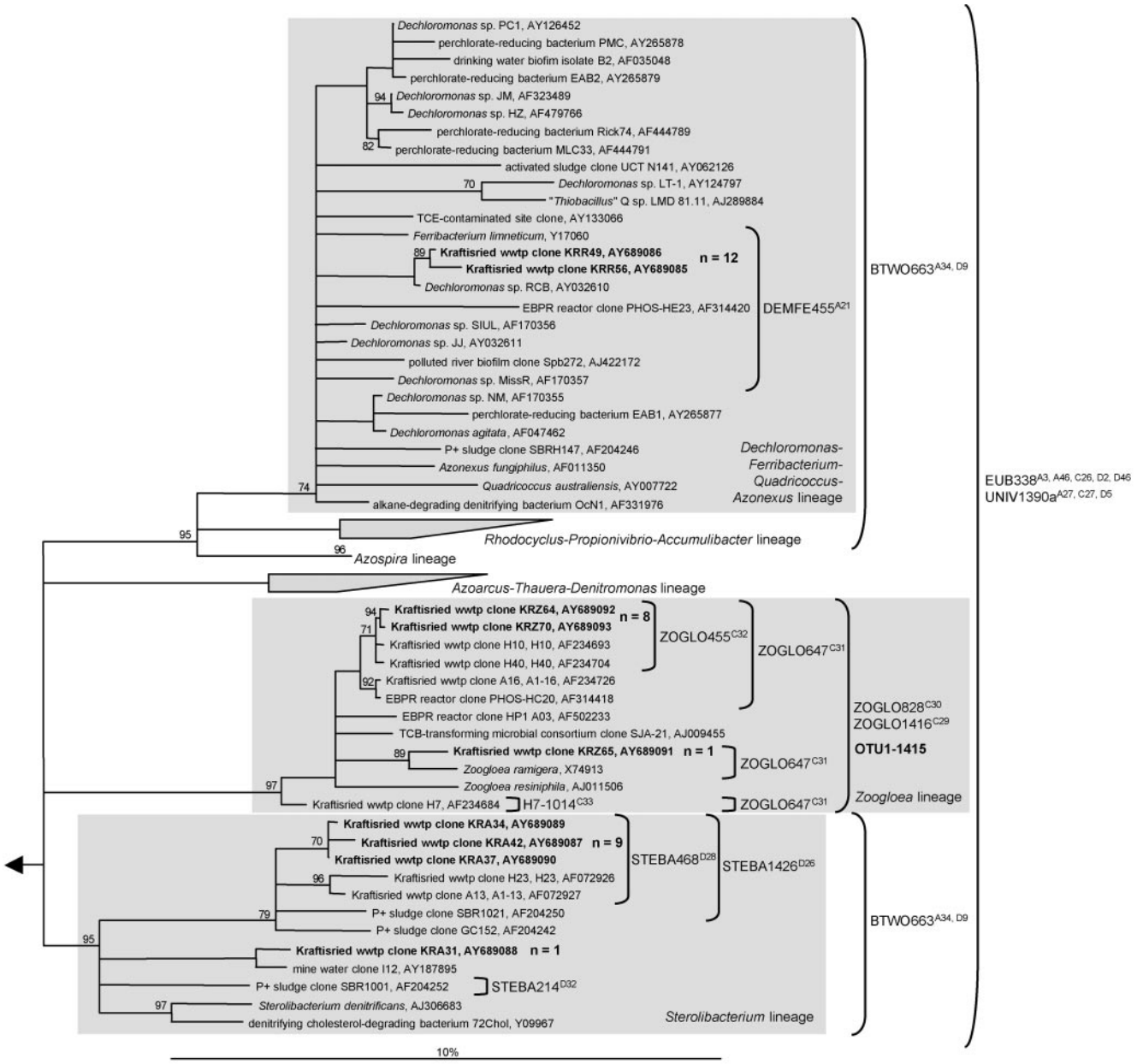

FIG. 4. 16S rRNA gene phylogenetic consensus tree based on maximum-likelihood analysis (Tree-puzzle) performed with a 50% conservation filter for the "*Betaproteobacteria*." The tree shows the affiliation of clone sequences (boldface type) retrieved from the sewage treatment plant Kraftisried by using "*Rhodocyclales*" subgroup-selective primer pairs A (KRA clones), R (KRR clones), and Z (KRZ clones) for PCR. The grey box shows affiliation to a "*Rhodocyclales*" lineage. The bar indicates 10% estimated sequence divergence. Polytomic nodes connect branches for which a relative order could not be determined unambiguously by applying neighbor-joining, maximum-parsimony, and maximum-likelihood treeing methods. The percent reliability value of each internal branch indicates how often the corresponding cluster was found among 50,000 intermediate trees during quartet puzzling. Values below 70% are not shown. Parentheses indicate the perfect-match target organisms of the probes. Probe S-*-OTU1-1415-a-A-20 (OTU1-1415) (Table 5) is depicted in bold and was used for quantitative FISH analysis. The microarray position is depicted after the probe name. Probes RHC630, RHC143, RHC222, RHC175a, and RHC175b, perfectly matching some of the Kraftisried clones, are not shown to enhance clarity.

the need for extensive empirical testing of the hybridization behavior of microarray probes. It should be further stressed that the ΔG threshold of -16 kcal mol^{-1} might only apply to the microarray set-up and the hybridization conditions used in this study.

RHC-PhyloChip application in activated sludge. To demonstrate the applicability of the RHC-PhyloChip for rapid screening of "*Rhodocyclales*" diversity in environmental samples, activated sludge from the nitrifying-denitrifying WWTP Kraftisried was analyzed. Kraftisried was chosen as a model

system because in 1996 members of diverse lineages of "*Rhodocyclales*" comprised more than one third of the entire bacterial biovolume in this WWTP (27).

Initially, 16S rRNA genes were amplified from Kraftisried DNA by using standard bacterial primers, fluorescently labeled, and hybridized with the RHC-PhyloChip. Surprisingly, the hybridization pattern obtained did not indicate the presence of members of the "*Rhodocyclales*" below the lineage level [some probes (BTWO663, ATD1459, RHC630, RHC175a, RHC143, RHC222, and RHAC855) targeting "*Rhodocyclales*" at broader phylogenetic levels showed positive signals but almost all probes of higher specificity were negative] (Fig. 3A). To find an explanation for this unexpected result, the relative abundance of "*Rhodocyclales*" in this WWTP was analyzed quantitatively by FISH. Compared to 1996, the relative abundance of "*Betaproteobacteria*" in the activated sludge from 2002 decreased from 47 to 18% of all bacteria detectable by FISH (Table 5). Similarly, the abundance of members of the "*Rhodocyclales*" decreased dramatically between the two samples. While the activated sludge from 1996 contained significant amounts of "*Rhodocyclales*" detectable by probes AT1458, S-*-OTU1-1415-a-A-20, and S-*-OTU3-0445-a-A-20, [each targeting a "*Rhodocyclales*" group found previously in this WWTP (27)] (Table 5), less than 1% of the cells hybridized with these probes in the WWTP sample from 2002.

To increase the sensitivity of the RHC-PhyloChip, three "*Rhodocyclales*"-subgroup-selective primer pairs called A, R, and Z (together targeting almost all "*Rhodocyclales*") (Table 3) were designed and applied for amplification of 16S rRNA genes prior to microarray hybridization. Although these new primers were selected to amplify 16S rRNA gene fragments of the maximum possible length, the target sites of some RHC-PhyloChip probes are outside the amplified 16S rRNA gene region (Table 4), and these probes must thus be ignored during interpretation of hybridization patterns.

Each "*Rhodocyclales*"-subgroup-selective primer pair was used separately for amplification of Kraftisried activated sludge DNA at low stringency (Table 3) to allow potential primer binding to 16S rRNA genes of "*Rhodocyclales*" having mismatches in the primer target sites. PCR products of the expected length were obtained for primer pairs R and Z, but no primer pair A PCR product was observed after gel electrophoresis. The "*Rhodocyclales*" subgroup-selective PCR amplicons obtained were fluorescently labeled and hybridized with two separate RHC-PhyloChips. The RHC-PhyloChip hybridization patterns of the R and Z amplicons differed from each other and from the pattern obtained by using general bacterial primer pairs (Fig. 3A). In more detail, the hybridization pattern obtained with primer pair R indicated the presence of bacteria related to the genera *Ferribacterium* and *Dechloromonas*, whereas the hybridization pattern obtained with primer pair Z pointed to the presence of *Zoogloea* species.

A composite microarray fingerprint of the "*Rhodocyclales*" community present in activated sludge from Kraftisried was created by merging the separate microarray hybridization patterns obtained with the "*Rhodocyclales*"-subgroup-selective and the common bacterial 16S rRNA gene amplicons (Fig. 3A). Besides *Ferribacterium*/*Dechloromonas*-related bacteria and *Zoogloea* species, this composite microarray fingerprint additionally indicated the presence of members of the *Sterolibacterium* lineage (Fig. 3B).

The microarray results were confirmed independently by cloning and sequencing of the 16S rRNA gene PCR products obtained with the three "*Rhodocyclales*" subgroup-selective primers. It should be noted that cloning of PCR products amplified from Kraftisried DNA with primer pair A was successful, although only small amounts of PCR product could be retrieved (see above). All 16S rRNA gene clones obtained with primer pairs Z and A were closely related to clones already found in the Kraftisried WWTP in 1996 (27) and belonged to the genus *Zoogloea* and the *Sterolibacterium* lineage, respectively (Fig. 4). In contrast, all 16S rRNA gene sequences obtained by using primer pair R clustered with members of the genera *Dechloromonas* and *Ferribacterium* (Fig. 4), which were not detected by the 16S rRNA full-cycle approach in Kraftisried WWTP samples from 1996 (27). The phylogeny of all retrieved 16S rRNA gene sequences was in perfect agreement with the microarray results. Furthermore, the sequenced 16S rRNA genes have perfectly matched target sites for the probes that showed a positive signal in the RHC-PhyloChip analyses (Figs. 3 and 4).

The microarray hybridizations, the retrieved 16S rRNA sequences, and the quantitative FISH data collected in this study provide corroborating evidence that substantial changes have occurred within the "*Rhodocyclales*" community in Kraftisried since the first bacterial community analysis of this WWTP (27). The dramatic decline in "*Rhodocyclales*," assumed to be the major denitrifiers in this system (27), from 35% of the total bacterial biovolume in 1996 to less than 1% in 2002 may have been caused by the seasonal implementation of a partial ammonium stripping step prior to biological nitrogen removal in 1999. This physical sewage treatment step reduces the ammonia concentration and increases the salt concentration in the sewage and probably had dramatic consequences for the population structure of nitrifiers (data not shown) and potentially denitrifying heterotrophs in the activated sludge.

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