APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1991, p. 1468–1477
0099-2240/91/051468-10$02.00/0

Vol. 57, No. 5

Designation of Streptomycete 16S and 23S rRNA-Based Target Regions for Oligonucleotide Probes

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Received 6 November 1990/Accepted 4 March 1991

The 16S and 23S rRNA of various *Streptomyces* species were partially sequenced and screened for the presence of stretches that could define all members of the genus, groups of species, or individual species. Nucleotide 929 (*Streptomyces ambofaciens* nomenclature [J. L. Pernodet, M. T. Alegre, F. Boccard, and M. Guerineau, Gene 79:33–46, 1989]) is a nucleotide highly unique to *Streptomyces* species which, in combination with flanking regions, allowed the designation of a genus-specific probe. Regions 158 through 203 of the 16S rRNA and 1518 through 1645 of the 23S rRNA (helix 54 [Pernodet et al., Gene 79:33–46, 1989]) have a high potential to define species, whereas the degree of variation in regions 982 through 998 and 1102 through 1122 of the 16S rRNA is less pronounced but characteristic for at least certain species. Alone or in combination with each other, these regions may serve as target sites for synthetic oligonucleotide probes and primers to be used in the determination of pure cultures and in the characterization of community structures. The specificity of several probes is demonstrated by dot blot hybridization.

The potential of large rRNAs to serve as a most valuable source for both delineating phylogenetic relationships (31, 51) and taxon identification (36, 52) has been exploited over the last 12 years. The number of partial and full 16S and 23S RNA sequences are in the hundreds (complete and almost complete 16S rRNA sequences have been collected by Neefs et al. [23], while numerous partial 16S rRNA sequences are reported in various journals). The primary structures of these rRNA molecules contain stretches of sequence conserved to various degrees and, at least for the thoroughly investigated prokaryotic 16S rRNAs, their positions are known. The more highly conserved regions are the basis for phylogenetic analysis (52) and for the design of universal oligonucleotide probes and primers used for the identification and amplification, respectively, of rDNA from members of higher taxa (35). The variable regions, on the other hand, are of doubtful phylogenetic significance (45). Depending on the degree of variability, these stretches, in principle, allow the allocation of isolates to lower taxonomic ranks (i.e., genus, species, and strains) by sequence analysis (7, 51), oligonucleotide probing (5, 6, 9, 10, 19, 37, 49), or polymerase chain reaction (PCR) diagnosis (2). These strategies have revolutionized the unraveling of community structures (8, 25, 26, 44) and the diagnosis of human and animal pathogenic microorganisms (2, 5, 27, 37).

In this report, we present rRNA sequences of the genus *Streptomyces* (as recently defined [50]). The biotechnological importance of streptomycetes demands screening methods which concentrate on the recognition of novel strains in environmental samples; for such studies, a combination of molecular approaches (13) and established screening programs should have definite advantages over established screening programs alone.

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MATERIALS AND METHODS

Organisms and growth conditions. Nonmarine streptomycete strains were cultivated in medium 65, as indicated in the catalog of strains of the German Collection of Microorganisms (DSM), Braunschweig, Germany. Marine strains were grown in the same medium supplemented with 25% (vol/vol) artificial seawater (21). The fact that these isolates are members of the genus *Streptomyces* was determined by morphological and physiological criteria (46).

Phenotypic characterization of soil isolates included morphological properties such as the formation of aerial mycelium, the position of spores, numerical analysis of 60 characteristics (48), and the determination of murein type (30) and fatty acid pattern (14). Data were compared to those obtained from a large number of reference organisms (20, 47, 48).

All organisms investigated are listed in Table 1. The order of the first 62 strains corresponds to the order in which their rRNA was blotted onto the membrane filter used for probe application (see Fig. 1).

Sequence and dot blot analysis. All steps, including lysis of the cells with a French press, selective precipitation of crude RNA by 3 M sodium acetate, and conditions used in the dideoxy sequence analysis of cDNA generated from rRNA by using avian myeloblastosis virus reverse transcriptase (Promega) and terminal deoxynucleotidyl transferase (Boehringer) have been previously described in detail (37). The following oligonucleotide primers were used: 16S rRNA (*Escherichia coli* nomenclature [3], in 5′ to 3′ direction) positions 343 through 357 (CTGCTGCCTCCCGTA; melting temperature [T_m], 50°C), positions 1100 through 1115 (AGG GTTGCGCTCGTTG; T_m, 52°C), and positions 1190 through 1209 (GGGCATGATGACTTGACGTC; T_m, 62°C); and 23S

TABLE 1. Organisms investigated in this study

Species or isolate[a]	Strain	Origin[b]	Abbreviation[c]
1. *S. spectabilis*	5512^T	SW	mst
2. *S. cinereus*	43033^T	DSM	mci
3. *S. diastaticus*	40496^T	DSM	mdi
4. *S. caesius*	40419^T	DSM	mca
5. *S. griseorubens*	5160	SW	mgb
6. Soil isolate	ID 1148	DSM	P
7. *S. alboviridis*	40326^T	DSM	mav
8. *S. minutiscleroticus*	5301^T	SW	mmi
9. "*S. scabies*"	40078	DSM	msb
10. *S. griseus* subsp. *griseus*	40236^T	DSM	mgg
11. *S. albus*	40313^T	DSM	mal
12. *S. prasinopilosus*	5098	SW	mpr
13. *S. spiralis*	MC9H^T	SW	msr
14. *S. phaeoviridis*	5285^T	SW	mpv
15. *S. griseosporeus*	5562^T	SW	mgr
16. *S. melanogenes*	5192^T	SW	mme
17. *S. griseoviridis*	5229^T	SW	mgv
18. *S. coerulescens*	5146^T	SW	mcr
19. *S. brasiliensis*	43159^T	DSM	mbr
20. *S. cyaneus*	40108^T	DSM	mcy
21. *S. baldaccii*	0819^T	DPDU	vba
22. *S. salmonis*	00098	DPDU	vsa
23. *S. rutilum*	41494	DSM	vru
24. *S. griseoverticillatum*	41490	DSM	vgr
25. *S. biverticillatum*	41488	DSM	vbi
26. *S. rubroverticillatum*	41489	DSM	vrb
27. *S. cinnamoneum* subsp. *cinnamoneum*	0093^T	DPDU	vcc
28. *S. cinnamoneum* subsp. *albosporum*	40897^T	DSM	vcb
29. *S. cinnamoneum* subsp. *lanosum*	40898^T	DSM	vcl
30. *S. cinnamoneum* subsp. *sparsum*	40899^T	DSM	vcs
31. *S. cinnamoneum* subsp. *azacolutum*	0074	DPDU	vca
32. *S. moro-okaense*	40503^T	DSM	vok
33. *S. olivoreticuli* subsp. *cellulophilum*	0278	DPDU	vor
34. "*S. mediocidicum*"	4285	JCM	vmd
35. "*S. rimofaciens*"	4880	JCM	vri
36. "*S. reticuli*"	4130	JCM	vrt
37. *S. netropsis*	2283	IFAM	vne
38. *S. hachijoense*	2011^T	DSM	vhj
39. *S. taitoense*	41499^T	DSM	vta
40. *S. purpureus*	43360	DSM	mka
41. *S. exfoliatus*	40060^T	DSM	mex1
42. *S. lavendulae*	40069^T	DSM	mlv
43. *S. violascens*	183^T	SW	mvi
44. *S. kitasatoensis*	41495	DSM	vkt
45. *S. abikoense*	40831^T	DSM	vab
46. "*S. tropicalensis*"	40520	DSM	vtr
47. *S. griseocarneum*	40004^T	DSM	vgc
48. "*S. kobense*"	5006	JCM	vko
49. *S. luteoreticuli*	0081	DPDU	vlu1
50. *S. saraceticus*	5241	SW	msa
51. *S. hydrogenans*	5586	SW	mhd
52. *S. sapporonensis*	41493^T	DSM	vso
53. *S. griseus* subsp. *formicus*	773	DSM	mgf
54. *S. flocculus*	5327^T	SW	mfl
55. Soil isolate	ID 1150	DSM	R
56. *S. hygroscopicus*	N736	SW	mhy
57. *Streptomyces* sp.	40509	DSM	vlu2
58. *Streptomyces* sp.	4030	JCM	mex2
59. "*S. verticillus*"	4924	JCM	vvt
60. *S. thioluteum*	41486	DSM	vth
61. *S. mobaraense*	2282	IFAM	vmo
62. *S. ladakanum* subsp. *ladakanum*	40587^T	DSM	vla
S. ambofaciens	23877^T	ATCC	mam
S. indianense	43803^T	DSM	min
S. violaceoruber	A3(2)		mco
S. violaceoruber	TK21		mli
Soil isolate	ID 39	DSM	B
Marine isolate	3094	AWI	3094
Marine isolate	3215	AWI	3215
Marine isolate	3257	AWI	3257
Marine isolate	3423	AWI	3423
Marine isolate	3497	AWI	3497
Marine isolate	3595	AWI	3595
Marine isolate	3797	AWI	3797
Marine isolate	4164	AWI	4164
Marine isolate	4181	AWI	4181
Marine isolate	4358	AWI	4358
63. *Frankia* sp.	Ag45/Mut	ML	
64. *Streptosporangium corrugatum*	43316^T	DSM	
65. *Mycobacterium leprae*	LTB	Borstel	
66. *Nocardia asteroides*	43003	DSM	
67. *Microbispora rosea*	43025	DSM	
68. *Thermomonospora mesophila*	43048^T	DSM	
69. *Amycolatopsis methanolica*	11946^T	NCIB	
70. *Tsukamurella aurantiaca*	20162^T	DSM	
71. *Bifidobacterium asteroides*	20089^T	DSM	
72. *Staphylococcus aureus*	20231^T	DSM	
73. *Bacillus subtilis*	347	DSM	
74. *Lactobacillus fermentum*	20052^T	DSM	
75. *Clostridium lituseburense*	797^T	DSM	
76. *Escherichia coli*	1671	IFAM	

[a] The numbers at left correspond to the rRNA dot blot matrix in Fig. 1 and 2. *S., Streptomyces.*

[b] AWI, Alfred Wegener Institut für Polar-und Meereskunde, Bremerhaven, Germany; DPDU, Instituto di difesa delle Piante, Universita degli studi di Udine, Udine, Italy; DSM, German Collection of Microorganisms, Braunschweig, Germany; JCM, Japan Collection of Microorganisms, Riken, Wako, Saitama, Japan; Borstel, Forschungsinstitut Borstel, Institut für experimentelle Biologie und Medizin, Borstel, Germany; ML, Mary Lechevalier, Waksman Institute of Microbiology, Rutgers, The State University of New Jersey, Piscataway, N.J.; SW, Stan Williams, Department of Botany, University of Liverpool, Liverpool, United Kingdom; NCIB, National Collection of Industrial Bacteria, Aberdeen, United Kingdom; IFAM, Institut für Allgemeine Mikrobiologie, Christian-Albrechts-Universität, Kiel, Germany; ATCC, American Type Culture Collection.

[c] The letter v in an abbreviation indicates that the organism has until recently been classified as a member of the genus *Streptoverticillium.*

rRNA (*Streptomyces ambofaciens* nomenclature [28]) positions 1643 through 1659 (CTACCTTCCTGCGTCAC; T_m, 54°C) and positions 1705 through 1719 (CGACGGATTT [A,G]CCTA; T_m, 44 to 46°C). The T_ms were calculated according to the method of Wallace and Miyada (43).

Dot blot hybridization of crude rRNA with ^{32}P-labeled DNA oligonucleotides was done as described previously (37).

Nucleotide sequence accession number. The EMBL accession numbers for the 16S rRNA fragments of each of the 13 streptomycetes reported here are as follows: *S. diastaticus*, X53161; *S. albus*, X53163; *S. brasiliensis*, X43162; *S. baldaccii*, X53164; *S. salmonis*, X53169; *S. cinnamoneum* subsp. *cinnamoneum*, X53171; *S. cinnamoneum* subsp. *azacolutum*, X53165; *S. olivoreticuli* subsp. *cellulophilum*, X53166; *S. purpureus*, X53170; *S. lavendulae*, X53173; *S.*

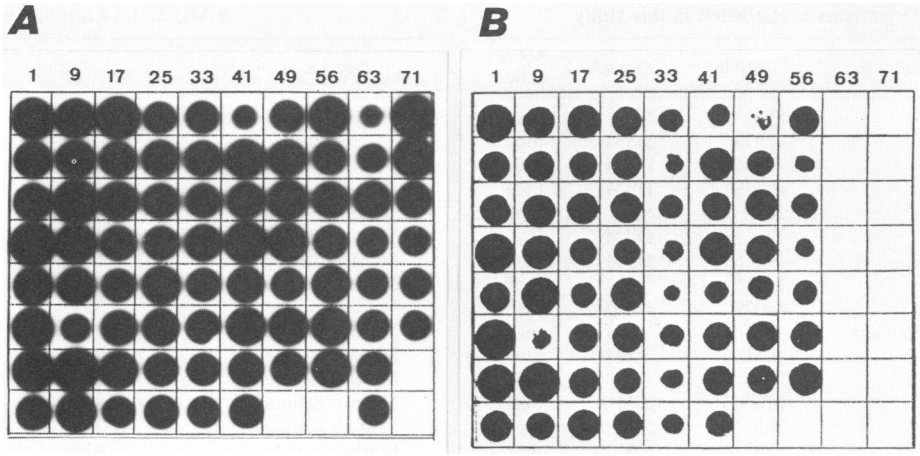

FIG. 1. Autoradiogram of a dot blot hybridization between crude rRNA of various *Streptomyces* and non-*Streptomyces* strains and ^{32}P-labeled DNA oligonucleotide probes. A total of 50 ng of rRNA was blotted and hybridized with a probe in a buffer consisting of 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 3× Denhardt solution, and 0.1% sodium biphosphate at 20°C below the T_m of the probe. After 2 h, filters were washed with 6× SSC at 25°C below the T_m for 3 min and at more stringent temperatures for 10 min. The numbers refer to organisms listed in Table 1. (A) Universal eubacterial probe (17). Hybridization temperature, 28°C; final washing temperature, 33°C. (B) *Streptomyces*-specific probe. Hybridization temperature, 34°C; final washing temperature, 53°C.

TABLE 2. Distribution of *Streptomyces* strains to variation groups of region alpha[a] of the 16S rRNA

Variation group	Sequence	Strains[b]
α1	CGGCCAGAGAUGGUCGC	vba, vsa, vru, vgr, vbi, vrb, vcc, vcb, vcl, vcs, vca, vok, vor, vmd, vri, vrt, vne, vhj, vkt, vab, vko, vso, vta, vlu2, vth
α2	GCGCUAGAGAUAGUGCC	mka, vla, vgc, vtr
α3	GCAUUAGAGAUAGUGCC	mam, mlv, vlu1, R, mme, mvi, mhd, 3094
α4	GCAUCAGAGAUGGUGCC	mli, mci, mdi, mca, mgb, B, P, mgg, mst, mco, mav, mmi, msb, 3215, 4164, 4181, 4358
α5	GCAUUAGAGAUGGUGCC	3423
α6	GCAUCAGAGAUGGUUCC	3257
α7	ACCCUGGAGACAGGGUC	mbr, msa, mgv, msr
α8	GCCGUAGAGAUACGGCC	mal
α9	CAUCCAGAGAUGGGUGC	vvt, vex, vmo
α10	CCAUUAGAGAUAGUCCC	mex
α11	CGUCUAGAGACAGGCGC	mpr
α12	CGUCUGGAGACAGGCGC	3497, 3595
α13	CGUCUGGAGAGAGGGUC	mcr
α14	CCUCUGGAGAGAGGGGC	mhy
α15	CGUCCAGAGAUGGGCGC	mgr, min
α16	GCCGUAGAGAUACGUCC	mfl
α17	GCUCUGGAGACAGGCGC	mpv
α18	CGGCCAGAGAUGGUUGC	mgf
α19	GCUGCAGAGAUGUGGCC	3797

[a] Positions 982 to 998, according to the *S. ambofaciens* sequence.
[b] Abbreviations are defined in Table 1.

TABLE 3. Distribution of *Streptomyces* strains to variation groups of region beta[a] of the 16S rRNA

Variation group	Sequence	Strains[b]
β1	AGCAUGCUUCGGGGUGAUG	vba, vsa, vru, vgr, vbi, vrb, vcc, vcb, vcl, vcs, vca, vok, vor, vmd, vri, vrt, vne, vhj, vkt, vab, vko, vso, vta, mka, vtr, vgc, mlv, mvi, vlu1, mhd, 3215, 4164, 4181, mst, mgb, P, B, mav, msb, mgg, 3423, 3257, msa, mex
β2	AGCAUGCCUUUCGGGGUGAUG	vla, vmo, vth, vvt, vex, vlu2, mhy
β3	AGCAAGCCCUUCGGGGUGUUG	mam, mli
β4	AGCAACUCCUUUCGGGGAGGUUG	mal
β5	AGCAAGCCUUCGGGUGUUG	mco
β6	AGCAGGCCCUUGUGGUGCUG	mbr, mdi, mcr, mgv, mme, mgr, mpv, msr, min, 3497, 3595, 3797
β7	AGCAAGCUUCGGGGUGUUG	mpr, mca
β8	AGCAACUCUUCGGAGGUUG	R, 4358
β9	AGCAACUCUCUUCGGAGGGGUUG	mci, mmi
β10	AGCACGUCUUCGGGAUgGUG	mgf
β11	AGCAACUCUUCGGGAGGUUG	mfl
β12	AGCAAGCCCUUCGGGGNUUG	3094

[a] Positions 1102 to 1122, according to the *S. ambofaciens* sequence.
[b] Abbreviations are defined in Table 1.

abikoense, X53168; *S. luteoreticuli*, X53172; and *S. ladakanum* subsp. *ladakanum*, X53167. Sequences for *S. ambofaciens* M27245 and *S. violaceoruber* Y00411 have been published previously.

RESULTS AND DISCUSSION

Recently, the complete 16S rDNA sequences of three *Streptomyces* species (*S. lividans* [41] and *S. coelicolor* [1] [both species are now classified as *S. violaceoruber*] and *S. ambofaciens* [28]) and the partial 16S rRNA sequences of 14 additional species of the genera *Streptomyces* and *Streptoverticillium* (50) have been published. On the basis of the intergeneric relationships of 17 members of these two genera and supported by numerical phenotypic analysis (48) and common chemotaxonomic properties, the unification of the two genera under *Streptomyces* was proposed (50). The generic name *Streptomyces* will therefore be used for all *Streptoverticillium* species throughout the following sections.

Identification of *Streptomyces* isolates. In order to investigate the potential of the molecular strategy to identify streptomycete isolates by the sequence analysis of the variable regions of the 16S rRNA, three colonies of *Streptomyces*-like appearance exhibiting different pigmentation were isolated from an agar plate inoculated with a compost heap sample taken from the botanical garden of the Christian-Albrechts-Universität, Kiel, Germany. In parallel, the isolates were investigated for traditional morphological and other phenotypic properties. The presence of LL-diaminopimelic acid in whole cell hydrolysates and the fatty acid pattern (15) confirmed that the isolates were members of the genus *Streptomyces*. Phenotypic characterization by numerical analysis gave the following results.

Isolate R (DSM ID 1150) belongs to the gray series, with spiral spore chains and red-pigmented substrate mycelium. Melanin was not produced. Although the fatty acid pattern and numerical phenetic data indicated a remote relationship to *S. violaceoruber* (synonyms: *S. lividans*, *S. coelicolor* A3, *S. lazureus*, and *S. caesius*), a more precise allocation to any of the described species was not possible.

Isolate B (DSM ID 1151) forms a whitish- to yellowish-pigmented aerial mycelium. Spore chains are straight to flexuous. Monopodial branched aerial hyphae (treelike) were occasionally observed. Melanin was not produced. Phenotypic characterization allowed the allocation of the isolate to *S. olivoverticillatum* (formerly *Streptoverticillium*; see below).

Isolate P (DSM ID 1148) produces grey straight to flexuous spore chains which show monopodial branching. Diagnostic pigments were not produced. Numerical analysis did not place the strain in any of the major clusters defined by Williams and coworkers (48). Minor cluster analysis revealed high similarity to *S. olivoverticillatum*, although isolate P does not exhibit the typical morphology of members of this species.

Analysis of 16S ribosomal RNA. In addition to the three 16S rRNA fragments (205 through 510, 595 through 895, and 970 through 1367) published recently for 13 streptomycete species (50), the 5′ terminal part of the molecule was sequenced as well. The reason for gaps in the sequences between positions 511 and 594 and between 896 and 969 is the lack of optimal binding of the routinely used "universal" primer hybridizing to a region around position 690 and a methylated rRNA-dependent termination of cDNA synthesis around position 966/967, respectively.

Genus-specific probe. A survey of the partial sequences for a genus-specific signature, which in turn could be used for the development of a genus-specific oligonucleotide probe, failed. On the other hand, the 16S rRNA oligonucleotide

TABLE 4. Distribution of *Streptomyces* strains to alpha-beta combination groups[a] of 16S rRNA

Group	Sequence combination	Strains[b]	No. of strains
1	α1/β1	vba, vsa, vru, vgr, vbi, vrb, vcc, vcb, vcl, vcs, vca, vok, vor, vmd, vri, vrt, vne, vhj, vkt, vab, vko, vso, vta	23
2	α1/β2	vlu2, vth	2
3	α2/β1	mak, vtr, vgc	3
4	α2/β2	vla	1
5	α3/β1	mlv, mvi, vlu1, mhd	4
6	α3/β3	mam	1
7	α3/β6	mme	1
8	α3/β8	R	1
9	α4/β1	3215, 4164, 4181	3
10	α4/β8	4358	1
11	α3/β12	3094	1
12	α4/β1	mst, mgb, P, B, mav, msb, mgg	7
13	α4/β3	mli	1
14	α4/β5	mco	1
15	α4/β6	mdi	1
16	α4/β7	mca	1
17	α4/β9	mci, mmi	2
18	α5/β1	3423	1
19	α6/β1	3257	1
20	α7/β1	msa	1
21	α7/β6	msr, mbr, mgv	3
22	α8/β4	mal	1
23	α9/β2	vex, vvt, vmo	3
24	α10/β1	mex	1
25	α11/β7	mpr	1
26	α12/β6	3497, 3595	2
27	α13/β6	mcr	1
28	α14/β2	mhy	1
29	α15/β6	mgr, min	2
30	α16/β11	mfl	1
31	α17/β6	mpv	1
32	α18/β10	mgf	1
33	α19/β6	3797	1

[a] See Tables 2 and 3.
[b] Abbreviations are defined in Table 1.

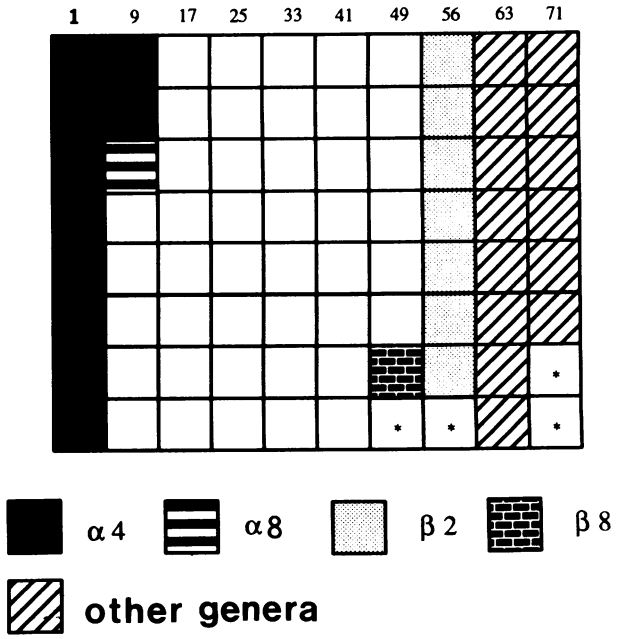

FIG. 2. Schematic presentation of the Hybond-N-filter used in RNA-DNA dot blot hybridization. The numbers refer to organisms listed in Table 1. Organisms are grouped according to their membership in the same alpha or beta sequence group, some of which are highlighted. After each hybridization step, the filter was washed at temperatures high enough to remove the labeled probe; removal was controlled by autoradiography before the filter was rehybridized with a new probe (see Fig. 1 and 3). An asterisk indicates that the position was not occupied.

catalogs of four members of *Streptomyces* (39) contain the sequence CUUAAUUCG, found so far only in streptomycetes and in members of the order *Planctomycetales* (38) but never in the rRNA catalogs of other taxa of *Actinomycetales*, which instead possess the version AUUAAUUCG (all other gram-positive bacteria have the sequence UUUAAU UCG). The 5′ terminal C residue has also been found in *Thermoproteus tenax* and *Sulfolobus acidocaldarius*, but their flanking regions show significant differences to those of streptomycetes (23). The position of the oligonucleotide within the three published complete sequences (929 through 937, according to the *S. ambofaciens* nomenclature) indicated that this stretch was part of an unsequenced region. For the designation of a probe (5′ GCGTCGAATTAAGCCA CA 3′), the flanking regions were therefore derived from the three published complete sequences.

The specificity of the genus-specific DNA probe was tested against crude rRNA from 77 *Streptomyces* strains representing 55 species, 2 soil isolates, and 10 marine isolates. rRNA from 14 species representing other actinomycete genera, gram-positive bacteria with a low DNA G+C content, and gram-negative bacteria served as nonspecific controls. The order of strains on the Hybond-N-filter is indicated in Table 1. The presence of crude rRNA was tested with a universal 16S rRNA primer (positions 786 to 803, *E. coli* nomenclature; Fig. 1A). The fact that the target site for the genus-specific probe differed by only a single nucleotide from those of members of related genera (C versus A residues) made it necessary to apply very stringent washing conditions. The discriminating temperature was 1°C below the T_m of the hybrids. Under these conditions, all *Streptomyces* rRNA still gave a strong signal, whereas the probe failed to hybridize with any of the reference rRNAs (Fig. 1B). The probe also reacted positively with rRNA from the nine marine streptomycetes (not shown), the two soil isolates, and *Kitasatosporia* strains (not shown), which are now considered members of the genus *Streptomyces* as well (45a).

Group- and species-specific 16S rDNA probes. In order to develop target sites for species-specific probes that could also be used for priming sites in PCR-mediated diagnostic rDNA amplification, the rRNA primary structure was checked for relevant regions in the 3′ half of the molecule. Two regions were identified which showed promising degrees of variation, regions 982 to 998 (alpha region) and 1102 to 1122 (beta region) (*S. ambofaciens* nomenclature). Analysis of 77 strains from 55 *Streptomyces* species, however, proved that these regions were only of restricted specificity. While 19 different variations were found in the alpha region (with 25 strains in α1; Table 2), only 12 variations were found in the beta region (with 44 strains in β1; Table 3). Interestingly, 23 of the former *Streptoverticillium* species exhibited the same alpha-beta sequence combination (Table 4), which indicates that these species are more closely related to each other than to the majority of the other *Streptomyces* species.

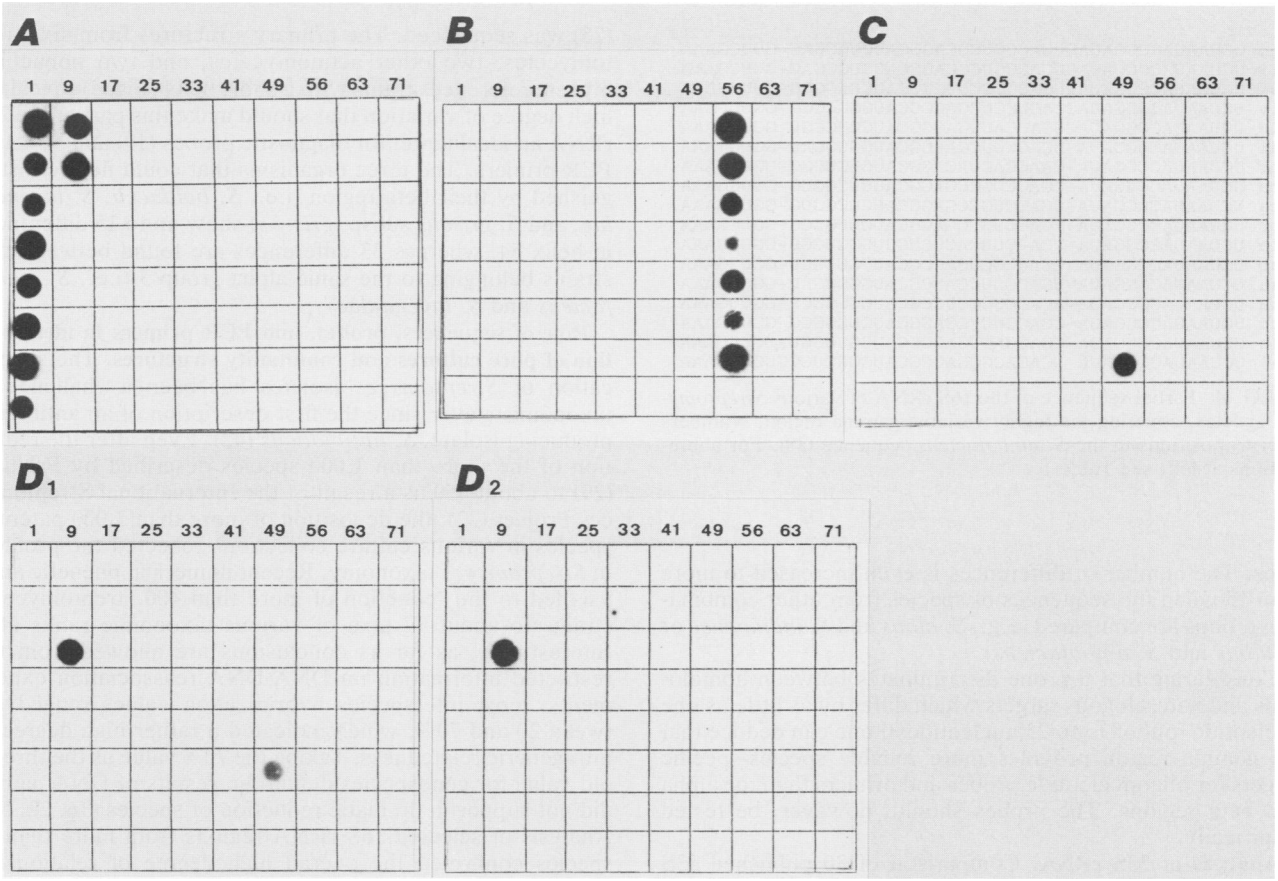

FIG. 3. Autoradiogram of dot blot hybridization between crude rRNA of organisms, as shown in Fig. 2 and group- and species-specific oligonucleotide probes. Conditions are the same as in the legend to Fig. 1. Temperatures given in parenthesis below are (in order) hybridization and final washing temperatures. (A) Probe α4 (34 and 52°C); (B) probe β2 (42 and 60°C); (C) soil isolate R probe β8 (34 and 52°C); (D_1) *S. albus* probe α8 (36 and 48°C, less stringent conditions); (D_2) *S. albus* probe α8 (36 and 55°C, stringent conditions).

Sixteen species and five marine isolates are characterized by a unique alpha-beta sequence combination, while nine other combinations are found in clusters of up to seven strains. Almost all of the marine strains possess an alpha-beta combination not found in terrestrial strains. This finding may point toward separate species status for most of the North Atlantic streptomycetes. With respect to the 200-nucleotide stretch between positions 950 and 1150, the soil isolates P and B were identical, sharing the same alpha-beta combination with five described *Streptomyces* species. A tentative phylogenetic tree based on this sequence information establishes the two isolates as phylogenetic neighbors of *S. griseorubens*, *S. alboviridis*, *S. griseus* subsp. *griseus*, and "*S. scabies*." These organisms constitute a separate line within the "streptoverticillia branch" (50). Although this data base is not substantial enough to be used reliably for phylogenetic purposes, the genetic analysis nevertheless is basically in accord with the numerical phenetic characterization which also linked strains B and P to strains formerly classified as streptoverticillia. Soil isolate R, on the other hand, has a unique alpha-beta combination, and the tentative phylogenetic analysis groups these organisms adjacent to the *S. cinereus* and the *S. violaceoruber* lines of descent. At least for the latter species, the phylogenetic and phenetic analyses are in accord. The four subspecies of *S. cinnamoneum* exhibit the same sequence variation (α1/β1), although the subspecies *azacolutum* and *cinnamoneum* possess different positions in the phylogenetic tree, which is based on a more complete 16S rRNA data base (50).

The specificity of four probes targeting two different alpha and beta regions each was tested with the same dot blot filter used in the experiment above. The distribution of the target sequences within the dot blot matrix is depicted schematically in Fig. 2. Figure 3A to C shows autoradiograms displaying the specificity of the probes. Figure 3D_1 and D_2 documents the influence of the washing temperature on the specificity of the α8 probe; whereas under less stringent conditions (4°C below T_m, which refers to the melting point of the homologous probe-target reassociation duplex) the probe directed against *S. albus* (T_m, 56°C) also binds to the rRNA from *S. flocculus* (T_m, 54°C), the latter signal is removed when the temperature is raised to 1°C below the T_m of the homologous hybrid.

The most highly variable region within the 16S rRNA, however, is located within a stretch of 30 nucleotides between positions 150 and 200 (gamma region). A total of 17 strains of 16 species were analyzed (Fig. 4), including 6 strains which could not be distinguished by their alpha or beta regions (combination group 1) (Table 4). While each of two pairs of organisms, i.e., *S. baldaccii* and *S. cinnomoneum* subsp. *azacolutum* and the two strains of *S. violaceoruber*, have identical sequences in the gamma region, other members of combination group 1 can be distinguished by sequences which differ from each other by up to 11 nucleo-

```
    158                                                        203
mco UCUAAUACCGGAUACUGACCCUCGCAGGCAUCUGCGAG-GUUCGAAA
mli UCUAAUACCGGAUACUGACCCUCGCAGGCAUCUGCGAG-GUUCGAAA
mam UCUAAUACCGGAUACUGAUCCGCUUGGGCAUCCAGGCG-GUUCGAAA
mlv UCUAAUACCGGAUACCACUCCUCGCCCGCAUGGGCGGG-GGUUGAAA
min UCUAAUACCGGAUACUGACCACUGAGGGCAUCCUCGGU-GGUUGAAA
mdi UCUAAUACCGGAUA-UGACCGUCCAUCGCAUGGUGGAU-GGUGUAAA
mbr UCUAAUACCGGAUA-CAACCACUACAGGCAUCUGUGGG-UGUGGAAA
vba UCUAAUACCGGAUA-CGACCUGCCGAGGCAUCUCGGUG-GGUGGAAA
vca UCUAAUACCGGAUA-CGACCUGCCGAGGCAUCUCGGUG-GGUGGAAA
vor UCUAAUACCGGAUA-CGACCUGCCGAGGCAUCUUGGCG-GGUGGAAA
vla UCUAAUACCGGAUA-UCA-CUUCACCCUCCUGGGUGGG-GGUUGAAA
vab UCUAAUACCGGAUA-CGACCGCUGACCGCAUGGUUGGU-GGUGGAAA
vsa UCUAAUACCGGAUA-UGACCGUCCAUCGCAUGGUGGAU-GGUGUAAA
mka UCUAAUACCGGAUA-ACACCGGCCUCCGCAUGGGGGCU-GGUUGAAA
vcc UCUAAUACCGGAUA-CGACCUGCCGAGGCAUCUCGGCG-GGUGGAAA
vlu UCUAAUACCGGAUA-CGACUACUGACCGCAUGGUUGGU-GGUGUAAA
mal UCUAAUACCGGAUA-UGACACGGGAUCGCAUGGUCUCCGUGUGGAAA
```

FIG. 4. Partial sequence of the 16S rRNA of various *Streptomyces* species, covering the highly variable gamma region. Numbers refer to positions in the *S. ambofaciens* sequence (38). For abbreviations at left, see Table 1.

tides. The number of differences is even increased to more than 15 when the sequences of species from other combination groups are compared (e.g., *S. albus* and *S. indianense* or *S. albus* and *S. ambofaciens*).

Considering that a probe discriminates between homologous and heterologous targets which differ by as little as one nucleotide (out of 15 to 25 nucleotides), one can deduce that the gamma region provides more reliable species-specific targets for oligonucleotide probes and primers than the alpha and beta regions. The probes should, however, be tested empirically.

Analysis of 23S rRNA. Comparison of 30 published 23S rRNA sequences revealed that helix 54 occurs in an extremely extended version in all members of the order *Actinomycetales*. In order to check the sequence variation within this helix, the region between position 1518 and 1637 (28) was sequenced. The primary structures from six streptomycetes, two other actinomycetes, and two nonactinomycete reference strains with a short helix (Fig. 5) reveal the high degree of variation that should make this part of the 23S rRNA an ideal target for diagnostic probes (19) and selective PCR primers. The three organisms that could not be distinguished by their beta region (i.e., *S. baldaccii*, *S. lavendulae*, and *S. griseus* subsp. *griseus*) show up to 15 differences in helix 54, whereas 33 differences are found between two strains belonging to the same alpha group 3 (i.e., *S. ambofaciens* and *S. lavendulae*).

Role of sequences, probes, and PCR primers in identification of pure cultures and community structures. The specification of *Streptomyces* isolates has been a challenge to taxonomists ever since the first description of an antibiotic-producing isolate, *S. antibioticus* (42). Even after the reduction of the more than 1,000 species described by Pridham (29) to about 450 as a result of the International Streptomyces Project (32), the deposition of more than 3,000 patented species in various culture collections reflected the problem in *Streptomyces* taxonomy. Recent numerical phenetic analysis led to the reduction of more than 400 streptomycetes strains to about 77 taxa of various taxonomic ranks (48). Interestingly, as far as conclusions are allowed from the restricted information on DNA-DNA reassociation experiments, most interspecies hybridization values ranged between 20 and 70%, which indicated a rather high degree of intrageneric relatedness. Taking the 70% value as the threshold value for genospecies definition, results of DNA pairing did not support a dramatic reduction of species (16, 22, 50). Analysis of selected 16S rRNA regions from more than 55 species confirmed the overall high degree of relationship between most of the described *Streptomyces* species. Decisions, however, about species delineation cannot be made on the basis of 16S rRNA homology values alone, because differences between the sequences are mainly found in

```
     1518
vba  UACCCGCUUUGAAACGCCCAAUACUGAAUCAGGCGAUGCUAAGUCCGUGAAGCCGCCCUGAGCU....C
mgg  UACCCGCUUUGAAACGCCCAAUAUCGAAUCAGGCGAUGCUAAGUCCGUGAAGCCGCCCUGGAGU....C
vla  UACCCGCUUUGAAACGCCCAAUAUCGAAUCAGGCGAUCGUAAGUCCGUGAAGcCGUUCCGGACC....C
mlv  UACCCGCUUUGAAACGCCCAAUAUCGAAUCAGGCGAUGCUAAGUCCGUGAAGCcGUUCCGGCC.....C
mam  UACCCGCUGUGAAGCGUCAAACAUCGAGCAUCGUGAUGCUAAGGCCGUGAAGCCGCCCUGAUCU....C
mal  UACCCGCUAUGCAGCGUCAAACAUCGAAUCAGGUGAUGCUAAGUCCGUGAUACCGCUUGUGcc.....C
pth  UACCCGUGACaGUGCGCCC.GUGUCGAGGCCGGUGAUGCUGACCAUCC.GAACCUCCUUCCUGG....C
mlu  UACCAGUGAAGAACCGCCC.AUGCUGAGCCG.GUGAUACUAACCGCCC.GAACCAUCCCGAACCUCGUC
bsu  UACCACCUCCUCACC.....AUUUGAGCAA.....................................
eco  UACUUGGUGUUACU.............GCGA.....................................

                                                                    1645
vba  UUCGGAGCGUAGGGGAGUGGUGGAGCCGACGAUCCAGACUUGUAGUAGGUAAGCGA.....UGGGGUG
mgg  UUCGGACAA.AGGGGAGUGGUGGAGCCGACGAACCAGACUUGUAGUAGGUAAGCGA.....UGGGGUG
vla  UUCGGGGAA.UGGAAAGUGGUGGAGCCGACGAUCCAGAC----------------------------
mlv  UUCGGGGAA.AGGAAAGUGGUGGAGCCGACGAACCAGaCUUGUAGUAGGUAAGCGA.....UGGGGUG
mam  UUCGGAGUUGAGGGGAGUGGUGGAGCCGCCGGACCAAGCGGUUAGUAGGUGAGUGA.....UGGGGUG
mal  UUUGCCGGn.nCGGGUUUGGUGGAGCCGACGGCCCGAGC----------------------------
pth  UUCGGGCUnGGUUGUGGGGGG..AGCGnGGGACCCGAUCCGGUAGUAGGCAACGCA.....UGGGGUG
mlu  UUUGACGGGGUCGGUGAUGGGG.AGCGCGGGACCUGAACCGGGG..AGGCAAGCGCAUUAACAGGUGUG
bsu  ...........................................................UGGGGGG
eco  ...........................................................AGGGGGG
```

FIG. 5. Helix 54 of the 23S rRNA of six *Streptomyces* species and two non-*Streptomyces* species. Two nonactinomycete species, lacking the long version of helix 54, are included for comparison. Numbers refer to positions in the *S. ambofaciens* sequence (28). For abbreviations at left, see Table 1.

variable regions known to be of restricted significance to phylogenetic relationships (51).

The major achievement that originated from the sequence analysis was the determination of the degree of variability. In most examples, the sequences of at least one of the three recognized 16S rRNA regions show differences sufficient to identify described species. Helix 54 of the 23S rRNA offers an even greater potential for species identification, but more data on the closely related group of the former *Streptoverticillium* species need to be generated. Unless a much more complete data set of 16S and 23S rRNA, and possibly of intracistronic spacers, becomes accessible, no decision can be made about whether or not a defined sequence is representative for a single species or a species group.

The same problems occur when an attempt is made to apply the knowledge about sequence variation to the development of diagnostic DNA oligonucleotide probes and PCR primers. Despite the availability of a substantial data base and a rather high degree of sequence variation within different regions, three main problems are encountered with molecular strategies for the identification of pure cultures of *Streptomyces* spp. or of streptomycetes in community structures. First, the target regions for PCR primers may not be sufficiently unique to discriminate between closely related sequences (34); unlike conditions for probes, those for primer annealing cannot be made strictly stringent (i.e., at the T_m of the hybrid) when the two primers used in the amplification reaction differ in their melting points (melting points of primers can, however, be adjusted by varying the length of the primers [personal communication of a referee of this study]). Identification of species on the basis of size fragments of PCR products alone, even when generated with apparently specific primers, may be problematic when the whole range of diversity within a primer target region has not yet been detected. Second, application is restricted to those taxa for which sequence information is available; members of a taxon under investigation which differ in the sequence(s) of the target region(s) will not be detected when probes and primers fail to hybridize under the conditions applied. This results in an underestimation of the whole range of genetic variation that may exist among members of a taxon. Third, different taxa may exhibit identical target regions; consequently, they may be treated as being identical to the target species, leading to an overestimation of target organisms during quantification of taxa in environmental samples. This reason makes extensive verification of probes necessary.

In general, the likelihood of these phenomena occurring is increased in those taxa which harbor a high number of phylogenetically closely related members, the genus *Streptomyces* being a prime example. One way to circumvent these problems is the combined application of different probes and primers. Basically, depending on the goal, three approaches may be used, each of them starting with the amplification of *Streptomyces* rDNA. Various methods are available for the extraction of DNA from pure cultures (12) or directly from the environment, and the reader is referred to the pertinent literature (11, 24, 33, 40). Several of the steps outlined below concerning the 16S rRNA have already proven valuable, as shown with streptomycetes (13, 48a) or other organisms (2, 9). Likewise, other target sites may become more reliable (possible in intergenic spacer regions and 23S rDNA), and different combinations may turn out to be more meaningful than those described.

Conclusion. The following summarizes the various strategies to be applied in the identification of *Streptomyces* species and streptomycetes in the environment. Although we have as yet not shown the experimental proof for the efficiency of the *Streptomyces*-specific PCR primers outlined below, the uniqueness of the sequences makes it likely that they can be used as reliably as those used in a study on relatives of *Streptomyces* species, namely, members of the genus *Mycobacterium* (2).

Identification of a pure culture. Identification of a pure culture may be made by the following methods: (i) amplification of 16S rDNA by using reverse primer 30 (binding to the 3′ terminus of the codogenic strand) and reverse transcriptase (RT) primer 1540 (binding to the 3′ terminus of the noncodogenic strand [18]) and subsequent check for *Streptomyces* identity of the cDNA by probing with the genus-specific probe 970 (49); (ii) sequence analysis of regions 150 to 210 and 980 to 1150 of the amplified DNA by using relevant "universal" (6, 37) reverse and/or RT primers and comparison of the sequences of the alpha, beta, and gamma regions with the data base (this study); (iii) amplification and sequence analysis of the 23S rDNA between regions 1463 and 1719 (including helix 54) by using a set of primers with the composition 5′GGACCTAAGGCGAGGCCG3′ and 5′CGACGGATTT[A,G]CCTA3′ and comparison of the sequences with the data base.

Identification of a defined species, for which sequence information is available, within an environmental sample. Identification of a defined species, for which sequence data are available, within an environmental sample may be made by the following methods: (i) amplification of 16S rDNA either by two primers or multiplex analyses (more reliable when the target region of the specific primer differs only slightly from those of related strains) (4) or by the nested primer strategy (i.e., by using reverse primer 30 as a universal primer, the first cycles could be performed with beta RT primer 1100, which would subsequently be replaced by the alpha RT primer 1000; likewise, simultaneous use of alpha and beta primers should result in the formation of fragments of two different sizes which can be visualized by agarose electrophoresis) and (ii) verification of the formation of species-specific fragments by probing with the specific gamma probe.

Detection of a *Streptomyces* population within an environmental sample. Detection of a *Streptomyces* population within an environmental sample may be made by the following methods: (i) amplification of the 16S rDNA genes from DNA isolated directly from the environmental sample by using primers with flanks which contain restriction enzyme recognition sites (alternatively, regions encompassing the 3′ terminus of the 16S rDNA and the 5′ half of the 23S rDNA, or the complete 23S rRNA, could be amplified; assuming that the intergene spacer in streptomycetes is about 300 nucleotides long, as in *S. ambofaciens* [28], it can readily be sequenced by using the 16S reverse primer 1492-1510 [*E. coli* nomenclature] and the 5′ 23S rRNA primer 5′TGCCAAG GCATCCACC3′ [positions 23 to 38 {28}]) and (ii) cloning of the amplified 16S ribosomal cDNA and selection of clones which give a positive signal with the *Streptomyces* probe, sequence analysis of the three variable alpha to gamma regions, and comparison of sequences with data bases. (iii) When sequences indicate the presence of novel isolates, new probes have to be designed to screen the collection of isolates, which are enriched and isolated in parallel with the molecular study of an ecosystem.

ACKNOWLEDGMENTS

We thank Elisabeth Wellington, Warwick, United Kingdom, for advice in strain selection; Stan Williams, Liverpool, United King-

dom, and Romano Locci, Udine, Italy, for providing us with strains; and Birgit Petermann and Kirsten Zenz, Kiel, Federal Republic of Germany, for the isolation of *Streptomyces* strains.

This work was supported in part by the German Collection for Microorganisms, Stöckheim, Federal Republic of Germany, and in part by the Australian Research Council.

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