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A Phylogenetic Analysis of the Family *Pseudonocardiaceae* and the Genera *Actinokineospora* and *Saccharothrix* with 16S rRNA Sequences and a Proposal To Combine the Genera *Amycolata* and *Pseudonocardia* in an Emended Genus *Pseudonocardia*

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The 16S rRNAs of 15 species of actinomycetes belonging to the genera *Actinokineospora* and *Saccharothrix* and the family *Pseudonocardiaceae*, including *Amycolatopsis*, *Amycolata*, *Pseudonocardia*, *Saccharomonospora*, and *Saccharopolyspora* species, were sequenced by using reverse transcriptase. The sequences were analyzed along with the sequences of reference actinomycetes by using distance matrix and parsimony methods. The wall chemotype IV genus *Actinokineospora* was found to be closely related to species of the genus *Saccharothrix* which have chemotype III walls. Together, these two genera formed a clade which was closely related to members of the family *Pseudonocardiaceae* which have chemotype IV walls. However, the phylogenetic branching pattern did not unambiguously resolve whether the members of all three taxa should be placed in a single family. We suggest, therefore, that the genera *Actinokineospora* and *Saccharothrix* should remain outside the family *Pseudonocardiaceae* until additional sequence or phenotypic data are available to decide the issue. The sequences of species belonging to the genera *Amycolata* and *Pseudonocardia* were always recovered as a mixed group in phylogenetic trees, and we propose that these organisms should be classified in an emended genus *Pseudonocardia*. This proposal is strongly supported by previously published lipid, ribosomal protein, and ultrastructure data.

The family *Pseudonocardiaceae* was proposed on the basis of the results of a 16S rRNA sequence analysis and currently contains the genera *Actinopolyspora*, *Amycolata*, *Amycolatopsis*, *Kibdelosporangium*, *Pseudonocardia*, *Saccharopolyspora*, and *Saccharomonospora* (1, 12, 13). Representatives of this family vary in morphology and in other phenotypic characteristics, but all of the current members have chemotype IV walls (43) (i.e., they contain *meso*-diaminopimelic acid in their peptidoglycan, and arabinose and galactose are diagnostic sugars in whole-cell hydrolysates [10, 32]). Recently, a member of a new wall chemotype IV genus, *Actinokineospora*, was isolated from a Japanese soil sample (25). This organism is interesting because it produces motile zoospores from an aerial mycelium, a characteristic shared by relatively few actinomycetes (41) and by none of the currently recognized members of the family *Pseudonocardiaceae* (10).

Members of the genus *Saccharothrix* contain *meso*-diaminopimelic acid in their peptidoglycan and galactose, but not arabinose, among their whole-cell sugars (37). This combination of characteristics is referred to as wall chemotype III (43) and is also found in members of the genera *Nocardiopsis*, *Actinosynnema*, and *Streptoalloteichus* (41). The genus *Saccharothrix* was originally thought to be most closely related to the genus *Nocardiopsis* on the basis of morphology (37). Chemotaxonomic studies have subsequently revealed that the genus *Saccharothrix* and *Nocardiopsis dassonvillei* (the type species of the genus *Nocardiopsis*) are not related, but that some *Nocardiopsis* strains should be transferred to the genus *Saccharothrix* (24, 36). Furthermore, the type species of the genus *Saccharothrix*, *Saccharothrix australiensis*, has been shown to be related to members of the family *Pseudonocardiaceae* on the basis of its 16S rRNA sequence (1). *Saccharothrix* strains have lipid compositions (1, 4, 24, 37) similar to those of certain members of this family (11, 53), so there is some support for the hypothesis that there is a close relationship. However, it was deemed premature by Bowen et al. (1) to transfer the genus *Saccharothrix* to the family *Pseudonocardiaceae*. The main reason for this was the difference in the wall chemotypes of the two taxa; wall chemotypes have a history of being reliable indicators of higher relationships among actinomycetes (41). It was also felt that the relationship might prove to be unstable. Previously, the position of *Amycolata autotrophica*, which was thought to be a member of the family *Pseudonocardiaceae* on the basis of chemotaxonomic evidence (12, 13), had changed depending on which sequences were analyzed. The membership of this organism in the family *Pseudonocardiaceae* was confirmed only when additional closely related sequences were included in the analysis (1).

In this study we determined 16S rRNA sequences of a single strain of *Actinokineospora riparia* and of four *Saccharothrix* species in order to clarify the relationships of these taxa to the family *Pseudonocardiaceae* and to other actinomycetes. Previously, only one or two 16S rRNA sequences have been determined for most of the genera in the *Pseudonocardiaceae*, so we also examined additional *Amycolata*, *Amycolatopsis*, *Pseudonocardia*, *Saccharopolyspora*, and *Saccharomonospora* species.

MATERIALS AND METHODS

Bacterial strains. The strains whose sequences were determined in this study were *Actinokineospora riparia* IFO 14541^T (T. Hasegawa, Institute for Fermentation, Osaka, Japan) (T =

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type strain), *Amycolata alni* VKM AC901^T (Department of Type Cultures, Institute of Microbiology, Moscow, Russia), *Amycolata saturnea* DSM 43195^T (Deutsche Sammlung von Mikroorganismen), *Amycolata hydrocarbonoxydans* DSM 43281^T, *Amycolatopsis orientalis* subsp. *orientalis* DSM 40040^T, *Amycolatopsis mediterranei* ATCC 13685^T (American Type Culture Collection, Rockville, Md.), *Pseudonocardia compacta* DSM 43592^T, *Saccharomonospora caesia* INMI 19125^T (Institute of Microbiology, Moscow, Russia), *Saccharomonospora* sp. strain A1206 (J. Lacey, Rothamsted Experimental Station, Harpendon, United Kingdom), *Saccharopolyspora gregorii* A85^T (J. Lacey), *Saccharopolyspora* sp. strain A215 (J. Lacey), *Saccharothrix coeruleofusca* DSM 43679^T, *Saccharothrix longispora* DSM 43749^T, *Saccharothrix mutabilis* subsp. *mutabilis* DSM 43853^T, and *Saccharothrix mutabilis* subsp. *capreolus* DSM 40225^T. For most strains biomass was prepared in shake flasks containing tryptone soy broth and incubated at 30°C for 30 to 72 h. *Saccharomonospora* sp. strain A1206 was grown in tryptone soy broth at 45°C for 30 h. The cells were harvested by centrifugation, washed once with cold TES buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA; pH 7.2), and stored at −20°C.

RNA extraction and sequencing. rRNA was extracted from approximately 2 g of biomass by using acid-phenol (pH 5.5) (12, 58) and was sequenced by using reverse transcriptase and previously described procedures (8, 12, 19, 39). Sequencing primers 704 (5′-TCTGCGCATTTCACCGCTAC) and 926 (5′-CCGTCAATTCCTTTGAGTTT) were slightly modified to hybridize more efficiently with actinomycete rRNA. For some strains it was difficult to obtain clear sequences from rRNA templates (typically in sequences initiated from primers 536 and 926). In these cases the sequences of the corresponding regions were confirmed by directly sequencing PCR-amplified rRNA genes (9).

Data analysis. The sequences which we determined were deposited in the EMBL data base and were aligned manually with reference sequences of other actinomycetes by using secondary structure as a guide. Two alignments were used. The first alignment comprised 981 bases of sequence from 73 actinomycetes representing 38 genera (1, 5, 6, 12, 13, 40, 44a, 48, 54, 55); in this alignment *Atopobium minutum* was used as an outgroup taxon (40). This alignment included the positions which were available for all of the taxa, and it excluded some of the more variable regions which cannot be aligned with confidence for distantly related organisms. The stretches of sequence used corresponded to positions 225 to 449, 494 to 833, 853 to 999, 1044 to 1124, and 1160 to 1352 (*Escherichia coli* 16S rRNA sequence numbering system [2]). The aim of this analysis was to determine the positions of *Saccharothrix* spp. and *Actinokineospora riparia* relative to members of the family *Pseudonocardiaceae*. Pairwise similarity values were calculated and converted to distances by using the model of Jukes and Cantor (33) in the DNADIST program (in PHYLIP, version 3.5c [16]). A tree was produced by using the neighbor joining method (51) in the NEIGHBOR program (PHYLIP, version 3.5c). Bootstrap proportions (BPs) (based on the data for 100 replicates [15, 17, 30]) for groups in a 50%-majority-rule consensus tree were calculated by using the SEQBOOT, NEIGHBOR, and CONSENSE programs (PHYLIP, version 3.5c).

The second alignment comprised 1,157 bases of sequence (333 variable positions) from *Actinokineospora riparia*, members of the family *Pseudonocardiaceae*, and *Saccharothrix* species. The aim of this analysis was to investigate the species level relationships among these taxa. This alignment contained some of the previously deleted variable positions which could be aligned with more confidence for these closely related taxa. The alignment comprised continuous stretches of sequence from positions 192 to 459 and 479 to 1373 (2). Distance matrix analyses were performed as described above. Parsimony analyses were performed by using the heuristic search option (PAUP, version 3.1.1) (56). The sites used for parsimony analyses were limited to informative sites, of which there were 174, including insertions and deletions, as a fifth state.

Nucleotide sequence accession numbers. The sequences determined in this study were deposited in the EMBL data base under accession numbers X76953 to X76967.

RESULTS

Relationships among the family *Pseudonocardiaceae*, the genera *Actinokineospora* and *Saccharothrix*, and other actinomycetes. Figure 1 shows a part of the distance tree obtained from the first analysis (981 bases, 73 actinomycetes), which was performed to determine the relative positions of the genus *Saccharothrix*, *Actinokineospora riparia*, and members of the family *Pseudonocardiaceae*. BPs were calculated in order to estimate the levels of support for particular groups from the sequence data analyzed (15, 30), and some of these BPs are shown at relevant nodes of the tree in Fig. 1. The strains shown in Fig. 1 formed a group that was distinct from the other actinomycetes analyzed (the complete tree is not shown) in 71 of 100 trees. The other wall chemotype III taxa included in the analysis (but not shown in Fig. 1), *Nocardiopsis dassonvillei*, *Actinomadura madurae*, and *Microtetraspora* spp., formed a clade (BP 79) which was clearly separated from the genus *Saccharothrix*.

The following three clades that have moderate to strong support can be recognized in Fig. 1: (i) group a, the "Actinoplanetes" group (20, 46), comprising in this analysis *Micromonospora chalcea*, *Ampullariella regularis*, *Dactylosporangium thailandense*, and *Dactylosporangium aurantiacus* (BP 100) (44a); (ii) group b, the mycolic acid-containing wall chemotype IV taxa (BP 74); and (iii) group c, a group comprising the *Saccharothrix* strains, *Actinokineospora riparia*, and members of the family *Pseudonocardiaceae* (BP 74). In a small number of bootstrap replicates (BP 10) the genera *Actinokineospora* and *Saccharothrix* formed an association with the mycolic acid-containing actinomycetes. The branching order for groups a, b, and c was unstable and varied with the choice of outgroup taxa and the method of analysis.

A close relationship between species of the wall chemotype III genus *Saccharothrix* and the wall chemotype IV species *Actinokineospora riparia* was observed in most trees (BP 78). However, no strong sister group relationships were detected between individual genera in the family *Pseudonocardiaceae*. With one exception, species belonging to the different genera were observed together in the consensus tree, but their levels of bootstrap support were generally low (Fig. 1). This was caused by the movement, in some trees from bootstrap replicates, of deeply branching strains from one genus to another. Species belonging to the genera *Amycolata* and *Pseudonocardia* were always mixed together.

Relationships between species in the family *Pseudonocardiaceae* and the genera *Actinokineospora* and *Saccharothrix*. Figure 2 is an unrooted tree showing the branching patterns for species belonging to the *Pseudonocardiaceae*, *Actinokineospora riparia*, and *Saccharothrix* species. The topology in Fig. 2 was produced by analyzing longer stretches of sequence, including some of the most variable regions of 16S rRNA. The larger number of sequence positions compared did not improve the resolution of sister group relationships for genera belonging to the family *Pseudonocardiaceae*. The distances between nodes

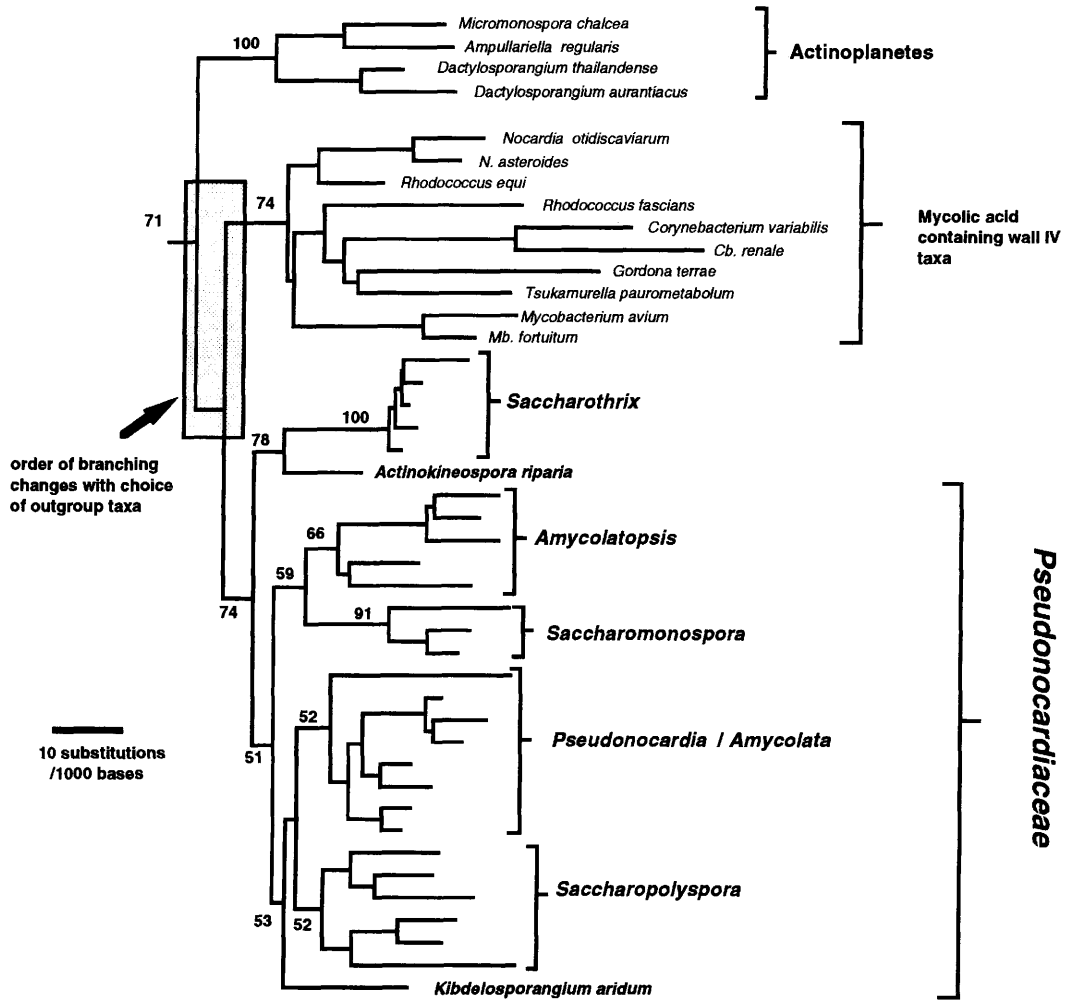

FIG. 1. Distance tree produced by using the neighbor-joining method (51) and showing the relationships among the genera *Saccharothrix* and *Actinokineospora*, members of the family *Pseudonocardiaceae*, and reference actinomycetes. The numbers at nodes indicate the levels of bootstrap support (based on the data for 100 replicates) for the groups to the right of the nodes. Bar = estimated (33) 10 substitutions per 1,000 bases. For the sources of the reference sequences used see the text.

separating the genera were generally short (Fig. 2), and different topologies were obtained in different analyses. The use of additional variable positions did improve bootstrap support for the genera *Saccharopolyspora* (BP 98) and *Saccharomonospora* (BP 100). A coherent genus *Saccharothrix* was also strongly supported (BP 100) by the rRNA sequence data used in this analysis. The two subspecies *Saccharothrix mutabilis* subsp. *capreolus* and *Saccharothrix mutabilis* subsp. *mutabilis* were separated by only a short distance, thus confirming that these organisms are very closely related.

The *Pseudonocardia* and *Amycolata* strains were again intermixed and formed a clade with strong support (BP 98). The position of *Pseudonocardia thermophila* (the type species of the genus *Pseudonocardia*) was affected by the method of analysis, but this taxon always appeared near the base of the group. *Pseudonocardia compacta* clustered with *Amycolata autotrophica* (the type species of the genus *Amycolata*) and "*Streptomyces nitrificans*" in most trees (BP 99). A close relationship between *Amycolata hydrocarbonoxydans* and "*Nocardia petroleophila*" was also strongly supported (BP 100). The position of *Kibdelosporangium aridum* was unstable, but this taxon was most commonly located near the genera *Pseudonocardia* and *Amycolata*.

There was only a low level of support for the existence of a coherent genus *Amycolatopsis* on the basis of the data for the sequence positions included in the second analysis. *Amycolatopsis fastidiosa* and *Amycolatopsis methanolica* seldom (BP 28) grouped with other three *Amycolatopsis* species (which formed a clade in all analyses), including the type species of the genus, *Amycolatopsis orientalis*. However, experiments in which PAUP, version 3.1.1, was used to constrain all five *Amycolatopsis* spp. to a clade produced an optimal solution which was only two steps longer (647 to 649 steps) than the trees obtained in an unconstrained analysis.

DISCUSSION

One of the main aims of this study was to clarify the relationship of the genus *Saccharothrix* to the family *Pseudonocardiaceae* and to determine whether this genus should be transferred to the family *Pseudonocardiaceae*. The results of previous analyses of fewer strains had suggested that this might

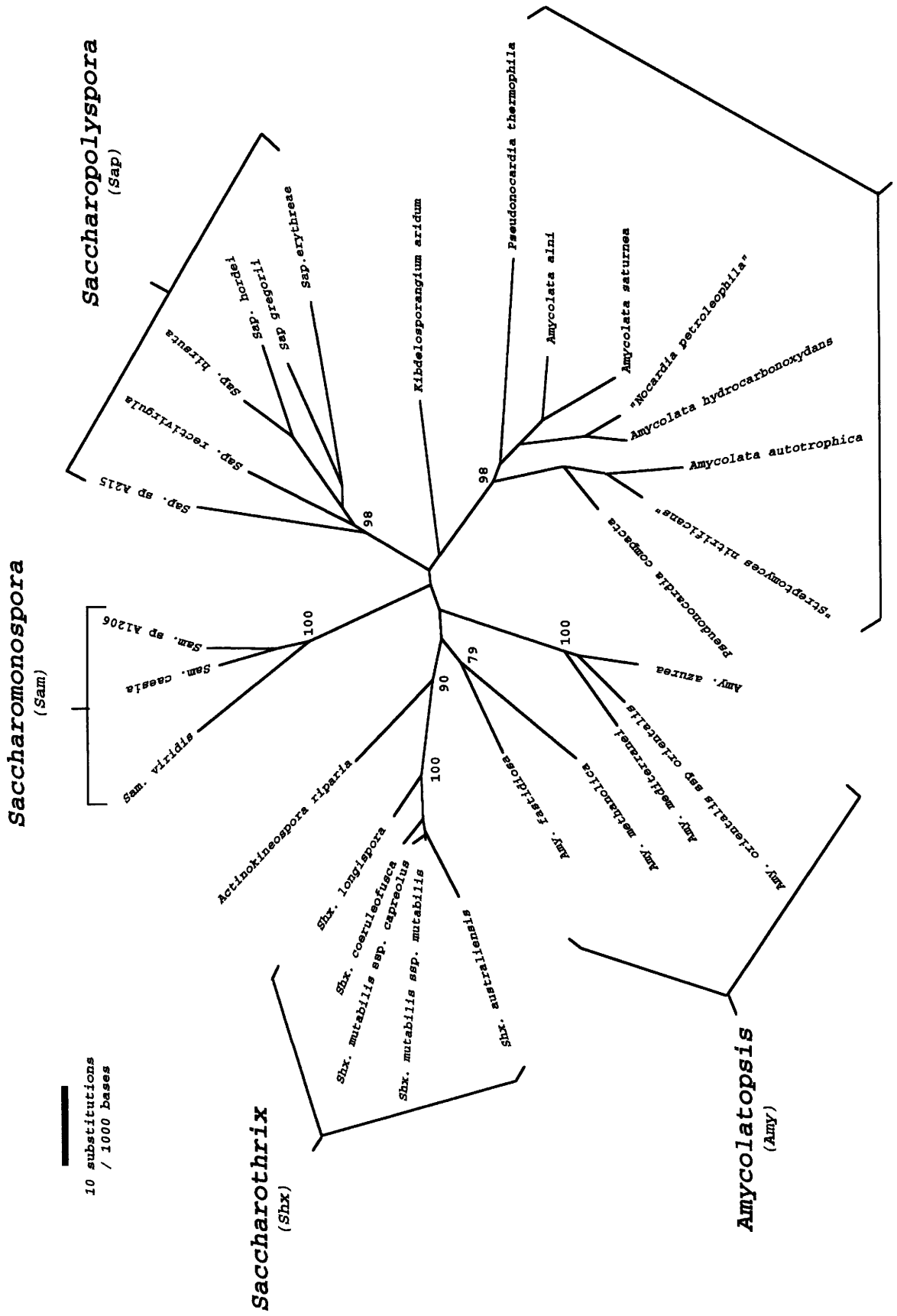

FIG. 2. Unrooted distance tree showing the relationships among members of the genera *Saccharothrix* and *Actinokineospora* and members of the family *Pseudonocardiaceae*. Bar = 10 substitutions per 1,000 bases. BPs based on the data for 100 replicates are indicated at the relevant nodes.

eventually be necessary (1). Our results confirmed that the two taxa are very closely related to each other. The sequence data also demonstrated that the recently described wall chemotype IV genus *Actinokineospora* (25) is more closely related to the genus *Saccharothrix* than to members of the family *Pseudonocardiaceae*. In most analyses the genera *Actinokineospora* and *Saccharothrix* formed a clade which was a sister group of the *Pseudonocardiaceae* (as shown in Fig. 1). The nearest neighbor of these three taxa could not be determined from the sequences compared. The nearest neighbor varied from the mycolic acid-containing wall chemotype IV taxa (the genera *Mycobacterium*, *Corynebacterium*, *Nocardia*, and *Rhodococcus* and their relatives) to the wall chemotype II Actinoplanetes group, which included *Dactylosporangium*, *Micromonospora*, and *Ampullariella* strains in this analysis. Wall chemotype II strains are defined as organisms that contain *meso*-diaminopimelic acid and glycine in their peptidoglycan and arabinose and xylose as whole-cell sugars (43). A relationship between the Actinoplanetes group and the genera *Mycobacterium*, *Rhodococcus*, and *Nocardia* was observed previously when 16S rRNA oligonucleotide catalogs were compared (18). The results of our analyses based on additional sequence information confirmed and extended this observation and showed that the organisms in Fig. 1 form a coherent phylogenetic group.

Previously published data for chemotaxonomic properties also support the hypothesis that there is a close relationship between the genera *Actinokineospora* and *Saccharothrix* and the family *Pseudonocardiaceae*. The members of all of these taxa contain similar fatty acids and menaquinones (1, 3, 4, 11, 24, 25, 35, 44), and the polar lipid patterns found in the genera *Actinokineospora* (25) and *Saccharothrix* (24, 37) are similar to the patterns found in the genera *Amycolatopsis* (44), *Kibdelosporangium* (53), and *Saccharomonospora* (11). As mentioned above, the only taxonomically significant difference between the genus *Saccharothrix* and all of these taxa is the absence of arabinose in whole-cell hydrolysates of members of the genus *Saccharothrix* (1, 24, 37). However, detailed investigations of members of the genus *Saccharothrix* (57) have recently shown that some strains may actually contain arabinose in whole-cell hydrolysates. It has also been demonstrated that the location and amount of arabinose may vary significantly among members of the family *Pseudonocardiaceae* (10, 32, 53, 57). It therefore appears that the distinction between these particular wall chemotype IV actinomycetes and wall chemotype III actinomycetes may not be as taxonomically significant as once thought (1, 10).

The question remains whether the close relationship between the genera *Actinokineospora* and *Saccharothrix* and the family *Pseudonocardiaceae* should be recognized by transferring these genera to the family. Many members of these taxa produce bioactive compounds (10), and it should be useful to industry, and for understanding the evolution of biosynthetic pathways, to clearly indicate that these organisms share a "recent" common ancestry. Balanced against this is the need for stability; i.e., is the enlarged family likely to split at some later date when more sequences are available for analysis? In our opinion the phylogenetic branching pattern itself does not unequivocally support transfer. In a small number of bootstrap samples, the genera *Actinokineospora* and *Saccharothrix* were more closely related to the mycolic acid-containing wall chemotype IV taxa than to members of the family *Pseudonocardiaceae*. Taxa which have combinations of taxonomic characteristics which suggest that they may be related to the genera *Saccharothrix* or *Actinokineospora* include the genera *Actinosynnema* and *Streptoalloteichus* (41). Moreover, very few of the closely related mycolic acid-containing wall chemotype IV taxa or Actinoplanetes have had their 16S rRNAs sequenced, and much of the genetic diversity of these groups may remain to be sampled. Since it is now a simple matter to amplify and sequence 16S rRNA genes of actinomycetes, it seems only a matter of time before many more sequences are available for analysis. In the interest of nomenclatural stability, we suggest that it is worth waiting to see whether such additional sequences affect the association between the genera *Actinokineospora* and *Saccharothrix* and the family *Pseudonocardiaceae* before making any formal taxonomic proposals. Having said this, we also believe that it is worth mentioning that if new sequences do serve to separate these taxa, it may still not be easy to find characteristics by which the taxa can be easily differentiated at the family level.

Figure 2 shows the detailed interrelationships among *Actinokineospora* and *Saccharothrix* species and members of the different genera in the family *Pseudonocardiaceae*. There was strong support for the integrity of the genera *Saccharopolyspora*, *Saccharomonospora*, and *Saccharothrix*, but weak support from 16S rRNA sequences for the genus *Amycolatopsis*. The relationship among *Amycolatopsis orientalis* (the type species of the genus *Amycolatopsis*), *Amycolatopsis mediterranei*, and *Amycolatopsis azurea* was strongly supported by the results of both parsimony and distance matrix analyses. However, *Amycolatopsis fastidiosa* and *Amycolatopsis methanolica* were separated by long branches from these three species and in some analyses (Fig. 2) were recovered as separate lineages. All five *Amycolatopsis* species exhibit many similarities in phenotype and chemical profile characteristics (7, 28, 44), and there is no clear indication that *Amycolatopsis fastidiosa* and *Amycolatopsis methanolica* are misclassified. Furthermore, in this study we found that only slightly less parsimonious trees were recovered when all five species were constrained to form a clade. It may be that additional sequences may stabilize the positions of *Amycolatopsis fastidiosa* and *Amycolatopsis methanolica* and thus clarify the relationships of these taxa to other members of the genus.

The 16S rRNA sequence data suggest that *Saccharomonospora* sp. strain A1206 and *Saccharopolyspora* sp. strain A215 are candidates for new species in their respective genera. Both strains were isolated from stored grains by Lacey (21, 38). Over the past few years there have been a number of additions to the genera *Saccharomonospora* and *Saccharopolyspora*, including *Saccharomonospora azurea* (49), *Saccharomonospora glauca* (22), *Saccharomonospora cyanea* (50), and *Saccharopolyspora spinosa* (45). These organisms were not included in our analysis, and we suggest that before *Saccharomonospora* sp. strain A1206 and *Saccharopolyspora* sp. strain A215 are classified as new species, they should be compared with these taxa.

One of the most interesting results of this study is that sequences of species belonging to the genera *Pseudonocardia* and *Amycolata* were always mixed together. Thus, there is strong evidence from the 16S rRNA sequence data that these taxa should be combined in a redefined genus, *Pseudonocardia*, since this genus name has nomenclatural priority over the genus name *Amycolata* (26, 27, 29, 44). The genera *Pseudonocardia* and *Amycolata* are the only members of the family *Pseudonocardiaceae* which contain MK-8(H_4) as the major menaquinone (11, 44), and the members of both taxa contain phosphatidycholine (11, 42, 44). Representatives of the genera *Amycolata* and *Pseudonocardia* produce ribosomal AT-L30 proteins which have very similar electrophoretic mobilities (47). The members of both genera also produce a unique (within the family *Pseudonocardiaceae*) electron-dense outer layer to the substrate and aerial mycelia, and there are similarities in spore shape and dehiscence (34). On the basis of

16S rRNA sequence data (1), it is also apparent that "*Nocardia petroleophila*" IFAM 78 (31) and "*Streptomyces nitrificans*" IFAM 379 (52) should be transferred to the genus *Pseudonocardia.*

On the basis of the results of this investigation and previously published data (1, 11, 23, 26, 27, 29, 34, 43, 44, 47), we propose that the genus name *Amycolata* (44) should be recognized as a junior synonym of the genus name *Pseudonocardia* (26).

Emended description of the genus *Pseudonocardia.* The vegetative mycelium varies in thickness (0.4 to 2.0 μm) and in the degree of branching. Aerial mycelium may or may not be present. Both types of mycelium exhibit cell division in different directions, and in some species the mycelium is covered by an electron-dense outer layer. Spore sizes vary, and spores are normally smooth; chains of spores are formed by acropetal budding or septation from the substrate or aerial mycelium.

Nonmotile. Biochemically versatile. Some species are facultative autotrophs, and some strains can oxidize hydrocarbons. *Pseudonocardia thermophila* can degrade cellulose.

The major menaquinone is tetrahydrogenated with eight isoprene units. Phosphatidylethanolamine and phosphatidylcholine are the major phospholipids. Arabinose and galactose are found in whole-cell hydrolysates. The guanine-plus-cytosine content of the DNA ranges from 68 to 79 mol%. The members of this genus form a coherent group on the basis of 16S rRNA sequence data. The type species is *Pseudonocardia thermophila*; the type strain of this species is strain ATCC 19285.

The following species should be transferred to the genus *Pseudonocardia*: *Amycolata autotrophica*, *Amycolata alni*, *Amycolata saturnea*, *Amycolata hydrocarbonoxydans*, "*Streptomyces nitrificans*," and "*Nocardia petroleophila*." The descriptions of these species have been published previously (14, 31, 44, 52).

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