Deoxyribonucleic Acid Homologies and Ribosomal Ribonucleic Acid Similarities Among Sporeforming Members of the Order *Actinomycetales*

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We investigated the relatedness of 24 named species belonging to 18 sporeforming genera of the order *Actinomycetales* with respect to deoxyribonucleic acid-deoxyribonucleic acid homology and ribosomal ribonucleic acid cistron similarity. A total of 21 of these species grouped in three deoxyribonucleic acid homology clusters in which the homology values ranged between 20 and 45%. The first cluster contained *Actinoplanes*, *Ampullariella*, *Amorphosporangium*, *Micromonospora*, and *Dactylosporangium*; the second cluster contained *Streptosporangium*, *Planomonospora*, and *Planobispora*; and the third cluster included *Streptomyces*, *Chainia*, *Kitasatoa*, *Streptovorticillium*, *Microellobospora*, *Elytrosporangium*, and *Actinosporangium*. The close molecular genetic relationship of the organisms in each cluster was supported by several physiological and biochemical properties of taxonomic value. The ribosomal ribonucleic acid cistron similarity data did not indicate that any two of these clusters were specifically related to each other. *Microtetrastroma viridis* DSM 43175, *Spirillospora albida* DSM 43034, and *Actinomadura madurae* DSM 43067 grouped separately from all of the other organisms investigated.

During the last 15 years there has been increasing interest in the phylogeny of the sporeforming members of the order *Actinomycetales* and in their relationship to nonsporeforming, gram-positive organisms with similar guanine-plus-cytosine (G+C) contents in their deoxyribonucleic acids (DNAs). However, the classification of families and genera presented in *Bergey's Manual of Determinative Bacteriology*, 8th ed. (17), is still unsatisfactory; these taxa were established mainly on the basis of features that were known to be of little value in the elucidation of the natural relationships among organisms (i.e., motility of spores, presence or absence of aerial mycelia and sporangia, and shape of sporangia). Most of the studies on the genetic relationships of these organisms have been performed by using DNA-DNA reassociation techniques (1, 11, 30, 33, 51), approaches that yield rough quantitative expressions of the overall genetic sequence homologies among organisms. Since these methods can detect only the closest genealogical relationships, they are of limited value in determining the relationships of more distantly related species and of genera and families. Most studies have included species of *Streptomyces* only (30, 33, 51), which is quite understandable from a commercial point of view; species of other genera have been neglected al-

most completely. The most detailed study published so far (11) included 17 species belonging to 12 genera and revealed that some of the genera appeared to be as closely related as the species of one genus.

After the conserved character of the nucleotide sequences of ribosomal nucleic acids was detected (9, 10, 31), reassociation experiments in which ribosomal ribonucleic acids (rRNA's) and rRNA cistrons were used were introduced in taxonomic studies (31, 34, 35). However, in contrast to the large number of gram-negative organisms investigated (5, 7, 8, 16, 18, 36), only a few gram-positive organisms have been studied (12, 19, 21, 29, 32), and there has been only one publication (32) dealing with sporeforming members of the order *Actinomycetales*.

In a third method which is of great value for measuring phylogenetic distances, the ribonuclease T₁-resistant oligonucleotides of 16S rRNA's are compared. The similarity coefficients obtained from binary comparisons of oligonucleotide catalogs (S_{AB} values) have been used successfully to generate a phylogenetic tree of the bacterial kingdom (13).

The phylogenetic positions of four sporeforming members of the *Actinomycetales* (namely, *Dactylosporangium aurantiacum*, *Actinoplanes philippinensis*, *Ampullariella regularis*,

and *Streptomyces griseus*) and various coryneforms, mycobacteria, and nocardiae have been described previously (13, 48, 49; E. Stackebrandt, W. Ludwig, K. H. Schleifer, and H. J. Gross, J. Mol. Evol., in press). These data have revealed that the sporeformers do not group separately from the nonsporeforming members of the *Actinomycetales* but are closely related to them.

To broaden the base of these studies, we investigated various representatives of the families *Actinoplanaceae*, *Streptomycetaceae*, and *Micromonosporaceae* by the three approaches mentioned above. In this report we present data obtained from DNA-DNA and DNA-rRNA reassociation experiments. The phylogenetic positions of various sporeforming members of the *Actinomycetales* (gram-positive organisms with high G+C contents in their DNAs) as determined by a comparative analysis of their 16S rRNA's will be described elsewhere.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All of the organisms investigated were obtained from the German Collection of Microorganisms (DSM) (Table 1). *Ampullariella campanulata* DSM 43148, *Ampullariella digitata* DSM 43149, and *Ampullariella lobata* DSM 43150 were grown in brain heart infusion medium (Difco Laboratories, Detroit, Mich.). All other organisms were grown in a broth containing 1% sucrose, 0.2% yeast extract, 0.5% peptone, 0.2% KNO₃, 0.1% K₂HPO₄, 0.05% MgSO₄·7H₂O, 0.05% KCl, and 0.001% FeSO₄. After sterilization, this medium was allowed to cool slowly. All strains were grown aerobically at 30°C. For the isolation of high-molecular-weight DNA, mass cultures were obtained from a total of 6 liters of medium from four 5-liter Erlenmeyer flasks, each containing 1.5 liters of medium. For the isolation of labeled DNA, cells were grown in 100-ml portions of medium in 500-ml flasks; the organic components of the medium used were reduced to one-half the concentrations listed for the above-described medium. To label RNA, the reduced medium was used, but yeast extract was omitted. To label DNA and RNA, 0.75 mCi of [³H]thymidine and 0.5 mCi of [³H]uracil, respectively, were added to the medium. Cells were harvested after 2 to 4 days. Labeled strains were harvested when the uptake of isotopes by the cells reached a maximum. The cells were harvested by filtering the broth through Whatman no. 1 filter paper. The purity of each preparation was checked microscopically.

The presence of LL-diaminopimelic acid or meso-diaminopimelic acid in cell walls was determined by the method of Rhuland et al. (39). Cell walls were prepared and hydrolyzed as described previously (41).

Isolation of nucleic acids. To isolate high-molecular-weight DNA, 30 g of precooled cells was suspended in 10 ml of a 1 M NaCl solution containing 1 mg of proteinase K (Boehringer Mannheim Corp., New York, N.Y.) and 1 ml of a 25% sodium dodecyl

sulfate solution. To isolate labeled DNA, 5 g of precooled cells was suspended in 2 ml of the same solution. Cells were broken by passing 5-ml quantities of the cell suspension through a French pressure cell at 8,000 lb/in². The lysate was collected in 100 ml (10 ml for labeled cells) of 1 M NaCl and was then heated to 60°C. After 9 ml (1 ml for labeled cells) of 25% sodium dodecyl sulfate was added the lysate was incubated for 10 min at 60°C. The procedures used for the isolation and purification of both kinds of DNA have been described previously (28).

Labeled RNA was isolated from cells suspended in 1 ml of a buffer containing 2 mM tris(hydroxymethyl)aminomethane, 1 mM magnesium acetate·4H₂O, 6 mM KCl, 100 µg of proteinase K per ml, and 1 mg of Macaloid (an anionic silica clay used for the inhibition of nucleases; Baroid Div., National Lead Co., Houston, Tex.) per ml. Breaking of the cells by a French press, phenolization of the lysate, and separation of the 16S and 23S rRNA's by one-dimensional sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis were performed by procedures described elsewhere (Stackebrandt et al., in press). After electrophoresis, the RNA was localized by ultraviolet light. Nucleic acids appeared as dark bands after the gel was placed on a thin-layer plate containing a fluorescent indicator (254 nm). The rRNA-containing gel material was cut out and embedded in agarose (Bio-Rad Laboratories, Richmond, Calif.) (0.5% in 0.1 M ammonium formate) in a plastic tube (13 by 1.5 cm) for electrophoretic elution. Electrophoreses were carried out at 45 mA/tube for 5 and 12 h for the isolation of 16S and 23S rRNA's, respectively. The final steps in the isolation procedure included purification of eluted RNA from a cellulose CF 11 (Whatman) column (14) with 1 M NaCl. The RNA was precipitated with 2 volumes of ethanol and was collected by centrifugation. The yields of nucleic acids were determined spectrophotometrically at 260 nm. The specific activities of [³H]-DNA ranged from 9.6×10^3 to 1.25×10^5 cpm/µg of DNA, and those of [³H]RNA ranged from 3.5×10^4 to 6.3×10^4 cpm/µg of RNA.

Nucleic acid reassociation procedures. DNA-DNA homology studies were performed by using the membrane filter technique (6, 15, 27). Preparation of DNA-containing filters (diameter, 5.5 mm), denaturation of the DNA by alkali, and fragmentation of [³H]DNA were performed by the methods of Meyer and Schleifer (28). Each hybridization was carried out with 0.1 µg of fragmented [³H]DNA in 200 µl of 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M trisodium citrate, pH. 7.0) containing 35% formamide against filter-bound DNA for 24 h at 60°C. Between 7 and 10 µg of DNA was bound to each filter, depending on the strain. The amount of filter-bound DNA was determined as described by Burton (2).

DNA-rRNA reassociations were performed by the method of De Ley and De Smedt (4), with the following modifications. The experiments were done simultaneously with four filters. Each filter contained 7 to 10 µg of DNA, depending on the strain, and was incubated with 0.5 µg of [³H]rRNA in 200 µl of 5× SSC containing 25% formamide for 18 h at 60°C. A separate vial was used for each filter. After hybridization, the RNA-containing solution was pipetted off, and the

TABLE 1. *DNA relatedness among various sporeforming members of the Actinomycetales*

Source of unlabeled DNA	Reassociation values (%) with the following sources of labeled DNA								DNA homology cluster
	<i>Actinoplanes philippinensis</i> DSM 43019	<i>Actinoplanes italicus</i> DSM 43146	<i>Ampullariella regularis</i> DSM 43151	<i>Micromonospora echinospora</i> DSM 43141	<i>Streptosporangium roseum</i> DSM 43021	<i>Planomonospora parontospora</i> DSM 43177	<i>Chainia antibiotica</i> DSM 43032	<i>Kitasatoa kawaiensis</i> DSM 43360	
<i>Actinoplanes philippinensis</i> DSM 43019	100	36	27	19	10	6	10	8	I
<i>Actinoplanes missouriensis</i> DSM 43046	29	34	37	21	9	6	10	11	I
<i>Actinoplanes utahensis</i> DSM 43147	46	31	31	18	14	5	12	10	I
<i>Actinoplanes italicus</i> DSM 43146	42	100	27	18	11	7	13	10	I
<i>Ampullariella regularis</i> DSM 43151	28	23	100	23	10	6	11	10	I
<i>Ampullariella lobata</i> DSM 43150	38	31	49	23	14	5	11	10	I
<i>Ampullariella digitata</i> DSM 43149	42	35	41	29	6	5	12	10	I
<i>Ampullariella campanulata</i> DSM 43148	36	35	42	20	13	4	11	11	I
<i>Amorphosporangium auranticolor</i> DSM 43031	25	23	23	19	8	7	8	8	I
<i>Micromonospora echinospora</i> DSM 43141	18	18	19	100	13	8	12	12	I
<i>Dactylosporangium aurantiacum</i> DSM 43157	19	19	20	24	11	10	13	7	I
<i>Streptosporangium roseum</i> DSM 43021	12	10	12	11	100	31	11	10	II
<i>Planomonospora parontospora</i> DSM 43177	8	11	10	11	33	100	13	12	II
<i>Planobispora longispora</i> DSM 43041	6	5	7	5	38	33	10	12	II
<i>Streptomyces griseus</i> DSM 40236	7	6	7	8	8	5	26	31	III
<i>Chainia antibiotica</i> DSM 43032	11	9	12	10	8	6	100	32	III
<i>Kitasatoa kawaiensis</i> DSM 43360	6	6	8	7	12	5	40	100	III
<i>Microellobosporia cinerea</i> DSM 43033	6	5	7	6	10	5	36	28	III

TABLE 1—Continued

Source of unlabeled DNA	Reassociation values (%) with the following sources of labeled DNA								DNA homology cluster
	<i>Actinoplanes philippinensis</i> DSM 43019	<i>Actinoplanes italicus</i> DSM 43146	<i>Ampullariella regularis</i> DSM 43151	<i>Micromonospora echinospora</i> DSM 43141	<i>Streptosporangium roseum</i> DSM 43021	<i>Planomonospora parvontospora</i> DSM 43177	<i>Chainia antibiotica</i> DSM 43032	<i>Kitasatoa kauaiensis</i> DSM 43360	
<i>Streptovorticillium baldacii</i> DSM 40845	7	5	7	8	9	5	37	31	III
<i>Elytrosporangium brasiliense</i> DSM 43159	6	6	9	8	10	7	34	28	III
<i>Actinosporangium violaceum</i> DSM 43350	8	9	7	8	7	8	35	41	III
<i>Streptomyces</i> species ^a	12	12	12	11	9	6	23	27	III
<i>Spirillospora albida</i> DSM 43034	12	11	12	11	11	12	12	11	
<i>Microtetraspora viridis</i> DSM 43175	6	10	7	10	12	10	13	11	
<i>Actinomadura madurae</i> DSM 43067	6	6	9	7	12	6	12	11	
" <i>Micrococcus conglomeratus</i> " CCM 2134 ^b	5	6	4	5	6	4	6	2	

^a The *Streptomyces* species was received as *Actinoplanes armeniacus* DSM 43125. As with all members of DNA homology cluster III, this strain contained LL-diaminopimelic acid in its cell walls.

^b This name is not on the Approved Lists of Bacterial Names (43) and has not been validly published since 1 January 1980; hence it is without standing in nomenclature. CCM, Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia.

filters were washed once with cold 5× SSC and incubated with 1 ml of a ribonuclease solution (2.5 µg of ribonuclease A [Serva, Heidelberg, Germany] and 50 U of ribonuclease T₁ [Calbiochem, La Jolla, Calif.]) for 45 min at 37°C. Then the filters were incubated in 1 ml of 5× SSC containing 25% formamide for 10 min at 60°C, washed twice with 5× SSC, and dried in a desiccator for 2 h at 50°C. After the bound radioactivity was measured, the filters were washed with toluene and dried. To determine of melting points [$T_{m(e)}$] of the hybrids, three of the filters used in one reassociation experiment were placed in one vial containing 1 ml of 1.5× SSC–20% formamide. The thermal stability of the duplexes was determined between 60 and 95°C by using previously described methods (4). One filter was used to determine the amount of DNA; this value was needed to calculate the amount of rRNA binding to 100 µg of DNA (percent rRNA binding).

RESULTS

DNA-DNA reassociation studies. The conditions used in the DNA-DNA reassociation studies (25°C below T_m at 60°C) were identical

to those reported previously in taxonomic studies on gram-positive organisms having high G+C contents in their DNAs (i.e., on members of *Arthrobacter* and *Brevibacterium* [12, 44], *Cellulomonas* [46], and *Oerskovia* [45]). The T_m was calculated on the basis of an average G+C content of 72 mol% (3, 17). Table 1 summarizes the reassociation values for 24 sporeforming species belonging to 18 genera of *Actinomycetales*, as listed in *Bergey's Manual* (17). The average binding efficiency in the homologous system was 32%. With [³H]DNAs from representatives of the genera *Actinoplanes*, *Ampullariella*, *Micromonospora*, *Streptosporangium*, *Planomonospora*, *Kitasatoa*, and *Chainia*, 21 of the 24 species fell into three homology clusters defined by homology values of more than 18%. Values for hybridization between organisms from different clusters ranged from 4 to 15%. These values were only slightly higher than the values for hybridization between any of the seven reference sporeforming *Actinomycetales* strains and "Mi-

crococcus conglomeratus" (not on the Approved Lists of Bacterial Names [43] and not validly published since 1 January 1980; hence, without standing in nomenclature), which served as an unrelated organism with a high G+C content (4 to 9%). Three *Actinomycetales* strains could not be assigned positively to any of the three groups; these were *Spirillospora albida* DSM 43034, *Actinomadura madurae* DSM 43067, and *Microtetraspora viridis* DSM 43175 (5 to 16%).

Homology clusters I to III could not be defined by species of one single genus. Cluster I containing species belonging to the genera *Actinoplanes* and *Ampullariella*, as well as *Amorphosporangium auranticolor* DSM 43031, *Dactylosporangium aurantiacum* DSM 43157, and *Micromonospora echinospora* DSM 43141. The homology values obtained between the [³H]DNAs from *Actinoplanes philippinensis* DSM 43019, *Actinoplanes italicus* DSM 43146, and *Ampullariella regularis* DSM 43151 and filter-bound DNAs from various species of these five genera ranged from 20 to 49%.

Cluster II contained *Streptosporangium roseum* DSM 43021, *Planomonospora parontospora* DSM 43177, and *Planobispora longispora* DSM 43041 (homology values, 31 to 38%), and cluster III contained *Streptomyces griseus* DSM 40236, *Chainia antibiotica* DSM 43032, *Kitasatoa kauaiensis* DSM 43360, *Microellobosporia cinerea* DSM 43033, *Streptovorticillum baldaccii* DSM 40845, *Elytrosporangium brasiliense* DSM 43159, and *Actinosporangium violaceum* DSM 43350 (homology values, 23 to 41%).

DNA-rRNA reassociation studies. Nucleic acid reassociation studies between DNA and the highly conserved 23S and 16S rRNA's, as well as a comparative analysis of 16S rRNA's and physiological and biochemical data, have shown that organisms sharing DNA-DNA homology values of more than 20% obtained under optimal hybridization conditions can be regarded as closely related species at the genus level (21, 45; W. Ludwig, K. H. Schleifer, G. E. Fox, E. Seewaldt, and E. Stackebrandt, J. Gen. Microbiol., in press). On the other hand, organisms with homology values below 15% cannot be generally excluded as members of one genus, as shown by studies of *Staphylococcus* (Ludwig et al., in press), *Arthrobacter* and *Cellulomonas* (48), and *Oerskovia* (45). In these investigations, organisms sharing DNA homologies of about 15% could be placed in the same genus.

To investigate the degree of relationship between the strains of the homology clusters described above and strains that were obviously not related to any of these clusters, we chose the

DNA-rRNA reassociation technique. One representative of each of the three DNA homology clusters was labeled with [³H]uracil; 16S rRNA's were isolated from *Actinoplanes philippinensis* DSM 43019 (cluster I) and *Kitasatoa kauaiensis* DSM 43360 (cluster III), and 23S rRNA was isolated from *Streptosporangium roseum* DSM 43021 (cluster II).

Reassociation experiments were performed with various sporeforming and nonsporeforming gram-positive bacteria which had high G+C values (more than 65 mol%) and produced mycelia, rods, and coccoids. *Staphylococcus aureus* ATCC 12600 was included as a gram-positive reference organism that had a low G+C content (33 mol%).

The 23S and 16S rRNA's of the three strains investigated were isolated by one-dimensional slab gel electrophoresis. Figure 1 shows the appearance of the two RNA bands of *Streptosporangium roseum* DSM 43021. No breakdown products of either of the two RNAs were detected. However, it was important for the purity of the RNA to use log-phase cells which were disrupted and phenolized immediately after harvesting (see above). The 16S rRNA isolated by this technique has been proved to be pure by fingerprinting ³²P-labeled ribonuclease T₁-resistant oligonucleotides (Stackebrandt et al., in press).

The degree of relatedness was expressed in the following two ways: by the thermal stability of the duplexes [*T*_{m(e)}] and by the percentage of [³H]RNA bound per 100 µg of DNA (Table 2). Figure 2 shows some examples of melting curves based on 23S rRNA from *Streptosporangium roseum* DSM 43021.

In general, the amount of [³H]rRNA that dissociates from filter-bound DNA at 60°C during

FIG. 1. Distribution of 16S and 23S rRNA's from *Streptosporangium roseum* DSM 43021 after electrophoresis on a one-dimensional sodium dodecyl sulfate-polyacrylamide slab gel.

TABLE 2. *Properties of DNA-rRNA duplexes produced with ³H-labeled rRNA's from Actinoplanes philippinensis DSM 43019, Streptosporangium roseum DSM 43021, and Kitasatoa kauaiensis DSM 43360 and DNAs from various sporeforming and nonsporeforming members of the Actinomycetales*

Sequence no. ^a	Strain	DNA from:		³ H-labeled rRNA from:			
		<i>Actinoplanes philip- pinensis</i> DSM 43019		<i>Streptosporangium roseum</i> DSM 43021		<i>Kitasatoa kauaiensis</i> DSM 43360	
		% rRNA binding	$T_{m(r)}$ (°C)	% rRNA binding	$T_{m(r)}$ (°C)	% rRNA binding	$T_{m(r)}$ (°C)
1	<i>Actinoplanes philippinensis</i> DSM 43019	0.122	82.5	0.045	69.3	0.031	70.0
2	<i>Actinoplanes italicus</i> DSM 43146	0.091	77.8	0.051	72.0	0.033	71.0
3	<i>Ampullariella regularis</i> DSM 43151	0.090	78.1	0.064	69.5	0.060	70.7
4	<i>Ampullariella lobata</i> DSM 43150	0.098	78.5	0.082	72.7	0.054	71.1
5	<i>Ampullariella campanulata</i> DSM 43148	0.120	79.2	0.069	72.7	0.055	70.9
6	<i>Amorphosporangium aurantiacolor</i> DSM 43031	0.082	76.2	0.060	71.7	0.040	68.8
7	<i>Micromonospora echinospora</i> DSM 43141	0.067	76.2	0.060	72.0	0.045	68.7
8	<i>Dactylosporangium aurantiacum</i> DSM 43157	0.060	76.4	0.044	70.5	0.036	70.3
9	<i>Streptosporangium roseum</i> DSM 43021	0.080	72.2	0.125	82.9	0.044	71.2
10	<i>Planomonospora parontospora</i> DSM 43177	0.070	71.0	0.145	75.3	0.089	69.7
11	<i>Planobispora longispora</i> DSM 43041	0.078	72.8	0.162	76.4	0.105	71.0
12	<i>Kitasatoa kauaiensis</i> DSM 43360	0.085	71.8	0.103	70.1	0.158	80.7
13	<i>Streptomyces griseus</i> DSM 40236	0.068	70.6	0.068	71.3	0.077	78.5
14	<i>Chainia antibiotica</i> DSM 43032	0.092	72.5	0.110	69.7	0.098	76.9
15	<i>Microellobospora cinerea</i> DSM 43033	0.052	71.8	0.083	71.9	0.097	76.2
16	<i>Elytrosporangium brasiliense</i> DSM 43159	0.058	72.8	0.141	71.8	0.128	76.8
17	<i>Actinosporangium violaceum</i> DSM 43350	0.062	71.0	0.078	70.5	0.095	77.9
	<i>Spirillospora albida</i> DSM 43034	0.068	71.5	0.101	71.2	0.067	67.3
	<i>Microtetraspora viridis</i> DSM 43175	0.052	72.1	0.079	72.7	0.051	70.4
	<i>Actinomadura madurae</i> DSM 43067	0.035	69.7	0.038	71.1	0.026	70.1
	<i>Oerskovia turbata</i> NCIB 10587 ^b	0.040	72.4	0.030	70.4	0.041	70.3
	<i>Cellulomonas cartae</i> DSM 20106 ^c	0.019	71.6	0.019	71.0	0.021	70.6
	<i>Cellomonas flavigena</i> DSM 20109	0.048	71.4	0.044	71.6	0.047	71.3
	" <i>Micrococcus conglomeratus</i> " CCM 2134 ^d	0.037	69.7	0.040	69.0	0.044	70.0
	<i>Micrococcus nishinomiyaensis</i> CCM 2140	0.017	71.2	0.016	68.0	0.018	69.7
	<i>Micrococcus sedentarius</i> DSM 20317	0.038	71.2	0.041	72.0	0.049	68.2
	<i>Corynebacterium betae</i> DSM 20141	0.023	71.9	0.036	71.5	0.023	69.7
	<i>Microbacterium lacticum</i> DSM 20172	0.018	70.2	0.023	71.2	0.022	69.4

TABLE 2—Continued

Sequence no. ^a	Strain	³ H-labeled rRNA from:					
		Actinoplanes philippinensis DSM 43019		Streptosporangium roseum DSM 43021		Kitasatoa kauaiensis DSM 43360	
		% rRNA binding	$T_{m(e)}$ (°C)	% rRNA binding	$T_{m(e)}$ (°C)	% rRNA binding	$T_{m(e)}$ (°C)
	<i>Agromyces ramosus</i> DSM 43045	0.028	70.6	0.037	70.6	0.029	69.1
	<i>Arthrobacter globiformis</i> DSM 20124	0.033	72.5	0.038	70.9	0.038	69.3
	<i>Bifidobacterium bifidum</i> DSM 20082	0.025	71.2	0.023	67.0	0.022	67.3
	" <i>Bifidobacterium lactentis</i> " DSM 20223 ^d	0.035	70.4	0.035	66.6	0.037	66.3
	<i>Bifidobacterium cuniculi</i> ATCC 27916	0.044	71.0	0.055	66.8	0.044	66.9
	<i>Staphylococcus aureus</i> ATCC 1260	0.038	63.8	0.041	62.2	0.042	63.0

^a See Fig. 3 through 5.^b NCIB, National Collection of Industrial Bacteria, Aberdeen, Scotland.^c This name is not on the Approved Lists of Bacterial Names (43), but it has been validly published since 1 January 1980 (47).^d These names are not on the Approved Lists of Bacterial Names (43) and have not been validly published since 1 January 1980; hence, they are without standing in nomenclature.

FIG. 2. Examples of melting curves of duplexes between ³H-labeled 23S rRNA from *Streptosporangium roseum* DSM 43021 and DNAs from various bacteria. The counts per minute released at each temperature are expressed as percentages of the total counts released between 60 and 95°C. Line 1, *Streptosporangium roseum* DSM 43021; line 2, *Planobispora longispora* DSM 43041; line 3, *Planomonospora parontospora* DSM 43177; line 4, *Actinoplanes philippinensis* DSM 43019; line 5, *Kitasatoa kauaiensis* DSM 43360; line 6, *Bifidobacterium bifidum* DSM 20082; line 7, *Staphylococcus aureus* ATCC 12600.

the melting process depends upon the degree of relatedness of the organisms serving as the sources of [³H]rRNA and DNA (8, 21, 32). The fact that [³H]rRNA dissociates at 60°C is due to

the conditions used to determine the $T_{m(e)}$ [1.5× SSC containing 20% formamide for the determination of $T_{m(e)}$ compared with 5× SSC containing 25% formamide for hybridization).

Figures 3 through 5 show the correlation between $T_{m(e)}$ and rRNA binding for each reference RNA in similarity maps, as originally devised by De Smedt and De Ley (8).

As reported in previous publications on rRNA similarities, the $T_{m(e)}$ values of the duplexes are more reliable indicators of relationships than the percentages of rRNA binding (8, 21, 32). Closely related organisms (as determined by physiological, biochemical, and nucleic acid reassociation studies) exhibit a much smaller range in the $T_{m(e)}$ values of the duplexes than in the percentages of rRNA binding. These latter values vary with genome size and with the number of rRNA cistrons per genome (7). The $T_{m(e)}$ values of the homologous duplexes ranged from 80.7 to 82.5°C, whereas those of the heterologous duplexes formed between the reference rRNA and DNAs from members of the same DNA homology cluster ranged from 75.3 to 79.2°C. Neither the percentage of rRNA binding nor the $T_{m(e)}$ depended upon whether 16S or 23S rRNA was used as a reference. The $T_{m(e)}$ values between the reference rRNA and DNAs from members of either of the other two homology clusters were significantly lower (69.3 to 72.8°C). These values were similar to those obtained for representatives of *Arthrobacter*, *Micrococcus*, *Cellulomonas*, *Oerskovia*, and the plant pathogen coryneforms (68 to 72°C). Only the bifidobacteria showed a lower degree of rRNA similarity

FIG. 3. Similarity map of duplexes between ^3H -labeled 16S rRNA from *Actinoplanes philippinensis* DSM 43019 and DNAs from various members of the Actinomycetales. Strains belonging to DNA homology clusters I through III are represented by their sequence numbers (Table 2). Nonsporeforming organisms that formed a genetic cluster as determined by DNA-DNA homology studies (44, 46) and comparative analyses of their 16S rRNA's (48) are marked with identical symbols. DNA homology values for bifidobacteria were obtained from E. Lauer (unpublished data), and values for *Microbacterium* and related organisms were obtained from H. Döpfer (unpublished data). Abbreviations: *Actinomad. mad.*, *Actinomadura madurae* DSM 43067; *Arthr. glob.*, *Arthrobacter globiformis* DSM 20124; *Bifodob.*, *Bifidobacterium* (*Bifidobacterium bifidum* DSM 20082, *Bifidobacterium lactentis* DSM 20223, and *Bifidobacterium cuniculi* ATCC 27916); *Cellulom.*, *Cellulomonas* (*Cellomonas cartae* DSM 20106, *Cellomonas flavigena* DSM 20109, and *Oerskovia turbata* NCIB 10587); *Micrococc.*, *Micrococcus* (*Micrococcus con-*

66.3 to 71.2°C). As expected, duplexes of *Staphylococcus aureus* ATCC 12600 and members of the Actinomycetales had the lowest $T_{m(e)}$ (63°C). *Micromonospora echinospora* DSM 43141, which shared low DNA-DNA homology with members of DNA cluster I, produced high rRNA similarity values with *Actinoplanes philippinensis* DSM 43019 (76.2°C). *Actinomadura madurae* DSM 43067, *Spirillospora albida* DSM 43034, and *Microtetraspora viridis* DSM 43175 did not group with any of the three clusters. The $T_{m(e)}$ values for the duplexes obtained for the DNAs from these organisms and the [^3H]-rRNA's from the reference organisms ranged between 67.5 and 72.7°C.

DISCUSSION

DNA-DNA homology values of more than 20% and obtained under optimal conditions (26) have been reliable indicators of a high degree of similarity among the DNA nucleotide sequences of the strains investigated; that is, strains with such homology values have to be regarded as closely related organisms (12, 40, 44, 45). This was strikingly evident with the species included in our DNA homology cluster I. Neither in *Actinoplanes* nor in *Ampullariella* did the species group separately from the species of the other genus. *Actinoplanes philippinensis* and *Actinoplanes italicus* are as closely related to various species of *Ampullariella* as *Ampullariella regularis* is related to various species of *Actinoplanes*. *Amorphosporangium auranticolor*, *Dactylosporangium aurantiacum*, and *Micromonospora echinospora* are more distantly related, as shown by slightly lower homology values to *Actinoplanes* and *Ampullariella*. The most distinctive features separating *Actinoplanes*, *Ampullariella*, *Amorphosporangium*, and *Dactylosporangium* are the morphologies of sporangia and spores. These characteristics certainly do not carry enough genetic weight to justify a genus, but they may be appropriate for differentiation at the species level.

The genetic relatedness of *Actinoplanes*, *Ampullariella*, and *Dactylosporangium* has been established by Farina and Bradley (11). With [^3H]DNA from *Actinoplanes philippinensis* as a reference, *Actinoplanes*, *Ampullariella*, and *Dactylosporangium* could be clearly distinguished from the second group containing *Strep-*

glomeratus CCM 2134, *Micrococcus nishinomiyaensis* CCM 2140, and *Micrococcus sedentarius* DSM 20317; *Microbact.*, *Microbacterium* (*Microbacterium lacticum* DSM 20172, *Corynebacterium betae* DSM 20141, and *Agromyces ramosus* DSM 43045); *Microtet. vir.*, *Microtetraspora viridis* DSM 43175; *Spirillosp. alb.*, *Spirillospora albida* DSM 34034.

FIG. 4. Similarity map of duplexes between ^3H -labeled 23S rRNA from *Streptosporangium roseum* DSM 43021 and DNAs from various members of the Actinomycetales. For additional information, see legend to Fig. 3.

tosporangium, *Spirillospora*, *Planomonospora*, and *Planobispora*. In addition, we have found that *Amorphosporangium* and *Micromonospora* should be transferred to this genetically defined group. The close biochemical and physiological relationships among *Micromonospora*, *Amorphosporangium*, and the other members of this group are obvious. Although it does not form distinct spore vesicles, *Micromonospora* resembles *Actinoplanes* and its relatives in peptidoglycan type, sugar composition of the cell wall, and lack of aerial mycelia (Table 3). Furthermore, *Micromonospora* and *Actinoplanes* have been found to be specifically related in comparative physiological studies (20), as well as in comparative analyses of their 16S rRNA's (Stackebrandt et al., manuscript in preparation).

Farina and Bradley (11) concluded that those organisms which form motile spores, such as *Planobispora*, *Planomonospora*, and *Spirillospora*, appear to be as closely related to *Streptosporangium* (characterized by nonmotile spores) as they are to *Actinoplanes*. Because no reference DNAs of any of the former organisms were

FIG. 5. Similarity map of duplexes between ^3H -labeled 16S rRNA from *Kitasatoa kauaiensis* DSM 43360 and DNAs from various members of the Actinomycetales. For additional information, see legend to Fig. 3.

used in their experiments, the high degree of relatedness that actually exists among *Streptosporangium*, *Planomonospora*, and *Planobispora* could not be detected. However, our data show that *Spirillospora albida* DSM 43034 is not closely related to *Streptosporangium*, *Planomonospora*, or *Planobispora* despite a high degree of similarity in their biochemical and physiological features (Table 3).

The separation of streptomycetes from the sporangium-forming Actinomycetales based on morphological and biochemical criteria (Table 3) was supported by nucleic acid reassociation studies (1, 11), comparative physiology studies (20), phage typing experiments (37, 38), and comparative analyses of 16S rRNA's (13, 49). On the other hand, there has been considerable controversy concerning the validity of the genera closely related to *Streptomyces* (3). *Chainia*, *Streptoverticillium*, *Elytrosporangium*, and *Ac-*

TABLE 3. *Morphological and biochemical properties of the Actinomycetales genera investigated*

Genus ^a	DNA homology cluster	Peptidoglycan type ^b	Sugar composition	Presence of:		
				Aerial mycelium ^c	Motility of spores ^c	Sporangia ^c
<i>Actinoplanes</i>	I	<i>m</i> -Dpm (+Gly), ^d (II) ^e	Xylose, arabinose ^e	— ^f	+	+
<i>Ampullariella</i>	I	<i>m</i> -Dpm (+Gly), (II) ^e	Xylose, arabinose ^e	—	+	+
<i>Amorphosporangium</i>	I	<i>m</i> -Dpm (+Gly), (II) ^e	Xylose, arabinose ^e	—	+	+
<i>Dactyloporangium</i>	I	<i>m</i> -Dpm (+Gly), (II) ^e	Xylose, arabinose ^e	—	+	+
<i>Micromonospora</i>	I	<i>m</i> -Dpm (+Gly), (II) ^e	Xylose, arabinose ^e	—	—	—
<i>Streptosporangium</i>	II	<i>m</i> -Dpm, (A1γ), ^g (III) ^e	3- <i>O</i> -methyl-D-galactose ^h	+	—	+
<i>Planomonospora</i>	II	<i>m</i> -Dpm (A1γ), ^g (III) ^e	3- <i>O</i> -methyl-D-galactose ^h	+	+	+
<i>Planobispora</i>	II	<i>m</i> -Dpm (A1γ), ^g (III) ^e	3- <i>O</i> -methyl-D-galactose ^h	+	+	+
<i>Streptomyces</i>	III	LL-Dpm-Gly, (A3γ), ^g (I) ^e	Not characteristic ^e	+	—	—
<i>Kitasatoa</i>	III	LL-Dpm-Gly, (A3γ), ^g (I) ^e	Not characteristic ^e	+	+	+
<i>Chainia</i>	III	LL-Dpm-Gly, (A3γ), ^g (I) ^e	Not characteristic ^e	+	—	—
<i>Microellobosporia</i>	III	LL-Dpm-Gly, (A3γ), ^g (I) ^e	Not characteristic ^e	+	—	+
<i>Elytrosporangium</i>	III	LL-Dpm-Gly, (A3γ), ^g (I) ^e	Not characteristic ^e	+	+	—
<i>Streptoverticillium</i>	III	LL-Dpm-Gly, (A3γ), ^g (I) ^e	Not characteristic ^e	+	—	—
<i>Actinosporangium</i>	III	LL-Dpm-Gly, (A3γ), ^g (I) ^e	Not characteristic ^e	+	—	+
<i>Spirillospora</i>		<i>m</i> -Dpm, (A1γ), ^g (III) ^e	3- <i>O</i> -methyl-D-galactose ^h	+	+	+
<i>Actinomadura</i> (<i>A. madurae</i> type)		<i>m</i> -Dpm, (A1γ), ^g (III) ^e	3- <i>O</i> -methyl-D-galactose ^h	+	—	—
<i>Microtetraspora</i>		<i>m</i> -Dpm ⁱ	Not characteristic ^j	+	—	—

^a As listed in *Bergey's Manual of Determinative Bacteriology*, 8th ed. (17).^b Dpm, Diaminopimelic acid (hydroxydiaminopimelic acid may be present in members of DNA homology cluster I [25]).^c According to Cross and Goodfellow (3).^d (+Gly), L-Alanine is replaced by glycine in position 1 of the subunit (42).^e According to Lechevalier and Lechevalier (25).^f Except for *Actinoplanes armeniacus*.^g According to Schleifer and Kandler (42).^h According to Lechevalier and Gerber (24).ⁱ The cell wall of *Microtetraspora viridis* reportedly contains *meso*-diaminopimelic acid, traces of LL-diaminopimelic acid, glycine, and lysine (52).^j According to Thiemann et al. (52).

inosporangium have been regarded as synonyms of *Streptomyces* by some taxonomists, whereas others have favored the elevation of morphologically defined groups into genera merely to reduce the complexity of *Streptomyces* (3, 23). DNA-DNA reassociation experiments with *Streptomyces venezuelae* and *Elytrosporangium flavea* have shown that these two organisms are as closely related as *Streptomyces venezuelae* and other streptomycetes (1). As pointed out by Prauser (37), the broad range of S-phase activity against strains of all *Streptomycetaceae* genera can be taken as an indication of the high similarity of the genetic material. Our data clearly show that the separation of the members of the *Streptomycetaceae* into different genera on the basis of morphological criteria

is unjustified; representatives of the different genera investigated here appear to be moderately related species within the genus *Streptomyces*. What has already been observed for the genera in DNA homology clusters I and II is also true for the *Streptomycetaceae*; namely, these taxa were established as a result of overclassification, which was mainly due to an overestimation of the value of morphological features.

Determinations of DNA-rRNA similarities as expressed in the average thermal stabilities of duplexes [$T_{m(e)}$] have given no indication that the three DNA homology clusters are specifically related to one another (Fig. 6). The $T_{m(e)}$ values of DNA homology clusters I through III (69 to 72°C) are in the same range as those

FIG. 6. Dendrogram of relationships derived from average linkage clustering of the $T_{m(e)}$ values of DNA-rRNA duplexes listed in Table 2. The enclosed areas represent the ranges of $T_{m(e)}$ values of the reference DNA homology clusters.

obtained for the reference rRNA and DNAs from *Spirillospora albida*, *Microtetraspora viridis*, and *Actinomadura madurae*, as well as from *Arthrobacter globiformis*, micrococci, cellulomonads, the plant pathogen coryneforms, and *Microbacterium lacticum*. The lower $T_{m(e)}$ values of the duplexes between the reference rRNA and DNAs from bifidobacteria caused by a higher degree of dissimilarity of the nucleotide sequences of the rRNA cistrons indicate that the bifidobacteria are more distantly related to the *Actinomycetales* and the coryneform bacteria.

The branching pattern shown in Fig. 6 is similar to the pattern derived from a comparative analysis of 16S rRNA's (13, 49). *Streptomyces griseus*, *Dactylosporangium aurantiacum*, and the group of nonsporeforming *Actinomycetales* made up of *Arthrobacter*, *Micrococcus*, *Cellulomonas*, and *Microbacterium* share a similarity coefficient (S_{AB} value) of about 0.5. Members of *Arthrobacter* are more closely related (S_{AB} values, about 0.6). This specific relationship is not evident in Fig. 6 because no reference rRNA of any of the bacteria was prepared for this study. On the other hand, bifidobacteria share lower S_{AB} values with *Streptomyces*, *Dactylosporangium*, and *Arthrobacter* (S_{AB} values, 0.4). A more detailed description of the phylogeny of sporeforming *Actinomycetales* derived from a comparison of the oligonucleotide sequences of the 16S rRNA's will be presented elsewhere.

We are aware that the number of organisms included in this study is small compared with the large number of described species. On the other hand, the species which we investigated represent the major types of sporeforming *Actinomycetales* listed in *Bergey's Manual*, 8th ed. (17). Many more DNA-DNA reassociation studies on other species, especially on the type strains not used in our studies, will be necessary before the taxonomic rank of the three DNA

homology clusters can be determined with confidence.

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REPRINT REQUESTS

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