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 9076^T and *Thermoactinomyces candidus* KCTC 9557^T had the same 16S rDNA sequences and *Thermoactinomyces thalpophilus* KCTC 9789^T and *Thermoactinomyces sacchari* KCTC 9790^T 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). Park *et al.* (1993) also showed that the type strain of *Thermoactinomyces vulgaris* is phylogenetically related to the genus *Bacillus* based on 5S rRNA sequences. Accordingly, it has been

proposed that genus *Thermoactinomyces* should be placed within the family *Bacillaceae* (Stackebrandt & Woese, 1981; Park *et al.*, 1993). Nevertheless, the morphological characteristics of producing aerial and substrate mycelia have led to classification of the genus *Thermoactinomyces* becoming confused.

Thermoactinomyces species are aerobic, Gram-positive and thermotolerant, with the exception of one mesophilic species, *Thermoactinomyces peptonophilus* (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

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 & Agre, 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; Unsworth & Cross, 1980) and *Thermoactinomyces vulgaris* (Tsiklinesky, 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 thermophilus*' (Waksman, 1961), '*Thermoactinomyces antibioticus*' (Craveri *et al.*, 1964) and '*Thermoactinomyces albus*' (Lacey & Cross, 1989), were known but have not been validly described. '*Thermoactinomyces antibioticus*' and '*Thermoactinomyces albus*' 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.*, 1998). However, forward primer 373F (5'-AATGGG-CGCAAGCCTGAT-3'; positions 373–390 in *Escherichia coli* 16S rRNA numbering) was replaced by reverse primer 704R (5'-TCTRCGNATTTCCACCNTAC-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 EX-CEL II DNA sequencing kit (Epicentre 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* NCFB 2751^T), X60640 (*Bacillus stearothermophilus* NCDO 1768^T), X60646 (*Bacillus subtilis* NCDO 1769^T), V00348 (*E. coli*), AB007908 (*Lactobacillus delbrueckii* JCM 1002^T), M58837 (*Lactococcus lactis* ATCC 19435^T), X60632 (*Paenibacillus polymyxa* NCDO 1774^T), Z38003 (*Saccharomonospora glauca* DSM 43769^T), Z38007 (*Saccharomonospora viridis* NCIMB 9602^T), AB002521 (*Streptococcus pyogenes* ATCC 12344^T) and AF002262 (*Thermomonospora curvata* JCM 3096^T). 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-

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

KCTC, Korean Collection for Type Cultures, Taejeon, Korea; JCM, Japan Collection of Microorganisms, Institute of Physical and Chemical Research, Saitama, Japan; IFO, Institute for Fermentation, Osaka, Japan; ATCC, American Type Culture Collection, Manassas, VA, USA; CCRC, Culture Collection and Research Center, Taiwan.

Species	Strain	Accession no.
<i>Thermoactinomyces candidus</i>	KCTC 9557 ^T	AF138732
<i>Thermoactinomyces dichotomicus</i>	KCTC 3667 ^T (= JCM 9688 ^T)	AF138733
<i>Thermoactinomyces intermedius</i>	KCTC 9646 ^T (= IFO 14230 ^T)	AF138734
<i>Thermoactinomyces peptonophilus</i>	KCTC 9740 ^T (= ATCC 27302 ^T)	AF138735
<i>Thermoactinomyces putidus</i>	KCTC 3666 ^T (= JCM 8091 ^T)	AF138736
<i>Thermoactinomyces sacchari</i>	KCTC 9790 ^T (= CCRC 13341 ^T)	AF138737
<i>Thermoactinomyces thalpophilus</i>	KCTC 9789 ^T (= CCRC 12549 ^T)	AF138738
<i>Thermoactinomyces vulgaris</i>	KCTC 9076 ^T	AF138739
' <i>Thermoactinomyces glaucus</i> '	KCTC 9645 (= IFO 12530)	AF139879
' <i>Thermoactinomyces monosporus</i> '	KCTC 3673 (= IFO 14050)	AF139880

structured by using the neighbour-joining method (Saitou & Nei, 1987) as implemented within the NEIGHBOR program of the PHYLIP package. The stability of relationships was assessed by a bootstrap analysis of 1000 data sets by using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE of the PHYLIP package.

RESULTS

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 thalpophilus* 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

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.

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 and *Thermoactinomyces intermedius* KCTC 9646^T are shown to be

closely related and, particularly, 16S rDNA sequences of *Thermoactinomyces vulgaris* KCTC 9076^T and *Thermoactinomyces candidus* KCTC 9557^T were the same (Fig. 1). Since *Thermoactinomyces candidus* KCTC 9557^T was distinguished from *Thermoactinomyces vulgaris* KCTC 9076^T 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^T and *Thermoactinomyces sacchari* KCTC 9790^T, 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 3667^T and *Thermoactinomyces peptonophilus* KCTC 9740^T 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 9076^T, *Thermoactinomyces candidus* KCTC 9557^T and *Thermoactinomyces intermedius* KCTC 9646^T and the cluster containing *Thermoactinomyces putidus* KCTC 3666^T, *Thermoactinomyces sacchari* KCTC 9790^T and *Thermoactinomyces thalpophilus* KCTC 9789^T 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 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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