NOTE

**DNA–DNA relatedness among
Thermoactinomyces species:
Thermoactinomyces candidus as a synonym of
Thermoactinomyces vulgaris and
Thermoactinomyces thalpophilus as a synonym
of *Thermoactinomyces sacchari***

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DNA–DNA relatedness of all validly described *Thermoactinomyces* species was determined to infer the genetic relationships between them. The levels of DNA–DNA relatedness among the type strains of *Thermoactinomyces* species ranged from 2.5 to 92.8%. Based on DNA relatedness data, the type strains of *Thermoactinomyces intermedius*, *Thermoactinomyces putidus*, *Thermoactinomyces dichotomicus* and *Thermoactinomyces peptonophilus* were considered to be distinct species of the genus *Thermoactinomyces*. However, the relationship between the type strains of *Thermoactinomyces vulgaris* and *Thermoactinomyces candidus* and the relationship between the type strains of *Thermoactinomyces sacchari* and *Thermoactinomyces thalpophilus* were re-evaluated from levels of DNA–DNA relatedness. The independent DNA relatedness values between *Thermoactinomyces vulgaris* KCTC 9076^T and *Thermoactinomyces candidus* KCTC 9557^T were 90.8 and 92.8%. *Thermoactinomyces thalpophilus* KCTC 9789^T and *Thermoactinomyces sacchari* KCTC 9790^T exhibited independent values of 85.6 and 87.3%. Accordingly, on the basis of DNA–DNA relatedness data together with 16S rDNA sequence data determined recently, it is proposed that *Thermoactinomyces candidus* should be considered as a synonym of *Thermoactinomyces vulgaris* and *Thermoactinomyces thalpophilus* be considered as a synonym of *Thermoactinomyces sacchari*.

Keywords: *Thermoactinomyces* species, DNA–DNA relatedness, taxonomic re-evaluation

Thermoactinomyces species are taxonomically very interesting taxa. Morphological properties of forming aerial and substrate mycelia led *Thermoactinomyces* species to be classified in actinomycetes. However, taxonomic data, such as the ability to produce dipicolinic-acid-containing endospores (Cross *et al.*, 1968; Lacey & Vince, 1971), low G + C content (Lacey & Cross, 1989), menaquinone profiles (Collins *et al.*, 1982; Tseng *et al.*, 1990) and 5S rRNA (Park *et al.*, 1993) and 16S rRNA oligonucleotide sequences (Stackebrandt & Woese, 1981), suggested that *Thermoactinomyces* species should no longer be classified within the order *Actinomycetales* and should be placed within the family *Bacillaceae*. Recently, our phylo-

genetic inference based on 16S rDNA sequences showed clearly that *Thermoactinomyces* species are phylogenetically related to the family *Bacillaceae* (Yoon & Park, 2000). 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; Lacey & Cross, 1989) and *Thermoactinomyces*

vulgaris (Tsiklinsky, 1899), the type species of the genus. However, these species have mainly been differentiated by morphological and physiological characteristics (Lacey & Cross, 1989). The 16S rDNA sequence analysis showed that some *Thermoactinomyces* species are phylogenetically closely related and may have to be taxonomically re-evaluated (Yoon & Park, 2000). In particular, *Thermoactinomyces vulgaris* KCTC 9076^T and *Thermoactinomyces candidus* KCTC 9557^T shared identical 16S rDNA sequences. *Thermoactinomyces intermedius* KCTC 9646^T also exhibited relatively high 16S rDNA similarity values 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 position corresponding to one ambiguous nucleotide (C or T) of *Thermoactinomyces sacchari* KCTC 9790^T. In current bacterial systematics, DNA–DNA relatedness data are now recognized as being the most important for inferring the relationships between species belonging to a genus (Wayne *et al.*, 1987), together with phylogenetic inference based on 16S rDNA sequence comparison. The current phylogenetic definition of a species states that strains having approximately 70% or greater DNA similarity are members of the same species; that is, there is general agreement that a level of DNA–DNA relatedness of 70% is the threshold value for defining species (Wayne *et al.*, 1987). Therefore, the aims of this study were to determine the levels of DNA–DNA relatedness among validly described *Thermoactinomyces* species and to investigate with the result of DNA–DNA relatedness, together with the result of 16S rDNA sequence analysis, whether current taxonomic status of *Thermoactinomyces* species is correct or not.

The following type strains of the eight validly described *Thermoactinomyces* species were used for this study: *Thermoactinomyces candidus* KCTC 9557^T, *Thermoactinomyces dichotomicus* KCTC 3667^T, *Thermoactinomyces intermedius* KCTC 9646^T, *Thermoactinomyces peptonophilus* KCTC 9740^T, *Thermoactinomyces putidus* KCTC 3666^T, *Thermoactinomyces sacchari* KCTC 9790^T, *Thermoactinomyces thalpophilus* KCTC 9789^T and *Thermoactinomyces vulgaris* KCTC 9076^T. Chromosomal DNAs were isolated as described previously (Yoon *et al.*, 1996), with the exception that ribonuclease T1 was used together with ribonuclease A. DNA–DNA hybridization to determine genomic relatedness was performed fluorometrically by the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and microdilution wells. Brief procedure is as follows: each 25 µl of purified DNA samples (200 µg ml⁻¹) was denatured by boiling for 5 min and then diluted to 10 µg ml⁻¹ with cold PBS containing 0.1 M MgCl₂. Each diluted DNA suspension was distributed to 5 wells in microplate (Nunc) at 100 µl per well and the plate incubated for 8 h at 30 °C. Excess DNA sus-

pensions were discarded and the plate was dried for 30 min at 45 °C. The plate was prehybridized for 10 min and then hybridized with biotinylated DNA at 45 °C overnight. The plate was washed three times with 1 × SSC and 100 µl streptavidin-D-galactosidase (Gibco-BRL) (1000-fold diluted with 0.5% BSA-PBS) was added to each well. The plate was incubated at 37 °C for 30 min and washed three times with 1 × SSC. Finally, 100 µl of 4-methylumbelliferyl-β-D-galactopyranoside (substrate 10 mg dimethylformamide ml⁻¹ diluted 100-fold with PBS) was added to the wells and the plate was incubated at 37 °C for 30 min. The fluorescence intensities were read with a Labsystems fluoroskan II at a wavelength of 360 nm for excitation and 450 nm for emission. The highest and lowest values in each sample were excluded and the remaining three values were used for calculation of similarity value. DNA relatedness values are the mean of three values.

The genus *Thermoactinomyces* has had a very confusing taxonomic history; it consists currently of eight validly described species. *Thermoactinomyces vulgaris* was the first proposed species of the genus *Thermoactinomyces* (Tsiklinsky, 1899), and prior to 1964, six *Thermoactinomyces* species had been described. Among them, three species, '*Thermoactinomyces glaucus*' (Henssen 1957), '*Thermoactinomyces thermophilus*' (Waksman 1961) and '*Thermoactinomyces monosporus*' (Waksman and Corke 1953), are *nomina dubia*. One species, *Thermoactinomyces viridis* (Schuurmans *et al.*, 1956), was reclassified in a new genus, *Saccharomonospora*, as *Saccharomonospora viridis* by Nonomura & Ohara (1971). Two other species, *Thermoactinomyces vulgaris* and *Thermoactinomyces thalpophilus*, were placed as synonyms by Küster & Locci (1964). There were only two species, *Thermoactinomyces vulgaris* and *Thermoactinomyces sacchari*, listed in 8th edition of *Bergey's Manual of Determinative Bacteriology* (Küster, 1974). Since then, there were many changes in the genus *Thermoactinomyces* including the transfer of *Actinobifida dichotomica* to *Thermoactinomyces dichotomicus* (Cross & Goodfellow, 1975), the description of *Thermoactinomyces candidus* (Kurup *et al.*, 1975), *Thermoactinomyces intermedius* (Kurup *et al.*, 1980), *Thermoactinomyces peptonophilus* (Nonomura & Ohara, 1971) and *Thermoactinomyces putidus* (Lacey & Cross, 1989) as new species and the revival of *Thermoactinomyces thalpophilus* (Lacey & Cross, 1989).

The inclusion of additional strains led to *Thermoactinomyces vulgaris* becoming a variable species (Flockton & Cross, 1975). This species acquired some characters that were not present in the original description of Tsiklinsky (1899). For example, the ability to utilize starch, which was previously not observed, is found in the description of *Thermoactinomyces vulgaris* of the 8th edition of the Manual (Küster, 1974). Kurup *et al.* (1975) classified the isolates lacking amylase as *Thermoactinomyces candidus* and the isolates producing amylase as *Thermo-*

Table 1. Levels of DNA–DNA relatedness among the type strains of *Thermoactinomyces* species

Species	1	2	3	4	5	6	7	8
1. <i>T. vulgaris</i> KCTC 9076 ^T	100	92.8	48.7	6.4	6.3	5.0	5.6	3.0
2. <i>T. candidus</i> KCTC 9557 ^T	90.8	100	49.5	4.3	5.1	3.7	5.0	2.8
3. <i>T. intermedius</i> KCTC 9646 ^T	47.4	51.2	100	3.9	4.4	3.8	4.8	2.5
4. <i>T. sacchari</i> KCTC 9790 ^T	3.4	3.7	4.2	100	85.6	7.7	4.4	2.7
5. <i>T. thalpophilus</i> KCTC 9789 ^T	4.6	4.0	4.7	87.3	100	13.5	4.0	2.8
6. <i>T. putidus</i> KCTC 3666 ^T	4.0	3.7	4.8	11.9	10.5	100	5.0	2.5
7. <i>T. dichotomicus</i> KCTC 3667 ^T	4.8	4.0	4.2	3.7	3.4	4.3	100	4.5
8. <i>T. peptonophilus</i> KCTC 9740 ^T	3.2	2.7	3.0	2.8	2.5	3.1	4.8	100

actinomyces vulgaris. The currently available type strain of *Thermoactinomyces vulgaris* was that isolated by Erikson (1953) as *Micromonospora vulgaris* strain D, which is listed as strain KCC A-0162 in the Approved Lists of Bacterial Names (Skerman *et al.*, 1980). This strain corresponded to the original concept of *Thermoactinomyces vulgaris* by Tsiklinsky (1899) and the concept of *Thermoactinomyces candidus*. The two species were hence regarded as synonyms by Lacey & Cross (1989), but this thought has not been accepted. Revival of *Thermoactinomyces thalpophilus* was with the isolates named as *Thermoactinomyces vulgaris* by Kurup *et al.* (1975). In addition, '*Thermoactinomyces antibioticus*' and '*Thermoactinomyces albus*' had been known, but they were described to be synonyms of *Thermoactinomyces thalpophilus* and *Thermoactinomyces vulgaris*, respectively (Lacey & Cross, 1989). It is described in the 1st edition of *Bergey's Manual of Systematic Bacteriology* that the taxonomic status of *Thermoactinomyces vulgaris*, *Thermoactinomyces candidus* and *Thermoactinomyces thalpophilus* is confused and there can be little doubt that the three species are closely related (Lacey & Cross, 1989). However, recent 16S rDNA sequence analysis showed a little different results (Yoon & Park, 2000). The type strain of *Thermoactinomyces candidus* was shown to be phylogenetically closely related to the type strain of *Thermoactinomyces vulgaris*, but *Thermoactinomyces thalpophilus* KCTC 9789^T is more closely related to *Thermoactinomyces sacchari* KCTC 9790^T rather than *Thermoactinomyces vulgaris* KCTC 9076^T.

The type strains of all validly described *Thermoactinomyces* species used in this study exhibited levels of DNA–DNA relatedness ranging from 2.5 to 92.8% (Table 1). On the basis of DNA–DNA relatedness values between *Thermoactinomyces* species obtained, the taxonomic status of *Thermoactinomyces* species was elucidated. As shown in Table 1, DNA–DNA relatedness between other *Thermoactinomyces* species, except for the relationships between *Thermoactinomyces vulgaris* and *Thermoactinomyces candidus* and between *Thermoactinomyces thalpophilus* and *Thermoactinomyces sacchari*, exhibited such values confirming their status as distinct species. It was proven from DNA–DNA relatedness values that *Thermoactino-*

myces intermedius is the species distinct from other *Thermoactinomyces* species as well as *Thermoactinomyces vulgaris* and *Thermoactinomyces candidus* that exhibit relatively high 16S rDNA similarities. Also, *Thermoactinomyces putidus* KCTC 3666^T exhibited DNA relatedness values below 13.5% to other *Thermoactinomyces* species, *Thermoactinomyces dichotomicus* KCTC 3667^T below 5.6% to other *Thermoactinomyces* species and *Thermoactinomyces peptonophilus* KCTC 9740^T below 4.5% to other *Thermoactinomyces* species (Table 1). *Thermoactinomyces dichotomicus* KCTC 3666^T and *Thermoactinomyces peptonophilus* KCTC 9740^T have already been found to exhibit such low levels of 16S rDNA similarity that they can be considered as distinct species of the genus *Thermoactinomyces* (Yoon & Park, 2000). *Thermoactinomyces dichotomicus* KCTC 3666^T exhibited levels of 16S rDNA similarity ranging from 90.8 to 94.8% (Table 1). Levels of 16S rDNA similarity between *Thermoactinomyces peptonophilus* KCTC 9740^T and the type strains of other *Thermoactinomyces* species were 90.8–91.8% (Table 1). According to the current species definition in bacterial systematics (Stackebrandt & Goebel, 1994; Wayne *et al.*, 1987), *Thermoactinomyces intermedius*, *Thermoactinomyces putidus*, *Thermoactinomyces dichotomicus* and *Thermoactinomyces peptonophilus* must be distinct species of the genus *Thermoactinomyces*. However, the relationships between *Thermoactinomyces vulgaris* and *Thermoactinomyces candidus* and between *Thermoactinomyces thalpophilus* and *Thermoactinomyces sacchari* should be taxonomically reconsidered. *Thermoactinomyces vulgaris* KCTC 9076^T and *Thermoactinomyces candidus* KCTC 9557^T revealed independent DNA–DNA relatedness values of 90.8 and 92.8%, which demonstrates that they are members of the same species (Wayne *et al.*, 1987). *Thermoactinomyces thalpophilus* KCTC 9789^T and *Thermoactinomyces sacchari* KCTC 9790^T revealed independent values of 85.6% and 87.3%, indicating that the two are also members of the same species (Wayne *et al.*, 1987). It has already been shown that *Thermoactinomyces vulgaris* KCTC 9076^T and *Thermoactinomyces candidus* KCTC 9557^T and *Thermoactinomyces thalpophilus* KCTC 9789^T and *Thermoactinomyces sacchari* KCTC 9790^T have

16S rDNA similarities of 100%, respectively. Accordingly, on the basis of DNA–DNA relatedness and the results of 16S rDNA sequence analysis, it is correct that *Thermoactinomyces vulgaris* and *Thermoactinomyces candidus* should be unified as one species and *Thermoactinomyces thalophilus* and *Thermoactinomyces sacchari* as one species. According to international rule in which the oldest legitimate epithet has the priority, we propose that *Thermoactinomyces candidus* should be considered as a synonym of *Thermoactinomyces vulgaris* and *Thermoactinomyces thalophilus* be considered as a synonym of *Thermoactinomyces sacchari*.

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