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Reassociation of Deoxyribonucleic Acids from *Actinoplanes* and Other Actinomycetes

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The ability of deoxyribonucleic acid (DNA) isolated from a number of actinomycetes to reassociate with reference DNA from *Actinoplanes philippinensis* or *Streptomyces venezuelae* has been measured. All of the DNA preparations except for those from *Nocardia erythropolis* and *Thermomonospora viridis* contained 70 to 73 moles per cent guanine and cytosine. DNA from two species of *Actinoplanes*, two species of *Dactylosporangium*, and *Ampullariella digitata* formed extensive thermally stable duplexes with the *Actinoplanes philippinensis* reference. DNA from streptomycetes formed duplexes with the *A. philippinensis* reference, but these duplexes possessed low thermal stability. DNA from *N. erythropolis* and *T. viridis* did not bind significant amounts of this reference DNA. Only DNA from *Streptomyces albus*, *Streptoverticillium baldaccii*, and *Microellobosporia flavea* appreciably bound the *Streptomyces venezuelae* reference. Our results separate the actinomycetes forming sporangia into two groups: the first group contained *Actinoplanes*, *Dactylosporangium*, and *Ampullariella;* the second group contained *Planomonospora*, *Spirillospora*, and *Streptosporangium*.

Many actinomycete cultures forming spores in sporangia have been isolated during the past decade. The first actinomycete taxon characterized by sporangium formation was *Actinoplanes* (6). The family *Actinoplanaceae* initially included actinomycetes with flagellated sporangiospores, named *Actinoplanes*, and actinomycetes with nonmotile sporangiospores, named *Streptosporangium* (7). Krassilnikov (15) proposed that the family *Actinoplanaceae* should only include the actinomycetes forming motile, flagellated sporangiospores, and he created a new family *Streptosporangiaceae* for those with nonmotile spores. Many new genera have been added to both families in recent years (26). All of these genera were recognized and defined on the basis of their morphological characteristics.

Recent advances in biochemical and genetic concepts and techniques now allow for preliminary appraisal of relatedness at the molecular level (5). There are, however, no formal guidelines by which molecular information derived from these empirical techniques can be correlated with taxonomic groupings. Nevertheless, Tewfik and Bradley (21) have shown that deoxyribonucleic acid (DNA) renaturation analyses reveal relationships among *Streptomyces* and *Nocardia*. Similarly, Brenner et al. (5) have compared the relationships of a number of enteric bacteria and genetic hybrids based on genetic analyses and on quantitative DNA reassociation studies. The successful application of this art to specific problems is dependent on a number of variables, including (i) ionic strength of the reannealing medium, (ii) incubation temperature during renaturation, and (iii) the complexity and size of the genome (29). Accordingly, not only must the extent of DNA reassociation be considered in assessing specificity of these reactions, but also the thermal stability of the duplexes.

In the present study, the relationships among *Actinoplanes* and many other actinomycete genera have been determined, based upon the nucleotide composition of their DNA, and the ability to form hybrid DNA molecules with particular reference DNA preparations. Our results have established that the genera *Actinoplanes*, *Dactylosporangium*, and *Ampullariella* are closely related. The genera *Planomonospora*, *Planobispora*, and *Spirillospora* which also form motile sporangiospores, are less closely related to the *Actinoplanes* group.

MATERIALS AND METHODS

The organisms used in this study belong to the following genera: *Actinoplanes* (6), *Ampullariella* (8, 9), *Dactylosporangium* (24), *Microellobosporia*

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(11), *Micromonospora* (20), *Nocardia* (27), *Planobispora* (25), *Planomonospora* (23), *Spirillospora* (8), *Streptomyces* (2, 28), *Streptosporangium* (7), *Streptoverticillium* (1, 3), and *Thermomonospora* (14).

The culture designations and their sources are given in Table 1. The stock cultures were propagated on peptone-yeast extract agar (22) and oatmeal agar (12). *Thermomonospora viridis* was grown on nutrient agar supplemented with 0.2% yeast extract. The incubation temperature was 30 C for all cultures, except *Dactylosporangium* strains which were incubated at 37 C, and *T. viridis* which was incubated at 45 C.

Mycelia for DNA extraction were grown in peptone-yeast extract broth, harvested, and lysed by methods reported previously (22). *Actinoplanes*, *Ampullariella*, *Dactylosporangium*, *Micromonospora*, *Nocardia*, *Planobispora*, *Planomonospora*, *Spirillospora*, and *Streptosporangium* mycelia were lysed by mechanical and enzymatic action (21). For *Microellobosporia*, *Streptoverticillium*, and *Thermomonospora*, lysozyme and Pronase digestion was sufficient. After lysis, DNA was extracted by the Marmur procedure (17). The nucleotide composition of DNA preparations was estimated from the hyperchromic shift upon thermal denaturation (13); the DNA preparations were diluted in 0.1 × SSC (0.15 M NaCl plus 0.015 M sodium citrate; 19). The degree of genetic homology between *Actinoplanes* and other DNA samples was assessed by measuring the extent of in vitro hybrid formation between single-stranded DNA molecules of different origin; the nitrocellulose membrane filter technique developed by Warnaar and Cohen (30) was used.

Streptomyces venezuelae S13 and *Actinoplanes philippinensis* DNA were labeled with uracil-2-^{14}C (21). The specific activity of the labeled DNA was about 3,500 counts per min per μg of DNA. *S. venezuelae* S13 was chosen as one reference DNA because it had been used in previous studies on DNA homologies among *Streptomyces* species (21, 22). *A. philippinensis* DNA was chosen as the other reference DNA because it was the first actinomycete described as forming spores in sporangia. It is the type species of the family *Actinoplanaceae*. If available, type cultures have been selected for this study.

All of the general procedures followed for hybridization experiments were as described by Monson et al. (19). Filters charged with approximately 50 μg of denatured DNA were incubated with 1 μg of denatured ^{14}C-labeled DNA in 1.25 × SSC containing 0.02 M tris(hydroxymethyl)aminomethane (Tris), adjusted to *p*H 8, at 70 C for 24 hr.

The thermal stability of the duplexes formed was also determined (5). Filters were prepared, loaded with DNA, and incubated at 70 C for 24 hr as before. After reannealing was complete, the filters were removed, rinsed briefly with 0.003 M Tris-hydrochloride (*p*H 9.4), and immersed in 10 ml of 1 × SSC at the incubation temperature for 10 min. The labeled DNA was eluted by incubating the filters for 15 min in 2.5 ml of 1 × SSC in scintillation vials at temperature increments of 5 C. Next 15 ml of a Triton X-100,

TABLE 1. *Deoxyribonucleic acid base composition and source of the principal cultures*

Organisms	Mole per cent guanine + cytosine	Source [a]
Actinoplanes philippinensis CBS	72	J. E. Thiemann
A. missouriensis CBS	72	J. E. Thiemann
A. utahensis CBS	72	J. E. Thiemann
Ampullariella digitata CBS-19169	73	J. E. Thiemann
Dactylosporangium aurantiacum D/748; ATCC-23491	73	J. E. Thiemann
D. thailandense D/499; ATCC-23490	71	J. E. Thiemann
Microellobospora flavea ATCC-15332	71	ATCC
Micromonospora species 401	73	Virginia Commonwealth University Collection
Nocardia erythropolis 2	62	J. N. Adams
Planomonospora parontospora B/677; ATCC-23863	72	J. E. Thiemann
Planobispora longispora Pb/1075; ATCC-23867	71	J. E. Thiemann
P. rosea Pb/1435; ATCC-23866	70	J. E. Thiemann
Spirillospora albida CBS	71	J. E. Thiemann
S. albida 1030	72	J. E. Thiemann
Streptomyces albus IPV 1298	72	E. Baldacci
S. venezuelae S13	72	Virginia Commonwealth University Collection
Streptosporangium roseum 27b	71	J. Couch
Streptoverticillium baldaccii IPV 1339	71	E. Baldacci
Thermomonospora viridis ATCC-15386	69	ATCC

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toluene scintillation fluid (666 ml of toluene, 333 ml of Triton X-100, 5.5 g of 2,5-diphenyl-oxazole, and 125 mg of *p*-bis-2'(5-phenyl-oxazolyl)-benzene) was added to the vials, and the radioactivity was determined. Up to 30% aqueous salt solution can be counted in this fluid with only a small loss in efficiency from quenching. The total of homologous counts per minute was obtained by summing all the counts per minute for the homologous system remaining after the 10-ml wash at 70 C.

RESULTS

The nucleotide composition of selected actinomycete DNA preparations, as determined from thermal denaturation profiles, ranged between 62 and 73 mole per cent guanine plus cytosine (GC). Only *Nocardia erythropolis* and *T. viridis* possessed DNA with a GC content significantly less than 70% (Table 1).

With *S. venezuelae* S13 DNA as the reference in reassociation experiments, the relative amount of radioisotope bound by DNA from members of the *Actinoplanaceae* ranged between 10 and 21%. *Streptoverticillium baldaccii* and *Streptomyces albus* DNA preparations showed 58 and 31% homology, respectively, with *S. venezuelae* DNA (Table 2). Although *Microellobosporia flavea* DNA bound 30% as much ^{14}C-labeled reference DNA as did the homologous *S. venezuleae* DNA, the intergeneric duplexes possessed a melting temperature at which half of the bound reference DNA was eluted (T_m) of 78 C, whereas the intrageneric duplexes had T_m values of 83 to 89 C. With *A. philippinensis* DNA as the reference, the relative amount of radioisotope bound by DNA from *Micromonospora* sp., *Actinoplanes missouriensis*, *A. uthahensis*, *Dactylosporangium thailandense*, *D. aurantiacum*, and *Ampullariella digitata* ranged between 51 and 75%. The relative amount of labeled DNA bound by *S. venezuelae*, *S. albus* and *S. baldaccii* DNA ranged between 45 and 50%. DNA from *Spirillospora albida*, *Planomonospora parontospora*, *Planobispora rosea*, and

TABLE 2. *Reassociation of deoxyribonucleic acids (DNA) from various morphologically distinct actinomycetes with that of Streptomyces venezuelae*

Test organism	Relative DNA bound	T_m[a]
	%	C
Streptomyces venezuelae	100 (17)[b]	89
Streptoverticillium baldaccii	58	ND[c]
Streptomyces albus	31	83
Microellobosporia flavea	30	78
Micromonospora species 401	23	71
Actinoplanes philippinensis	21	79
Spirillospora albida 1030	19	75
Planomonospora parontospora	18	78
Actinoplanes missouriensis	17	ND
Ampullariella digitata	16	80
Planobispora longispora	15	82
Dactylosporangium aurantiacum	14	72
Thermomonospora viridis	13	76
Actinoplanes utahensis	12	85
Streptosporangium roseum	12	81
Dactylosporangium thailandense	10	ND
Nocardia erythropolis	8	77
Myxococcus xanthus	5	80
Escherichia coli	3	85
Blank filter	10 count/min	

[a] Temperature at which half of the bound reference DNA is eluted.

[b] Absolute per cent binding of the reference DNA is given in parentheses.

[c] Not done.

TABLE 3. *Homology of deoxyribonucleic acid (DNA) from various morphologically distinct actinomycetes with that of Actinoplanes philippinensis*

Test organism	Relative DNA bound	T_m[a]
	%	C
Actinoplanes philippinensis	100 (21%)[b]	100
A. missouriensis	75	ND[c]
A. utahensis	68	100
Dactylosporangium thailandense	66	ND
D. aurantiacum	65	99
Ampullariella digitata	59	97
Micromonospora species	51	91
Streptomyces venezuelae	50	77
S. albus	47	78
Streptoverticillium baldaccii	45	ND
Planomonospora parontospora	33	75
Streptosporangium roseum	32	97
Planobispora rosea	32	80
Spirillospora albida 1030	32	80
S. albida	29	82
Thermomonospora viridis	22	75
P. longispora	21	86
Microellobosporia flavea	13	86
Myxococcus xanthus	12	72
Nocardia erythropolis	10	82
Escherichia coli	2	80

[a] Temperature at which half of the bound reference DNA is eluted.

[b] Absolute per cent binding of the reference DNA is given in parentheses.

[c] Not done.

Streptosporangium roseum relatively bound 29 to 32% of the reference *A. philippinensis* DNA (Table 3).

The DNA duplexes formed between *A. philippinensis* and *A. utahensis*, *D. aurantiacum* or *Ampullariella digitata* were quite stable, with T_m values of 97 to 100 C. The duplexes formed between *Micromonospora* DNA and *A. philippinensis* DNA were heterogeneous, that is, one fraction of these duplexes was stable but another fraction could be eluted at 80 C (Fig. 1). With DNA from *S. venezuelae* as the reference, stable duplexes were formed only with other streptomycete DNA. The 70 C incubation temperature used for the reassociation process permitted extensive formation of duplexes with low thermal stability (Tables 2 and 3).

DISCUSSION

Our determinations of the DNA base compositions of a variety of actinomycetes for the species examined in common were similar to those of Yamaguchi (32). Yamaguchi reported the following GC values: *A. philippinensis*, 72%; *A. utahensis*, 73%; *Ampullariella digitata*, 72%; *M. flavea*, 70%; *Spirillospora albida*, 73%; *Streptomyces albus*, 72%; and *Streptosporangium roseum*, 71%. Craveri et al. (10) and Manachini et al. (18) determined the DNA base composition of various mesophilic and thermophilic actinomycetes. The species that they considered were different from ours; however, comparing genera, they obtained per cent GC values that were a few per cent higher than ours. This consistent difference probably reflects their use of a particular formula for computation of per cent GC (10) rather than interpolation from a standard curve. *T. viridis* was the only species that we examined in common. They reported that the DNA of *T. viridis* strain IMAM 5 had 74% GC, and DNA of *T. viridis* strain IPV 704 had 75% GC. We found that the DNA from *T. viridis* strain ATCC 15386 contained 69% GC. According to their results, DNA of thermophilic actinomycetes, which grew optimally at 50 to 60 C, had a low per cent GC, that is, 43 to 53% GC.

From our results and from the data reported in the literature, it is clear that the actinomycetes forming sporangiospores have very similar DNA base compositions and that they are not distinguishable from other related actinomycetes by this criterion (21, 22, 32). It is necessary, therefore, to use alternative methods for discovering relationships, for example, by the formation of DNA hybrid molecules. By using ^{14}C-labeled DNA from *Streptomyces venezuelae* S13 as the reference, we found that all of the genera examined in the families *Actinoplanaceae* and *Streptosporangiaceae* showed little homology with this reference. The amount of DNA that they bound ranged from 11 to 21% of that bound by the homologous system. *Streptoverticillium baldaccii* DNA, however, bound substantial amounts of the *Streptomyces venezuelae* reference DNA, indicating a close relationship between these two organisms. *M. flavea* DNA bound nearly as much reference DNA as *S. albus* DNA, but the intergeneric duplexes were more readily dissociated by heat.

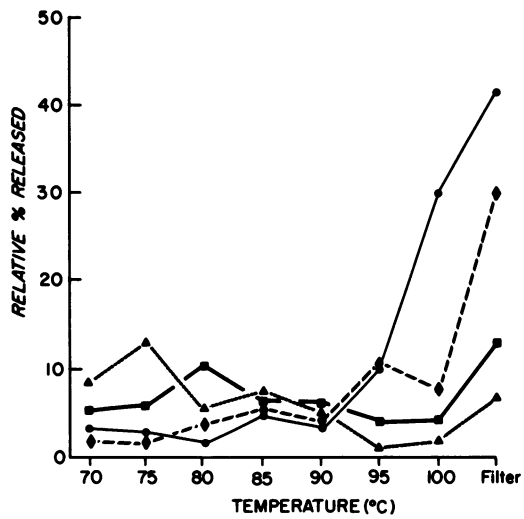

FIG. 1. *Thermal stability of DNA duplexes formed with Actinoplanes philippinensis reference DNA at 70 C. Relative per cent released = (counts per minute released)/(total homologous counts per minute) × 100. A. philippinensis, ●; Actinoplanes utahensis, ◆; Micromonospora species, ■; Streptomyces venezuelae, ▲.*

The primary purpose of the present work was to study the relationships among *Actinoplanes* and other actinomycetes. By using *A. philippinensis* as the reference DNA, the actinomycete genera studied can best be arranged in the following way: *Dactylosporangium*, *Ampullariella*, and *Micromonospora* showing substantial homology with *Actinoplanes; Streptomyces* and *Streptoverticillium* showing less, but significant binding of the reference *A. philippinensis* DNA; *Planomonospora*, *Planobispora*, *Streptosporangium*, and *Spirillospora* showing less affinity to *Actinoplanes; Thermomonospora* and *Nocardia* showing little or no homology with the reference DNA. The thermal stability of the duplexes formed by DNA from *Dactylosporangium* or *Ampullariella* with the *Actinoplanes* reference indicates that the relative binding observed in these assays reflects well-matched nucleotide pairing. Streptomycete

DNA, however, formed duplexes of low thermal stability. A decrease in thermal stability of 20 C means, on the average, that 30% of the base pairs are mismatched (29). Accordingly, DNA from *Micromonospora* sp. is much more like that of the *Actinoplanes* reference than streptomycete DNA, even though their relative binding values were similar under the experimental conditions used.

Yamaguchi (32), working with various morphologically distinct actinomycetes, failed to find consistent patterns of binding of test DNA with *Streptomyces griseus* reference DNA. The lower incubation temperature could be responsible for the greater nonspecific binding in Yamaguchi's study than in our analyses. Even at 70 C, we observed extensive formation of DNA duplexes with low thermal stability. This indicates that there was considerable mismatching of nucleotides during the reassociation process. Thermal elution profiles differentiate between mismatched and well-matched duplexes (5).

A comparison between taxonomic arrangements based on DNA homologies and on cell wall composition of actinomycetes is most significant. Yamaguchi (31) and Lechevalier and coworkers (4, 16) arranged the actinomycete genera into different cell wall types. Both groups of workers considered *Actinoplanes*, *Ampullariella*, and *Micromonospora* related on this basis; they also placed *Spirillospora* and *Streptosporangium* together in another group. In addition, *Streptomyces*, *Streptoverticillium*, and *Microellobosporia* were grouped together; *Nocardia* was placed in a separate category. The cell wall analyses and DNA homologies lead to the same conclusions. These studies confirm the usefulness of both methods.

Our results separate the actinomycetes forming sporangia into two groups: the first including *Actinoplanes*, *Dactylosporangium*, and *Ampullariella;* the second including *Planomonospora*, *Planobispora*, *Spirillospora*, and *Streptosporangium*. Accordingly, the proposal of Krassilnikov to include in the family *Actinoplanaceae* only the actinomycetes forming sporangia containing motile spores, and to reserve the family *Streptosporangiaceae* for those with no motile spores is not supported by our study or by the cell wall analyses (4, 16, 31). *Planomonospora*, *Planobispora*, and *Spirillospora*, all having motile spores, appear to be as closely related to *Streptosporangium roseum*, which is characterized by nonmotile spores, as they are to the *Actinoplanes* group.

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