Phylogenetic Analysis of Mycolic Acid-Containing Wall-Chemotype IV Actinomycetes and Allied Taxa by Partial Sequencing of Ribosomal Protein AT-L30

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The phylogenetic relationships among 30 mycolic acid-containing wall chemotype IV actinomycete strains and 12 strains belonging to allied taxa were examined by determining the amino acid sequences of the ribosomal AT-L30 proteins of these organisms. Sequencing 20 N-terminal amino acids of AT-L30 preparations revealed that the members of the genera containing mycolic acid-containing actinomycetes form two clusters; the first cluster contains the genera *Nocardia*, *Rhodococcus*, *Gordona*, and *Tsukamurella*, and the second cluster contains the genera *Corynebacterium* and *Mycobacterium*. The genus *Nocardia* was placed in a clade containing the genus *Rhodococcus*. The data showed that *Tsukamurella paurometabolum* is closely related phylogenetically to the genus *Gordona*. The phylogenetic clusters identified were entirely consistent with the proposal of Goodfellow that the family *Nocardiaceae* should encompass the mycolate-containing, cell wall type IV actinomycete genera *Nocardia*, *Rhodococcus*, *Gordona*, and *Tsukamurella*. The genera *Actinomyces* and *Micrococcus* exhibited AT-L30 amino acid sequence characteristics intermediate between those of actinomycetes and those of typical eubacteria. The genera *Nocardia*, *Gordona*, *Mycobacterium*, *Actinoplanes*, and *Micromonospora* were each a taxon that consisted of phylogenetically coherent species. In contrast, the genera *Rhodococcus* and *Corynebacterium* are taxa that consist of phylogenetically distantly related species. In general, my results are consistent with previous 16S rRNA sequencing results, but significant differences were also found. My data, together with previous AT-L30 sequencing data, show that phylogenetic relationships among taxa can be determined by using markers other than the ribosomal gene sequences.

Microorganisms containing mycolic acids in their cell walls have many properties in common (13) and form a recognizable suprageneric group (28, 49, 52). These organisms have chemotype IV walls (26) and can be assigned to the genera *Corynebacterium*, *Gordona*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, and *Tsukamurella* by using a combination of chemical and morphological markers (12). *Nocardia* produce abundant or sparse aerial mycelia, but *Corynebacteria* do not produce mycelia. *Mycobacteria* and *rhodococci* do not usually form aerial hyphae, with a few exceptions. The taxonomy of the actinomycetes containing mycolic acids has undergone significant revisions based on data obtained by chemical, numerical, and molecular systematic methods; such revisions have occurred in the genera *Rhodococcus* (11–13, 15), *Corynebacterium* (27), *Gordona* (51), *Tsukamurella* (4), and *Mycobacterium* (17, 18, 46, 60). The genus *Nocardia* is the type genus of the family *Nocardiaceae*. The genus *Gordona* is characterized by the absence of mycelia and slight or strong acid fastness, and members of this genus have characteristics intermediate between those of the genus *Nocardia* and those of the genus *Mycobacterium* (54). The taxonomy of the genus *Gordona* and the related genus *Tsukamurella* has been clarified by using chemical, molecular systematic, and numerical taxonomic methods (16).

Traditionally, taxonomic studies have relied heavily on morphological characteristics, but numerical taxonomy and chemotaxonomic characteristics have played important roles in determining taxonomic relationships. DNA hybridization and 16S rRNA similarity data are also important, especially for determining phylogenetic relationships at both the generic level and the subgeneric level.

Since 1989, Ochi and coworkers have developed a method for identifying and classifying actinomycetes (30–32). This ap-

proach is based on analysis of ribosomal AT-L30 proteins (homologous to *Escherichia coli* L30 protein); i.e., the electrophoretic mobilities of the AT-L30 proteins and their N-terminal amino acid sequences are determined. The efficacy of this approach has been demonstrated in studies of the phylogeny of actinomycete genera such as *Streptomyces*, *Streptoverticillium*, *Streptosporangium*, *Actinomadura*, *Microbispora*, *Microtetraspora*, and *Thermoactinomyces*, in addition to the genera belonging to the family *Pseudonocardiaceae* (31, 34, 36, 38–42). This approach has also proven to be effective for studying the phylogeny of bacteria other than actinomycetes, including the genera *Bacillus*, *Staphylococcus*, *Escherichia*, and *Pseudomonas* and allied taxa (33, 35). In this study I examined the taxonomic structure of wall chemotype IV actinomycetes and allied taxa by using the AT-L30 protein analysis method. The aims of this study were (i) to clarify the phylogenetic relationships among the genera *Nocardia*, *Rhodococcus*, *Mycobacterium*, *Corynebacterium*, and *Tsukamurella* and (ii) to clarify the taxonomic status of members of the family *Actinoplanaceae* and actinobacteria as represented by the genera *Actinomyces* and *Micrococcus* within the gram-positive bacteria.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1. Almost all of the strains were type strains that were obtained from the Japan Collection of Microorganisms, Wako, Saitama, Japan, the Institute of Fermentation, Osaka, Japan, and the American Type Culture Collection, Rockville, Md. The strains of *Corynebacterium diphtheriae* and *Corynebacterium xerosis* which I studied are not the type strains of these species. It should be noted that *C. xerosis* ATCC 7711, which was used in this study, is known to be genomically distinct from the type strain of this species, strain ATCC 373 (5). Most of the strains were grown to the mid-exponential phase in soluble starch-Polypeptone-yeast extract medium (31) at 30°C; *Actinomyces viscosus*, *Actinomyces naeslundii*, and *Actinomyces pyogenes* were grown for 3 days at 37°C under microaerobic conditions in a medium described by Lämmler and Blobel (24).

Preparation of total ribosomal proteins and two-dimensional PAGE. The methods used to prepare total ribosomal proteins and the methods used for

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TABLE 1. Strains used in this study

Species or subspecies	Strain	Genus in which the strain was classified previously
<i>Actinomyces naeslundii</i>	JCM 8349 ^T (= ATCC 12104 ^T) ^a	<i>Corynebacterium</i>
<i>Actinomyces pyogenes</i>	CCM 5753 ^T (= ATCC 19411 ^T)	
<i>Actinomyces viscosus</i>	JCM 8353 ^T (= ATCC 15987 ^T)	
<i>Actinoplanes digitatis</i>	JCM 3060 ^T (= ATCC 15349 ^T)	<i>Ampullariella</i>
<i>Actinoplanes philippinensis</i>	JCM 3001 ^T (= ATCC 12427 ^T)	
<i>Actinoplanes regularis</i>	JCM 3062 ^T (= DSM 43151 ^T)	
<i>Actinoplanes utahensis</i>	JCM 3122 ^T (= ATCC 14539 ^T)	<i>Ampullariella</i>
<i>Bacillus subtilis</i>	JCM 1465 ^T (= ATCC 6051 ^T)	
<i>Bacillus megaterium</i>	JCM 2506 ^T (= ATCC 14581 ^T)	
<i>Corynebacterium amycolatum</i>	JCM 7447 ^T (= NCFB 2768 ^T)	<i>Microbacterium</i>
<i>Corynebacterium cystitidis</i>	JCM 3715 ^T (= ATCC 29593 ^T)	
<i>Corynebacterium diphtheriae</i>	JCM 1310 (= ATCC 11913)	
<i>Corynebacterium flavesces</i>	JCM 1317 ^T (= ATCC 10340 ^T)	<i>Micrococcus</i>
<i>Corynebacterium glutamicum</i>	JCM 1318 ^T (= ATCC 13032 ^T)	
<i>Corynebacterium vitrum</i>	JCM 1323 ^T (= ATCC 10234 ^T)	
<i>Corynebacterium xerosis</i>	JCM 1324 (= ATCC 7711)	<i>Brevibacterium</i>
<i>Escherichia coli</i>	K-12	
<i>Gordona amarae</i>	JCM 3171 ^T (= ATCC 27808 ^T)	
<i>Gordona bronchialis</i>	JCM 3198 ^T (= ATCC 25592 ^T)	<i>Nocardia</i>
<i>Gordona rubropertinctus</i>	JCM 3204 ^T (= ATCC 14352 ^T)	
<i>Gordona sputi</i>	JCM 3228 ^T (= ATCC 29627 ^T)	
<i>Gordona terrae</i>	JCM 3206 ^T (= ATCC 25594 ^T)	<i>Rhodococcus</i>
<i>Micrococcus agilis</i>	JCM 2584 ^T (= ATCC 966 ^T)	
<i>Micromonospora carbonacea</i> subsp. <i>carbonacea</i>	JCM 3139 ^T (= ATCC 27114 ^T)	
<i>Micromonospora chalybeata</i>	JCM 3031 ^T (= ATCC 12452 ^T)	<i>Nocardia</i>
<i>Micromonospora echinospora</i> subsp. <i>echinospora</i>	JCM 3073 ^T (= ATCC 15837 ^T)	
<i>Micromonospora purpurea</i>	IFO 12575 ^T (= ATCC 15835 ^T)	
<i>Mycobacterium austroafricanum</i>	JCM 6369 ^T (= ATCC 33464 ^T)	<i>Nocardia</i>
<i>Mycobacterium chelonae</i> subsp. <i>chelonae</i>	JCM 6388 ^T (= ATCC 35752 ^T)	
<i>Mycobacterium diernhoferi</i>	JCM 6371 ^T (= ATCC 19340 ^T)	
<i>Mycobacterium fortuitum</i> subsp. <i>acetamidolyticum</i>	JCM 6368 ^T (= ATCC 35931 ^T)	<i>Nocardia</i>
<i>Mycobacterium morioakaense</i>	JCM 6375 ^T (= ATCC 43059 ^T)	
<i>Nocardia asteroides</i>	JCM 3384 ^T (= ATCC 19247 ^T)	
<i>Nocardia brasiliensis</i>	IFM 0236 ^T (= ATCC 19296 ^T)	<i>Nocardia</i>
<i>Nocardia farcinica</i>	JCM 3088 ^T (= ATCC 3318 ^T)	
<i>Nocardia nova</i>	IFM 0290 ^T (= ATCC 33726 ^T)	
<i>Nocardia otitidiscaviarum</i>	JCM 3377 ^T (= ATCC 14629 ^T)	<i>Amycolata</i>
<i>Pseudonocardia satumnea</i>	JCM 3187 ^T (= ATCC 15104 ^T)	
<i>Pseudonocardia thermophila</i>	JCM 3095 ^T (= ATCC 19285 ^T)	
<i>Rhodococcus coprophilus</i>	JCM 3200 ^T (= ATCC 29080 ^T)	<i>Corynebacterium</i>
<i>Rhodococcus equi</i>	JCM 1311 ^T (= ATCC 25729 ^T)	
<i>Rhodococcus erythropolis</i>	JCM 3201 ^T (= ATCC 4277 ^T)	
<i>Rhodococcus luteus</i>	JCM 6162 ^T (= ATCC 35014 ^T)	<i>Nocardia</i>
<i>Rhodococcus rhodnii</i>	JCM 3203 ^T (= ATCC 35071 ^T)	
<i>Rhodococcus rhodochrous</i>	JCM 3202 ^T (= ATCC 13808 ^T)	
<i>Rhodococcus ruber</i>	JCM 3205 ^T (= ATCC 25593 ^T)	<i>Gordona</i>
<i>Saccharomonospora viridis</i>	JCM 3036 ^T (= ATCC 15386 ^T)	
<i>Staphylococcus aureus</i>	JCM 2151 (= ATCC 6538P)	
<i>Streptomyces coelicolor</i>	A3(2) ^b	<i>Rhodococcus</i>
<i>Streptomyces lavendulae</i> subsp. <i>lavendulae</i>	IFO 12789 ^T (= ATCC 19777 ^T)	
<i>Tsukamurella paurometabolum</i>	JCM 3226 (= ATCC 25938) ^c	

^a T = type strain.^b This strain is a misclassified strain of *Streptomyces violaceoruber* (23, 37).^c This strain was the type strain of *Rhodococcus aurantiacus* (58).

polyacrylamide gel electrophoresis (PAGE) have been described in detail previously (20, 30).

Determination of the N-terminal amino acid sequences. The N-terminal amino acid sequences of AT-L30 protein samples were determined by using a model 470A protein sequencer (Applied Biosystems, Foster City, Calif.) as described previously (39, 40). Determination of a maximum of 25 N-terminal amino acids was possible when 100 to 150 pmol of an AT-L30 preparation was used. These 25 N-terminal amino acids represent nearly one-half of the total AT-L30 protein, assuming that the L30 protein from *E. coli* and the AT-L30 proteins from actinomycetes are the same size.

Clustering of the strains. A dendrogram was constructed on the basis of levels of amino acid sequence homology by using the multiple-alignment method of Higgins et al. (19).

RESULTS

Amino acid sequences of AT-L30 proteins. (i) Actinomycetes with type IV cell walls. AT-L30 proteins from five to seven species of each of five genera (the genera *Nocardia*, *Rhodococcus*, *Corynebacterium*, *Mycobacterium*, and *Gordona*) and one *Tsukamurella* species were investigated first (Fig. 1). To express quantitatively the levels of similarity of the amino acid sequences (SAS values), I determined the frequency of appearance of a particular amino acid in 20 N-terminal amino acids of each AT-L30 protein for every combination of strains tested.

	1	5	10	15	20	25
<i>N. asteroides</i>	Ala-Asp-Leu-Lys-Val-Thr-Gln-Ile-Lys-Ser-Thr-Ile-Gly-Ala-Lys-Ala-Asn-Gln-Lys-Asp					
<i>N. otitidiscaviarum</i>	Ala-Glu-Leu-Lys-Val-Thr-Gln-Ile-Lys-Ser- ? -Ile-Gly-Ala-Lys-Ala-Asn-Gln- ? -Glu					
<i>N. nova</i>	Ala-Gln-Leu-Lys-Val-Thr-Gln-Ile-Lys-Ser-Thr-Ile-Gly-Ala-Lys-Lys-Asn-Gln- ? -Glu					
<i>N. brasiliensis</i>	Ala-Asp-Leu-Lys-Val-Thr-Gln-Ile-Lys-Ser-Thr-Ile-Gly-Ala-Lys-Gln-Asn-Gln- ? -Asp					
<i>N. farcinica</i>	Ala-Asp-Leu-Lys-Val-Thr-Gln-Ile-Lys-Ser-Thr-Ile-Gly-Ala-Lys- ? -Asn-Gln- ? -Asp					
<i>R. rhodochrous</i>	Ala-Glu-Leu-Lys-Ile-Thr-Gln-Ile-Lys-Ser-Thr-Ile-Gly-Ala-Lys-Ser-Asn-Gln- ? -Asp					
<i>R. rhodnii</i>	Ala-Asn-Ile-Lys-Ile-Thr-Gln-Ile-Lys-Ser-Thr-Ile-Gly-Thr-Lys-Gln-Asn-Gln- ? -Asp-Ser-Leu- ? -Thr-Leu					
<i>R. ruber</i>	Ala-Glu-Leu-Lys-Ile-Thr-Gln-Val-Lys-Ser-Thr-Ile-Gly-Ala-Lys-Ser-Asn-Gln- ? -Asp					
<i>R. erythropolis</i>	Ala-Asp-Leu-Lys-Val-Thr-Gln-Ile-Lys-Ser-Ile-Ile-Gly-Thr-Lys-Gln-Asn- ? - ? -Asp					
<i>R. coprophilus</i>	Ala-Glu-Leu-Lys-Ile-Thr-Gln-Ile-Lys- ? -Thr-Ile-Gly- ? -Lys- ? -Asn-Gln- ? -Asp					
<i>R. luteus</i>	Ala-Gln-Leu-Lys-Val-Thr-Gln-Ile-Lys-Ser-Thr-Ile-Gly-Gln-Lys-Ala-Asn-Gln- ? -Asp-Ser-Leu- ? -Thr-Leu					
<i>R. equi</i>	Ala-Gln-Leu-Lys-Val-Thr-Gln-Val-Lys-Ser-Thr-Ile-Gly-Thr-Lys-Gln-Asn- ? - ? -Asp-Ser- ? - ? -Thr-Leu					
<i>M. diernhoferi</i>	Ala-Glu-Leu-Lys-Ile-Thr-Gln-Val-Arg-Gly-Thr-Ile-Gly-Ala-Arg- ? -Lys-Gln- ? -Glu					
<i>M. fortuitum</i>	Ala-Glu-Leu-Lys-Ile-Thr-Gln-Val-Arg-Gly-Thr-Ile-Gly-Ala-Arg- ? -Lys-Gln- ? -Glu					
<i>M. chelonae</i>	Ala-Asp-Val-Lys-Ile-Thr-Gln-Val-Arg-Ser-Thr-Ile-Gly-Ala-Arg- ? -Lys- ? - ? -Glu					
<i>M. moriokaense</i>	Ala-Glu-Leu-Lys-Ile-Thr-Gln-Val-Arg-Ser-Thr-Ile-Gly-Ala-Arg- ? -Lys- ? - ? -Glu					
<i>M. austroafricanum</i>	Ala-Glu-Leu-Lys-Ile-Thr-Gln-Val-Arg-Ser-Thr-Ile-Gly-Ala-Arg- ? -Lys-Gln- ? -Glu					
<i>C. diphtheriae</i>	Ala-----Leu-Lys-Ile-Thr-Gln-His-Lys-Gly-Leu-Val-Gly-Ala-Asn-Pro-Lys-Gln- ? - ? -Asn					
<i>C. flavescens</i>	Ala-----Leu-Lys-Ile-Thr-Gln-Val-Lys-Gly-Leu-Val-Gly-Thr-Lys-Pro- ? - ? - ? -Ala-Asn					
<i>C. cystitidis</i>	Ala-----Leu-Lys-Ile-Thr-Leu-Lys-Arg-Gly-Lys-Ile-Gly-Thr-Lys-Pro- ? - ? - ? - ? -Asn					
<i>C. vitrumen</i>	Ala-----Leu-Lys-Ile-Thr-Gln-Thr-Lys-Gly-Leu-Val-Gly-Ala-Asn-Pro-Lys-Gln- ? -Lys-Asn					
<i>C. glutamicum</i>	Ala-----Leu-Lys-Ile-Thr-Gln-Ile-Lys-Gly-Thr-Val-Gly-Thr-Lys-Pro- ? - ? - ? - ? -Asn					
<i>C. xerosis</i>	Ala-----Leu-Lys-Ile-Thr-Gln-Leu-Arg-Gly-Thr-Ala-Gly-Thr-Lys-Gln-Asn-Gln-Lys-Asp-Asn					
<i>C. amycolatum</i>	Ala-----Leu-Lys-Ile-Thr-Gln-Val-Arg-Gly-Thr-Ala-Gly-Thr-Glu-Gln-Lys- ? -Lys-Asp-Ser-Leu- ? -Thr-Leu					
<i>G. bronchialis</i>	Ala-Glu-Leu-Lys-Ile-Thr-Gln-Val-Lys-Gly-Thr-Ile-Gly-Thr-Lys-Lys-Asn-Gln- ? -Asp-Ser-Leu- ? -Thr-Leu					
<i>G. rubropertinctus</i>	Ala-Glu-Leu-Lys-Ile-Thr-Gln-Val-Lys-Gly-Thr-Ile-Gly-Thr-Lys-Lys-Asn-Gln- ? -Asp-Ser-Leu- ? -Thr-Leu					
<i>G. terrae</i>	Ala-Glu-Leu-Lys-Ile-Thr-Gln-Val-Lys-Gly-Thr-Ile-Gly-Thr-Lys-Lys-Asn-Gln- ? -Asp-Ser-Leu- ? -Thr-Leu					
<i>G. amarae</i>	Ala-Glu-Leu-Lys-Ile-Thr-Gln-Val-Lys-Gly-Thr-Ile-Gly-Thr-Lys-Lys-Asn-Gln- ? -Asp-Ser-Leu- ? -Thr-Leu					
<i>G. sputi</i>	Ala-Gln-Leu-Lys-Ile-Thr-Gln-Val-Lys-Gly-Thr-Ile-Gly-Ala-Lys-Ser-Asn-Gln- ? -Asp-Ser-Leu- ? -Thr-Leu					
<i>T. paurometabolum</i>	Ala-Glu-Leu-Lys-Ile-Thr-Gln-Val-Arg-Gly-Thr-Ile-Gly-Thr-Lys-Lys-Asn-Gln- ? -Asp-Ser-Leu- ? -Thr-Leu					

FIG. 1. Primary structures of the N termini of AT-L30 proteins from various species of the genera belonging to mycolic acid-containing wall chemotype IV actinomycetes. The question marks indicate amino acids that were not determined. The dotted lines indicate deletions. The amino acids which characterize each genus are enclosed in boxes. Abbreviations: N, *Nocardia*; R, *Rhodococcus*; M, *Mycobacterium*; C, *Corynebacterium*; G, *Gordona*; T, *Tsukamurella*.

Smaller numbers of amino acids were compared in several combinations. Nondetermined positions were not weighted when SAS values were calculated, but deletions were weighted. Only the results of significant comparisons are shown in Table 2. As shown in Fig. 1 and Table 2, high levels of amino acid sequence homology were detected within the genus *Nocardia* (SAS values, 83 to 100%), the genus *Mycobacterium* (SAS values, 82 to 100%), and the genus *Gordona* (SAS values, 84 to 100%). In contrast, low levels of sequence homology were

detected in the genus *Rhodococcus* (SAS values were as low as 67%) and in the genus *Corynebacterium* (SAS value, 50%). In addition to simply comparing the levels of homology of amino acid sequences, I identified amino acids that were located at specific positions and characterized each genus; these amino acids are enclosed in boxes in Fig. 1. Despite the low levels of sequence homology detected within the genera *Rhodococcus* and *Corynebacterium*, these two genera were characterized by amino acids at specific positions. It is especially noteworthy

TABLE 2. SAS values for the amino acid sequences of AT-L30 proteins^a

Species	SAS values (%) with:																							
	<i>Nocardia asteroides</i>	<i>Nocardia otitidiscaviarum</i>	<i>Nocardia nova</i>	<i>Nocardia brasiliensis</i>	<i>Nocardia farcinica</i>	<i>Rhodococcus rhodochrous</i>	<i>Rhodococcus rhodnii</i>	<i>Rhodococcus ruber</i>	<i>Rhodococcus erythropolis</i>	<i>Rhodococcus coprophilus</i>	<i>Rhodococcus luteus</i>	<i>Rhodococcus equi</i>	<i>Mycobacterium diernhoferi</i>	<i>Mycobacterium chelonae</i>	<i>Corynebacterium diphtheriae</i>	<i>Corynebacterium cystitidis</i>	<i>Corynebacterium glutamicum</i>	<i>Corynebacterium amycolatum</i>	<i>Gordona bronchialis</i>	<i>Gordona amarae</i>	<i>Gordona sputi</i>	<i>Tsukamurella paurometabolum</i>	<i>Actinoplanes philippinensis</i>	<i>Micromonospora chalybea</i>
<i>Nocardia otitidiscaviarum</i>	89																							
<i>Nocardia nova</i>	84	89																						
<i>Nocardia brasiliensis</i>	95	83	84																					
<i>Nocardia farcinica</i>	100	88	89	100																				
<i>Rhodococcus rhodochrous</i>	84	83	79	84	89																			
<i>Rhodococcus rhodnii</i>	74	67	68	79	78	79																		
<i>Rhodococcus ruber</i>	79	78	74	79	83	95	74																	
<i>Rhodococcus erythropolis</i>	83	76	72	89	88	72	78	67																
<i>Rhodococcus coprophilus</i>	88	87	81	88	88	100	88	94	80															
<i>Rhodococcus luteus</i>	89	83	84	84	89	79	74	74	78	88														
<i>Rhodococcus equi</i>	78	71	78	83	82	72	78	78	83	80	83													
<i>Mycobacterium diernhoferi</i>	56	65	61	56	56	67	50	72	41	69	50	53												
<i>Mycobacterium chelonae</i>	59	56	59	59	59	59	53	65	47	53	47	53	82											
<i>Corynebacterium diphtheriae</i>	50	53	50	50	53	56	44	56	41	60	44	41	65	50										
<i>Corynebacterium cystitidis</i>	44	47	44	44	47	50	50	50	50	62	44	50	60	47	53									
<i>Corynebacterium glutamicum</i>	63	60	63	63	67	69	69	63	63	85	63	63	60	47	73	67								
<i>Corynebacterium amycolatum</i>	60	35	39	50	47	50	56	56	50	60	45	61	65	59	56	60	67							
<i>Gordona bronchialis</i>	68	67	68	68	72	79	74	84	67	94	68	78	72	53	56	63	75	67						
<i>Gordona amarae</i>	68	67	68	68	72	79	74	84	67	94	68	78	72	53	56	63	75	67	100					
<i>Gordona sputi</i>	74	67	74	74	78	84	68	89	61	88	74	78	72	59	61	56	69	61	84	84				
<i>Tsukamurella paurometabolum</i>	63	61	63	63	67	74	68	79	61	88	63	72	78	59	50	69	69	72	95	95	79			
<i>Actinoplanes philippinensis</i>	74	72	68	79	78	68	68	63	78	75	74	72	50	47	39	50	50	50	58	58	58	63		
<i>Micromonospora chalybea</i>	68	67	68	68	72	63	63	68	72	69	68	78	56	53	39	56	50	56	74	74	63	79	79	
<i>Streptomyces coelicolor</i>	60	61	63	68	67	68	68	74	72	75	68	83	50	53	44	50	56	53	68	68	74	63	63	63

^a The SAS values for pairs of organisms based on 20 N-terminal amino acids (or fewer amino acids for several pairs) of the AT-L30 proteins were determined from the data shown in Fig. 1, 4, and 5. Undetermined positions were not weighted in the SAS value calculations.

that in the genus *Corynebacterium* there was a consistent deletion of the amino acid at position 2. On the basis of the SAS values I constructed a dendrogram (Fig. 2). Representative strains of mycolateless wall type IV genera belonging to the family *Pseudonocardaceae* (*Pseudonocardia thermophila*, *Pseudonocardia saturnea*, and *Saccharomonospora viridis*) were included on the dendrogram by using data from a previous study (34). Two separate groups of wall chemotype IV actinomycetes were identified on this dendrogram; group 1 contained the genera *Corynebacterium* and *Mycobacterium*, and group 2 contained the genera *Nocardia*, *Rhodococcus*, *Gordona*, and *Tsukamurella*. Figure 2 also shows that the genera *Nocardia* and *Gordona* each consists of phylogenetically coherent species. The sequences of *Gordona bronchialis*, *Gordona rubropertinctus*, *Gordona terrae*, and *Gordona amarae* were completely homologous. Five rapidly growing species of the genus *Mycobacterium* that I examined were also found to be phylogenetically coherent. Surprisingly, five of the *Nocardia* species tested were all placed in a clade containing the genus *Rhodococcus*. Figure 3 shows the results of a comparison of the amino acid sequences of AT-L30 or L30 proteins obtained from wall chemotype IV actinomycetes and two *Streptomyces* species, as well as *Bacillus subtilis* and *E. coli* (included as typical bacteria). The genera *Nocardia* and *Rhodococcus* shared common characteristics only with the genus *Streptomyces*, as shown by the boxes in Fig. 3, whereas the genera *Corynebacterium* and *My-*

cobacterium exhibited characteristics intermediate between the characteristics of the genus *Streptomyces* and the characteristics of typical bacteria.

(ii) **Family Actinoplanaceae.** I detected high levels of sequence homology within the genera *Actinoplanes* (SAS values, 89 to 100%) and *Micromonospora* (SAS values, 83 to 100%) (Fig. 4). These genera, which are members of the family *Actinoplanaceae* (21, 43), were closely related phylogenetically to each other and, to a lesser extent, to the genera *Nocardia* and *Rhodococcus* (Fig. 2). The genera *Actinoplanes* and *Micromonospora* shared more characteristics with the genus *Streptomyces* than with the genus *Bacillus* (Fig. 4).

(iii) **Actinobacteria.** The genera *Actinomyces* and *Micrococcus* have been classified in a group called the actinobacteria (10). The three species of the genus *Actinomyces* tested (*Actinomyces viscosus*, *Actinomyces naeslundii*, and *Actinomyces pyogenes*) exhibited relatively high levels of homology in their AT-L30 amino acid sequences (SAS values, 77 to 100%), although only five N-terminal amino acids were determined in *Actinomyces pyogenes* (Fig. 5). The AT-L30 amino acid sequences of members of the genus *Actinomyces* are characterized by a number of deletions, which sharply distinguish this taxon from allied taxa (Fig. 5). The members of the genus *Actinomyces* and *Micrococcus agilis* exhibited characteristics that were intermediate between the characteristics of actinomycetes and the characteristics of typical bacteria in terms of

FIG. 2. Clustering based on AT-L30 protein SAS values. The dendrogram was drawn by using the data in Fig. 1, 4, and 5.

not only characteristic amino acids (Fig. 5) but also levels of sequence homology (Fig. 2). Another *Micrococcus* species, *Micrococcus luteus*, the type species of the genus, was also investigated, but preparing ribosomal proteins from this species was very difficult. Therefore, this organism was not included in this study. *Micrococcus agilis* and *Staphylococcus aureus*, both of which are a cocci, exhibited a low level of sequence homology (47%) (Fig. 5).

FIG. 3. Comparison of the N termini of AT-L30 proteins of mycolic acid-containing genera, typical bacteria, and members of the genus *Streptomyces*. The data for *Bacillus subtilis*, *Streptomyces lavendulae*, and *Streptomyces coelicolor* are from previous studies (33, 39). The data for *E. coli* are from reference 45.

FIG. 4. Primary structures of the N termini of AT-L30 proteins from species belonging to the genera *Actinoplanes* and *Micromonospora*, *Bacillus subtilis*, and *Streptomyces coelicolor*.

DISCUSSION

Previously (31), I created a sound system for classifying members of the family *Nocardiaceae*. The genera *Nocardia*, *Rhodococcus*, *Gordona*, and *Tsukamurella* could be clearly separated on the basis of the electrophoretic mobilities of their AT-L30 proteins. This study was an expansion of that work, in which I dealt with more taxa and used amino acid sequences of AT-L30 proteins. There was generally good agreement between the results of the AT-L30 analyses (Fig. 2) and the results of 16S rRNA sequence analyses conducted in various laboratories (7, 9, 22, 29, 48, 50, 52, 53, 59); the results of 16S rRNA sequence analyses have been summarized by Goodfellow (10) in *Bergey's Manual of Systematic Bacteriology*, vol. 4. However, a few discrepancies have also been found. The most significant difference is that in AT-L30 analyses the genera *Corynebacterium* and *Mycobacterium* were placed in a single clade on the phylogenetic tree (Fig. 2), whereas in 16S rRNA analyses the genus *Mycobacterium* was placed in a clade containing nocardioform actinomycetes together with the genera *Nocardia* and *Rhodococcus* (8, 52). On the basis of the results of 16S rRNA cataloging analyses, Fox and Stackebrandt (8) suggested that the family *Mycobacteriaceae* should include the genera *Mycobacterium*, *Nocardia*, and *Rhodococcus* and that with the genus *Corynebacterium* should be in a separate family, the *Corynebacteriaceae*. On the other hand, there is also the suggestion of Goodfellow (12) and Goodfellow and Lechevalier (14) that the mycolic acid-containing wall chemotype IV actinomycete genera *Nocardia*, *Rhodococcus*, *Gordona*, and *Tsukamurella* should be classified in the family *Nocardiaceae* Castellani and Chalmers 1919. My AT-L30 analysis results are in good agreement with the proposals of Goodfellow and Goodfellow and Lechevalier. This is not surprising since the proposal of Fox and Stackebrandt was based on 16S rRNA cataloging data rather than sequencing data. Another significant difference is that the phylogeny obtained from AT-L30 analyses did not recognize the *Corynebacterium-Mycobacterium-Nocardia* group as a distinct group among the other acti-

FIG. 5. Primary structures of the N termini of AT-L30 proteins from species belonging to the genera *Actinomyces* and *Micrococcus* and other microorganisms.

nomycetes, as demonstrated by the results of chemotaxonomic studies and 16S rRNA analyses (8, 59). In the AT-L30 analyses the family *Nocardiaceae* formed a clade together with the families *Pseudonocardiaceae* and *Actinoplanaceae*, and the *Corynebacterium-Mycobacterium* group was in a separate clade (Fig. 2). In 16S rRNA cataloging and sequence analyses the *Corynebacterium-Mycobacterium-Nocardia* group was distinct from the actinoplanetes and pseudonocardiaceae, although these two taxa were near neighbors of the *Corynebacterium-Mycobacterium-Nocardia* group (8, 59). Another less significant difference is that in AT-L30 analyses the members of the genus *Nocardia* examined were all recovered in a cluster within the clade containing the genus *Rhodococcus* (Fig. 2), but in 16S rRNA analyses the genera *Nocardia* and *Rhodococcus* occupied separate phylogenetic positions (22, 51). My results imply that among the mycolic acid-containing taxa, the genera *Nocardia* and *Rhodococcus* are very closely related. Conceivably, the marginal separation of these two taxa on the basis of the electrophoretic mobilities of their AT-L30 proteins (31) and the historical difficulty of identifying rhodococci (Table 1) are reflections of a complex phylogenetic picture.

Tsukamurella paurometabolum JCM 3226, which previously was classified in the genus *Rhodococcus*, was found to be closely related to the genus *Gordona* in both AT-L30 analyses (Fig. 2) and 16S rRNA analyses (22, 59). Despite their relatedness, the separate taxonomic status of the genera *Tsukamurella* and *Gordona* is evident from the different electrophoretic mobilities of their AT-L30 proteins (relative electrophoretic mobility of 22.0 versus 84.5), as demonstrated in a previous study (31). The separate status of the genera *Tsukamurella* and *Rhodococcus* is also evident from differences in the electrophoretic mobilities of their AT-L30 proteins (31).

It is interesting that the genera *Actinomyces* and *Micrococcus* exhibited characteristics intermediate between the characteristics of the actinomycetes and the characteristics of typical bacteria (Fig. 5). Intra- and intergeneric relationships of *Actinomyces* species were determined recently by comparing 16S rRNA sequences (47). Although the genus *Micrococcus* was previously classified in the family *Micrococcaceae*, together with the genus *Staphylococcus*, a 5S rRNA sequence analysis revealed that the genus *Micrococcus* is distantly related to the genus *Staphylococcus* but closely related to actinomycetes (6). My results agreed with the 5S rRNA analysis results.

As summarized in Table 1, the taxonomy of the mycolate-containing wall chemotype IV actinomycetes has undergone extensive revisions. *Nocardia amarae* was transferred recently to the genus *Gordona* as *Gordona amarae* by Klatte et al. (22) on the basis of both chemotaxonomic and genetic data. These authors found that the genus *Gordona* is phylogenetically coherent, in agreement with the AT-L30 data (Fig. 2). *Gordona sputi* was previously classified in the genus *Rhodococcus* (57), but Stackebrandt et al. (51) included this organism in the revived genus *Gordona* on the basis of 16S rRNA analysis data. My results agreed with this classification (Fig. 2). *Rhodococcus equi* is an important equine pathogen and was previously classified in the genus *Corynebacterium*. Although the members of the genus *Rhodococcus* generally contain longer mycolic acids than the members of the genus *Corynebacterium*, several rhodococci, including *R. equi*, have mycolic acids whose lengths overlap the lengths of the mycolic acids of corynebacteria (1, 3). The AT-L30 data support the current taxonomic status of *R. equi* (Fig. 2). *Actinomyces pyogenes*, which was previously classified in the genus *Corynebacterium* (44), is also an important veterinary pathogen. *Corynebacterium glutamicum*, which was previously classified in the genus *Micrococcus*, is an important organism in the glutamic acid fermentation industry.

My AT-L30 data provide strong support for the current taxonomic status of *Actinomyces pyogenes* and *C. glutamicum* (Fig. 2 and 5). It is especially interesting that *Corynebacterium amycolatum*, a relatively new species proposed by Collins et al. (2), differs from other coryneform bacteria by lacking mycolic acid. Despite such a significant difference, inclusion of this organism in the genus *Corynebacterium* is strongly supported not only by the high level of amino acid sequence similarity to other *Corynebacterium* species, but also by a distinctive characteristic of this genus, a deletion of the amino acid at position 2 (Fig. 1). Although the revised taxonomic positions of the organisms mentioned above were based mainly on chemotaxonomic data and 16S rRNA sequence data, there is good agreement between the AT-L30 data and the numerical phenetic analysis data; with both approaches *Rhodococcus rhodochrous*, *Rhodococcus ruber*, and *Rhodococcus coprophilus* were found to be closely related, while *Rhodococcus erythropolis*, *Rhodococcus luteus*, and *Rhodococcus rhodnii* occupied rather isolated taxonomic positions within the genus (15) (Fig. 2).

Taxonomists have argued over the species and generic concepts in bacteria. My findings concerning the heterogeneity of AT-L30 amino acid sequences in a single genus are important. Species belonging to the genera *Nocardia* and *Rhodococcus* can be difficult to identify, although several diagnostic schemes are available for this purpose (14). Thus, there still is a need for reliable and simple tests for species identification. Among the *Nocardia* species, *Nocardia brasiliensis*, *Nocardia nova*, and *Nocardia otitidiscaviarum* are well-established taxa (12). *Nocardia asteroides*, as defined by Gordon, however, has been found to be markedly heterogeneous on the basis of DNA relatedness, numerical taxonomy, phage sensitivity, and immunology data (12). *N. nova* is the new name for a subgroup of *N. asteroides* defined by numerical taxonomic data (55). It is noteworthy that each of these well-defined *Nocardia* species has an AT-L30 amino acid sequence that is typical of the species (Fig. 1 and 2). On the other hand, there is controversy concerning the epithet of *Nocardia farcinica* in relation to the epithet of *N. asteroides*, as described by Lechevalier (25) and Tsukamura (56). My AT-L30 data did not discriminate between these two species (Fig. 2). *Nocardia brevicatena*, *Nocardia carnea*, *Nocardia transvalensis*, and *Nocardia vaccinii* are species that have been studied less extensively. Subjecting these less well-defined taxa to an AT-L30 analysis may be an effective way to establish their species status. Thus, the AT-L30 sequence method appears to be useful for bacterial taxonomy not only at the generic level but also at the species level. However, identification at the species level has to be confirmed by testing more species and more than one strain per species. Also, this technique is limited for species identification as *Gordona* species cannot be differentiated (Fig. 1 and 2).

As demonstrated by comparative sequencing of various families of ribosomal proteins obtained from species belonging to the genus *Pseudomonas* or allied genera, L30 proteins (homologous to actinomycete AT-L30 proteins) have been found to exhibit the lowest levels of conservation in terms of N-terminal sequences among the ribosomal proteins examined (35). Apparently, the variability in amino acid sequences among species even in a single genus is a reflection of this characteristic of AT-L30 proteins. The low number of variable positions (usually less than 15) observed in amino acid sequence analyses may be insufficient to accurately reflect phylogenetic relationships and thus may limit this technique. However, as a counterpart to rRNA sequence analysis, this method is a useful method for investigating bacterial taxonomy, especially in polyphasic approaches, as demonstrated in this and previous papers.

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