Phylogenetic analysis of *Saccharothrix* and related taxa: proposal for *Actinosynnemataceae* fam. nov.

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Partial sequences for 16S rDNA were determined for strains of the genus *Saccharothrix*, including most described species, as well as strains of the described species of the related genera *Kutzneria*, *Actinokineospora* and *Actinosynnema*. These were aligned with published sequences for other species of these genera, as well as those of the genera '*Actinoalloteichus*', '*Asiosporangium*', *Lentzea*, *Kutzneria*, *Streptoalloteichus* and representative taxa from other actinomycete families. Phylogenetic analysis of the sequence data showed that species of the genera *Actinokineospora*, *Actinosynnema*, *Lentzea* and *Saccharothrix* are members of the same clade, and distinct from the *Pseudonocardiaceae*. It is proposed that a new family be created within the class *Actinobacteria* for these genera, to be called the *Actinosynnemataceae* on the basis of the oldest described genus within this family, *Actinosynnema* Hasegawa *et al.* The chemotaxonomic properties of all the genera to be placed within the new family have similar cell wall type (type III), whole-cell sugars (generally galactose although mannose and rhamnose may be present), phospholipid type (PII) and menaquinones [MK-9(H₄) predominant].

Keywords: *Saccharothrix*, actinomycetes, *Actinosynnemataceae* fam. nov.

INTRODUCTION

The genus *Saccharothrix* was described (Labeda *et al.*, 1984) to accommodate actinomycete strains that morphologically resemble the genus *Nocardopsis*, but that can be chemotaxonomically differentiated from members of this genus based on type III cell wall chemistry (*meso*-diaminopimelic acid, *meso*-DAP), rhamnose and galactose as diagnostic whole-cell sugars, a type PII phospholipid pattern (phosphatidylethanolamine as key diagnostic phospholipid), and presence of MK-9 (H₄) as the predominant menaquinone. The actual phylogenetic position of this genus has not been certain, although it was suggested by Warwick *et al.* (1994), on the basis of a phylogenetic study of 16S rRNA sequences, that this genus should be placed phylogenetically with the *Pseudonocardiaceae*. In the recent proposal for the hierarchic classification of actinomycete taxa in the new class *Actinobacteria*

(Stackebrandt *et al.*, 1997), members of the genus *Saccharothrix* are again loosely grouped with the *Pseudonocardiaceae*. Yassin *et al.* (1995) noted the close phylogenetic relationship of their new genus *Lentzea* to members of the genera *Actinosynnema*, *Kutzneria* and *Saccharothrix*. In order to clarify the phylogenetic position of this genus, as well as to study its phylogenetic continuity, 16S rDNA sequence data were determined for representative type strains of species of *Saccharothrix* and allied genera selected from the ARS Culture Collection.

METHODS

Strains, cultivation and maintenance. All strains included in this study are held in the ARS Culture Collection (NRRL), National Center for Agricultural Utilization Research, Peoria, IL, USA. The strains selected for this are shown in Table 1. Primary storage of strains is as lyophilized ampules of mycelial and spore suspensions in sterile beef serum held at 4 °C. Working stock cultures were maintained on slants of ATCC Medium No. 172 (Cote *et al.*, 1984) and stored at 4 °C until needed. Biomass for extraction of DNA was grown as 7 d streak cultures on ATCC Medium 172 agar plates.

Abbreviation: DAP, diaminopimelic acid.

The GenBank accession numbers for the 16S rDNA sequences determined in this paper are AF114797–AF114815.

Table 1. Strains for which 16S rDNA sequence data were determined in this study

Strain	GenBank no.
<i>Actinokineospora diospyrosa</i> NRRL B-24047 ^T (= IFO 15665 ^T)	AF114797
<i>Actinokineospora globicatena</i> NRRL B-24048 ^T (= IFO 15664 ^T)	AF114798
<i>Actinokineospora inagensis</i> NRRL B-24050 ^T (= IFO 15663 ^T)	AF114799
<i>Actinokineospora riparia</i> NRRL B-16432 ^T	AF114802
<i>Actinosynnema pretiosum</i> subsp. <i>pretiosum</i> NRRL B-16060 ^T	AF114800
<i>Kutzneria kofuensis</i> NRRL B-24061 ^T (= JCM 3157 ^T)	AF114801
<i>Saccharothrix aerocolonigenes</i> NRRL B-3298 ^T	AF114804
<i>Saccharothrix australiensis</i> NRRL 11239 ^T	AF114803
<i>Saccharothrix coeruleofusca</i> NRRL B-16115 ^T (= ATCC 35108 ^T = DSM 43679 ^T)	AF114805
<i>Saccharothrix cryophilis</i> NRRL B-16238 ^T	AF114806
<i>Saccharothrix espanaensis</i> NRRL 15764 ^T	AF114807
<i>Saccharothrix flava</i> NRRL B-16131 ^T (= ATCC 29533 ^T = DSM 43885 ^T)	AF114808
<i>Saccharothrix longispora</i> NRRL B-16116 ^T (= ATCC 35109 ^T = DSM 43749 ^T)	AF114809
<i>Saccharothrix syringae</i> NRRL B-16468 ^T (= DSM 43886 ^T)	AF114812
<i>Saccharothrix texasensis</i> NRRL B-16107	AF114815
<i>Saccharothrix texasensis</i> NRRL B-16134 ^T	AF114814
<i>Saccharothrix waywayandensis</i> NRRL B-16159 ^T	AF114813
<i>Saccharothrix</i> species NRRL B-16108	AF114810
<i>Saccharothrix</i> species NRRL B-16133	AF114811

Chemotaxonomic analysis. The chemotaxonomic profile of *Lentzea albidocapillata* was determined using previously described methods (Grund & Kroppenstedt, 1989) for menaquinones, fatty acids and whole-cell sugars.

DNA extraction. Genomic DNA was isolated and purified by a modification of the procedure of Rainey *et al.* (1996). Cells scraped from solid growth media (1–2 loopfuls) were placed in a 1.5 ml microfuge tube with a small amount of 0.1 mm zirconia/silica beads (Biospec Products) and were ground with a conical disposable pestle (Kontes Glass Company). The homogenate was suspended in 400 µl saline-EDTA buffer (150 mM NaCl, 10 mM EDTA; pH 8.0), 10 µl 1% (w/v) proteinase K and 10 µl 25% (w/v) SDS was added, mixed and incubated at 55–60 °C for 10 min. The resulting preparation was extracted sequentially with an equal volume of phenol, and an equal volume of chloroform. DNA was recovered from the aqueous phase by using a Prep-A-Gene kit (Bio-Rad). The purified DNA was eluted from the binding matrix in 30 µl sterile distilled water.

Amplification and direct sequencing of the 16S rRNA gene. The 16S rDNA was amplified by the PCR according to the method of Rainey *et al.* (1996) using the primers 27f (GAGTTTGATCCTGGCTCAG) and 1525r (AGAAA-GGAGGTGATCCAGCC). PCR products were purified and concentrated using a Gene-Clean II kit (Bio 101) and were eluted into 30 µl sterile distilled water. The sequencing reactions were performed with a PRISM Ready-Reaction DyeDeoxy terminator cycle sequencing kit by using AmpliTaq FS (Applied Biosystems) and a Perkin-Elmer Cetus model 2400 thermal cycler according to the protocol and thermal profile recommended by Applied Biosystems. The sequencing primers used were: 343r (CTGCTGCCTCCC-GTA), 357f (TACGGGAGGCAGCAG), 519r [G(T/A)-ATTACCGCGGC(T/G)GCTG], 536f [CAGC(C/A)GCC-GCGGTAAT(T/A)C], 803f (ATTAGATACCCTGGTA-G), 907r (CCGTCAATTCATTTGAGTTT), 1114f (GCA-ACGAGCGCAACCC) and 1385r [CGGTGTGT(A/G)-

CAAGGCCCC]. Sequencing reaction mixtures were purified as recommended by Applied Biosystems and were electrophoresed on a 6% (w/v) polyacrylamide sequencing gel for 9 h using either a 373A or 377 model automated DNA sequencer (Applied Biosystems).

Phylogenetic analysis. The 16S rDNA sequences obtained in this study were manually aligned with actinomycete reference sequences obtained from the Ribosomal Database Project (Maidak *et al.*, 1994) and GenBank. Many of the reference sequences obtained from the databases were partial sequences and thus the data set used for analysis consisted of the sequences generated in this study and actinomycete reference sequences contained information for 1384 nucleotide positions. The programs contained in the PHYLIP package of Felsenstein (1993) were used to calculate evolutionary distances by the method of Kimura (1980), and linkages by the neighbour-joining method of Saitou & Nei (1987). The topographies of the trees resulting from these analyses were evaluated by bootstrap analysis of the data with 100 resamplings. The sequences were also subjected to likelihood analysis using fastDNAmI (Olsen *et al.*, 1994) and PAUP 3.1 (Swofford, 1993) was used for parsimony analysis. Evolutionary trees were displayed and printed using version 1.5 of the TREEVIEW program of Page (1996).

Nucleotide sequence accession numbers. The 16S rDNA sequences determined in this study have been deposited in the GenBank database under the accession numbers shown in Table 1. The accession numbers for the sequences of the strains used as representatives of the main actinomycete groups are as follows: '*Actinoballoteichus cyanogriseus*' IFO 14455, AB006178; *Actinomadura kijaniata* DSM 43764^T (= type strain), X97890; *Actinomadura madurae* DSM 43067^T, X97889; *Actinoplanes philippinensis* DSM 43019^T, X93187; *Actinosynnema mirum* DSM 43827^T, X84447; *Amycolatopsis mediterranei* ATCC 13685^T (= DSM 43304^T = IFO 13415^T = IMET 7651^T), X76957; *Amycolatopsis methanolica* NCIB 11946^T, X54274; *Amycolatopsis orientalis*

DSM 40040^T (= NCIMB 9378^T), X76958; *Arthrobacter globiformis* DSM 20124^T (= ATCC 8010^T = NCIMB 8907^T), M23411; '*Asiosporangium albidum*' IFO 16102, AB006176; *Atopobium minutum* NCFB 2751^T (= DSM 20586^T = ATCC 33267^T = VPI 9428^T), X67148; *Bifidobacterium bifidum* ATCC 29521^T (= DSM 20456^T), M38018; *Dactylosporangium aurantiacum* DSM 43157^T, X93191; *Kibdelosporangium aridum* subsp. *aridum* ATCC 39323^T, X53191; *Kutzneria viridogrisea* DSM 43850^T (= ATCC 25242^T), X70429; *Lentzea albidocapillata* DSM 44073^T, X84321; *Microbacterium lacticum* IFO 14135^T (= NCIMB 8540^T = ATCC 8180^T = DSM 20427^T = NCFB 747^T), D21343; *Microtholus phosphovorius*, JCM 9379^T, D26169; *Micromonospora chalicea* DSM 43026^T, X92594; *Microtetrastroma glauca* DSM 43311^T, X97891; *Nonomuria salmonae* DSM 43678^T, X97892; *Mycobacterium tuberculosis*, NCTC 7416^T, X58890; *Nocardia asteroides* ATCC 19247^T, Z36934; *Nocardioideus albus* DSM 43109^T (= ATCC 27980), X53211; *Nocardioopsis dassonvillei* DSM 43111^T, X97886; *Propionibacterium freudenreichii* subsp. *freudenreichii* DSM 20271^T (= ATCC 6207^T = NCTC 10470^T = NCIB 5959^T), X53217; *Pseudonocardia alni* VKM AC901^T, X76954; *Pseudonocardia halophobica* DSM 43089^T, Z14111; *Pseudonocardia petroleophila* ATCC 15777^T (= DSM 43193^T), X80596; *Saccharomonospora azurea* IFO 14651^T, Z38017; '*Saccharomonospora caesia*' INMI 19125^T, X76960; *Saccharomonospora cyanea* IFO 14841^T, Z38018; *Saccharomonospora glauca* DSM 43769^T, Z38003; *Saccharomonospora viridis* Goodfellow SB-33, Z38005; *Saccharopolyspora gregorii* NCIMB 12823^T, X76962; *Saccharopolyspora hirsuta* ATCC 27875^T (= NCIB 11079^T), M20388; *Saccharothrix mutabilis* subsp. *capreolus* DSM 40225^T, X76965; *Saccharothrix mutabilis* subsp. *mutabilis* DSM 43853^T, X76966; *Sporichthya polymorpha* DSM 46113, X72377; *Streptoalloteichus hindustanus* IFO 15115^T, D85497; *Streptomyces ambifaciens* ATCC 23877^T, M27245; *Streptomyces griseus* KCTC 9080^T, X61478; *Streptosporangium longisporum* DSM 43180^T, X89944; *Streptosporangium roseum* DSM 43021^T, X89947; *Thermocrisum agreste* DSM 44070^T, X79183; *Thermocrisum municipale* DSM 44069^T, X79184; *Thermomonospora curvata* DSM 43183^T, X97893.

RESULTS

Phylogenetic analyses

Almost complete 16S rDNA sequences were determined for type strains of the genera *Actinokineospora*, *Kutzneria* and *Saccharothrix* for which these data had not been previously reported. In addition, similar data were determined for *Saccharothrix australiensis* NRRL 11239^T, *Saccharothrix coeruleofusca* NRRL B-16115^T and *Saccharothrix longispora* NRRL B-16116^T, for which sequence information had been previously reported (Warwick *et al.*, 1994) as well as *Saccharothrix* species NRRL B-16108 and NRRL B-16133.

The phylogenetic dendrogram shown in Fig. 1 was derived from evolutionary distances by the neighbour-joining method. A total of 1384 nucleotides between positions 99 and 1440 (*Escherichia coli* numbering; Brosius *et al.*, 1978) were used for the analysis. Phylogenetic analyses using maximum-likelihood and parsimony methods generated very similar results and are not shown.

DISCUSSION

Intragenetic structure of the genus *Saccharothrix*

The intragenetic relationship among the species of *Saccharothrix* in this study based on 16S rDNA sequences, as seen in Fig. 1, is somewhat surprising since the genus appears phylogenetically heterogeneous. On the basis of the phylogenetic relationships observed in this study, *Saccharothrix sensu stricto* would appear to consist of *Saccharothrix australiensis* NRRL 11239^T (the type species), *Saccharothrix coeruleofusca* NRRL B-16115^T, *Saccharothrix espanaensis* NRRL 15764^T, *Saccharothrix longispora* NRRL B-16116^T, *Saccharothrix mutabilis* subsp. *capreolus* DSM 40225^T, *Saccharothrix mutabilis* subsp. *mutabilis* DSM 43853^T, *Saccharothrix syringae* NRRL B-16468^T and *Saccharothrix texasensis* NRRL B-16134^T, as well as the unnamed *Saccharothrix* strains NRRL B-16108 and NRRL B-16133. The strains of *Saccharothrix texasensis* sequenced, NRRL B-16107 and NRRL B-16134^T, were previously shown to have 89% DNA relatedness and not surprisingly had 100% agreement in their 16S rDNA sequences. *Saccharothrix wayway-andensis* NRRL B-16159^T appears to be phylogenetically more closely related to '*Asiosporangium albidum*' IFO 16102 and *Lentzea albidocapillatus* DSM 44073^T than the other *Saccharothrix* species, while *Saccharothrix aerocolonigenes* NRRL B-3298^T, *Saccharothrix flava* NRRL B-16131^T and *Saccharothrix cryophilis* NRRL B-16238^T do not seem to belong to *Saccharothrix* or any other described actinomycete genus. The latter strain, however, appears to be more related to the sporangiate genera '*Actinoalloteichus*', *Streptoalloteichus* or *Kutzneria*. The 16S rDNA sequence data do explain anomalies previously observed in DNA relatedness studies of the genus *Saccharothrix* (Labeda & Lechevalier, 1989; Labeda & Lyons, 1989) where strains NRRL B-3298^T, NRRL B-16159^T and NRRL B-16238^T exhibited extremely low DNA relatedness (<20%) to any of the other *Saccharothrix* strains. DNA relatedness values below 20% observed between strains could be interpreted as indicating that they belong to different genera, but in the case of the aforementioned *Saccharothrix* strains, the chemotaxonomic and morphological properties observed placed them within the description for the genus *Saccharothrix*.

Phylogenetic relationship of the genus *Saccharothrix* and related genera with the other actinomycetes

Although it has been suggested in the past (Embley *et al.*, 1988; Warwick *et al.*, 1994) that the genus *Saccharothrix* should be placed in the family *Pseudonocardiaceae*, the phylogenetic position of the genus *Saccharothrix* and the allied genera *Actinokineospora*, *Actinosynnema* and *Lentzea* relative to the genera representative of other actinomycete families was consistent and distinct from the *Pseudonocardiaceae*, as can be seen in Fig. 1, regardless of phylogenetic analysis methods used. The relationship of this clade

Fig. 1. For legend see facing page.

Table 2. Chemotaxonomic characteristics of members of the proposed family *Actinosynnemataceae* and related genera

For all genera, the cell wall diamino acid was *meso*-DAP and the cell wall chemotype was III.

Taxon	Whole-cell sugar pattern	Phospholipid type	Phospholipid*	Predominant menaquinone	Fatty acid
' <i>Actinoalloteichus</i> '†	Ribose, mannose, galactose	PII	PME	MK-9(H ₄), MK-8(H ₄), MK-9(H ₂)	iso, anteiso
<i>Actinokineospora</i>	Galactose, mannose, rhamnose	PII	PE	MK-9(H ₄)	Straight-chain, monounsaturated, iso, anteiso
<i>Actinosynnema</i>	Galactose, mannose	PII	PE, PI, PIM, DPG	MK-9(H ₄), MK-9(H ₆)	Straight-chain, monounsaturated, anteiso
' <i>Asiosporangium</i> '‡	Arabinose, xylose	ND	ND	ND	ND
<i>Kutzneria</i> §	Trace, galactose, rhamnose	PII	PE, DPG, PG, PI	MK-9(H ₄)	Straight-chain, unsaturated, iso, anteiso
<i>Lentzea</i>	Galactose	PII	PE, DPG, PG, PI	MK-9(H ₄)	Straight-chain, monounsaturated, iso, anteiso
<i>Saccharothrix</i>	Galactose, rhamnose, mannose	PII, PIV	PE, PIM, PI, DPG, PG	MK-10(H ₄), MK-9(H ₄)	Straight-chain, monounsaturated, iso, anteiso
<i>Streptoalloteichus</i>	Galactose, mannose, rhamnose	PII	PE	MK-9(H ₆), MK-10(H ₆)	ND

ND, Not determined.

* DPG, diphosphatidyl glycerol; PE, phosphatidyl ethanolamine; PG, phosphatidyl glycerol; PI = phosphatidyl inositol; PIM = phosphatidyl inositol mannosides; PME, phosphatidyl methylethanolamine.

† Data from Itoh *et al.* (1987).

‡ Data from Tamura & Hatano (1998).

§ Data from Stackebrandt *et al.* (1994).

to the other actinomycete families studied, including the *Pseudonocardaceae*, is supported by a bootstrap value of 77% and provides good evidence for the creation of a new family for these genera. The relationship to the *Pseudonocardioaceae* was consistent for all phylogenetic analysis methods used. The levels of 16S rDNA sequence similarity between the taxa in the proposed new family and the members of other actinomycete lineages were 100–95.1% and 93.4–79.1%, respectively. A diagnostic nucleotide signature pattern of TA (823–975), GC (824–874) was also observed for all of the strains proposed for inclusion into the family *Actinosynnemataceae*.

The phylogenetic position of *Kutzneria kofuensis*, *Kutzneria viridogrisea*, *Streptoalloteichus hindustanus*, *Saccharothrix cryophilis* and '*Actinoalloteichus cyano-griseus*' is not clear at this time. These sporangiate taxa have the appropriate chemotaxonomic profiles (i.e. *meso*-DAP, galactose and possibly rhamnose and mannose as diagnostic sugars, PII phospholipid pattern, and menaquinones with nine isoprenoid units) and exhibit 16S rDNA sequence similarities ranging from 96.1 to 92.1% with the taxa in the proposed new family, but the low bootstrap values observed do not support inclusion of these taxa into the *Actinosynnemataceae* and they also do not have the nucleotide signature pattern observed for the family. *Saccharothrix cryophilis* NRRL B-16238 was recently observed to produce sporangia, and thus all of these related genera produce sporangia. Maximum-parsimony analysis of the data implied a close relationship of these genera with the *Pseudonocardaceae*, while the other algorithms used demonstrated linkage with the

Actinosynnemataceae. It is likely that these taxa represent a new lineage separate from either family, but this probably can only be clarified by isolating additional representatives and sequencing their 16S rDNA.

An evaluation of the chemotaxonomic characteristics reported in the descriptive literature for all of these genera, seen in Table 2, also supports the creation of a new family, since these genera have cell wall type III (*meso*-DAP), all have galactose in their whole-cell sugar pattern, most have mannose and/or rhamnose, phosphatidylethanolamine is a common constituent of the phospholipid pattern (type PII), and all contain menaquinones with nine isoprenoid units (MK-9). It is proposed that this family be named the *Actinosynnemataceae* for the oldest named genus in the family, *Actinosynnema* Hasegawa, Lechevalier and Lechevalier 1978 (Hasegawa *et al.*, 1978). A formal description of the family follows:

Description of *Actinosynnemataceae* fam. nov. Labeda and Kroppstetdt

Actinosynnemataceae (Ac.ti.no.syn.ne.mat'ace.ae. M.L. n. *Actinosynnema* type genus of the family, pl. *Actinosynnemata*; L. ending -*ceae* ending to denote a family; M.L. n. *Actinosynnemataceae* the *Actinosynnema* family).

Aerobic, catalase-positive actinomycetes comprising the genera *Actinokineospora*, *Actinosynnema*, *Lentzea* and *Saccharothrix*. Vegetative mycelium branches, diameter approximately 0.5–0.7 µm; aerial mycelium

Fig. 1. Phylogenetic dendrogram reconstructed from evolutionary distances (Kimura, 1980) by the neighbour-joining method (Saitou & Nei, 1987), indicating the position of members of the family *Actinosynnemataceae* compared to the family *Pseudonocardaceae* and representatives of the main lineages of the order *Actinomycetales*. Bar, 0.1 nucleotide substitutions per site.

is produced and fragments into chains of smooth-surfaced, rod-shaped elements which may be motile. Synnemata or dome-like bodies may also be formed on the agar surface. Vegetative mycelium may also fragment into coccoidal rod-shaped elements. Gram-positive. Not acid-fast. Resistant to lysozyme. Soils and plant materials are typical habitats. The genera from a coherent phylogenetic unit on the basis of partial 16S rDNA sequences. Type III cell wall composition (*meso*-diaminopimelic acid). Whole-cell sugars pattern consists of galactose and may also include rhamnose and mannose. Phospholipid pattern is type PII with a significant amount of phosphatidylethanolamine. The principal menaquinone consists of nine isoprenoid units (MK-9), although the degree of unsaturation may vary. The G+C content of the DNA ranges from 68 to 76 mol %.

ACKNOWLEDGEMENTS

The able technical assistance of E. N. Hoekstra in the isolation and purification of DNA, PCR amplification of 16S rDNA, and sequence determinations for this study is gratefully acknowledged.

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