Clarification of the relationship between the members of the family *Thermomonosporaceae* on the basis of 16S rDNA, 16S–23S rRNA internal transcribed spacer and 23S rDNA sequences and chemotaxonomic analyses

Zhenshui Zhang,¹ Takuji Kudo,² Yuki Nakajima² and Yue Wang¹

Author for correspondence: Yue Wang. Tel: +65 7783207. Fax: +65 7791117.
e-mail: mcbwangy@imcb.nus.edu.sg

¹ Microbial Collection and Screening Laboratory, Institute of Molecular and Cell Biology, National University of Singapore, 30 Medical Drive, Singapore 117609

² Japan Collection of Microorganisms, RIKEN (The Institute of Physical and Chemical Research), 2–1 Hirosawa, Wako-shi, Saitama, 351–0198 Japan

To resolve relationships between members of the family *Thermomonosporaceae*, phylogenetic analyses using three sets of nucleotide sequences from 16S rDNA, 23S rDNA and the 16S–23S internal transcribed spacer (ITS) were carried out. Nearly all species of the family were included in this study. On the basis of congruous phylogenetic results and chemotaxonomic data, the following proposals are made. First, *Actinomadura libanotica*, *Actinomadura aurantiaca*, *Actinomadura glomerata* and *Actinomadura longicatena* are transferred to the genus *Actinocorallia* as *Actinocorallia libanotica* comb. nov., *Actinocorallia aurantiaca* comb. nov., *Actinocorallia glomerata* comb. nov. and *Actinocorallia longicatena* comb. nov., respectively. All the species of this genus are phylogenetically coherent and of phospholipid type PII (presence of phosphatidylethanolamine), distinguishing them from other *Actinomadura* species that are of phospholipid type PI (absence of diagnostic phospholipids). Second, *Excellospora viridilutea* is transferred to the genus *Actinomadura* as *Actinomadura viridilutea* comb. nov. As a result of the proposed transfers, the family *Thermomonosporaceae* now contains four genera *Thermomonospora*, *Actinomadura*, *Actinocorallia* and *Spirillospora*. The genus *Actinocorallia* and family *Thermomonosporaceae* are redescribed.

Keywords: *Thermomonosporaceae*, *Actinomadura*, *Actinocorallia*, *Excellospora*, phylogeny

INTRODUCTION

In a previous 16S rDNA sequence-based phylogenetic analysis of the members of the family *Thermomonosporaceae*, it was observed that *Thermomonospora curvata*, *Excellospora viridilutea*, *Actinocorallia herbida* and *Spirillospora albida* were intermixed with *Actinomadura* species in a clade distantly separated from their phylogenetic neighbours, the members of the families *Streptosporangiaceae* and *Nocardiopsiaceae*

Abbreviations: DPG, diphosphatidylglycerol; ITS, internal transcribed spacer; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIM, phosphatidylinositol mannoside.

The GenBank accession numbers for the 16S rDNA, 16S–23S rDNA ITS and 23S rDNA sequences of members of the family *Thermomonosporaceae* are given in Table 1.

(Zhang *et al.*, 1998). This intermixing causes confusion in the taxonomic positions of and relationships between members of five actinomycete genera, raising the necessity of further investigation. The close relatedness of *Thermomonospora curvata*, *Excellospora viridilutea*, *Actinocorallia herbida* and *Spirillospora albida* with *Actinomadura* species has also been noticed in some earlier studies on the basis of phylogenetic and chemotaxonomic results (Goodfellow, 1992; Kropenstedt & Goodfellow, 1992; Zhang *et al.*, 1998). However, no transfer has been proposed, largely because each of the four species exhibits some features that appear to justify their independent genus status.

Thermomonospora was proposed by Henssen in 1957 for thermophilic actinomycete strains characterized by the formation of single spores on aerial mycelium.

After recent reclassification of the genus *Thermomonospora* (Zhang *et al.*, 1998), the type species *Thermomonospora curvata* and *Thermomonospora chromogena* are now the only two members left in this genus. *Thermomonospora curvata* seems to have the closest phylogenetic relatedness with *Actinomadura echinospora* (Zhang *et al.*, 1998). However, this relationship is not conclusive due to the lack of significant bootstrap value support. *Thermomonospora chromogena* has been recently found to contain two distinct types of rRNA operons which renders its phylogenetic relationships with other actinomycetes uncertain (Yap *et al.*, 1999). However, it is quite certain that *Thermomonospora chromogena* is located outside of the family *Thermomonosporaceae* (Zhang *et al.*, 1998; Yap *et al.*, 1999).

The genus *Excellospora* was introduced to accommodate three thermophilic species (Agre & Guzeva, 1975). The type species *Excellospora viridilutea* was cited in the Approved Lists of Bacterial Names (Skerman *et al.*, 1980), while the other two species '*Excellospora rubrobrunea*' and '*Excellospora viridinigra*' were considered invalid. Previous studies by several groups of researchers showed that '*Excellospora rubrobrunea*' and '*Excellospora viridinigra*' had many properties in common both with one another and with *Actinomadura madurae* and related strains (Greiner-Mai *et al.*, 1987; Meyer, 1989; Kroppenstedt *et al.*, 1990). The two species were, therefore, transferred to the genus *Actinomadura* as *Actinomadura rubrobrunea* (Kroppenstedt *et al.*, 1990). Currently, the only chemotaxonomic property used to distinguish *Excellospora viridilutea* from *Actinomadura* species is the presence in *Excellospora* strains of higher amounts of branched fatty acids, particularly *iso*-17:0, and lower amounts of 10-methyl acids than in typical *Actinomadura* strains. However, it is rather puzzling that although the three *Excellospora* species share similar fatty acid profiles among many other properties, '*Excellospora rubrobrunea*' and '*Excellospora viridinigra*', but not the type species *Excellospora viridilutea*, were transferred to *Actinomadura*. In the 16S rDNA tree, *Excellospora viridilutea* is located among *Actinomadura* species (Zhang *et al.*, 1998). Thus, the taxonomic position of *Excellospora viridilutea* is questionable and needs further clarification.

In 1963, Couch proposed the genus *Spirillospora*. Bearing a sporangium-like structure similar to that of *Streptosporangium*, *Spirillospora* was temporarily placed in the family *Streptosporangiaceae* (Goodfellow, 1992). Chemotaxonomic data showed that the type species *Spirillospora albida* (type strain ATCC 15331^T = JCM 3041^T) is more similar to *Actinomadura* species in the profiles of phospholipids, predominant menaquinones and fatty acids than to *Streptosporangium* species (Goodfellow, 1992; Kroppenstedt & Goodfellow, 1992). *Spirillospora albida* was found to be very closely related to some *Actinomadura* species in the 16S rDNA tree (Zhang *et*

al., 1998). Despite all the similarities to *Actinomadura* species, *Spirillospora* species are characterized by having a unique property, the formation of sporangia containing motile spores.

The genus *Actinocorallia herbida* was proposed by Iinuma *et al.* (1994). This genus is characterized by cell wall type III/C (containing *meso*-diaminopimelic acid without diagnostic sugar) and the growth of unique coralloid sporophores arising from the substrate mycelia; on the tips of the sporophores long chains of non-motile spores are borne. In the 16S rDNA tree *Actinocorallia herbida* was found to aggregate tightly with *Actinomadura aurantiaca* and *Actinomadura libanotica* forming a clade distant from the one containing the rest of the *Actinomadura* species, *Thermomonospora curvata*, *Excellospora viridilutea* and *Spirillospora albida*. Interestingly, *Actinomadura libanotica* grows synnemata (Meyer, 1979), spore-bearing structures resembling those formed by *Actinocorallia herbida*, but *Actinomadura aurantiaca* does not form a similar structure (Lavrova & Preobrazhenskaya, 1975). Thus, the question arises whether *Actinocorallia herbida* should be a member of *Actinomadura* or whether the two *Actinomadura* species should be transferred to the genus *Actinocorallia*.

16S rDNA sequences have been used extensively in determining phylogenetic relationships between organisms (Woese, 1987; Woese *et al.*, 1990; Amann *et al.*, 1995). Due to their highly conserved nature, closely related organisms are often found to have nearly identical 16S rRNA gene sequences (Stackebrandt & Goebel, 1994), limiting its power in resolving close relationships. One solution to this problem is to use other evolutionarily conserved genes, such as 23S rRNA genes. Congruity of the results obtained from using multiple gene sets will provide more reliable data for inferring organismal relationships. Analysis of genes or DNA sequences exhibiting faster evolution rates, such as the internal transcribed spacers (ITSs) of rRNA operons, is also of important value in elucidating close relationships (Gurtler & Stanisich, 1996; Zhang *et al.*, 1997).

In this study, we conducted phylogenetic analyses using DNA sequences of 16S and 23S rDNA and 16S–23S rRNA gene spacers to further investigate the phylogenetic positions of *Thermomonospora curvata*, *Excellospora viridilutea*, *Actinocorallia herbida* and *Spirillospora* species and their relationships with *Actinomadura* species.

METHODS

Organisms and culture conditions. The actinomycete strains used in this study were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA), IFO (Institute for Fermentation, Osaka, Japan), DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and JCM (Japan Collection of Microorganisms, Wako, Japan). Strain names and GenBank

Table 1 Actinomycete species used in this study

Only accession numbers for sequences determined in this study are given.

Species	Source	GenBank accession no.		
		16S	ITS	23S
<i>Actinocorallia herbida</i>	IFO 15485 ^T		AF134109	AF134086
<i>Actinomadura atramentaria</i>	IFO 14695 ^T		AF134089	AF134071
<i>Actinomadura aurantiaca</i>	JCM 8201 ^T	AF134066	AF134090	AF134072
<i>Actinomadura citrea</i>	IFO 14678 ^T		AF134091	
<i>Actinomadura coerulea</i>	JCM 3320 ^T		AF134092	
<i>Actinomadura cremea</i> subsp. <i>cremea</i>	JCM 3308 ^T	AF134067	AF134094	AF134073
<i>Actinomadura cremea</i> subsp. <i>rifamycin</i>	IFO 14183 ^T		AF134093	AF134074
<i>Actinomadura echinospora</i>	IFO 14042 ^T		AF134095	AF134075
<i>Actinomadura fibrosa</i>	ATCC 49459 ^T	AF163114	AF163125	AF163136
<i>Actinomadura formosensis</i>	JCM 7474 ^T		AF134096	
<i>Actinomadura fulvescens</i>	IFO 14347 ^T		AF134097	AF134076
<i>Actinomadura glomerata</i>	JCM 9376 ^T	AF134068	AF134098	AF134077
<i>Actinomadura hibisca</i>	JCM 9627 ^T	AF163115	AF163126	AF163137
<i>Actinomadura kijaniata</i>	JCM 3306 ^T		AF134099	
<i>Actinomadura libanotica</i>	IFO 14095 ^T		AF134100	AF134078
<i>Actinomadura livida</i>	JCM 3387 ^T	AF163116	AF163127	AF163138
<i>Actinomadura longicatena</i>	JCM 9377 ^T	AF163117	AF163128	AF163139
<i>Actinomadura luteofluorescens</i>	IFO 13057 ^T		AF134101	AF134079
<i>Actinomadura macra</i>	IFO 14102 ^T		AF134102	AF134080
<i>Actinomadura madurae</i>	JCM 7436 ^T		AF134103	
<i>Actinomadura oligospora</i>	ATCC 43269 ^T	AF163118	AF163129	AF163140
<i>Actinomadura pelletieri</i>	JCM 3388 ^T	AF163119	AF163130	AF163141
<i>Actinomadura rubrobrunea</i>	IFO 14622	AF134069	AF134104	AF134081
<i>Actinomadura rugatobispora</i>	IFO 14382 ^T		AF134105	AF134082
<i>Actinomadura spadix</i>	JCM 3146 ^T	AF163120	AF163131	AF163142
<i>Actinomadura umbrina</i>	JCM 6837 ^T	AF163121	AF163132	AF163143
<i>Actinomadura verrucososporea</i>	IFO 14100 ^T		AF134106	AF134083
<i>Actinomadura vinacea</i>	JCM 3325 ^T	AF134070	AF134107	AF134084
<i>Actinomadura viridis</i>	JCM 3112 ^T		AF134108	AF134085
<i>Actinomadura yumaensis</i>	JCM 3369 ^T	AF163122	AF163133	AF163144
<i>Excellospora viridilutea</i>	JCM 3398 ^T		AF134110	AF134087
<i>Spirillospora albida</i>	JCM 3041 ^T		AF134111	AF134088
<i>Spirillospora rubra</i>	JCM 6875 ^T	AF163123	AF163134	AF163145
<i>Spirillospora</i> sp.	JCM 3123 ^T	AF163124	AF163135	AF163146
<i>Thermomonospora curvata</i>	JCM 3096 ^T		AF134112	

nucleotide sequence accession numbers are listed in Table 1. For the preparation of genomic DNA, the cells were grown in Bennett's medium (Atlas, 1993) to exponential phase. For the phospholipid and fatty acid analyses, the cells were grown to late exponential phase in glucose yeast extract medium (10 g yeast extract, 10 g D-glucose in 1 l distilled water, pH 7.2).

Preparation of genomic DNAs. The genomic DNA of the actinomycetes was prepared as described previously (Wang *et al.*, 1996).

PCR amplification, cloning and sequencing. The PCR amplification, cloning and sequencing of 16S rDNAs were done as described previously (Wang *et al.*, 1996). The 16S–23S rRNA gene spacer and the 5' one-third of the 23S rRNA genes were amplified by PCR using a pair of primers, one targeting a conserved region at the end of the 16S rRNA

gene and the other a conserved block within the 23S rRNA gene. The sequences of the two oligonucleotides were as follows: 5'-GGTTGGATCCACCTCCTT-3', corresponding to nt 1525–1542 (*Escherichia coli* 16S rRNA gene numbering; Brosius *et al.*, 1978), and 5'-ACCAGTGAGC-TATTAGCG-3' (nt 1090–1107). After cloning of the PCR products, the M13 forward and reverse universal primers were used for sequencing the ends of each clone. The internal regions were sequenced in both orientations by using the following two sets of oligonucleotide primers targeting two conserved sequences within 23S rDNA. The first set of primers targeting nt 45–60 (*Escherichia coli* 23S rRNA gene numbering) were 23S-40f (5'-CCGATGAAGGACGTGG-GA-3') and 23S-40r (5'-TCCCACGTCCTTCATCGG-3'), and the second set of primers targeting nt 456–472 were 23S-460f (5'-CCTTTCCCTCACGGTACT-3') and 23S-460r (5'-AGTACCGTGAGGGAAAGG-3'). Being aware of

possible existence of sequence heterogeneity between different copies of rRNA operons (Clayton *et al.*, 1995; Wang *et al.*, 1997; Yap *et al.*, 1999), we picked three clones from each PCR reaction for sequence analysis. Only low levels of heterogeneity (<1%) were observed between different clones from a few organisms and the heterogeneity had no effect on the results of phylogenetic analysis.

Sequence alignment and phylogenetic analysis. Multiple sequence alignment and computation of sequence similarities were carried out by using the CLUSTAL method of the DNASTAR program (Madison, WI, USA). The sequence alignments were also verified according to the consensus secondary structures of the 16S and 23S rRNA molecules (Gutell *et al.*, 1994). Evolutionary distance matrices were generated by the method of Jukes & Cantor (1969). Phylogenetic trees were constructed by using both the neighbour-joining method of Saitou & Nei (1987) and the maximum-parsimony method (Swofford & Begle, 1993). The confidence level of phylogenetic tree topology was evaluated by using the bootstrap method (Felsenstein, 1985). The software for the bootstrap analysis is contained in the CLUSTAL v phylogenetic analysis software package (Higgins, 1992).

Chemotaxonomic analyses. Phospholipids were analysed by the method of Minnikin *et al.* (1984). Cellular fatty acid methyl esters were prepared by the direct transmethylation method with methanolic hydrochloride and analysed by GLC as described by Suzuki & Komagata (1983). Samples for the analysis of whole-cell sugars were prepared as described by Lechevalier & Lechevalier (1970) and the composition was determined by using the HPLC method of Mikami & Ishida (1983).

RESULTS

To get a more complete representation of species belonging to the family *Thermomonosporaceae*, we included in this study all the *Actinomadura* species listed in *Bergey's Manual of Determinative Bacteriology* (Holt *et al.*, 1994), two new *Actinomadura* species, *Actinomadura glomerata* and *Actinomadura longicatena* (Itoh *et al.*, 1995), *Spirillospora albida*, *Spirillospora rubra*, an undefined *Spirillospora* strain, JCM 3123^T, *Excellospora viridilutea* and *Thermomonospora curvata* (Table 1). We obtained the 16S rDNA sequences from all the species which were not included in the previous study. We also determined the sequences of approximately 1.2 kb of the 5' end of 23S rDNAs and the 16S–23S rRNA gene spacers.

Analysis of 16S rDNA sequences

A pairwise comparison of almost complete 16S rDNA sequences (nt 59–1491 and data not shown) shows that *Excellospora viridilutea* is 99.5% identical to *Actinomadura rubrobrunea* which was previously a member of the genus *Excellospora* (Kroppenstedt *et al.*, 1990), indicating their close relationship. *Spirillospora albida* exhibits highest similarity (97.5%) with *Spirillospora* strain JCM 3123^T; however, *Spirillospora rubra* shares about 94% similarity with *Spirillospora albida* and strain JCM 3123^T, a level lower than its similarity to several *Actinomadura* species. *Thermomonospora curvata* displays highest similarity to *Actinomadura*

umbrina (94.2%) and a mean of 91.6% similarity (ranging from 90.3 to 93.2%) to all other species of the family *Thermomonosporaceae*.

Phylogenetic trees were reconstructed using the 16S rDNA sequences. The application of both the neighbour-joining and the maximum-parsimony methods and the change of outgroups produced nearly identical tree topology relating the members of the family *Thermomonosporaceae*. Fig. 1(a) shows a representative tree constructed using the neighbour-joining method. Within the clade of *Thermomonosporaceae*, intermixing of members from different genera is again demonstrated and some new relationships also emerge which were not observed previously. The three *Spirillospora* sequences fail to form one clade. Although *Spirillospora albida* and *Spirillospora* strain JCM 3123^T aggregate tightly, they are distantly related to *Spirillospora rubra* and located among *Actinomadura* species. *Excellospora viridilutea* exhibits the closest relatedness with *Actinomadura rubrobrunea*. The clade containing the two species is also placed among *Actinomadura* species. Four *Actinomadura* species, *Actinomadura libanotica*, *Actinomadura aurantiaca*, *Actinomadura glomerata* and *Actinomadura longicatena*, form a very stable clade (bootstrap value 999) with *Actinocorallia herbida*. This clade is separated from the one containing the rest of the *Actinomadura* species except *Actinomadura spadix* that stands alone and is distantly related to all other members of the family *Thermomonosporaceae*. *Thermomonospora curvata* aggregates with *Actinomadura echinospora* and *Actinomadura umbrina* forming a clade separated from other *Actinomadura* species. However, this clade is supported by a low bootstrap value of 337.

Analysis of 23S rDNA sequences

It is interesting to note that the similarity values scored between the different 23S rDNA sequences [~1200 bases corresponding to nt 1–1108 (*Escherichia coli* numbering)] are on average 2% lower than those between 16S rDNA sequences, indicating that this region of 23S rRNA genes has evolved faster than the 16S rRNA genes. The result of the pairwise sequence comparison is largely the same as that of the 16S rDNA sequence analysis. *Excellospora viridilutea* was again found to share with *Actinomadura rubrobrunea* the highest sequence similarity of 96%. The sequences of *Spirillospora albida* and *Spirillospora* strain JCM 3123^T, which share 95.4% similarity to each other, show only 92.7 and 92.8% similarity, respectively, to the sequence of *Spirillospora rubra*, a level lower than their relatedness with many *Actinomadura* species. *Thermomonospora curvata* exhibits 92.4 and 91.3% sequence similarity to *Actinomadura echinospora* and *Actinomadura umbrina*, respectively, and less than 90.5% to all other *Actinomadura* species.

The 23S rDNA sequences were used to reconstruct phylogenetic trees by both neighbour-joining and

Fig. 1. Neighbour-joining trees reconstructed by using 16S (a) and 23S (b) rDNA sequences. The regions from nt 56 to 1491 (*Escherichia coli* numbering) and from 1 to 1108 were used for constructing the 16S and 23S rDNA trees, respectively. Small regions with alignment ambiguities were excluded, i.e. nt 181–220, 453–481, 1007–1040 and 1133–1141 for the 16S rDNA sequences and 256–320, 348–358, 539–552 and 651–655 for the 23S rDNA sequences. Both trees were rooted by using the corresponding rDNA sequences of *Streptomyces coelicolor* A3(2) (GenBank accession no. AL079345). The numbers at the nodes are bootstrap values based on 1000 resamplings. Bootstrap values lower than 500 are not shown except a few very low values on the 23S rDNA tree to show the unreliable relationship between *Actinomadura* species of the clade containing *Actinocorallia herbida* and four *Actinomadura* species. The bars represent the number of inferred substitutions per 1000 nt. All the sequences determined in this study are listed in Table 1 and the rest of the sequences were retrieved from the GenBank and EMBL databases.

maximum-parsimony methods. The relationships described below were produced on both trees and Fig. 1(b) shows the neighbour-joining tree only. The relationships revealed in this tree are in good accord with those shown by the 16S rDNA tree in the following aspects. First, *Excellospora viridilutea* and *Actinomadura rubrobrunea* aggregate tightly. Second, *Thermomonospora curvata*, *Actinomadura echinospora* and *Actinomadura umbrina* form a fairly stable clade (bootstrap value 636) which is separated from the one containing the rest of the *Actinomadura* species except *Actinomadura spadix* which again shows a distant relationship to all other members of the family. Third, the tight clustering of *Actinocorallia herbida* with four *Actinomadura* species, *Actinomadura libanotica*, *Actinomadura aurantiaca*, *Actinomadura glomerata* and *Actinomadura longicatena*, is reproduced, even though the clade is located among *Actinomadura* species. However, this clade has a long branch and its location is not certain because all the higher level

clades have very low bootstrap values. The tight aggregation of these species was not affected by the use of different outgroups, though the location of this clade may change. Fourth, the three *Spirillospora* strains do not form one clade, and *Spirillospora rubra* is distantly related to *Spirillospora albida* and strain JCM 3123^T.

Analysis of 16S–23S rRNA ITS sequences

We sequenced three clones of the ITS from each organism and did not find significant heterogeneity. No tRNA gene was found in any of the spacer sequences. The lengths of the 16S–23S rDNA spacers of *Actinocorallia herbida*, *Actinomadura libanotica*, *Actinomadura aurantiaca*, *Actinomadura glomerata* and *Actinomadura longicatena* are similar, being 403, 385, 374, 381 and 385 bp, respectively. The spacer sizes of the rest of the *Actinomadura* species vary over a larger range from 387 to 553 bp. Due to the variations

Fig. 2. Thin-layer chromatograms of total phospholipids. The name of the actinomycete analysed is given above each pair of chromatograms. The reagents used for visualizing the phospholipids are indicated at the bottom of the figure.

in length between the spacers from different species, multiple sequence alignment is difficult. Nevertheless, a major part (~70%) of the sequences of the five *Actinocorallia*-related species can be aligned with limited ambiguity, which contains several blocks of sequences which are not present in the spacers of the rest of the *Actinomadura* species (alignment not shown). The levels of similarity between *Actinocorallia herbida*, *Actinomadura libanotica*, *Actinomadura glomerata*, *Actinomadura longicatena* and *Actinomadura aurantiaca* range from 47.3 to 62.2% with a mean value of 53.5%, which is markedly higher than the scores between these five species and the rest of the *Actinomadura* species ranging from 24 to 45.5% with a mean of 35%. This result is in good accordance with the results of phylogenetic analyses above, suggesting close relatedness of these five species and their distance to other *Actinomadura* species. *Excelspora viridilutea* once again was found to share the highest sequence similarity (79.5%) with *Actinomadura rubrobrunea*, which is considerably greater than the 30.5–59.4% range recorded between these two species and other

Actinomadura species. *Spirillospora albida* and strain JCM 3123^T exhibit about 70 and 50% sequence similarity with several *Actinomadura* species, respectively, and these values are much higher than those (<40%) scored between many *Actinomadura* species. *Spirillospora rubra* exhibits only 30–40% sequence similarity to all other species of the family.

Re-examination of some chemotaxonomic properties of *Actinocorallia herbida* and *Actinomadura libanotica*

We have observed that four *Actinomadura* species, *Actinomadura libanotica*, *Actinomadura aurantiaca*, *Actinomadura glomerata* and *Actinomadura longicatena*, consistently aggregate with *Actinocorallia herbida* forming one stable clade. Peculiarly, the phospholipid composition of all the species is of type PII, except that of *Actinomadura libanotica* which is of type PI (Kroppenstedt *et al.*, 1990) like the rest of the *Actinomadura* species; the whole-cell hydrolysates of the four *Actinomadura* species contain the diagnostic sugar madurose which was not reported to be present in *Actinocorallia herbida* (Inuma *et al.*, 1994). It appears that this phylogenetically well-defined group is chemotaxonomically heterogeneous. To address the possibility that the phospholipid type of *Actinomadura libanotica* and sugar type of *Actinocorallia herbida* were wrongly determined in earlier studies, we re-examined the phospholipids and sugars of *Actinomadura libanotica* (strains JCM 3284^T and DSM 43554^T obtained from two culture collections) and *Actinocorallia herbida*. Figure 2 shows the detection in *Actinomadura libanotica* of major amounts of diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE) and phosphatidylinositol (PI), components corresponding to phospholipid type PII, not type PI. We also detected the presence of madurose in *Actinocorallia herbida* (not shown). In addition, the description of the fatty acid composition of *Actinocorallia herbida* given by Inuma *et al.* (1994) was not complete, lacking information about the branched and 10-methyl-branched fatty acids. To fill in the missing information, we analysed the fatty acids of *Actinocorallia herbida* and found that its fatty acid pattern is very similar to that exhibited by *Actinomadura* species (Table 2) which was defined as type 3a (Kroppenstedt *et al.*, 1990).

DISCUSSION

Several relationships among the members of *Thermomonosporaceae* were consistently demonstrated by the use of the three DNA sequence sets. First, *Actinomadura libanotica*, *Actinomadura aurantiaca*, *Actinomadura glomerata* and *Actinomadura longicatena* consistently aggregate with *Actinocorallia herbida*, forming a very stable clade in both the 16S and 23S rDNA phylogenetic trees. This clade is separated from another clade containing the rest of the *Actinomadura*

Table 2 Cellular fatty acid composition

Organism*	Fatty acid composition (%)																				Reference†
	Normal									Iso						Anteiso		10-Methyl			
	14:0	16:0	16:1	18:0	18:1	15:0	17:0	17:1	19:1	14:0	16:0	16:1	18:0	18:1	15:0	17:0	17:0	10Me-16	10Me-17	10Me-18	
<i>Actinocorallia herbida</i> JCM 9647 ^T	1	18	9	3	15	2	4	7	1	1	13	1	1	2	TR	1	1	3	3	10	a
(<i>Actinocorallia herbida</i>)		26	15	4	27	4	8	16													b
<i>Actinomadura libanotica</i> JCM 3284 ^T	1	20	14	3	19	2	3	4	1	TR	8	1	1	2	TR	1	1	2	1	8	a
(<i>Actinomadura libanotica</i>)	2	22	9	5	17	4	7	7			12		1		1	3	1	1	4	4	c
(<i>Actinomadura aurantiaca</i>)	1	21	9	6	17	1	3	1		TR	17	1	2		TR	2	2	2	4	9	c
(<i>Actinomadura glomerata</i>)	2	28	4	5	20	3	6				9						4		2	15	d
(<i>Actinomadura longicatena</i>)	2	24	5	6	16	3	5				13						4		2	14	d
<i>Spirillospora albida</i> JCM 3041 ^T	3	24	3	3	21	TR	TR	TR		1	11		2	2				TR	TR	21	a
<i>Spirillospora</i> sp. JCM 3123 ^T	3	26	2	5	27	TR	1	1		1	10		3	2				TR	1	11	a
<i>Spirillospora rubra</i> JCM 6875 ^T	1	20	8	3	21	1	5	5	1	TR	5		4	2				1	2	11	a
(<i>Actinomadura madurae</i>)	2	34	3	3	18	1	2	1		1	20		3					TR	3	9	c
(<i>Actinomadura madurae</i>)	2	39	3	10	22	1	2	1			4		1	TR				TR	1	14	e

* Organisms in parentheses have been analysed previously.

† References: a, this study; b, Iinuma *et al.* (1994); c, Kroppenstedt *et al.* (1990); d, Itoh *et al.* (1995); e, Kudo *et al.* (1993).

species except *Actinomadura spadix* in the 16S rDNA tree, though this separation is not clear in the 23S rDNA tree due to unstable tree topology. Furthermore, the sequence similarity level of the 16S–23S spacers between these five species is much higher than between them and the rest of the *Actinomadura* species. In a re-examination of the phospholipid and whole-cell sugar compositions, we detected major amounts of DPG, PE and PI in *Actinomadura libanotica* and the presence of madurose in *Actinocorallia herbida*. Thus, *Actinomadura libanotica* is characterized by phospholipid type PII, not PI, and *Actinocorallia herbida* has sugar type B, not type C as reported by Kroppenstedt *et al.* (1990) and Iinuma *et al.* (1994). Now all the five species of this clade are characterized by having phospholipid type PII, distinguishing them from all other *Actinomadura* species containing type PI phospholipids (Kroppenstedt, 1990; Iinuma *et al.*, 1994; Itoh *et al.*, 1995). Based on both phylogenetic and chemotaxonomic evidence, we propose the transfer of four *Actinomadura* species to the genus *Actinocorallia*: *Actinomadura libanotica* as *Actinocorallia libanotica* comb. nov., *Actinomadura aurantiaca* as *Actinocorallia aurantiaca* comb. nov., *Actinomadura glomerata* as *Actinocorallia glomerata* comb. nov. and *Actinomadura longicatena* as *Actinocorallia longicatena* comb. nov.

On the basis of 16S rDNA sequence analysis Stackebrandt *et al.* (1997) proposed the family *Thermomonosporaceae*, in which the genus *Actinocorallia* was not included. They also proposed the following nucleotide signatures to be characteristic of the members of *Thermomonosporaceae*: 440–494 (C-G), 442–492 (G-C), four to seven extra bases between positions 453 and 479, 501–544 (C-G), 502–543 (G-C), 586–755 (C-G), 603–635 (U-A), 613–627 (C-G), 658–748 (C-U), 681–709 (C-G), 1003–1037 (A-G), 1116–1184 (C-G), 1355–1367 (A-U), 1422–1478 (G-C) and 1435–1466 (G-C). In all the *Actinocorallia* species we found all these nucleotide signatures except at 1003–1037 (G-C) and 1422–1478 (A-C). We also noticed that *Thermomonospora curvata*, *Spirillospora* species and a majority of *Actinomadura* species also have a G-C pair at 1003–1037; *Actinomadura fulvescens* and *Actinomadura oligospora* also have an A-C pair and *Thermomonospora curvata* has a G-T pair at 1422–1478. Thus, the initially proposed nucleotide signatures 1003–1037 (A-G) and 1422–1478 (G-C) should not be used as being characteristic of the members of *Thermomonosporaceae*. The rest of the nucleotide signatures for this family remain valid. *Actinocorallia* should be placed in the family *Thermomonosporaceae*.

Second, *Excellospora viridilutea* exhibits the closest relationship with *Actinomadura rubrobrunea* and they form a tight clade among other *Actinomadura* species in both the 16S and 23S rDNA trees. The levels of sequence similarity of all three DNA segments between the two species and other *Actinomadura* species are well within the range of similarity scores between

Actinomadura species. Therefore, there is neither a phylogenetic basis nor substantial chemotaxonomic differences to separate *Excellospora viridilutea* and *Actinomadura rubrobrunea* and to separate the two species from most *Actinomadura* species. We therefore propose the transfer of *Excellospora viridilutea* to the genus *Actinomadura* as *Actinomadura viridilutea* comb. nov.

Third, *Spirillospora rubra* is distantly related to *Spirillospora albida* and *Spirillospora* strain JCM 3123^T as well as to all other species in the family *Thermomonosporaceae*, suggesting that *Spirillospora rubra* may merit an independent genus status. *Spirillospora albida* is shown to be very closely related to some *Actinomadura* species. The high level of sequence similarity, especially between the 16S–23S rDNA spacers (above 70%) of *Spirillospora albida* and some *Actinomadura* species is particularly strong evidence for close relatedness. This level of similarity is significantly higher than most similarity values scored between different *Actinomadura* species. The phylogenetic data strongly suggest that *Spirillospora albida* has a very close evolutionary relationship with some *Actinomadura* species. So, should the two genera *Spirillospora* and *Actinomadura* be combined? It is still quite puzzling how *Spirillospora* species have developed complex sporangia containing motile spores, a property not found in any *Actinomadura* species. One explanation could be the lateral transfer (Lawrence & Ochman, 1998) from a distant source of a whole cluster of genes required for producing the unique morphology into the ancestor of *Spirillospora*. If this is the case, the morphological distinction should not be translated into a distant organismal relationship and the two genera may need to be combined. However, currently there is no evidence supporting this hypothesis. Further studies are needed to unravel the relationships between *Spirillospora* and *Actinomadura* species.

Fourth, *Actinomadura echinospora* and *Actinomadura umbrina* have been shown to form a clade with *Thermomonospora curvata*, which is separated from other *Actinomadura* species in both the 16S and 23S rDNA trees and supported by a significant bootstrap value in the latter. The result suggests close relatedness among these three species. However, further investigations are needed to find other taxonomic properties to unify these species.

Finally, *Actinomadura spadix* may merit an independent genus status. The results of a previous numerical study (Athalye *et al.*, 1985) and an electrophoretic mobility study of ribosomal protein AT-L30 (Ochi *et al.*, 1991) of *Actinomadura*-related actinomycetes also suggested a distant relationship between *Actinomadura spadix* and other *Actinomadura* species.

As the result of the proposed transfers above, the family *Thermomonosporaceae* now embraces four genera *Thermomonospora*, *Actinomadura*, *Spirillospora* and *Actinocorallia*.

Emendation of *Actinocorallia* (Iinuma *et al.*, 1994)

The description is taken from Iinuma *et al.* (1994), Lavrova & Preobrazhenskaya (1975), Meyer (1989), Kroppenstedt *et al.* (1990), Kudo (1997), Zhang *et al.* (1998) and this study. This genus is well defined on the basis of 16S and 23S rDNA sequence-based phylogenetic analyses and major chemotaxonomic properties, but exhibits some heterogeneity in morphological characteristics. Two species, *Actinocorallia herbida* and *Actinocorallia libanotica*, form synnemata bearing long chains of non-motile spores on their tips, while other species grow spore chains from aerial mycelium. Gram-positive and aerobic. Vegetative mycelia are branched but not fragmented. Mycolic acid is absent. All species are characterized by cell wall type III/B, phospholipid type PII, predominant menaquinones MK-9(H₄), MK-9(H₆) and MK-9(H₈), and fatty acid type 3a. The type species is *Actinocorallia herbida* and the type strain is IFO 15485^T (= JCM 9647^T).

Description of *Actinocorallia aurantica* (Lavrova & Preobrazhenskaya 1975) comb. nov.

The description of *Actinocorallia aurantica* (*Actinomadura* Lavrova & Preobrazhenskaya 1975) is similar to that given by Lavrova & Preobrazhenskaya (1975) for phenotypic characteristics, by Kroppenstedt *et al.* (1990) for chemotaxonomic properties and by Zhang *et al.* (1998) and this study for its phylogenetic position. Type strain is JCM 8201^T.

Description of *Actinocorallia glomerata* (Itoh *et al.*, 1995) comb. nov.

The description of *Actinocorallia glomerata* (*Actinomadura* Itoh *et al.*, 1995) is the same as that given by Itoh *et al.* (1995) for phenotypic characteristics and chemotaxonomic properties, and by this study for its phylogenetic position. Type strain is JCM 9376^T.

Description of *Actinocorallia libanotica* (Meyer 1979) comb. nov.

The description of *Actinocorallia libanotica* (*Actinomadura* Meyer 1979) is the same as that given by Meyer (1989) and Kudo (1997) for phenotypic characteristics, by Kroppenstedt *et al.* (1990) and this study for chemotaxonomic properties and by Zhang *et al.* (1998) and this study for its phylogenetic position. Type strain is IFO 14095^T.

Description of *Actinocorallia longicatena* (Itoh *et al.*, 1995) comb. nov.

The description of *Actinocorallia longicatena* (*Actinomadura* Itoh *et al.*, 1995) is the same as that given by Itoh *et al.* (1995) for phenotypic characteristics and chemotaxonomic properties, and by this study for its phylogenetic position. Type strain is JCM 9377^T.

Description of *Actinomadura viridilutea* (Agre and Guzeva 1975) comb. nov.

The description of *Actinomadura viridilutea* (*Excellorospira* Agre and Guzeva 1975) is the same as that given by Agre & Guzeva (1975) for phenotypic characteristics, by Kroppenstedt & Goodfellow (1992) for chemotaxonomic properties and by Zhang *et al.* (1998) and this study for its phylogenetic position. Type strain is JCM 3398^T.

Emendation of the family *Thermomonosporaceae* (Stackebrandt *et al.*, 1997)

The following descriptions are taken from earlier investigations (Henssen, 1957; Couch, 1963; McCarthy & Cross, 1984; Vobis & Kothe, 1989; Meyer, 1989; Kroppenstedt *et al.*, 1990; Goodfellow, 1992; Kroppenstedt & Goodfellow, 1992; Iinuma *et al.*, 1994; Kudo, 1997; Stackebrandt *et al.*, 1997; Zhang *et al.*, 1998) and this study. After the reclassification proposed by Zhang *et al.* (1998) and this paper, *Thermomonosporaceae* contains four genera, *Thermomonospora*, *Actinomadura*, *Actinocorallia* and *Spirillospora*. They form a distinct clade in the sub-order *Streptosporangineae* and are closely related to the members of *Streptosporangiaceae* and *Nocardiopsiaceae*. All species of *Thermomonosporaceae* share the same cell wall type (type III; meso-diaminopimelic acid), a similar menaquinone profile [4B2; MK-9(H₄), MK-9(H₆) and MK-9(H₈)], in which MK-9(H₆) is predominant, and fatty acid profile type 3a, differentiating them from members of *Streptosporangiaceae* and *Nocardiopsiaceae*. The presentation of the diagnostic sugar madurose is variable, but can be found in most species of this family. The polar lipid profiles are characterized as phospholipid type PI by the presence of PIM, PI, PG and DPG for most species of *Thermomonospora*, *Actinomadura* and *Spirillospora*. The members of *Actinocorallia* are characterized by phospholipid type PII because of the presence of PE. Aerobic, Gram-positive, non-acid-fast, chemo-organotrophic actinomycetes which produce a branched substrate mycelium bearing aerial hyphae that undergo differentiation into single or short chains of arthrospores to sporangia containing the zoospores. The G + C content of the DNA lies within the range 66–72 mol %. The pattern of 16S rDNA signatures consists of nucleotides at positions 440–494 (C-G), 442–492 (G-C), four to seven extra bases between positions 453 and 479, 501–544 (C-G), 502–543 (G-C), 586–755 (C-G), 603–635 (U-A), 613–627 (C-G), 658–748 (C-U), 681–709 (C-G), 1116–1184 (C-G), 1355–1367 (A-U) and 1435–1466 (G-C). The type genus is *Thermomonospora* (Henssen 1957).

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