Phylogenetic analysis of the genus *Thermoactinomyces* based on 16S rDNA sequences

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**INTRODUCTION**

The genus *Thermoactinomyces* was one of the earliest known actinomycete taxa that was first proposed with *Thermoactinomyces vulgaris*, the type species of the genus (Tsiklinsky, 1899). There was no doubt in recognizing *Thermoactinomyces* species as actinomycetes because of their morphological characteristics of forming aerial and substrate mycelia. However, some studies provided evidence that the genus *Thermoactinomyces* should no longer be classified within the order *Actinomycetales*. *Thermoactinomyces* species produce endospores as shown in bacilli (Cross et al., 1968, 1971; Lacey & Vince, 1971) and have lower G+C contents than those of actinomycetes (Lacey & Cross, 1989). 16S rRNA oligonucleotide sequencing revealed that the genus *Thermoactinomyces* is more closely related to *Bacillus* species than to actinomycetes (Stackebrandt & Woese, 1981). 16S rRNA sequences of the type strains of eight validly described species and two invalid species of the genus *Thermoactinomyces* were determined and phylogenetically analyzed. The validly described *Thermoactinomyces* species formed phylogenetic lineages related to the family *Bacillaceae*, as shown previously. However, the available strains of *Thermoactinomyces glaucus* and *Thermoactinomyces monosporus* exhibited their closest phylogenetic affinities not to the genus *Thermoactinomyces* but to the genus *Saccharomonospora*. Some *Thermoactinomyces* species were shown to be closely related from 16S rDNA sequence analysis. Particularly, *Thermoactinomyces vulgaris* KCTC 9076T and *Thermoactinomyces candidus* KCTC 9557T had the same 16S rDNA sequences and *Thermoactinomyces thalpophilus* KCTC 9789T and *Thermoactinomyces sacchari* KCTC 9790T showed 16S rDNA similarity value of almost 100%. From phylogenetic analysis based on 16S rDNA sequences, it is suggested that the genus *Thermoactinomyces* should be taxonomically re-evaluated using other useful taxonomic markers.

**Keywords:** *Thermoactinomyces* species, 16S rDNA sequence, phylogeny

The GenBank accession numbers for the 16S rDNA sequences reported in this paper are AF138732–AF138739, AF139879 and AF139880.
acids (Kroppenstedt, 1985). The G + C contents of DNA range from 52 to 54.8 mol % \((T_m)\) (Lacey & Cross, 1989). There are currently eight validly described Thermoactinomyces species, namely Thermoactinomyces candidus (Kurup et al., 1975), Thermoactinomyces dichotomicus (Krasil’nikov & Age, 1964; Cross & Goodfellow, 1973), Thermoactinomyces intermedius (Kurup et al., 1980), Thermoactinomyces peptonophilus (Nonomura & Ohara, 1971), Thermoactinomyces putidus (Lacey & Cross, 1989), Thermoactinomyces sacchari (Lacey, 1971), Thermoactinomyces thalpophilus (Waksman & Corke, 1953; Usworth & Cross, 1980) and Thermoactinomyces vulgaris (Tsiklinevsky, 1899). Before including the eight valid species, the genus Thermoactinomyces had very confusing taxonomic history (Lacey & Cross, 1989). In addition to the eight valid Thermoactinomyces species, some species, such as Thermoactinomyces glaucus (Henssen, 1957), Thermoactinomyces monosporus (Waksman & Corke, 1953), Thermoactinomyces peptonophilus (Waksman, 1961), Thermoactinomyces antibiosis (Craveri et al., 1964) and Thermoactinomyces albicus (Lacey & Cross, 1989), were known but have not been validly described. Thermoactinomyces antibiosis and Thermoactinomyces albicus were described to be synonyms of Thermoactinomyces thalpophilus and Thermoactinomyces vulgaris, respectively (Lacey & Cross, 1989). Strain(s) of Thermoactinomyces thermophilus are no longer available from the culture collections. The type strains of Thermoactinomyces glaucus and Thermoactinomyces monosporus are no longer available. However, one strain of Thermoactinomyces glaucus and one strain of Thermoactinomyces monosporus, which are found in catalogues of some culture collections, were those described by Fergus (1964) and Nonomura & Ohara (1969), respectively.

Thermoactinomyces species have also been noticed due to their pathogenicity. They have been implicated as causal agents in various forms of hypersensitivity pneumonitis (extrinsic allergic alveolitis), especially farmer’s lung disease and bagassosis (Pepys et al., 1963; Lacey, 1971; Lacey & Cross, 1989). Such disease are likely to appear in farmers that have been exposed to mouldy hay and cereal grains in which Thermoactinomyces species are known to be most abundant. However, Thermoactinomyces species are known to be found in a variety of natural sources, such as soil, rivers, dairy products and marine sediments, and even in humidifiers of air-conditioning systems (Lacey & Cross, 1989).

Despite confusing taxonomic history and their importance as pathogens, it is surprising that useful taxonomic methods being recently used, such as phylogenetic analysis based on 16S rDNA sequences, have not been applied to the genus Thermoactinomyces. The 16S rRNA sequences of three Thermoactinomyces species, Thermoactinomyces vulgaris, Thermoactinomyces candidus and Thermoactinomyces dichotomicus, were previously determined, but these sequences, especially 16S rRNA sequence of Thermoactinomyces dichotomicus, may contain many unreadable nucleotides and are therefore of little value. The aim of this study was to examine the 16S rDNA nucleotide sequences as one useful taxonomic marker for systematic study of the genus Thermoactinomyces. These sequences were thought to be very useful for inferring phylogenetic relationships between Thermoactinomyces species as well as between the genus Thermoactinomyces and other related genera.

**METHODS**

**Bacterial strains.** Table 1 summarizes the strains used in this study and the GenBank accession numbers for the 16S rDNA sequences. All strains, except Thermoactinomyces dichotomicus, Thermoactinomyces intermedius, Thermoactinomyces peptonophilus and Thermoactinomyces glaucus, were grown in shake flasks containing CYC broth (Cross & Attwell, 1974). Thermoactinomyces intermedius was grown in shake flasks containing Trypticase Soy Broth and Thermoactinomyces dichotomicus and Thermoactinomyces glaucus were grown in broth medium containing 0.1% yeast extract, 0.1% beef extract, 0.2% N-Z amine (type A) and 1% sucrose (pH 7.3). Thermoactinomyces peptonophilus was grown in broth medium containing 1.5% starch, 1% yeast extract and 0.05% MgSO_4_ in tap water (pH 7.6). All strains, except Thermoactinomyces peptonophilus, were grown at suitable temperatures between 45 and 55 °C. Thermoactinomyces peptonophilus was grown at 35 °C. The broth cultures were checked for purity before they were harvested by centrifugation.

**Chromosomal DNA isolation.** Chromosomal DNAs were isolated by the method described previously (Yoon et al., 1996).

**16S rDNA sequencing and phylogenetic analysis.** 16S rDNA sequencing was performed as described previously (Yoon et al., 1996). However, forward primer 373F (5’TATGGCCGGCAAGCCGTGAT-3’) positions 373–390 in Escherichia coli 16S rDNA numbering) was replaced by reverse primer 704R (5’-TCTRCGNATTTCACCNCTAC-3’; positions 704 to 685 in E. coli 16S rDNA numbering). In some cases sequencing reactions were performed with dITP from the DNA sequencing kit (Amersham) or the SequiTherm EXCEL II DNA sequencing kit (Epicentre Technologies) to relieve compression artefacts.

The 16S rDNA sequences determined were aligned with 16S rDNA gene sequences of other strains by using CLUSTAL w software (Thompson et al., 1994). Reference sequences were obtained from the GenBank database with the following accession numbers: X67148 (Atopobium minutum NCDO 2751), X60640 (Bacteriols stearothermophilus NCDO 1768), X60646 (Bacillus subtilis NCDO 1769), V00348 (E. coli), AB007908 (Lactobacillus delbrueckii JCM 1002), M58837 (Lactococcus lactis ATCC 19435), X60632 (Paenibacillus polymyxa NCDO 1774), Z38003 (Saccharomonospora viridica DSM 4379), Z38005 (Saccharomonospora viridica DSM 4379), AB007921 (Streptococcus pyogenes ATCC 12344) and AF002262 (Thermomonospora curtata JCM 3096). Gaps at the 5’ and 3’ ends of the alignment were omitted from further analysis. Evolutionary-distance matrices were calculated by using the algorithm of Jukes & Cantor (1969) with the DNADIST program within the PHYLIP package (Felsenstein, 1993). A phylogenetic tree was con-
Phylogeny of the genus *Thermoactinomyces*

**Table 1.** *Thermoactinomyces* species and strains used in this study and accession numbers of 16S rDNA sequences

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Thermoactinomyces candidas</em></td>
<td>KCTC 9557T</td>
<td>AF138732</td>
</tr>
<tr>
<td><em>Thermoactinomyces dichotomicus</em></td>
<td>KCTC 3667T (=JCM 9688T)</td>
<td>AF138733</td>
</tr>
<tr>
<td><em>Thermoactinomyces intermedium</em></td>
<td>KCTC 9646T (=IFO 14230T)</td>
<td>AF138734</td>
</tr>
<tr>
<td><em>Thermoactinomyces peptonophilus</em></td>
<td>KCTC 9740T (=ATCC 27302T)</td>
<td>AF138735</td>
</tr>
<tr>
<td><em>Thermoactinomyces putidus</em></td>
<td>KCTC 3666T (=JCM 8091T)</td>
<td>AF138736</td>
</tr>
<tr>
<td><em>Thermoactinomyces sacchari</em></td>
<td>KCTC 9790T (=CCRC 13341T)</td>
<td>AF138737</td>
</tr>
<tr>
<td><em>Thermoactinomyces thalophilus</em></td>
<td>KCTC 9789T (=CCRC 12549T)</td>
<td>AF138738</td>
</tr>
<tr>
<td><em>Thermoactinomyces vulgaris</em></td>
<td>KCTC 9076T</td>
<td>AF138739</td>
</tr>
<tr>
<td>‘<em>Thermoactinomyces glaucus</em>’</td>
<td>KCTC 9645 (=IFO 12530)</td>
<td>AF138789</td>
</tr>
<tr>
<td>‘<em>Thermoactinomyces monosporus</em>’</td>
<td>KCTC 3673 (=IFO 14050)</td>
<td>AF139880</td>
</tr>
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</table>

The phylogenetic tree was constructed using 16S rDNA sequences of two invalid *Thermoactinomyces* species, ‘*Thermoactinomyces glaucus*’ KCTC 9645 and ‘*Thermoactinomyces monosporus*’ KCTC 3673, were also compared with those of other *Thermoactinomyces* species. ‘*Thermoactinomyces glaucus*’ KCTC 9645 and ‘*Thermoactinomyces monosporus*’ KCTC 3673 had only a 1 bp sequence difference in their 16S rDNA sequences, but they exhibited very low 16S rDNA similarity values (less than 83%) with the type strains of validly described *Thermoactinomyces* species.

Almost complete 16S rDNA sequences of 10 species attributed to the genus *Thermoactinomyces* were determined. The 16S rDNAs were amplified by PCR and non-phosphorylated strands of PCR products, whose 5′-phosphorylated strands were selectively digested by λ-exonuclease, were used as templates for sequencing. The 16S rDNA sequences determined in this study correspond to the region between positions 28 and 1524 by comparison with *E. coli* 16S rRNA.

The levels of 16S rDNA similarity between the type strains of validly described *Thermoactinomyces* species were very broad, ranging from 90.8% to 100%. High levels of 16S rDNA similarity were found between some *Thermoactinomyces* species (Fig. 1). In particular, *Thermoactinomyces vulgaris* KCTC 9076T and *Thermoactinomyces candidas* KCTC 9557T shared identical 16S rDNA sequences. The two species were shown to have 22 bp sequence differences and 10 gaps, except ambiguous nucleotides, between their 16S rRNA sequences determined previously. *Thermoactinomyces intermedium* KCTC 9646T also exhibited relatively high 16S rDNA similarity value of 99.4% with *Thermoactinomyces vulgaris* KCTC 9076T and *Thermoactinomyces candidas* KCTC 9557T. *Thermoactinomyces sacchari* KCTC 9790T and *Thermoactinomyces thalophilus* KCTC 9789T had the same 16S rDNA sequences, except a single position corresponding to one ambiguous nucleotide (C or T) of *Thermoactinomyces sacchari* KCTC 9790T. *Thermoactinomyces peptonophilus* KCTC 9740T exhibited the lowest levels of 16S rDNA similarity (90.8–91.8%) with other validly described *Thermoactinomyces* species. This phylogenetic distinctiveness of *Thermoactinomyces peptonophilus* KCTC 9740T may have been guessed, considering that *Thermoactinomyces peptonophilus* KCTC 9740T has some physiological characteristics different from those of other *Thermoactinomyces* species (Lacey & Cross, 1989). The 16S rDNA sequences of two invalid *Thermoactinomyces* species, ‘*Thermoactinomyces glaucus*’ KCTC 9645 and ‘*Thermoactinomyces monosporus*’ KCTC 3673 had only a 1 bp sequence difference in their 16S rDNA sequences, but they exhibited very low 16S rDNA similarity values (less than 83%) with the type strains of validly described *Thermoactinomyces* species. The phylogenetic analysis showed that ‘*Thermoactinomyces glaucus*’ KCTC 9645 and ‘*Thermoactinomyces monosporus*’ KCTC 3673 cannot be members of the genus *Thermoactinomyces* (Fig. 1). ‘*Thermoactinomyces glaucus*’ KCTC 9645 and ‘*Thermoactinomyces monosporus*’ KCTC 3673 exhibited the highest 16S rDNA similarity values with the genus *Saccharomonospora*, especially with *Saccharomonospora glauca*. The 16S rDNAs of ‘*Thermoactinomyces glaucus*’ KCTC 9645 and ‘*Thermoactinomyces monosporus*’ KCTC 3673 showed only 1 bp and 2 bp sequence differences, respectively, with 16S rDNA of the type strain of *Saccharomonospora glauca*. The phylogenetic tree was constructed using 16S rDNA/16S rRNA sequences of *Thermoactinomyces* species determined, the representatives of the family *Bacillaceae*, some related taxa and some actinomycete species (Fig. 1). The phylogenetic tree showed that the type strains of validly described *Thermoactinomyces* species form a distinct radiation of the cluster encompassed by the genus *Thermoactinomyces* (Fig. 1). The tree indicates...
that the genus *Thermoactinomyces* are much more phylogenetically related to the family *Bacillaceae* than to the actinomycetes, as shown in previous studies (Stackebrandt & Woese, 1981; Park *et al*., 1993).

**DISCUSSION**

A phylogenetic study based on 16S rDNA sequences, together with chemotaxonomic and genomic analyses, is one of the most useful methods for inferring the relationships between genera or between species belonging to a genus (Vandamme *et al*., 1996). However, the genus *Thermoactinomyces* has scarcely been subjected to these methods and most species belonging to this genus have been characterized by mainly relying on morphological and physiological properties (Lacey & Cross, 1989). Accordingly, 16S rDNA sequences of the type strains of all valid species assigned to the genus *Thermoactinomyces* were determined and phylogenetically analysed in the present study. Our data confirmed previous findings that the genus *Thermoactinomyces* is phylogenetically related not to the actinomycetes but to the family *Bacillaceae* (Stackebrandt & Woese, 1981; Park *et al*., 1993). This study also showed the interspecific phylogenetic relationships of the genus *Thermoactinomyces* based on 16S rDNA sequences that were not revealed previously. Some species were found to be closely related by having high levels of 16S rDNA similarity between them, and some species exhibited relatively low levels of 16S rDNA similarity with other *Thermoactinomyces* species (Fig. 1).

![Fig. 1](image)

**Fig. 1.** Phylogenetic tree showing the positions of *Thermoactinomyces* species and representatives of some other taxa based on 16S rDNA sequences. The scale bar represents 1 nucleotide substitution per 100 nucleotides. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at the branch points. NCDO, National collection of dairy organisms, Reading, UK.
closely related and, particularly, 16S rDNA sequences of *Thermoactinomyces vulgaris* KCTC 9076T and *Thermoactinomyces candidus* KCTC 9557T were the same (Fig. 1). Since *Thermoactinomyces candidus* KCTC 9557T was distinguished from *Thermoactinomyces vulgaris* KCTC 9076T by differences of some physiological properties, it was proposed as a new species of the genus *Thermoactinomyces* (Kurup et al., 1975). However, *Thermoactinomyces candidus* was regarded as a synonym of *Thermoactinomyces vulgaris* in *Bergey’s Manual of Systematic Bacteriology* (Lacey & Cross, 1989) and, therefore, not listed in the manual but the thought has not been accepted. A very close phylogenetic relationship was also found between *Thermoactinomyces thalpophilus* KCTC 9789T and *Thermoactinomyces sacchari* KCTC 9790T, which show a 16S rDNA similarity value of almost 100% (Fig. 1). DNA–DNA relatedness is now recognized as being the most important criterion for defining species in current bacteriology (Wayne et al., 1987; Vandamme et al., 1996). The current phylogenetic definition of a species states that strains with approximately 70% or greater DNA–DNA relatedness are members of the same species (Wayne et al., 1987). From the results of 16S rDNA sequence analysis, DNA–DNA relatedness test is likely to be necessary for determining exact taxonomic relationships between some *Thermoactinomyces* species. It is apparent that *Thermoactinomyces dichotomicus* KCTC 3667T and *Thermoactinomyces peptonophilus* KCTC 9740T need not necessarily be subjected to a DNA–DNA relatedness test, because they show levels of nucleotide similarity that are low enough for them to be placed as distinct species within the genus *Thermoactinomyces* (Stackebrandt & Goebel, 1994). Two invalid species, *Thermoactinomyces glaucus* KCTC 9645 and *Thermoactinomyces monosporus* KCTC 3673, exhibited their closest phylogenetic affinities not to the genus *Thermoactinomyces* but to the genus *Saccharomonospora*, especially *Saccharomonospora glauca*. However, chemotaxonomic characterizations are also necessary to finally confirm the reclassification of the two species to the genus *Saccharomonospora*, since the genera *Thermoactinomyces* and *Saccharomonospora* are different in some chemotaxonomic properties such as predominant menaquinone profile and wall chemotype.

The results of the phylogenetic analysis exhibit some correlation with some physiological properties and predominant menaquinone profiles shown in the study of Tseng et al. (1990). The cluster containing *Thermoactinomyces vulgaris* KCTC 9076T, *Thermoactinomyces candidus* KCTC 9557T and *Thermoactinomyces intermedius* KCTC 9646T and the cluster containing *Thermoactinomyces putidus* KCTC 3666T, *Thermoactinomyces sacchari* KCTC 9790T and *Thermoactinomyces thalpophilus* KCTC 9789T show different predominant menaquinone profiles. The type strains of *Thermoactinomyces vulgaris*, *Thermoactinomyces candidus* and *Thermoactinomyces intermedius* were shown to contain MK-7 as the predominant menaquinones (Tseng et al., 1990). The type strains of *Thermoactinomyces sacchari* and *Thermoactinomyces thalpophilus* were shown to contain MK-8 and MK-9 as the predominant menaquinones (Tseng et al., 1990). The predominant menaquinone profile for the type strain of *Thermoactinomyces putidus* was not shown but *Thermoactinomyces putidus* JCM 3213 has MK-8 and MK-9 as the predominant menaquinones (Tseng et al., 1990). However, it should be considered that the study of Collins et al. (1982) showed different menaquinone profiles from those shown in the study of Tseng et al. (1990) for some *Thermoactinomyces* species. *Thermoactinomyces dichotomicus* can be distinguished from other *Thermoactinomyces* species by its morphological property of forming yellow to orange colonies. The predominant menaquinone profile of the type strain of *Thermoactinomyces dichotomicus* was MK-7 in study of Collins et al. (1982) but was not shown in study of Tseng et al. (1990). The type strain of *Thermoactinomyces peptonophilus* forms a line of descent distinct from other *Thermoactinomyces* (Fig. 1). It is mesophiile, unlike other *Thermoactinomyces* species, and has some physiological properties distinguishable from other *Thermoactinomyces* species (Lacey & Cross, 1989; Nonomura & Ohara, 1971). However, little is known about the chemotaxonomic properties, including the menaquinone profile, of *Thermoactinomyces peptonophilus* that may be necessary for investigating the taxonomic relationships with other *Thermoactinomyces* species. From the results of the phylogenetic analysis, together with morphological and physiological properties and predominant menaquinone profiles, it is supposed that the genus *Thermoactinomyces* may be heterogeneous group containing more than one genus. To solve this question, a comparative taxonomic study using additional phenotypic markers, especially chemotaxonomic markers, should be performed in the genus *Thermoactinomyces*.

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REFERENCES


