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

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The almost complete 16S rDNA sequences of the type strains of eight validly described species and two invalid species of the genus *Thermoactinomyces* were determined and phylogenetically analysed. 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

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 analysed. 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.

*Thermoactinomyces* species are aerobic, Gram-positive and thermotolerant, with the exception of one mesophilic species, *Thermoactinomyces peptonophilis* (Nonomura & Ohara, 1971). The genus *Thermoactinomyces* contains *meso*-diaminopimelic acid but no diagnostic sugars in the cell wall (Lacey & Cross, 1989), indicating that the wall chemotype is type III (Lechevalier & Lechevalier, 1970). Differences in the isoprenoid quinone profile of the genus *Thermoactinomyces* were seen between the studies of Collins et al. (1982) and Tseng et al. (1990). Collins et al. (1982) showed that this genus has unsaturated menaquinones with seven or nine isoprene units (MK-7 or MK-9) as the predominant menaquinones but Tseng et al. (1990) found unsaturated menaquinones with seven, or eight and nine isoprene units (MK-7 or MK-8 and MK-9) as the predominant menaquinones. The genus *Thermoactinomyces* has a cellular fatty acid profile containing major amounts of iso- and anteiso-branched fatty acids.

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 Thermoa
toactinomyces species, namely Thermoa
toactinomyces candidus (Kurup et al., 1975), Thermoa
toactinomyces dichotomius (Krasil’nikov & Agre, 1964; Cross & Goodfellow, 1973), Thermoa
toactinomyces intermedius (Kurup et al., 1980), Thermoa
toactinomyces peptonophilus (Nonomura & Ohara, 1971), Thermoa
toactinomyces putidus (Lacey & Cross, 1989), Thermoa
toactinomyces sacchari (Lacey, 1971), Thermoa
toactinomyces thalpophilus (Waksman & Corke, 1953; Unsworth & Cross, 1980) and Thermoa
toactinomyces vulgaris (Tsiklinesky, 1899). Before including the eight valid species, the genus Thermoa
toactinomyces had very confusing taxonomic history (Lacey & Cross, 1989). In addition to the eight valid Thermoa
toactinomyces species, some species, such as ‘Thermoa
toactinomyces glaucus’ (Henssen, 1957), ‘Thermoa
toactinomyces monosporus’ (Waksman & Corke, 1953), ‘Thermoa
toactinomyces thalpophilus’ (Waksman, 1961), ‘Thermoa
toactinomyces antibioticus’ (Craveri et al., 1964) and ‘Thermoa
toactinomyces abius’ (Lacey & Cross, 1989), were known but have not been validly described. ‘Thermoa
toactinomyces antibioticus’ and ‘Thermoa
toactinomyces abius’ were described to be synonyms of Thermoa
toactinomyces thalpophilus and Thermoa
toactinomyces vulgaris, respectively (Lacey & Cross, 1989). Strain(s) of ‘Thermoa
toactinomyces thalpophilus’ are no longer available from the culture collections. The type strains of ‘Thermoa
toactinomyces glaucus’ and ‘Thermoa
toactinomyces monosporus’ are no longer available. However, one strain of ‘Thermoa
toactinomyces glaucus’ and one strain of ‘Thermoa
toactinomyces monosporus’, which are found in catalogues of some culture collections, were those described by Fergus (1964) and Nonomura & Ohara (1969), respectively.

Thermoa
toactinomyces species have also been noticed due to their pathogenicity. They have been implicated as causal agents in various forms of hypersensitivity pneumonia (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 Thermoa
toactinomyces species are known to be most abundant. However, Thermoa
toactinomyces 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 Thermoa
toactinomyces. The 16S rRNA sequences of three Thermoa
toactinomyces species, Thermoa
toactinomyces vulgaris, Thermoa
toactinomyces candidus and Thermoa
toactinomyces dichotomius, were previously determined, but these sequences, especially 16S rRNA sequence of Thermoa
toactinomyces 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 Thermoa
toactinomyces. These sequences were thought to be very useful for inferring phylogenetic relationships between Thermoa
toactinomyces species as well as between the genus Thermoa
toactinomyces 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 Thermoa
toactinomyces dichotomius, Thermoa
toactinomyces intermedius, Thermoa
toactinomyces peptonophilus and ‘Thermoa
toactinomyces glaucus’, were grown in shake flasks containing CYC broth (Cross & Attwell, 1974). Thermoa
toactinomyces intermedius was grown in shake flasks containing Trypticase Soy Broth and Thermoa
toactinomyces dichotomius and ‘Thermoa
toactinomyces 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). Thermoa
toactinomyces 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 Thermoa
toactinomyces peptonophilus, were grown at suitable temperatures between 45 and 55 °C. Thermoa
toactinomyces 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., 1998). However, forward primer 373F (5' - AATGGG-CCGACAGCTTGT-3' ; positions 373–390 in Escherichia coli 16S rRNA numbering) was replaced by reverse primer 704R (5' - TCTRCGNNATTTCCACCTAC-3' ; positions 704 to 685 in E. coli 16S rRNA numbering). In some cases sequencing reactions were performed with dITP from the DNA sequencing kit (Amersham) or the SequiTherm EXCEL II DNA sequencing kit (Epitcience Technologies) to relieve compression artefacts.

The 16S rDNA sequences determined were aligned with 16S rRNA 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 NCDB 2751), X60640 (Bacillus steatherophilus NCDO 1768), X60646 (Bacillus subtilis NCDO 1769), V00348 (E. coli), AB007908 (Lactobacillus delbrueckii JCM 1002), M58837 (Lactococcus lactis ATCC 19435), X60632 (Paeni
cacillus polymyxa NCDO 1774), Z38003 (Saccharomonospora virida DSM 4370), Z38004 (Saccharomonospora virida DSM 4370), AB002521 (Streptococcus pyogenes ATCC 12344) and AF002262 (Thermomonospora curvata 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 candidus</em></td>
<td>KCTC 9557(^T)</td>
<td>AF138732</td>
</tr>
<tr>
<td><em>Thermoactinomyces dichotomicus</em></td>
<td>KCTC 3667(^T) (=JCM 9688(^T))</td>
<td>AF138733</td>
</tr>
<tr>
<td><em>Thermoactinomyces intermedius</em></td>
<td>KCTC 9646(^T) (=IFO 14230(^T))</td>
<td>AF138734</td>
</tr>
<tr>
<td><em>Thermoactinomyces peptonophilus</em></td>
<td>KCTC 9740(^T) (=ATCC 27302(^T))</td>
<td>AF138735</td>
</tr>
<tr>
<td><em>Thermoactinomyces putidus</em></td>
<td>KCTC 3666(^T) (=JCM 8091(^T))</td>
<td>AF138736</td>
</tr>
<tr>
<td><em>Thermoactinomyces sacchari</em></td>
<td>KCTC 9790(^T) (=CCRC 13341(^T))</td>
<td>AF138737</td>
</tr>
<tr>
<td><em>Thermoactinomyces thalophilus</em></td>
<td>KCTC 9789(^T) (=CCRC 12549(^T))</td>
<td>AF138738</td>
</tr>
<tr>
<td><em>Thermoactinomyces vulgaris</em></td>
<td>KCTC 9076(^T)</td>
<td>AF138739</td>
</tr>
<tr>
<td>‘<em>Thermoactinomyces glaucus</em>’</td>
<td>KCTC 9645 (=IFO 12530)</td>
<td>AF138979</td>
</tr>
<tr>
<td>‘<em>Thermoactinomyces monosporus</em>’</td>
<td>KCTC 3673 (=IFO 14050)</td>
<td>AF139880</td>
</tr>
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</table>

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 9076\(^T\) and *Thermoactinomyces candidus* KCTC 9557\(^T\) 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 intermedius* KCTC 9646\(^T\) also exhibited relatively high 16S rDNA similarity value of 99.4% with *Thermoactinomyces vulgaris* KCTC 9076\(^T\) and *Thermoactinomyces candidus* KCTC 9557\(^T\). *Thermoactinomyces sacchari* KCTC 9790\(^T\) and *Thermoactinomyces thalophilus* KCTC 9789\(^T\) had the same 16S rDNA sequences, except a single position corresponding to one ambiguous nucleotide (C or T) of *Thermoactinomyces sacchari* KCTC 9790\(^T\). *Thermoactinomyces peptonophilus* KCTC 9740\(^T\) 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 9740\(^T\) may have been guessed, considering that *Thermoactinomyces peptonophilus* KCTC 9740\(^T\) 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, 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. 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).

Based on the results of 16S rDNA sequence analysis, *Thermoactinomyces vulgaris* KCTC 9076^T*, *Thermoactinomyces candidus* KCTC 9557^T*, *Thermoactinomyces intermedius* KCTC 9646^T*, *Thermoactinomyces sacchari* KCTC 9790^T*, *Thermoactinomyces thalpophilus* KCTC 9789^T*, *Thermoactinomyces putidus* KCTC 3666^T*, *Thermoactinomyces dichotomicus* KCTC 3667^T*, *Thermoactinomyces peptonophilus* KCTC 9740^T*, *Bacillus subtilis* NCDO 1769^T*, *Bacillus stearothermophilus* NCDO 1768^T*, *Pae nibacillus polymyxa* NCDO 1774^T*, *Streptococcus pyogenes* ATCC 12344^T*, *Lactococcus lactis* ATCC 19435^T*, *Lactobacillus delbrueckii* ICM 1002^T*, *Saccharomonospora glauca* DSM 43769^T*, *Saccharomonospora viridis* NCIMB 9602^T*, *Thermomonospora curvata* ICM 3096^T*, *Atopobium minutum* NCDO 2751^T*, and *Escherichia coli*.

*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 9076<sup>T</sup> and *Thermoactinomyces candidus* KCTC 9557<sup>T</sup> were the same (Fig. 1). Since *Thermoactinomyces candidus* KCTC 9557<sup>T</sup> was distinguished from *Thermoactinomyces vulgaris* KCTC 9076<sup>T</sup> 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 9789<sup>T</sup> and *Thermoactinomyces sacchari* KCTC 9790<sup>T</sup>, 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).

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 9076<sup>T</sup>, *Thermoactinomyces candidus* KCTC 9557<sup>T</sup> and *Thermoactinomyces intermedius* KCTC 9646<sup>T</sup> and the cluster containing *Thermoactinomyces putidus* KCTC 3666<sup>T</sup>, *Thermoactinomyces sacchari* KCTC 9790<sup>T</sup> and *Thermoactinomyces thalpophilus* KCTC 9789<sup>T</sup> 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* species (Fig. 1). It is mesophilic, 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**


