Taxonomic Utility of Restriction Endonuclease Fingerprinting of Large DNA Fragments from *Streptomyces* Strains

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Using a method known as low-frequency restriction fragment analysis (LFRFA) (M. L. Beyazova and M. P. Lechevalier, *Int. J. Syst. Bacteriol.* 42:422–433, 1992), we determined the molecular weights of *AseI* restriction fragments of *Streptomyces* DNAs by pulsed-field gel electrophoresis. The levels of similarity of fragment patterns among strains were determined by using the simple matching coefficient, and clustering was performed by using the unweighted pair group with mathematical average algorithm. A total of 59 strains representing eight species and the numerically classified taxon *Streptomyces cyaneus* group A18 (S. T. Williams, M. Goodfellow, G. Alderson, E. M. H. Wellington, P. H. A. Sneath, and M. J. Sackin, *J. Gen. Microbiol.* 129:1743–1813, 1983) were studied. Forty-two strains (six species) formed eight clusters at levels of similarity of more than 80%; 17 strains (including the entire *S. cyaneus* group) were unclustered. Cluster 1 contained all of the *Streptomyces albus* strains studied plus two strains of *Streptomyces somaliensis* and two strains of *Streptomyces lavendulae*. Cluster 2 contained 8 of the 12 *Streptomyces fradiae* strains examined plus one strain each of *S. somaliensis* and *S. lavendulae*. Cluster 3 was heterogeneous in terms of species. Cluster 4 contained two *S. somaliensis* strains; cluster 5 contained three *S. fradiae* strains; cluster 6 contained three *Streptomyces rimosus* strains; and clusters 7 and 8 contained seven and three *Streptomyces ipomoea* strains, respectively. The *S. cyaneus* group strains exhibited no clustering among themselves or with the other species examined. Some *Streptomyces* species which exhibited high levels of similarity (85 to 95%) in physiological tests (e.g., *S. albus* and the *S. fradiae* strains in cluster 2) exhibited high levels of similarity in the LFRFA (84 and 81%, respectively). Other taxa (*S. cyaneus* group) which exhibited equally high levels of physiological similarity (90%) appeared to be unrelated as determined by the LFRFA. Species with lower levels of physiological similarity (e.g., *S. somaliensis* [75%], *S. lavendulae* [63%], and *S. rimosus* [68%]) exhibited low levels of LFRFA similarity (75, 64, and 54%, respectively). High levels of DNA-DNA relatedness (>90%) (*S. ipomoea*) were reflected in high levels of similarity as determined by the LFRFA (75 to 100%); lower levels of DNA-DNA relatedness (ca. 70%) (*S. cyaneus* group) were reflected in low levels of LFRFA similarity (strains not clustered). We concluded that the presently used physiological tests reflect too small a portion of the genome to be universally useful in streptomycete species characterization. In contrast, high levels of DNA-DNA relatedness (>90%) and high LFRFA similarity values will probably both be valuable in species delineation in actinomycetes.

Restriction enzymes which generate many DNA fragments yield patterns which are strain specific but are not useful in delineating species (7). When enzymes with low-frequency cleavage are used (28), the DNA fragments generated are fewer and larger, but must be separated by using special electrophoretic techniques, such as the pulsed-field technique. The molecular weights of such fragments can readily be determined by comparison with suitable standards, and the frequency and stability of specific restriction sites in genomic DNA can be determined. Since in the actinomycete genus *Streptomyces* the G+C content of the DNA is very high (69 to 78 mol%), it seemed likely that the AT-rich regions are conserved areas of importance in the genome. Using enzymes recognizing only AT-rich sites, we performed a low-frequency restriction fragment analysis (LFRFA) of a number of *Streptomyces* strains and in this paper describe the results obtained for 15 species of this genus.

MATERIALS AND METHODS

Bacterial strains. The strains included in this study are shown in Table 1.

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Media and growth conditions. All strains were maintained on Bennett agar slants (37) and grown in Trypticase soy broth (BBL) under static conditions at 28°C for 3 to 4 days.

Physiological tests. The physiological tests of Gordon (9, 10), Gordon and Horan (11), and Mishra et al. (30) were used.

Preparation of cells and inserts. For analysis, cells were collected, washed with 0.5 M EDTA (all of the EDTA used was at pH 8), ground, extracted with water-saturated phenol (27) for 20 min at 65°C, and then washed three times with CHCl₃-isoamyl alcohol (24:1) at room temperature. The resulting samples were treated with 2% sodium dodecyl sulfate (SDS)–5 mM EDTA at 65°C for 30 min, washed three times with 5 mM EDTA containing 1 mg of bovine serum albumin fraction V (Sigma Chemical Co.) per ml, and then washed three times with 10 mM EDTA. Finally, the cells were suspended in TE (10 mM Tris–1 mM EDTA) and mixed with low-melting-point temperature agarose (final agarose concentration, 0.5%), and these inserts were incubated for 3 to 4 h or overnight (the time was not critical) at 37°C in EC lysis buffer (35) containing 1 mg of lysozyme per ml. Next, the samples were digested at 37°C with 1 mg of subtilisin Carlsberg (Sigma Chemical Co.) per ml for 3 to 4 h or overnight with gentle shaking and washed with TE at 56°C.

Restriction of DNA. Restriction enzymes *DraI*, *AseI* (or its isoschizomer *AsnI*), and *SpeI* were purchased from Bethesda Research Laboratories, New England Biolabs, or Boehringer-Mannheim. Restriction digestions were per-

TABLE 1. *Streptomyces* strains investigated in this study

Species	Strain(s)
<i>S. albus</i>	ATCC 3004 ^T , ATCC 618, IMRU 1182, IMRU 1187, IMRU 1205, IMRU 3854
<i>S. coelicolor</i>	NRRL B-12032 (= A3/2)
<i>S. cyaneus</i> cluster ^a	
<i>S. cyaneus</i>	IMRU 3761 ^T
<i>S. afghanensis</i>	NRRL B-5621 ^T
<i>S. bellus</i>	NRRL B-2575 ^T
<i>S. coeruleorubidus</i>	NRRL B-2569 ^T
<i>S. coeruleorubidus</i>	NRRL 3045
<i>S. janthinus</i>	NRRL B-3365 ^T (= ISP 5206 ^T)
<i>S. purpureus</i>	NRRL B-12230 ^T
<i>S. roseoviolaceus</i>	NRRL B-12177 ^T
<i>S. fradiae</i>	IMRU 3535 ^T , ATCC 11903, IMRU 1186, IMRU 1199, IMRU 3554, IMRU 3556A, IMRU 3594, IMRU 3719, IMRU 3572, NRRL B-16471, NRRL B-16472, NRRL B-16473
<i>S. ipomoea</i>	ISP 5383 ^T , Clark Standard (= NRRL B-16443), Clark 7849, Clark 7851, Clark 7852, Clark 7857, Clark 7860, Clark 7861, Clark 7862, NRRL B-16445, NRRL B-16447, NRRL B-16480, NRRL B-16182, NRRL B-16183, NRRL B-16185, NRRL B-16188, NRRL B-16189
<i>S. lavendulae</i>	IMRU 3440-8 ^T , IMRU 467, IMRU 577, IMRU 3445, IMRU 3455, IMRU 3555, IMRU 3736
<i>S. lividans</i>	NRRL B-16148 (= John Innes 1326)
<i>S. rimosus</i>	IMRU 3558 ^T , IMRU 949, IMRU 3560, IMRU 3701, IMRU 3867, IMRU 3873
<i>S. somaliensis</i>	IMRU 1274 ^T , IMRU 632, IMRU 1270, IMRU 1272, IMRU 1280, IMRU 1295, IMRU 1298

^a Cluster A18 of Williams et al. (39).

formed by using the method of Smith and Cantor (35) and the manufacturers' recommendations.

PFGE. Pulsed-field gel electrophoresis (PFGE) was performed on 0.8% agarose gels in 1× Tris-borate buffer (pH 8) in a Beckman Geneline TAFE apparatus. In most cases the following programs were used: (i) for fragments having molecular sizes of 200 to 1,500 kb, a first stage consisting of 12 h at 150 V with a 70-s pulse time and a second stage consisting of 14 h at 150 V with a 2-min pulse time (the standard used was *Saccharomyces cerevisiae* chromosomes); and (ii) for fragments having molecular sizes of 50 to 500 kb, one stage consisting of 20 h at 140 mA with a 25-s pulse time in 1% Tris-acetate buffer (the standard used was λ concatemers). The gels were stained with ethidium bromide, visualized at 302 nm, and photographed with Polaroid type 55 or 57 film.

Plasmid detection. For plasmid detection samples were prepared as described above until the restriction step. Strains displaying bands in the 100- to 1,500-kb range were subjected to PFGE with the second program (see above) to distinguish between giant linear plasmids (GLPs) and supercoiled (SC) plasmids (14).

Numerical analysis. Similarity levels were calculated by using the Taxan program (version 2.0) of David Swartz, Biotechnology Institute, University of Maryland, and the

simple matching coefficient, and cluster analysis was performed by the unweighted average pair group method (36). Clusters were distinguished at levels of similarity of 80% or greater. Data were coded as 0 (negative) or 1 (positive).

RESULTS

Using LFRFA, we analyzed 59 *Streptomyces* strains belonging to 15 species. We grouped together seven of these species (Table 1) as representatives of the *Streptomyces cyaneus* cluster proposed by Williams et al. (39) on the basis of numerical taxonomic data. The *Streptomyces albus*, *Streptomyces lavendulae*, *Streptomyces rimosus*, and *Streptomyces somaliensis* strains were identified by Gordon (10), Gordon and Horan (11), and Mishra et al. (30) on the basis of phenotypic test results. Of the 12 *Streptomyces fradiae* strains examined by the LFRFA in this study, 9 were identified by Gordon (10) and Mishra et al. (30) on the basis of physiological characteristics, and 3 (NRRL B-16471, NRRL B-16472, and NRRL B-16473), a tylosin producer and two mutants, were tentatively assigned to *S. fradiae* by McGuire et al. (29). The *Streptomyces ipomoea* strains were all isolated from sweet potato rot; they included the type strain, ISP 5383, nine strains isolated by C. A. Clark or W. J. Martin in the United States (6), and two strains isolated by T. Suzui in Japan (23). The DNA fragments generated in this analysis ranged from 100 to 1,500 kb long. In all cases the fragments were well resolved by PFGE, and differences among strains could clearly be detected.

Pretreatment. In preliminary experiments it was found that the original procedure for preparing bacterial inserts for pulsed-field analysis described by Smith and Cantor (35) and used by us with modifications for strains belonging to the actinomycete genus *Frankia* (3) yields unrestricted, incompletely restricted, or degraded streptomycete DNA. The growth conditions used, pretreatment of cells, and extraction with water-saturated phenol (see Materials and Methods) provided the clear, interpretable, highly reproducible results desired with all of the *Streptomyces* strains examined thus far.

Plasmids. An examination of unrestricted DNAs from the strains used in this study subjected to PFGE analysis showed that GLPs or SC plasmids were often present (Table 2). Many strains of *S. ipomoea* and all of the *S. lavendulae* strains tested contained at least one GLP (Fig. 1). The largest linear plasmid (length, >1,000 kb) was found in *S. lavendulae* strain IMRU 467.

Restriction enzyme-specific patterns. We evaluated the potential usefulness of different restriction enzymes for generating low-frequency restriction fragments having lengths suitable for analysis. All of the patterns generated by *SpeI* (A'CTAG) were complex, containing numerous fragments (e.g., Fig. 2, *S. albus*). The *DraI* restrictase (TT-T'AAA) produced either simple patterns with three to five DNA fragments (Fig. 3, *S. albus*) or patterns with numerous fragments (Fig. 4, *S. ipomoea*). Some *S. somaliensis* strains seemed to have no detectable *DraI* sites at all (data not shown). Restriction with *AseI* (AT'TAAT) gave rise to between 3 to 15 fragments in the strains studied (Fig. 5 and 6 and Table 3). As most of the *AseI*-generated fragments were sufficiently numerous to provide meaningful discrimination among strains and they fell within the molecular size range amenable to good separation with PFGE, we used this enzyme for the analyses described below.

Strain- and/or species-specific patterns. There were no LFRFA patterns found that were specific for all strains of a

TABLE 2. Linear plasmid contents of the *Streptomyces* strains used in this study

Taxon	Strain	Plasmid size(s) (kb)
<i>S. fradiae</i>	IMRU 3556A	184, 250
	NRRL B-16471	440
	NRRL B-16472	440
<i>S. rimosus</i>	IMRU 3558 ^T	260
	IMRU 3701	260
	IMRU 949	<100
<i>S. cyaneus</i> cluster	NRRL B-12177	180
	NRRL B-12230	90
<i>S. lavendulae</i>	IMRU 467	370, 1,000
	IMRU 577	100 (SC plasmid?)
	IMRU 3440-8 ^T	275 (SC plasmid?)
	IMRU 3455	90, 275
	IMRU 3555	370, 400
	IMRU 3736	700
<i>S. ipomoea</i>	NRRL B-16483	270
	NRRL B-16485	<90
	NRRL B-16488	<90, 270
	NRRL B-16489	<90, 270
	NRRL B-16447	<90

given species. In most cases the patterns were strain specific. However, there were cases of identical or highly related patterns (similarity levels, >94%) found within groups of strains identified as *S. albus*, *S. fradiae*, *S. ipomoea*, *S. lavendulae*, *S. rimosus*, or *S. somaliensis*. None of the members of the *S. cyaneus* cluster examined exhibited a close relationship to other members of the cluster or to the strains of the other species examined.

Numerical analysis. For purposes of numerical analysis,

FIG. 1. Pulsed-field gel of *S. lavendulae* strains (unrestricted), showing positions of plasmids. Lane A, *Saccharomyces cerevisiae* chromosomal standard (chromosome sizes from bottom to top, 260, 290, 370, 460, 580, 650, 763, 835, 864, 991, 1,047, 1,285, and 1,500 kb); lane B, IMRU 467 (GLP); lane C, IMRU 577 (GLP); lane D, IMRU 3440-8^T (GLP); lane E, IMRU 3440-14 (GLP); lane F, IMRU 3445 (GLP); lane G, IMRU 3455 (upper band, GLP; lower band, SC plasmid); lane H, IMRU 3555 (GLP); lane I, IMRU 3736 (upper band, GLP; lower band, SC plasmid?).

FIG. 2. Pulsed-field gel of *S. albus* strains restricted with *SpeI*. Lane A, *Saccharomyces cerevisiae* chromosomal standard (see the legend to Fig. 1); lane B, ATCC 3004^T; lane C, IMRU 1182; lane D, IMRU 3854; lane E, IMRU 1205; lane F, ATCC 618; lane G, IMRU 1187; lane H, IMRU 3854.

because of (i) the inevitable error inherent in estimating the sizes of large fragments (35) and (ii) the possibility of deleted or amplified sequences, which are common in *Streptomyces* strains (4), we grouped together bands in 30-kb ranges except for bands larger than 1,080 kb, a highly compressed area for which 140-kb intervals were used (Table 3). Figure 7 shows a dendrogram giving the levels of relatedness for the various strains based on the numerical analysis of the data. Of the 59 strains tested, 42 (71%) were distributed among eight clusters related at similarity levels of 80% or greater; 17 (29%) of the strains were not clustered (Table 3).

All strains designated *S. albus* were restricted by both *DraI* and *AseI* in a manner similar to the restriction of type culture ATCC 3004, giving rise to three and four or five fragments, respectively (Fig. 5) (*DraI* data not shown). Most

FIG. 3. Pulsed-field gel of *S. albus* strains restricted with *DraI*. Lane A, *Saccharomyces cerevisiae* chromosomal standard (see the legend to Fig. 1); lane B, ATCC 3004^T; lane C, ATCC 618; lane D, IMRU 1182; lane E, IMRU 1205; lane F, IMRU 3854.

FIG. 4. Pulsed-field gel of *S. ipomoea* strains restricted with *Dra*I. Lane A, *Saccharomyces cerevisiae* chromosomal standard (see the legend to Fig. 1); lane B, NRRL B-16480; lane C, NRRL B-16483; lane D, NRRL B-16489; lane E, NRRL B-16445; lane F, NRRL B-16447.

of the strains also exhibited strain-specific digestion differences; the only exception was *S. albus* ATCC 618, which seemed to be identical to the type strain. All of the *S. albus* strains were found together in cluster 1 (Fig. 7 and Table 3).

Among the *S. somaliensis* strains, only one, IMRU 1280, was restricted by *Ase*I in a manner which gave a pattern very similar to that of the type strain, ATCC 1274. These two strains were grouped in cluster 1. The rest of the *S. somaliensis* strains showed more complicated digestion patterns and were scattered among clusters 2 through 4 or were not clustered. Between five and eight fragments were generated in all strains of *S. somaliensis* with *Ase*I; in contrast, these strains exhibited relative or complete insensitivity to *Dra*I,

FIG. 5. Pulsed-field gel of *S. albus* strains restricted with *Ase*I. Lane A, *Saccharomyces cerevisiae* chromosomal standard (see the legend to Fig. 1); lane B, ATCC 3004^T; lane C, IMRU 1182; lane D, IMRU 3854; lane E, IMRU 1205; lane F, ATCC 618; lane G, IMRU 1187.

FIG. 6. Pulsed-field gel of *S. ipomoea* strains restricted with *Ase*I. Lane A, *Saccharomyces cerevisiae* chromosomal standard (see the legend to Fig. 1); lane B, ISP 5383^T; lane C, Clark Standard (= NRRL B-16443); lane D, Clark 7849; lane E, Clark 7851; lane F, Clark 7852; lane G, Clark 7857; lane H, Clark 7860; lane I, Clark 7861.

with zero to three restriction fragments produced (data not shown).

When *Ase*I was used, two clusters (clusters 2 and 5) were observed with the *S. fradiae* strains (Fig. 7). The cluster 2 strains yielded three to six DNA fragments with this enzyme, whereas the cluster 5 strains yielded eight or nine fragments. The same two groups were distinguished by *Dra*I (data not shown). Differences were detected in the tylosin producer and its two mutants (NRRL B-16471, NRRL B-16472, and NRRL B-16473), but these strains clustered at a similarity level 93%.

All of the *S. ipomoea* strains except the type strain could be recognized by their similar *Ase*I digestion profiles (Fig. 6). They formed two closely related clusters (clusters 7 and 8) linked to each other at a similarity level of 76%. The cluster 7 strains yielded 10 to 13 DNA fragments, and the cluster 8 strains yielded 12 to 15 fragments, with many fragments in the low-molecular-size range (<200 kb). The type strain, ISP 5383, did not cluster with any of the other *S. ipomoea* strains studied.

*Ase*I digestions of *S. lavendulae* strains without preliminary plasmid elimination produced 9 to 12 fragments (data not shown). Plasmid-free samples yielded simpler strain-specific patterns (data not shown) having 3 to 10 fragments, and these patterns were used in the numerical analysis. Two strains, including the type strain, were found in cluster 1 and were related to each other at a similarity level of 97%. The other strains were found in clusters 2 and 3 or were unclustered.

All of the strains designated *S. rimosus* yielded 11 to 13 bands after *Ase*I digestion. Three of the *S. rimosus* strains (IMRU 3558^T, IMRU 3701, and IMRU 3873) were identical as determined with both *Ase*I and *Dra*I (data not shown) and formed a cluster by themselves (cluster 6) (Fig. 7). The other two strains studied were unclustered; they were related to each other at a similarity level of only 73% and were remote from the other *S. rimosus* strains in cluster 6.

An analysis of the *Ase*I digests of the *S. cyaneus* cluster strains showed that these strains were not related to each other or to the other strains studied; none of them were

TABLE 3. Distribution of *AseI* fragments of various sizes within the clusters of *Streptomyces* strains

Cluster	No. of strains	No. of fragments		Cluster composition (no. of strains of each species)	% of strains having <i>AseI</i> fragments in the following size ranges (kb) ^a :															
		Avg	Range		90– 119	150– 179	180– 209	210– 239	240– 269	270– 299	300– 329	330– 359	360– 389	390– 419	420– 449	450– 479	480– 509	510– 539	540– 569	
1	10	4.3	3–5	6 <i>S. albus</i> , 2 <i>S. somaliensis</i> , 2 <i>S. lavendulae</i>	0	0	0	20	0	60	10	30	0	0	0	10	50	30	20	
2	10	4.9	3–7	8 <i>S. fradiae</i> , 1 <i>S. lavendulae</i> , 1 <i>S. somaliensis</i>	0	10	80	0	100	0	0	0	10	0	10	10	10	0	10	
3	3	4.7	4–6	1 <i>S. fradiae</i> , 1 <i>S. somaliensis</i> , 1 <i>S. lavendulae</i>	0	100	33	33	0	0	0	0	0	66	0	0	0	0	0	
4	2	7.5	7–8	2 <i>S. somaliensis</i>	0	100	0	100	50	50	0	0	50	0	0	0	0	0	0	
5	3	8.3	8–9	3 <i>S. fradiae</i>	100	100	0	0	0	100	0	0	0	66	0	0	0	100	0	
6	3	13	13	3 <i>S. rimosus</i>	100	0	100	0	0	100	0	0	0	100	100	0	100	0	100	
7	8	12	11–13	8 <i>S. ipomoea</i>	86	0	57	100	100	100	0	86	0	0	100	28	72	72	0	
8	3	13	12–15	3 <i>S. ipomoea</i>	66	33	66	100	100	0	0	100	33	33	100	100	66	66	66	

^a Data for the 120- to 149-kb, 570- to 599-kb, and 1,020- to 1,049-kb ranges have been omitted because no *AseI* fragments with these sizes were observed for any strain.

found in any of the clusters. These strains yielded between 9 and 12 fragments.

DISCUSSION

The genus *Streptomyces* is the largest genus in terms of number of species among the actinomycetes, and its members are also the predominant actinomycetic forms in nature. Included in the genus are producers of antibiotics (e.g., *S. fradiae*, *S. rimosus*, *S. lavendulae*), as well as pathogens of sweet potato (*S. ipomoea*) and possibly of humans (*S. somaliensis* and *S. albus*). The taxonomy of the genus has been clarified by the finding that its members contain the L isomer of diaminopimelic acid in their peptidoglycans, a characteristic shared by only about 5% of the other actinomycete genera recognized (25). However, the genus contains more than 400 validly described species, the descriptions of which are often based on very few characters. The various attempts to deal with species definitions include the International *Streptomyces* Project (33), the work of Mishra et al. (30), and the work of Pridham and Tresner (32). All of these workers utilized morphological and physiological characteristics. The same types of criteria were used by numerical taxonomists who proposed clustering strains on the basis of their overall similarities by using various algorithms (38, 39). All of these researchers have contributed to our understanding of the complexity of the genus, but none has resolved the problem of species identification in a wholly satisfactory way. Because of this workers in the field have turned to other methods, including analysis of DNA relatedness, as a route to a better definition of species (23).

We have found another technique for grouping *Streptomyces* strains which appears to be complementary to both the phenotypic and DNA relatedness approaches, at least in some species. It is our feeling that LFRFA of the entire streptomycete genome may be a good approach to the study of the species problem. Since sequencing and DNA relatedness determinations are long and laborious procedures, LFRFA may be an alternative solution which is rapid, accurate, and reproducible and can take into account the plasmid content of a strain. In one previous study of streptomycetes in which this technique was used (24) four strains of *Streptomyces ambifaciens* were examined. It was found

that three strains clustered reasonably closely and one was different. Using LFRFA, we have already obtained promising results in defining species groups in the actinomycete genus *Frankia* (3). The method has been exploited with success in other bacteria, including brucellae (1), *Escherichia coli* (2, 5), mycobacteria (34), and pseudomonads (12).

The strains which we examined (Table 1) included the type strains of 15 species, including the type species of the genus *Streptomyces*, *S. albus*. Seven of these species have been grouped by phenetic methods in the so-called *S. cyaneus* cluster (cluster group A18 [39]), and their levels of DNA homology have been determined (20, 21). In the cases of *S. albus*, *S. fradiae*, *S. lavendulae*, *S. rimosus*, and *S. somaliensis*, we selected six to eight representative strains identified on the basis of physiological tests by Gordon. The strains and their origins have been described previously (10, 11, 30). Some of the species which we studied have recently been investigated by other workers using different methods, including 16S rRNA sequencing (*S. albus*, *Streptomyces coelicolor*, *S. lavendulae*, and *Streptomyces lividans* [40]), ribosomal protein patterns (*S. albus*, *S. coelicolor*, and *S. lavendulae* [8, 31]), and an enzyme-linked immunosorbent assay (*S. rimosus* [18]), although in most cases the strains tested differed from the strains which we used and it is difficult to compare the results of the various studies.

Restriction of streptomycete DNA in situ in agarose plugs for analysis by PFGE cannot be accomplished with the procedures currently in general use (35) or the modification developed by us for use with the genus *Frankia* (3). A simple but indispensable step in the pretreatment protocol for *Streptomyces* strains is water-saturated phenol extraction of the ground cells (see Materials and Methods), which results in a dramatic improvement in the restriction patterns. At the same time, the risk of DNA degradation which we and other workers (16, 41) have observed in many strains is eliminated or at least much reduced. Although we have not tried to elucidate the mechanism of phenol action, it can be speculated that this action is probably associated with complete denaturation of proteins inaccessible to the other reagents used in the procedure to eliminate protein interference (e.g., SDS, proteases, etc.).

TABLE 3—Continued

% of strains having <i>AseI</i> fragments in the following size ranges (kb) ^a :																			
600– 629	630– 659	660– 689	690– 719	720– 749	750– 779	780– 809	810– 839	840– 869	870– 899	900– 929	930– 959	960– 989	990– 1,019	1,050– 1,079	1,080– 1,219	1,220– 1,359	1,360– 1,499	>1,500	
0	0	10	40	20	0	0	0	0	0	0	0	20	70	0	0	10	20	0	
0	0	0	10	0	10	0	0	0	0	0	10	0	10	20	50	40	80	10	
33	0	0	0	0	0	0	0	33	0	33	0	0	33	0	33	33	0	0	
0	50	0	0	0	0	0	100	0	0	0	0	50	0	0	50	100	0	0	
0	0	33	0	0	0	0	33	0	0	100	0	0	0	0	0	100	100	0	
100	0	0	100	0	0	100	0	0	100	100	0	0	0	0	0	100	0	0	
14	0	0	72	28	0	100	0	0	0	0	0	0	0	0	0	72	100	0	
0	0	0	0	0	0	100	0	100	0	0	100	0	0	0	0	0	100	0

FIG. 7. Dendrogram constructed by unweighted average linkage cluster analysis of simple matching coefficient (S_{SM}) values for DNA fragment sizes expressed as percentages. Each of the *Streptomyces* strains was restricted with *AseI*, and the sizes of the resulting DNA fragments were calculated (see Materials and Methods). The prefixes of the strain designations indicate the species or taxon names, as follows: A, *S. albus*; C, *S. cyaneus* cluster; Co, *S. coelicolor*; F, *S. fradiae*; I, *S. ipomoea*; L, *S. lavendulae*; Li, *S. lividans*; R, *S. rimosus*; S, *S. somaliensis*. NC, not clustered.

Proper evaluation of the taxonomic implications of the method requires also taking into account the influence of certain factors. The first factor to be considered is the plasmid content of the strains. The existence of GLPs and/or SC plasmids could jeopardize the reliability of the patterns. As shown in Table 2, many of the streptomycete strains which we investigated contain GLPs. Finding such plasmids is not a new development. The detection of GLPs has been reported in *Nocardia opaca* (15) and at least eight antibiotic-producing *Streptomyces* spp. (13, 17), including two tylosin-producing *S. fradiae* strains used in our study. The value which we determined for GLP size in one of the strains which we used (440 kb) is in agreement with previously published data (ca. 400 kb [13]). We confirmed the finding that plasmids are also present in many *S. ipomoea* strains (19). Our data extend the number of *Streptomyces* strains known to carry GLPs. Our data also show that most of the *S. ipomoea* strains which we examined carry two or three plasmids and that all of the *S. lavendulae* strains in our study contain GLPs, including the largest GLP found (size, 1,000 kb).

Another factor which has major significance for LFRFA taxonomic evaluation is the choice of restriction enzyme(s). The use of rare-site restrictases (28) for generating interpretable patterns is a *conditio sine qua non*. As the genomes of streptomycetes have high G+C contents, the currently available restriction enzymes of choice are *DraI* (TTT'AAA), *AseI* (AT'TAAT), and *SpeI* (A'CTAG). However, other enzymes will undoubtedly be developed. We found that at this time *AseI* digests are most universally suited (both in number and distribution of fragments) for LFRFA.

The *AseI*- and *DraI*-generated LFRFA patterns of the *S. albus* strains showed that this taxon, defined phenotypically by Gordon (10) and Mishra et al. (30), is quite homogeneous. The strains studied have diverse origins (they are often clinical isolates); e.g., they have been obtained from human dental (IMRU 1182), pulmonary (IMRU 1187), and bovine (IMRU 1205) sources. The low-frequency restriction fragment length homogeneity within this group is in good agreement with the morphological and physiological homogeneity of the members of this group (30). Also included in the *S. albus* cluster were two strains of *S. somaliensis* and two strains of *S. lavendulae*, including the type strains of these two species. The members of these three species cannot be

FIG. 8. Pulsed-field gel of *S. lavendulae* strains digested with *SpeI*. Lane A, *Saccharomyces cerevisiae* chromosomal standard (see the legend to Fig. 1); lane B, IMRU 467; lane C, IMRU 577; lane D, IMRU 3440-8^T; lane E, IMRU 3445; lane F, IMRU 3455; lane G, IMRU 3555.

differentiated on the basis of their *DraI* LFRFA patterns since all of them yielded few or no fragments with this enzyme (Fig. 3) (data for *S. lavendulae* and *S. somaliensis* not shown). In contrast, the utilization of a third enzyme, *SpeI*, permitted the differentiation of *S. albus* and *S. lavendulae* strains. The *S. albus* patterns (Fig. 2) contain many small fragments (<500 kb) and few large fragments. In contrast, most *S. lavendulae* strains have fragments evenly distributed from 1,200 to 260 kb (Fig. 8). The exception is *S. lavendulae* IMRU 3555, which has a pattern like the *S. albus* pattern. This information indicates the possible utility of a two- or three-step LFRFA to obtain intracluster separation.

Cluster 2 is made up of eight *S. fradiae* strains, one *S. somaliensis* strain, and one *S. lavendulae* strain. Seven of the *S. fradiae* strains clustered closely at a similarity level 93%, and three of these strains (IMRU 3535^T, IMRU 3594, and IMRU 3719) produced identical patterns. These three strains, which were independently isolated, produce neomycin, as do IMRU 3572, IMRU 3554, IMRU 1186, and IMRU 1199. The second cluster containing *S. fradiae* strains (cluster 5) contains only the tylosin producer and two of its mutants and is related to the first cluster at a similarity level of only 70%. This finding may be consistent with the doubts expressed by McGuire et al. (29) in their original discussion about the appropriateness of the assignment of the strains to *S. fradiae*.

Relative low-frequency restriction fragment length homogeneity is also characteristic of clusters 7 and 8, which contain the 11 test strains belonging to the sweet potato-pathogenic species, *S. ipomoea* (6) (Table 4). These strains exhibit high levels of DNA homology (23). Only the type strain of the species (ISP 5383) clustered at some distance from the other *S. ipomoea* strains in the LFRFA; this strain has also been reported to exhibit relatively low levels of DNA relatedness to the other strains and to be not pathogenic for sweet potatoes (20). Thus, we feel that the taxo-

TABLE 4. Comparison of DNA-DNA relatedness and LFRFA similarity values for *S. ipomoea* strains

Strain	Country of origin	LFRFA cluster	% Similarity to NRRL B-16580 as determined by LFRFA	% DNA-DNA relatedness to NRRL B-16580 ^a
Clark 7849	United States	7	73	91
Clark 7851	United States	7	78	92
Clark 7860	United States	7	76	92
Clark 7862	United States	7	76	93
NRRL B-16445	United States	8	81	95
NRRL B-16480	Japan	8	100	100
NRRL B-16482	Japan	8	95	96
ISP 5358 ^T	United States	NC ^b	65	76

^a Data from reference 23.

^b NC, not clustered.

nomic potential of the LFRFA method demonstrated by the *S. albus* and *S. fradiae* results are reinforced by the results obtained with this species.

The situation with the strains designated *S. lavendulae*, *S. rimosus*, and *S. somaliensis* is more complicated. The *S. lavendulae* and *S. somaliensis* strains digested with *AseI* showed diverse patterns which taken by themselves could be interpreted as similar, depending on the strain, to the patterns of *S. albus*, *S. fradiae*, or each other. Our findings with *S. lavendulae* lend weight to a recent finding that *S. lavendulae* strains were heterogeneous as measured by DNA-DNA hybridization (20). The *S. rimosus* strains also exhibited LFRFA heterogeneity. Three presumably independent isolates of *S. rimosus* (IMRU 3558^T, IMRU 3701, and IMRU 3873) were identical, but these organisms exhibited low levels of similarity to the other two strains (58%).

In addition to the two strains of *S. somaliensis* in cluster 1 discussed above, which were related to each other at a similarity level of 94%, a second cluster (cluster 4) was composed of two other strains of this taxon related at a similarity level of 86%. The three remaining strains studied were found in clusters 2 and 3 and or were unclustered. As discussed above, it is possible that *Streptomyces* strains may be better differentiated by using a second enzyme in parallel with the first enzyme. It is also possible that, because strains classified as *S. somaliensis* are quite inactive physiologically in the tests of Mishra et al. (30), their apparent phenotypic similarity may not be based on real genomic similarity.

The presumably closely related strains of *S. coelicolor* and *S. lividans* are the strains which have been widely used in genetic studies of streptomycetes. Our finding that these organisms are related at a similarity level of only 73% confirmed the recent finding of Kieser et al. (16) that the two strains are not closely related, as determined by LFRFA. However, we did not encounter the problem of degradation of *S. lividans* DNA reported by Kieser et al. and Zhou et al. (41), possibly because of our method of growing and treating the cells to be analyzed.

Included in our study for comparative purposes was a group of strains which were defined by numerical taxonomy as the *S. cyaneus* cluster (38, 39). The results of DNA relatedness studies performed with these strains (21) suggest that they belong to two major groups and several minor groups. Among the eight strains which we studied which

TABLE 5. Levels of physiological homogeneity and LFRFA similarity for various *Streptomyces* species

Species	% Similarity as determined by:	
	Physiological tests ^a	LFRFA ^b
<i>S. albus</i>	85	84
<i>S. fradiae</i>	95	81
<i>S. somaliensis</i>	75	75
<i>S. cyaneus</i>	90	58
<i>S. lavendulae</i>	63	67
<i>S. rimosus</i>	68	54

^a Percentage of tests positive or negative for more than 90% of the strains. Data from references 10, 26, and 30.

^b Level of LFRFA similarity for the strains tested.

represented the two major groups found in the DNA relatedness study, we were not able to discern any clustering whatever. Every strain was unclustered. These strains were not related to any of the other strains belonging to the *S. cyaneus* cluster or to any other member of the taxa which we examined. It is possible that the DNA relatedness levels chosen to demonstrate relatedness (ca. 70%) were too low. This hypothesis is supported by the fact that the high level of similarity in the LFRFA found for the *S. ipomoea* strains that were analyzed in this study was reflected by the high level of DNA relatedness (ca. 90%) found in a recent study by the same authors (23) (Table 4).

However, since we found by using the tests of Mishra et al. (30) that the representatives of the *S. cyaneus* group that we studied are 90% homologous physiologically (Table 5) (26), we concluded that although the results of physiological tests may point to similarities among strains, such tests really measure activities coded for by such a small portion of the whole genome that the apparent phenotypic relationships may not be always borne out when groupings are based on genotypic characterization.

Among more than 100 strains analyzed in our LFRFA study of the genus *Frankia* (3) we encountered only 2 strains which had identical LFRFA patterns. We thus assumed that because the two strains involved had been isolated in the same laboratory, one was a reisolat of the other. However, the results obtained in this study provide some evidence that apparently independently isolated strains can be identical in their LFRFA patterns. Such strains include *S. albus* ATCC 3004^T and ATCC 618 and *S. fradiae* IMRU 3535^T and IMRU 3719 (IMRU 3594, which was identical to these strains, was isolated in the same laboratory as IMRU 3535^T). In contrast, the identical strains of *S. rimosus* (IMRU 3558^T, IMRU 3701, and IMRU 3873), although thought to be independent isolates, all came from the same industrial source. The *S. ipomoea* strains, which clustered at a similarity level of 100%, were all isolated in the same laboratory. Thus, it is still not totally clear whether identical LFRFA patterns imply clonal identity, but the preponderance of evidence from our two studies and the studies of other workers (2, 5) seems to suggest that they may.

It is, however, evident that some strains which have been grouped on the basis of phenotype (e.g., *S. albus*, *S. fradiae*, and *S. ipomoea*) are also closely related as determined by LFRFA. As shown in Table 5, with one exception (the *S. cyaneus* cluster), the less closely related a species group is phenotypically, the less closely related it is as determined by LFRFA.

We believe that our data emphasize the high potential

usefulness of LFRFA for the taxonomy of streptomycetes. At the species level, this method shows clearly that at least some species produce closely related patterns, which may become the basis of a future identification key. Strain differences, as well as similarities, can easily be monitored. It is our belief that, with appropriate additional data obtained by using other suitable restrictases, a good basis for the delineation of *Streptomyces* species may be established.

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