APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1994, p. 3236–3244
0099-2240/94/$04.00+0

Vol. 60, No. 9

PCR-Based Preparation of 23S rRNA-Targeted Group-Specific Polynucleotide Probes

WOLFGANG LUDWIG,* SILVIA DORN, NINA SPRINGER, GUDRUN KIRCHHOF, AND KARL-HEINZ SCHLEIFER

Lehrstuhl für Mikrobiologie, Technische Universität München, D-80290 Munich, Federal Republic of Germany

Received 28 February 1994/Accepted 6 June 1994

DNA coding for a variable region within domain III of bacterial 23S rRNA was used as the target for group-specific polynucleotide hybridization probes. The corresponding rDNA was amplified in vitro by the PCR technique in combination with a pair of primers specific for flanking conserved target sites. The amplified fragments were cloned or used directly as probes. RNA probes were generated by in vitro transcription of cloned or amplified rDNA. The probes were labeled by incorporating modified nucleotides during in vitro DNA amplification or in vitro transcription or by random priming. The use of in vitro transcribed single-stranded RNA probes instead of double-stranded DNA probes provided stronger hybridization signals. Group-specific probes were prepared from genomic DNAs or directly from cells of *Acinetobacter calcoaceticus*, *Alcaligenes faecalis*, *Aeromonas hydrophila*, *Nannocystis exedens*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Pseudomonas stutzeri*.

During the past decade, rRNAs have proven to be not only excellent phylogenetic marker molecules with which to elucidate the genealogical relationships of microorganisms (34) but also ideal target molecules for specific hybridization probes (29). The rRNA sequences contain diagnostic regions which are of identical composition in the molecules of phylogenetically related taxa and different in the rRNAs from all other organisms. The primary structures of rRNAs consist of stretches of evolutionarily highly and less conserved regions. Consequently, there are potential target sites for probes with wider and more restricted ranges of specificities. Specific rRNA targeted probes have been designed for individual species, groups of related species, subclasses, and domains (29). A variety of hybridization methods are available for the rapid identification of microorganisms. One of the most attractive techniques, whole-cell hybridization (1, 5, 7), allows differentiation at the cellular level. Moreover, abundance and spatial distribution of so far uncultured microbial cells can be analyzed directly in complex samples by using this method in combination with specific probes (2, 3, 30, 31).

A comprehensive data set of rRNA sequences is a prerequisite for the proper design of specific probes. Potential target sites for species- or group-specific probes can be detected by comparative analyses of the data set of aligned sequences. Further evaluation of these target sites has to be done with respect to potential intramolecular base pairing of the derived probe and the qualities of mismatches with nontarget rRNAs (29, 32). Nowadays, specific probes usually are designed as short oligonucleotides comprising 15 to 20 bases. In comparison with polynucleotide probes, the use of short oligonucleotide probes is advantageous in that target and nontarget sequences differing by a single nucleotide can be differentiated by applying appropriate experimental conditions (5, 20). In general, this facilitates the design of highly specific probes. However, especially at more variable sequence positions of rRNAs, multiple changes during the course of evolution may have resulted in the occurrence of identical substitutions in phylogenetically diverse organisms. The larger the number of such rapidly changing positions in the target site of a given probe, the higher the risk that the same target sequence occurs within the rRNA of an organism which phylogenetically does not belong to the targeted group. Therefore, the specificities of such probes have to be carefully evaluated by database analyses and by probing a large number of representative reference organisms.

Modern probe technologies are excellent tools for the identification of taxa in complex communities (2, 20, 30, 31). However, extensive analysis of the probably vast number of species in such communities would require a reasonably comprehensive set of probes and a comparable number of experimental steps. The use of probes specific for groups of organisms related at higher phylogenetic levels allows a rough but more rapid characterization of these samples. In a nested approach, the population analysis can be started with domain-specific probes and successively continued with group- to species-specific probes (29). However, it is often difficult to find a unique target site for the design of a group-specific oligonucleotide probe which is identical within the rRNA primary structures of all members of the given group of phylogenetically related organisms. A possible solution of this problem is the combined use of a minimum mixture of oligonucleotides specific for the different variants of a given rRNA target site among the representatives of the group. However, when analyzing natural communities, extensive sequencing would have to be done to identify a significant fraction of natural variations of the rRNA target sites among the members of a given phylogenetic group. Furthermore, for the design of mixed oligonucleotide probes, care has to be taken to ensure identical dissociation temperatures for all probe hybrids.

The specificity of polynucleotide probes (150 to 400 bases) (13) is not so dependent on complete identity of the target regions. The melting temperatures of polynucleotide hybrids are not as drastically changed by single mismatches as are the dissociation temperatures of oligonucleotide hybrids (32).

* Corresponding author. Mailing address: Lehrstuhl für Mikrobiologie, Technische Universität München, D-80290 Munich, Germany. Phone: 4989 2105 2370. Fax: 4989 2105 2360. Electronic mail address: ludwig@mbitum2.biol.chemie.tu-muenchen.de.

TABLE 1. Data for polynucleotide target site sequences determined in the present study or taken from complete 23S rRNA sequences

Organism	Accession no.	Clone	Length (bp)[a]	Mol% G+C		Source or reference
				Fragment[b]	23S rRNS	
P. aeruginosa	Y00432	pPae23III	255	51	53	32a
P. alcaligenes		pPal23III	254	50	ND[c]	This study
P. aureofaciens	X79997	pPau23III	255	47	ND	This study
P. chlororaphis	X79998	pPch23III	255	46	ND	This study
P. cichorii	X79999	pPci23III	255	48	ND	This study
P. fluorescens	X80001	pPfl23III	255	47	ND	This study
P. pseudoalcaligenes	X80002	pPps23III	255	50	ND	This study
P. putida	X80003	pPpu23III	255	50	ND	This study
P. stutzeri	X80004	pPst23III	255	49	52	This study
P. syringae		pPsy23III	255	48	ND	This study
Acinetobacter calcoaceticus	X80005	pAca23III	255	46	51	This study
Aeromonas hydrophila	X79994	pAhy23III	252	53	53	This study
E. coli	V00331	ND	252	52	53	6
Alcaligenes faecalis	X79995	pAfa23III	248	52	52	This study
Burkholderia cepacia	X16368	ND	248	55	53	15
P. diminuta	X80000	pPdi23III	148	54	54	This study
N. exedens	X79996	pNex23III	267	59	56	This study
Micrococcus luteus	X06484	ND	358	60	56	24a
Bacillus subtilis	K00637 M10606 X00007	ND	258	52	54	14a

[a] Lengths including amplification primer sequences in base pairs.
[b] Primer sequences are not included.
[c] ND, no data available.

Therefore, the use of polynucleotide probes should be advantageous if group-specific detection is needed. However, it is more expensive to synthesize polynucleotides than a mixture of oligonucleotides.

In the present study, polynucleotide target sites on bacterial 23S rRNAs have been tested for their general applicability for group-specific probes. The assay does not depend on sequence analysis or the availability of big databases, and the probes are prepared by a PCR approach at low cost.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The organisms which were used to prepare the probes and to evaluate their specificities are listed in Table 1. Cells of *Chondromyces apiculatus*, *Corallococcus coralloides*, *Cystobacter fuscus*, *Myxococcus xanthus*, and *Nannocystis exedens* strains were kindly provided by H. Reichenbach, GBF, Brunswick, Germany. The other bacteria were grown in media and under culture conditions as recommended in the Catalogue of Strains 1993 of the German Collection of Microorganisms and Cell Lines, Brunswick, Germany.

Purification of nucleic acids. Genomic DNAs were prepared by using the method of Marmur (21). Alternatively, a purification kit was used as specified by the manufacturer (Diagen, Hilden, Germany). Cellular RNAs were purified as described by Oelmüller et al. (23).

Nucleic acid sequence determination. The nucleotide sequences of cloned rDNA fragments were determined by using the T7Sequencing Kit (Pharmacia, Heidelberg, Germany).

Preparation and labeling of polynucleotide probes. The polynucleotides were amplified in vitro from bacterial rRNA genes (rDNA) by applying the PCR technique as described by Roller et al. (25). Variants of the primers (25) used in the present study are shown in Table 2.

The polynucleotides were labeled either radioactively ([α-^{32}P]dATP [NEN, Dreieich, Germany]) or nonradioactively (digoxigenin-dUTP [Boehringer, Mannheim, Germany]) by different approaches. (i) The labeled nucleotides were introduced during PCR amplification of rDNA fragments (18). (ii) The amplified rDNA fragments were labeled by the random-priming method with a random-primed DNA-labeling kit (Boehringer). (iii) The rDNA was amplified in vitro by using a primer pair containing *Not*I restriction sites (Table 2), subsequently digested by the restriction endonuclease *Not*I, and finally cloned in the vector pBluescript (Stratagene, La Jolla, Calif.) in *Escherichia coli* JM83. Purified plasmid DNA was

TABLE 2. Nucleotide sequences and target sites of oligonucleotide primers used for in vitro amplification of 23S rRNA gene fragments encoding the variable region of domain III

Primer	Sequence[a]	Target[b]
1900VN	5′-TATAgcggccgcMADGCGTAGNCGAWGG-3′	1366–1381
1900V	5′-MADGCGTAGNCGAWGG-3′	1366–1381
317RNT3	5′-ATATAATTAACCCTCACTAAAGgcggccgcGTGTCGGTTTNSGGTA-3′	1602–1617
317RN	5′-ATATgcggccgcGTGTCGGTTTNSGGTA-3′	1602–1617
317R	5′-GTGTCGGTTTNSGGTA-3′	1602–1617

[a] *Not*I restriction sites are indicated by lowercase letters. The T_3 RNA polymerase promoter sequence is underlined.
[b] The numbering of target positions is based on the numbering of the *E. coli* 23S rRNA sequence (6).

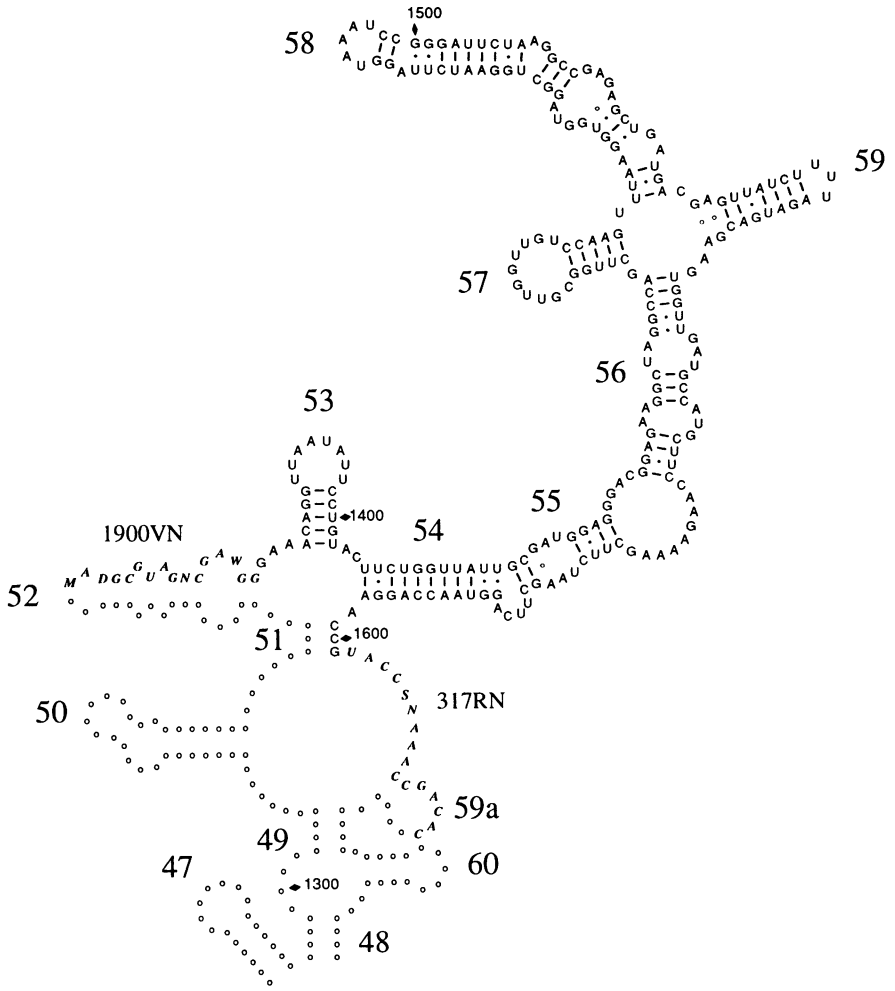

FIG. 1. Potential secondary-structure model of domain III of the 23S rRNA from *P. fluorescens*. Regions which had not been sequenced are schematically shown by circles according to a consensus secondary structure derived from published (17, 22) and unpublished complete 23S rRNA sequences from proteobacteria of the γ subclass. The numbering of bases corresponds to that of the homologous *E. coli* molecule (6). Helix numbering is as used in earlier models (15, 16, 19). The target sites of the primers used for in vitro amplification (1900VN and 317RN) are indicated by italics.

digested by *Not*I. The resulting rDNA fragments were electrophoretically separated from vector DNA (0.7% agarose). The rDNA was detected by its size (Table 1) after ethidium bromide staining of the gel and subsequently recovered by using the USBioclean Kit (United States Biochemicals, Bad Homburg, Germany). The rDNA fragments were labeled by applying the random-priming technique (see above). (iv) The rDNA was amplified in vitro by using the primer pair 317R and 1900VN (Table 2). The latter contained a *Not*I restriction site. The amplified rDNA was digested by *Not*I and cloned in the *Not*I- and *Eco*RV-digested vector pBluescript (Stratagene) in *E. coli* JM83. Recombinant plasmid DNA was linearized by restriction with *Not*I, and the rDNA was in vitro transcribed (RNA labeling kit; Boehringer) by using T_3 RNA polymerase (Boehringer) in combination with labeled nucleotides (see above). The lengths of the transcribed RNA fragments were checked by denaturing polyacrylamide gel electrophoresis (6% polyacrylamide, 8.3 M urea, 0.05 M Tris-borate [pH 8.3], 0.001 M Na_2 EDTA). (v) In vitro amplification of rDNA was carried out with the amplification primers 317R and 1900VNT3. The latter contained the T_3 RNA polymerase promoter sequence (Table 2). The amplified fragments were used as the template for in vitro transcription with the in vitro transcription kit (Boehringer).

Hybridizations. rRNA samples prepared from cells of target and reference organisms were transferred to Zetaprobe membranes (Bio-Rad, Munich, Germany) and to positively charged nylon membrane (Boehringer) as recommended by the manufacturers for hybridizations to radioactively labeled and digoxigenin-labeled probes, respectively. A Minifold (Schleicher & Schuell, Dassel, Germany) was used for nucleic acid transfers. Zetaprobe membranes were wetted in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]) containing 0.5% sodium dodecyl sulfate (SDS). Prehybridization was carried out at 55°C for 1 h in a solution containing 5× SSC, 5× Denhardt's solution (1× Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 1% (wt/vol) *N*-lauroyl sarcosine, and 62% (vol/vol) formamide. Hybridization of radioactively labeled polynucleotide probes was done under the same conditions for 16 h. The membranes were washed three times for 15 min at hybridization temperature in 2× SSC containing 0.1% (wt/vol) SDS.

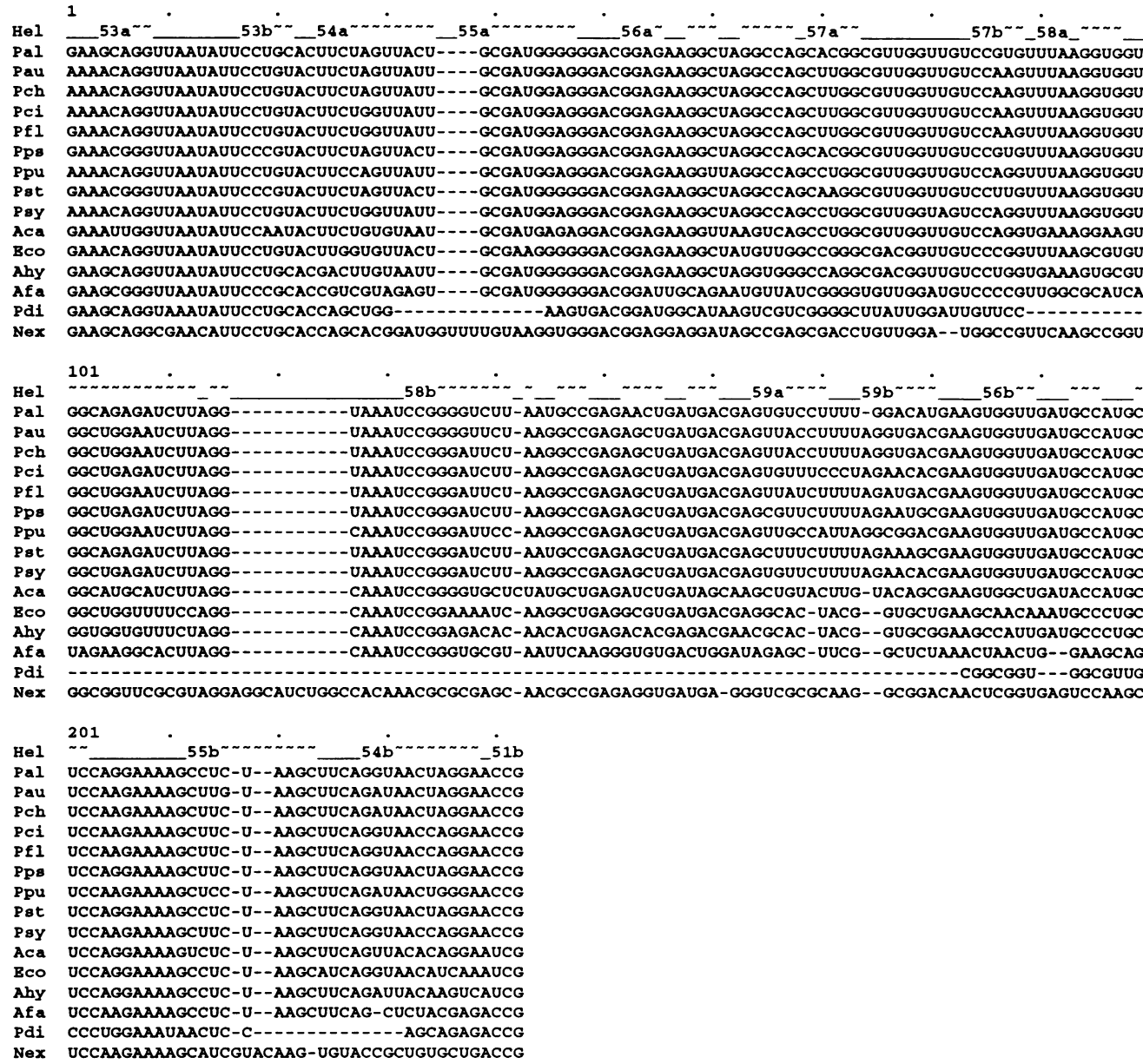

FIG. 2. Alignment of the 23S rRNA partial sequences determined in the present study. The corresponding 23S rRNA partial sequence from *E. coli* was included as a reference. Single-stranded regions or positions are indicated by solid lines within the secondary-structure mask (Hel, helix) whereas potentially base-paired residues are marked by tildes. The 5′ ends of helix halves are indicated by the helix numbers as in previously published secondary-structure models (15, 16, 19). The 5′ and 3′ helix halves are labeled as a and b, respectively. Abbreviations are as specified in Table 3.

When digoxigenin-labeled polynucleotide probes were used, prehybridization and hybridization were carried out at 55°C for 1 and 6 h, respectively, in 5× SSC containing 5% (wt/vol) blocking reagent (digoxigenin labeling and detection Kit; Boehringer), 0.1% (wt/vol) *N*-lauroyl sarcosine, 0.2% (wt/vol) SDS, and 62% (vol/vol) formamide. Washing was performed as described for radioactively labeled probes. The hybridizations with the oligonucleotide probe Eub338 (4) were performed under same conditions as specified for polynucleotide probes with the exceptions that formamide was omitted and the temperature was 42°C.

RESULTS AND DISCUSSION

The first 23S rRNA targeted probes (13, 14) were constructed by cloning and subcloning of large-subunit rRNA gene (rDNA) fragments. The recombinant plasmids or rDNA restriction fragments were empirically screened by hybridization to purified rRNA or genomic DNA from phylogenetically diverse bacteria. Given that only a few 23S rRNA sequences had been elucidated at that time, a more directed probe design relying on comparative sequence data analysis was impossible. However, a specific (cloned) polynucleotide probe (pHF360)

TABLE 3. Overall percent sequence similarities of the 23S rRNA target regions of group-specific polynucleotide probes (lower left) and complete 23S rRNA sequences (upper right)[a]

	Pae	Pal	Pau	Pch	Pci	Pfl	Pps	Ppu	Pst	Psy	Ahy	Eco	Aca	Afa	Bce	Pdi	Nex	Mlu	Bsu
Pae									95.3		85.5	84.6	86.8	81.9	82.6	78.1	76.5	71.5	74.3
Pal	84.7																		
Pau	90.0	88.8																	
Pch	90.0	88.8	99.1																
Pci	89.2	89.2	91.5	92.4															
Pfl	92.4	87.8	96.4	97.3	94.2														
Pps	87.0	92.8	89.2	90.1	91.9	91.0													
Ppu	85.7	84.7	92.8	93.7	87.9	91.0	86.1												
Pst	85.2	93.2	87.9	88.8	90.1	89.7	96.4	84.7			85.9	84.9	87.3	82.1	82.6	77.4	76.1	71.8	73.9
Psy	89.7	90.5	91.9	92.8	96.9	94.6	93.3	90.0	91.0										
Ahy	70.0	74.3	72.5	72.5	70.2	71.6	70.2	71.1	72.0	71.1		88.5	84.4	80.0	81.4	76.7	74.4	71.3	73.2
Eco	72.6	73.5	73.1	74.0	71.7	74.0	72.6	73.5	72.6	72.6	80.4		83.1	80.1	80.2	76.4	74.4	70.6	73.3
Aca	72.8	75.1	75.6	75.6	76.0	77.8	76.0	75.6	78.7	76.5	65.7	66.0		80.0	81.4	76.9	74.5	71.1	74.4
Afa	52.6	58.6	54.4	54.9	54.0	54.9	54.9	54.9	57.2	54.4	55.8	57.4	57.9		88.1	77.1	74.5	69.1	72.6
Bce	57.7	61.9	59.1	59.5	58.6	60.0	58.6	60.5	61.4	60.9	63.3	60.2	61.6	76.2		77.4	75.3	69.6	73.7
Pdi	45.1	52.2	46.0	46.9	47.0	47.8	46.9	48.7	49.6	46.9	54.9	50.4	44.2	57.5	58.4		74.9	71.4	73.0
Nex	57.0	59.0	60.0	60.2	58.3	60.0	57.4	63.0	58.3	58.3	54.7	53.0	48.9	48.8	48.8	51.2		72.5	74.6
Mlu	60.2	57.3	58.6	58.6	59.1	59.2	60.0	57.7	60.0	60.0	58.7	58.9	55.3	48.1	48.1	53.8	54.6		74.1
Bsu	63.1	63.6	64.0	64.5	63.5	64.0	64.0	63.5	63.6	64.0	60.0	60.1	60.2	56.1	56.1	59.4	55.6	61.8	
	Pae	Pal	Pau	Pch	Pci	Pfl	Pps	Ppu	Pst	Psy	Ahy	Eco	Aca	Afa	Bce	Pdi	Nex	Mlu	Bsu

[a] Abbreviations: Aca, *Acinetobacter calcoaceticus*; Afa, *Alcaligenes faecalis*; Ahy, *Aeromonas hydrophila*; Bce, *Burkholderia cepacia*; Bsu, *Bacillus subtilis*; Eco, *E. coli*; Mlu, *M. luteus*; Nex, *N. exedens*; Pae, *P. aeruginosa*; Pal, *P. alcaligenes*; Pau, *P. aureofaciens*; Pch, *P. chlororaphis*; Pci, *P. cichorii*; Pdi, *P. diminuta*; Pfl, *P. fluorescens*; Pps, *P. pseudoalcaligenes*; Ppu, *P. putida*; Pst, *P. stutzeri*; Psy, *P. syringae*.

could be constructed (13) for the genuine pseudomonads (24). When more bacterial 23S rRNA sequences were elucidated, it was recognized (15) that the rDNA fragment of pHF360 contained an evolutionarily only moderately conserved region of the 23S rRNA gene located in domain III. This is the longest coherent variable region within bacterial 23S rRNAs (15, 19, 28). There is no variable region within bacterial 16S rRNAs of comparable length. Large stable insertions of about 100 bases within domain III of 23S rRNAs have been described for gram-positive bacteria with a high DNA G+C content (25), whereas deletions of about 80 bases were detected within the same domain of 23S rRNA genes from proteobacteria of the α subclass (11, 15, 28).

Probe target site. Comparative sequence analyses of bacterial 23S rRNA genes revealed a large evolutionarily only moderately conserved region within domain III of the large-subunit rRNA (15, 19, 25, 28). DNA fragments covering the corresponding parts of 23S rRNA genes (homologous to bases 1366 to 1617 of the 23S rRNA from *E. coli*) (6) were amplified in vitro from a selection of proteobacteria specified in Table 1. The amplified fragments comprised 148 to 358 bp (Table 1). A potential secondary-structure model of domain III of 23S rRNA from *Pseudomonas fluorescens* is depicted in Fig. 1. This model is based on those published previously (15, 16, 19). The target sites of the amplification primers listed in Table 2 are rather conserved regions homologous to positions 1366 to 1381 and 1602 to 1617 of the *E. coli* 23S rRNA flanking the rDNA of interest (Fig. 1). However, these target sites are not completely invariant among bacterial 23S rRNAs. To allow the amplification of the corresponding rDNA from a wide spectrum of bacteria, we used degenerated primers (Table 1). Primer variants containing *Not*I restriction sites close to their 5′ ends were used for one or both target sites to allow forced cloning of the fragments and to improve the cloning efficiency, respectively. *Not*I restriction sites were chosen to avoid DNA cleavage within the amplified fragments. No *Not*I restriction sites have been detected within published (17, 22) and unpublished 23S rRNA sequences so far. The designations of the recombinant plasmids containing the rDNA fragments are listed in Table 1. The cloned rDNA fragments were sequenced, and the determined primary structures were inserted in an alignment of about 100 complete bacterial 23S rRNA sequences according to primary-structure and predicted secondary-structure similarities. The aligned partial sequences are shown in Fig. 2. Overall similarity values of the new partial sequences and the homologous parts of published 23S rRNA primary structures from selected reference organisms are given in Table 3. Overall similarity values derived from complete 23S rRNA sequences from the latter bacteria are shown for comparison. The matrix of similarity values in Table 3 illustrates the relatively low degree of sequence conservation within this particular 23S rRNA region. The highest value derived from comparison of complete sequences is 95.3% for the *P. aeruginosa-P. stutzeri* pair and corresponds to 85.2% similarity between the amplified fragments. The remarkably lower conservation in this region compared with that in the complete 23S rRNA molecules is even more evident from the comparison of the lowest values, i.e., 69.1% for the complete sequences of *Alcaligenes faecalis* and *Micrococcus luteus* versus 48.1% for the variable region.

Synthesis and labeling of the probes. The potential of empirically screened 23S rRNA gene fragments as specific

TABLE 4. Specificities of 23S rRNA targeted polynucleotide probes

Organism[a]	Phylogeny[a]	Hybridization to probe[a]:							
		ACA23III	AFA23III	AHY23III	NEX23III	PAE23III	PFL23III	PST23III	Eub338
P. aeruginosa DSM 50071	pbg	−	−	−	−	+	+	+	+
P. alcaligenes DSM 50342	pbg	−	−	−	0	+	+	+	+
P. aureofaciens DSM 50082	pbg	0	0	0	0	+	+	+	+
P. cichorii DSM 50259	pbg	0	0	0	0	+	+	+	+
P. chlororaphis DSM 50083	pbg	0	0	0	0	+	+	+	+
P. fluorescens DSM 50090	pbg	−	−	−	0	+	+	+	+
P. pseudoalcaligenes DSM 50188	pbg	0	0	0	0	+	+	+	+
P. putida DSM 291	pbg	0	0	0	0	+	+	+	+
P. stutzeri DSM 50227	pbg	0	0	0	0	+	+	+	+
P. syringae DSM 50252	pbg	0	0	0	0	+	+	+	+
Acinetobacter calcoaceticus DSM 30004	pbg	+	−	−	−	0	0	0	+
Aeromonas hydrophila DSM 30188	pbg	−	−	+	0	0	0	0	+
Stenotrophomonas maltophilia DSM 50170	pbg	0	0	0	0	−	−	−	+
"*P. diminuta*" DSM 1635	pba	−	−	−	−	−	−	−	+
Paracoccus denitrificans LMG 4218^T	pba	0	0	0	0	−	−	−	+
Achromobacter sp. strain DSM 30002	pbb	−	−	−	0	0	0	0	+
Acidovorax facilis DSM 649	pbb	0	0	0	0	−	−	−	+
Alcaligenes faecalis DSM 30030	pbb	−	+	−	−	0	0	0	+
Aquaspirillum metamorphum DSM 1837	pbb	−	−	−	0	0	0	0	+
Burkholderia caryophylli DSM 50341	pbb	0	0	0	0	−	−	−	+
Burkholderia cepacia DSM 50181	pbb	−	−	−	−	−	−	−	+
Burkholderia solanacearum DSM 1993	pbb	0	0	0	0	−	−	−	+
Comamonas acidovorans DSM 50251	pbb	−	−	−	0	−	−	−	+
Hydrogenophaga palleroni DSM 63	pbb	0	0	0	0	−	−	−	+
Zoogloea ramigera DSM 287	pba	−	−	−	0	0	0	0	+
Corallococcus coralloides 650^R	pbd	0	0	0	−	0	0	0	+
Corallococcus coralloides 651^R	pbd	0	0	0	−	0	0	0	+
Corallococcus coralloides 651^R	pbd	0	0	0	−	0	0	0	+
Chondromyces apiculatus 14^R	pbd	0	0	0	−	0	0	0	+
Chondromyces apiculatus 15^R	pbd	0	0	0	−	0	0	0	+
Cystobacter fuscus 16^R	pbd	0	0	0	−	0	0	0	+
M. xanthus 2^R	pbd	0	0	0	−	0	0	0	+
M. xanthus 10^R	pbd	0	0	0	−	0	0	0	+
M. xanthus 63^R	pbd	0	0	0	−	0	0	0	+
N. exedens 620^R	pbd	−	−	−	+	0	0	0	+
N. exedens 621^R	pbd	0	0	0	+	0	0	0	+
Bacillus globisporus DSM 4	gpl	0	0	0	0	−	−	−	+
Bacillus subtilis DSM 10	gpl	−	−	−	0	0	0	0	+
Enterococcus faecium DSM 20477	gpl	−	−	−	0	0	0	0	+
Staphylococcus carnosus DSM 20501^T	gpl	−	−	−	−	0	0	0	+
Micrococcus luteus DSM 20030	gph	0	0	0	0	−	0	0	+
Flavobacterium ferrugineum DSM 30193	cfb	−	−	−	0	0	0	0	+

[a] Abbreviations: +, hybrid detected; −, no hybrid detected; 0, not determined; Eub338, 16S rRNA targeted oligonucleotide probe specific for the domain *Bacteria* (1); cfb, *Cytophaga/Flavobacterium/Bacteroides* phylum; DSM, German Collection of Microorganisms and Cell Cultures, Brunswick, Germany; gph, gram-positive bacteria with high genomic DNA G+C content; gpl, gram-positive bacteria with low genomic DNA G+C content; LMG, Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium; pba, *Proteobacteria* α subclass; pbb, *Proteobacteria* β subclass; pbg, *Proteobacteria* γ subclass; pbd, *Proteobacteria* δ subclass; ^{R} Reichenbach, Brunswick, Germany.

hybridization probes for phylogenetic groups has already been demonstrated (13). On the basis of knowledge of the "conservation profile" of bacterial 23S rRNA primary structure derived from the comparison of about 100 complete 23S rRNA sequences (17, 22; unpublished data) from representatives of all bacterial phyla (34), the variable region within domain III was selected as a potential target site for group-specific polynucleotide probes. By applying the PCR technique in combination with site-specific primers, a more targeted approach was used in the present study to construct group-specific probes. The in vitro amplified DNA fragments corresponding to the large variable region of domain III of 23S rRNAs from *Aeromonas hydrophila* (AHY23III), *Acinetobacter calcoaceticus* (ACA23III), *Alcaligenes faecalis* (AFA23III), *N. exedens* (NEX23III), *P. aeruginosa* (PAE23III), *P. fluorescens* (PFL23III), and *P. stutzeri* (PST23III) were used as probes to test the general applicability of the region and the approach to construction of group-specific probes. Different approaches were used to prepare and to label the polynucleotide probes. The cloned rDNA or the amplified fragments were labeled by applying the random-priming technique. The disadvantage of using these techniques is that the resulting mixture contains labeled DNA fragments of different lengths covering only parts of the probe sequence. Thus, the specificity of the approach may be influenced by the fractions of different probe primary-structure fragments. In an alternative, more convenient approach, labeled nucleotides were introduced during in vitro amplification. However, both complementary strands are polymerized by using both these procedures. Thus, reannealing of complementary probe DNA strands is competing with probe-target hybridization, thereby decreasing the sensitivity of specific probe hybridization. These problems can be circumvented

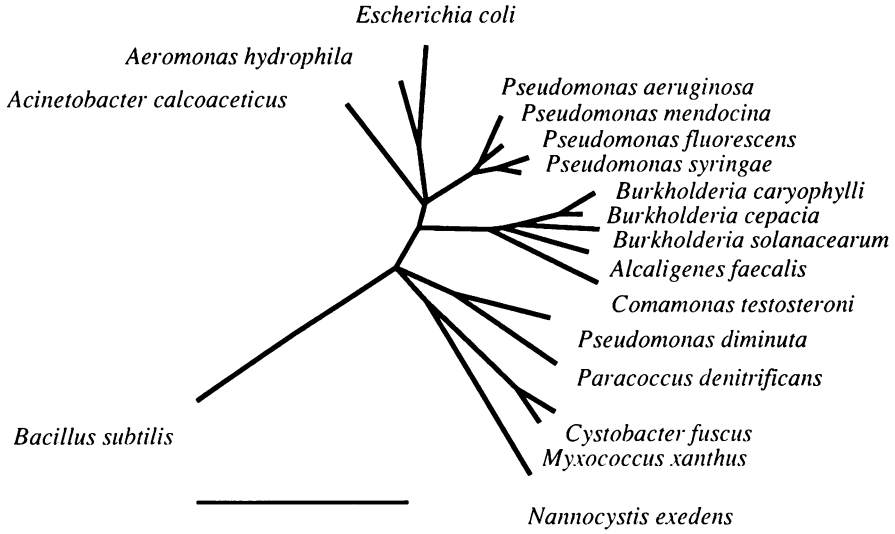

FIG. 3. Phylogenetic tree based on available (17, 22) complete 16S rRNA sequences from bacteria investigated in the present study. The distance matrix tree was reconstructed to include only positions which share the same composition in at least 50% of the entire data set. The program NEIGHBOR (12) was used for tree reconstruction. The bar indicates 10% estimated sequence divergence.

by applying the in vitro transcription assay for generating and labeling the probe nucleic acid. The resulting transcribed RNA probes are single stranded. Furthermore, RNA-DNA hybrids are more stable than DNA-DNA hybrids (26), allowing us to perform the hybridization experiments under more stringent conditions. It is advantageous to take care to transcribe an RNA complementary to the target rRNA. RNA-RNA hybrids are even more stable than RNA-DNA hybrids (26), and the high copy number of rRNA target molecules within bacterial cells enhances the sensitivity of the assay (29, 32). After linearization by digesting the recombinant pBluescript DNA with appropriate restriction enzymes, the cloned rDNA was transcribed with the corresponding RNA polymerases. Care has to be taken to completely digest the sample in order to prevent runoff transcripts of vector DNA. Furthermore, to ensure the desired specificity of the probes, it should be controlled electrophoretically so that full-length transcripts are generated. Using primers containing RNA polymerase promoters (Table 2) during in vitro amplification of the rDNA fragments subsequently allows direct in vitro transcription of the amplified template. In general, any of the above methods can be used, but the best results with respect to specificity and sensitivity of the assay were obtained by using in vitro transcribed RNA as probes.

Specificity of the probe. Phylogenetically closely (*P. aeruginosa*, *P. fluorescens*, and *P. stutzeri*) and moderately (*Acinetobacter calcoaceticus*, *Aeromonas hydrophila*) related species of the γ subclass of proteobacteria were arbitrarily chosen for probe design. As even less closely related members of the proteobacteria, *Alcaligenes faecalis* (β subclass) and *N. exedens* (δ subclass) were also included as sources of probes. Bacterial species which were included as reference organisms are listed in Table 4. Their phylogenetic affiliations to phyla and subclasses are given with the names. A 16S rRNA-based phylogenetic tree is shown in Fig. 3, illustrating the relatedness of those species from which rRNA sequences are available in public databases (17, 22). Unfortunately, only a few 16S rRNA sequences of the genuine pseudomonads (*P. aeruginosa*, *P. fluorescens*, *P. syringae*, and *P. mendocina*) have been published so far. However, the phylogenetic unity of the genuine pseudomonads (24) has been demonstrated by the results of DNA-RNA hybridizations (8–10) and 16S rRNA cataloguing data (35) and is supported by the similarity values of the 23S rRNA sequence fragments determined in the present study (Table 3). Therefore, the branching separating *P. aeruginosa* from *P. fluorescens* and *P. syringae* in the tree in Fig. 3 roughly reflects the phylogenetic depth of the group of genuine pseudomonads.

By applying the specified experimental conditions, the polynucleotide probes generated from rDNA of *P. aeruginosa*, *P. fluorescens*, and *P. stutzeri* hybridized to rRNAs (DNAs) from all genuine pseudomonads included in the present study but not to nucleic acids from any of the reference organisms. Consequently, the range of specificities of the "pseudomonad" probes is presumably defined by a level of phylogenetic relatedness which lies between the branch separating the genuine pseudomonads from the other species in the tree in Fig. 3. This is supported by the results obtained from hybridization experiments with the corresponding probes designed for phylogenetic groups represented by *Acinetobacter*, *Aeromonas*, *Alcaligenes*, and *Nannocystis* species, respectively. None of these four probes hybridized to nucleic acids from any of the included reference organisms. As can be seen in the tree (Fig. 3), the levels of relatedness among the four species and the other organisms are below that suggested for the "pseudomonad probe." Obviously, additional reference organisms have to be included to define the specificity range of the probes more exactly with respect to phylogenetic levels below that defining the group of genuine pseudomonads.

A more restricted range of specificity was reported for a probe in vitro transcribed from cloned rDNA (pPstu255) of *P. stutzeri* (27). In this case, only part of the cloned rDNA was used as template for in vitro transcription.

Conclusions. The usefulnes of the highly variable part of domain III of 23S rRNAs as target regions for group-specific polynucleotide probes was demonstrated for a selection of bacteria representing two phyla. By performing comparative analysis of complete bacterial 23S rRNAs from all phyla (17, 22; unpublished data), it was found that this region is comparably variable in all representatives of the different lines of

descent. Thus, it should be generally useful for the preparation of specific polynucleotide probes for groups of comparable phylogenetic depths at least among the *Bacteria*.

In comparison with short oligonucleotide probes, the melting temperatures of polynucleotide probe-target hybrids are not as drastically affected by single mismatches. Thus, the risk of loss of specificity as a result of single-base changes in the target sites of rRNAs from members of a phylogenetic group is reduced with polynucleotide probes. Similarily, the occurrence of single-base identities in target sites of molecules from unrelated organisms is less critical. However, in cases when a clear-cut definition of phylogenetic groups and their depths based on rRNA sequence differences is difficult, this may also be reflected by the probing results. By applying polynucleotide probes, weak hybridization signals may be expected from target rRNAs from "borderline" organisms lacking a sufficient fraction of diagnostic (group-defining) sequence positions, particularly within the probe target region. Since the stability of imperfectly matched probe-target hybrids is dependent on the experimental conditions, these have to be carefully controlled. The fact that probe specificities can be triggered by changing the stringency of hybridization (33) should, however, be regarded as an advantage of polynucleotide probes.

Whereas extensive sequence or database analyses have to be performed for the reliable design of oligonucleotide probes, knowledge of the corresponding sequences is not necessary for preparation of the described polynucleotide probes by PCR. The amplification primers (Table 2) have been successfully used to amplify the corresponding fragments from rDNA from members of various phyla (25; unpublished data). This may allow the rapid preparation of group-specific probes from small samples of cultivated or (after enrichment) uncultivated, known or unknown bacteria. Even more importantly, the approach is a promising tool for the rapid analysis of complex environmental communities (33).

ACKNOWLEDGMENT

This work was supported by Commission of the European Communities grant BIOT-CT91-0294.

REFERENCES

1. **Amann, R., W. Ludwig, and K. H. Schleifer.** 1992. Identification and *in situ* detection of individual bacterial cells. FEMS Microbiol. Lett. **100:**50–54.
2. **Amann, R., N. Springer, W. Ludwig, H. D. Görtz, and K. H. Schleifer.** 1991. Identification *in situ* and phylogeny of uncultured bacterial endosymbionts. Nature (London) **351:**161–164.
3. **Amann, R., J. Stromley, R. Devereux, R. Key, and D. A. Stahl.** 1992. Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms. Appl. Environ. Microbiol. **58:** 614–623.
4. **Amann, R. I., B. J. Binder, R. J. Olson, S. W. Chisholm, R. Devereux, and D. A. Stahl.** 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. **56:**1919–1925.
5. **Amann, R. I., L. Krumholz, and D. A. Stahl.** 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. **172:**762–770.
6. **Brosius, J., T. J. Dull, D. D. Sleeter, and H. F. Noller.** 1981. Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. J. Mol. Biol. **148:**107–127.
7. **DeLong, E. F., G. S. Wickham, and N. R. Pace.** 1989. Phylogenetic stains: ribosomal RNA based probes for the identification of single cells. Science **243:**1360–1363.
8. **De Vos, P., and J. De Ley.** 1983. Intra- and intergeneric similarities of *Pseudomonas* and *Xanthomonas* ribosomal ribonucleic acid cistrons. Int J. Syst. Bacteriol. **33:**487–509.
9. **De Vos, P., M. Goor, M. Gillis, and J. De Ley.** 1985. Ribosomal ribonucleic acid cistron similarities of phytopathogenic *Pseudomonas* species. Int J. Syst. Bacteriol. **35:**169–184.
10. **De Vos, P., A. Van Landschoot, P. Segers, R. Tytgat, M. Gillis, M. Bauwens, R. Rossau, M. Goor, B. Pot, K. Kersters, P. Lizzaraga, and J. De Ley.** 1989. Genotypic relationships and taxonomic localization of unclassified *Pseudomonas* and *Pseudomonas*-like strains by deoxyribonucleic acid:ribosomal ribonucleic acid hybridizations. Int J. Syst. Bacteriol. **39:**35–49.
11. **Dryden, S. C., and S. Kaplan.** 1990. Localization and structure analysis of the ribosomal RNA operons of *Rhodobacter sphaeroides*. Nucleic Acids Res. **18:**7267–7277.
12. **Felsenstein, J.** 1982. Numerical methods for inferring phylogenetic trees. Q. Rev. Biol. **57:**379–404.
13. **Festl, H., W. Ludwig, and K. H. Schleifer.** 1986. DNA hybridization probe for the *Pseudomonas fluorescens* group. Appl. Environ. Microbiol. **47:**49–55.
14. **Göbel, U. G., and E. J. Stanbridge.** 1984. Cloned *Mycoplasma* ribosomal RNA genes for the detection of mycoplasma contamination in tissue cultures. Science **226:**1211–1213.

14a. **Green, C. J., G. C. Stewart, M. A. Hollis, and B. S. Volu.** 1985. Nucleotide sequence of the *Bacillus subtilis* ribosomal RNA operon rrnB. Gene **37:**261–266.

15. **Höpfl, P., W. Ludwig, K. H. Schleifer, and N. Larsen.** 1989. The 23S ribosomal RNA higher-order structure of *Pseudomonas cepacia* and other prokaryotes. Eur. J. Biochem. **185:**355–364.
16. **Larsen, N.** 1992. Higher order interaction in 23S rRNA. Proc. Natl. Acad. Sci. USA **89:**5044–5048.
17. **Larsen, N., G. J. Olsen, B. L. Maidak, M. J. McCaughey, R. Overbeek, T. J. Macke, T. L. Marsh, and C. R. Woese.** 1993. The ribosomal database project. Nucleic Acids Res. **21**(Suppl.):3021–3023.
18. **Liesack, W., M. Menke, and E. Stackebrandt.** 1990. Rapid generation of vector-free digoxygenin-dUTP labeled probes for nonradioactive hybridization using the polymerase chain reaction (PCR) method. Syst. Appl. Microbiol. **13:**255–256.
19. **Ludwig, W., G. Kirchhof, N. Klugbauer, M. Weizenegger, D. Betzl, M. Ehrmann, C. Hertel, S. Jilg, R. Tatzel, H. Zitzelsberger, S. Liebl, M. Hochberger, D. Lane, P. R. Wallnöfer, and K. H. Schleifer.** 1993. Complete 23S ribosomal RNA sequences of gram-positive bacteria with a low DNA G+C content. Syst. Appl. Microbiol. **15:**487–501.
20. **Manz, W., R. Amann, W. Ludwig, M. Wagner, and K. H. Schleifer.** 1992. Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions. Syst. Appl. Microbiol. **15:**593–600.
21. **Marmur, J.** 1961. A procedure for the isolation of DNA from microorganisms. J. Mol. Biol. **3:**208–218.
22. **Neefs, J. M., P. De Rijk, Y. Van de Peer, S. Chapelle, and R. De Wachter.** 1993. Compilation of small ribosomal subunit RNA structures. Nucleic Acids Res. **21:**3025–3049.
23. **Oelmüller, U., N. Krüger, A. Steinbühl, and C. G. Friedrich.** 1990. Isolation of prokaryotic RNA and detection of specific mRNA with biotinylated probes. J. Microbiol. Methods **11:**73–78.
24. **Palleroni, N. J.** 1992 Present situation of the taxonomy of aerobic pseudomonads, p. 105–115. *In* E. Galli, S. Silver, and B. Witholt (ed.), *Pseudomonas*: molecular biology and biotechnology. American Society for Microbiology, Washington, D.C.

24a. **Regensburger, A., W. Ludwig, and K. H. Schleifer.** 1988. Complete nucleotide sequence of a 23S ribosomal RNA gene from *Micrococcus luteus*. Nucleic Acids Res. **16:**2344.

25. **Roller, C., W. Ludwig, and K. H. Schleifer.** 1992. Gram-positive bacteria with a high DNA G+C content are characterized by a common insertion within their 23S rRNA genes. J. Gen. Microbiol. **138:**1167–1175.
26. **Saenger, W.** 1984. Principles of nucleic acid structure. Springer-Verlag KG, Berlin.
27. **Schleifer, K. H., R. Amann, W. Ludwig, C. Rothemund, N. Springer, and S. Dorn.** 1992. Nucleic acid probes for the identification and in situ detection of pseudomonads, p. 127–134. *In* E. Galli, S. Silver, and B. Witholt (ed.), *Pseudomonas*: molecular biology and biotechnology. American Society for Microbiology,

Washington, D.C.
28. **Schleifer, K. H., and W. Ludwig.** 1989. Phylogenetic relationships among bacteria, p. 103–117. *In* B. Fernholm, K. Bremer, and H. Jörnwall (ed.), The hierarchy of life. Elsevier Science Publishers BV, Amsterdam.
29. **Schleifer, K. H., W. Ludwig, and R. Amann.** 1993. Nucleic acid probes, p. 464–499. *In* M. Goodfellow and O. McDonnell (ed.), Handbook of new bacterial systematics. Academic Press, Ltd., London.
30. **Spring, S., R. Amann, W. Ludwig, K. H. Schleifer, and N. Petersen.** 1992. Phylogenetic diversity and identification of nonculturable magnetotactic bacteria. Syst. Appl. Microbiol. **15:**116–122.
31. **Spring, S., R. Amann, W. Ludwig, K. H. Schleifer, H. van Gemerden, and N. Petersen.** 1993. Dominating role of an unusual magnetotactic bacterium in the microaerophilic zone of a freshwater sediment. Appl. Environ. Microbiol. **59:**2397–2403.
32. **Stahl, D. A., and R. Amann.** 1991. Development and application of nucleic acid probes, p. 205–242. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematic. John Wiley & Sons, Ltd., Chichester, United Kingdom.
32a. **Toschka, H. Y., P. Höpfl, W. Ludwig, K. H. Schleifer, N. Ulbrich, and V. A. Erdmann.** 1987. Complete nucleotide sequence of a 23S ribosomal RNA gene from *Pseudomonas aeruginosa*. Nucleic Acids Res. **15:**7182.
33. **Trebesius, K. H., R. Amann, W. Ludwig, K. Mühlegger, and K. H. Schleifer.** 1994. Identification of whole fixed bacterial cells with nonradioactive 23S rRNA-targeted polynucleotide probes. Appl. Environ. Microbiol. **60:**3228–3235.
34. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221–271.
35. **Woese, C. R., P. Blanz, and C. M. Hahn.** 1984. What isn't a pseudomonad: the importance of nomenclature in bacterial classification. Syst. Appl. Microbiol. **5:**179–195.