NOTE

Transfer of *Thermobacteroides leptospartum* and *Clostridium thermolacticum* as *Clostridium stercorarium* subsp. *leptospartum* subsp. nov., comb. nov. and *C. stercorarium* subsp. *thermolacticum* subsp. nov., comb. nov.

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16S rRNA sequencing and sequence analysis of the sole member of the genus *Thermobacteroides*, *Thermobacteroides leptospartum*, revealed that it was related to members of cluster III (according to the scheme of Collins *et al.* 1994) represented exclusively by cellulolytic *Clostridium* species. Phenotypic studies indicated that *Thermobacteroides leptospartum* was also able to grow on cellulose, providing further evidence of its affiliation to members of cluster III. Its closest phylogenetic relatives, *Clostridium thermolacticum* and *Clostridium stercorarium*, were almost equidistantly placed with a similarity value of 99%. DNA hybridization studies also indicated that *Thermobacteroides leptospartum*, *C. thermolacticum* and *C. stercorarium* were closely related to each other (values of over 95% homology). Similarities based on the comparison of the 16S rRNA gene sequences and DNA homology are sufficiently high to regard all three strains as subspecies of a single species. It is therefore proposed that *Thermobacteroides leptospartum* and *C. thermolacticum* be transferred to cluster III as *C. stercorarium* subsp. *leptospartum* subsp. nov., comb. nov. and *C. stercorarium* subsp. *thermolacticum* subsp. nov., comb. nov., respectively, thus automatically creating *C. stercorarium* subsp. *stercorarium* subsp. nov., comb. nov. The transfer of the sole member of *Thermobacteroides* invalidates the taxonomic status of the genus.

Keywords: *Thermobacteroides leptospartum*, *Clostridium thermolacticum*, *Clostridium stercorarium*, phylogeny, DNA homology

Several genera of thermoanaerobes including *Thermoanaerobium* (Zeikus *et al.*, 1979), *Thermoanaerobacter* (Wiegel & Ljungdahl, 1981), *Thermobacteroides* (Ben-Bassat & Zeikus, 1981) and *Fervidobacterium* (Patel *et al.*, 1985a) isolated in the early 1980s were proposed on the basis of their phenotypic characteristics alone. With the widespread application of rRNA sequencing and sequence analysis in phylogeny and taxonomy (Woese, 1987), extensive reorganization of the members of the genera *Thermoanaerobium* and *Thermoanaerobacter* was undertaken (Collins *et al.*, 1994;

Rainey *et al.*, 1993). This report evaluates the status of the genus *Thermobacteroides*, which was created in 1981 (Ben-Bassat & Zeikus, 1981) and was represented by *Thermobacteroides acetoethylicus* as the sole species. At that time, the creation of the new genus appeared to be justified from phenotypic characteristics, including the absence of an NADP-specific alcohol-aldehyde/ketone oxidoreductase in cell extracts of *Thermobacteroides acetoethylicus* which was present in phenotypically similar organisms such as *Thermoanaerobacter brockii* (formerly *Thermoanaerobium brockii*) and *Thermoanaerobacter thermohydrosulfuricum* (formerly *Clostridium thermohydrosulfuricum*) (Lamed & Zeikus, 1981). Subsequently, two additional *Thermobacteroides* species, *Thermobacteroides proteolyticus*

The GenBank accession number for the 16S rDNA sequence of *Thermobacteroides leptospartum* is AF266461.

(Ollivier *et al.*, 1985) and *Thermobacteroides leptospartum* (Toda *et al.*, 1988), were described. Sequence analysis of the 16S rRNA gene of *Thermobacteroides acetoethylicus* indicated that it was closely related to members of the genus *Thermoanaerobacter* (formerly *Thermoanaerobium*). *Thermobacteroides proteolyticus* formed a new single line of descent in the domain *Bacteria* and as a result was transferred out of the genus *Thermobacteroides* (Rainey *et al.*, 1993). The authors were unable to undertake studies on the phylogenetic relationship of the last remaining member of the genus, *Thermobacteroides leptospartum*, as the culture was unavailable to them at that time. We have now sequenced the 16S rRNA gene sequence of *Thermobacteroides leptospartum* and report on its phylogeny. We also report on the results of DNA hybridization studies between *Thermobacteroides leptospartum*, *Clostridium stercorarium* and *Clostridium thermolacticum*, the closest phylogenetic neighbours of *Thermobacteroides leptospartum*.

Thermobacteroides leptospartum (DSM 9219^T) and *C. thermolacticum* (DSM 2910^T) were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) and *C. stercorarium* (ATCC 35414^T) was purchased from the American Type Culture Collection (ATCC). The pH of the medium [composition in g l⁻¹: NH₄Cl, 1.0; K₂HPO₄, 0.3; KH₂PO₄, 0.3; KCl, 0.2; CaCl₂, 0.1; MgCl₂·6H₂O, 0.5; resazurin, 0.001; cysteine.HCl, 0.5; yeast extract (Difco), 2.0; Bactopeptone (Difco); trace elements, 10 ml] was adjusted to 7.2, rendered anaerobic, dispensed into vessels and sterilized as described by Patel *et al.* (1985b) and Fardeau *et al.* (1996). For routine culturing, an appropriate aliquot of pre-sterilized anaerobic glucose was injected into vessels containing the sterile basal medium to give a final concentration of 20 mM. Cellulose, casein and gelatin (10 g l⁻¹) were also tested as the energy sources. For this, the substrates were weighed in tubes before the basal medium was dispensed. All experiments were performed in duplicate, and all incubations were performed at 60 °C for up to 5 d unless otherwise indicated under nitrogen gas phase. Growth was monitored by measuring an increase in OD₆₀₀ over control tubes that lacked glucose, and also by quantifying end products resulting from carbohydrate fermentation. Fermentation products were determined as described by Fardeau *et al.* (1996). For DNA–DNA hybridization studies, DNA was isolated by chromatography on hydroxyapatite by the procedure of Cashion *et al.* (1977). DNA–DNA hybridization was carried out at the DSMZ as described by De Ley *et al.* (1970), with the modification described by Huss *et al.* (1983) and Escara & Hutton (1980) using a Gilford System model 2600 spectrometer equipped with a Gilford model 2527-R thermoprogrammer and plotter. Renaturation rates were computed with the TRANSFER.BAS program of Jahnke & Bahnweg (1986) and Jahnke (1992). Genomic DNA was purified from *Thermobacteroides leptospartum* as described by Redburn *et al.* (1993).

The universal primers Fd1 and Rd1 were used to obtain a PCR product of approximately 1.5 kb, corresponding to base positions 8–1542 based on *Escherichia coli* numbering of the 16S rDNA (Winker & Woese, 1991), using procedures described previously (Redburn & Patel, 1993; Andrews & Patel, 1996). PCR products were sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Kit containing AmpliTaq FS DNA polymerase and ABI 373A sequencer. A 10 µl reaction contained 35 ng PCR product, 4 µl cycle sequencing reaction mix, 3.2 pmol primer (Andrews & Patel, 1996) and 2.5 µg BSA. Thermal cycling was carried out using a Rapid Cycler (Idaho Technology) at a temperature transition slope of 2, an initial denaturation at 94 °C for 15 s, then 25 cycles of denaturation at 94 °C for 0 s, annealing at 50 °C for 10 s, and extension at 60 °C for 3 min. The new sequence data that were generated were aligned, and the almost full-length consensus 16S rRNA gene sequence was assembled and checked for accuracy manually using the alignment editor ae2 (Maidak *et al.*, 1999). These sequences were compared with other sequences in the GenBank database (Benson *et al.*, 2000) using BLAST (Altschul *et al.*, 1997) to determine if any newly released closely related sequences existed in the database. Pairwise evolutionary distances based on 1202 unambiguous positions were computed using DNADIST (Jukes & Cantor, 1969) and neighbour-joining programs that form part of the PHYLIP suite of programs (Felsenstein, 1993).

An almost full-length 16S rRNA gene of *Thermobacteroides leptospartum* consisting of 1518 nucleotides and corresponding to positions 14–1541 (according to *E. coli*) was sequenced. Sequence analysis indicated that it was a member of cluster III (as defined by Collins *et al.*, 1994), which consisted exclusively of thermophilic and mesophilic cellulolytic anaerobes. The most related members of cluster III were *C. thermolacticum* (Le Ruyet *et al.*, 1985) and *C. stercorarium* (Madden, 1983), which were almost equidistantly placed with a similarity value of 99%. Previously Rainey *et al.* (1993) had shown that *C. thermolacticum* and *C. stercorarium* were closely related but *Thermobacteroides leptospartum* was not included in the analysis as the culture was not available to the authors. Stackebrandt & Goebel (1994) have suggested that if a 16S rRNA similarity value of greater than 97% is obtained, then DNA–DNA hybridization studies should be performed. If the DNA hybridization value of such studies is > 70%, then the strains must be regarded as members of the same species (Wayne *et al.*, 1987). DNA–DNA hybridization studies revealed that all the three strains are closely related with each other (95%) and therefore belong to the same species.

C. stercorarium and *C. thermolacticum* are both described as cellulolytic micro-organisms, but this metabolic activity has not been reported for *Thermobacteroides leptospartum*. Our results indicate that *Thermobacteroides leptospartum* degrades Avicel cellu-

Table 1. Salient and distinguishing features of *Clostridium stercorarium*, *Clostridium thermolacticum* and *Thermobacteroides leptospartum*

All strains ferment glucose, cellobiose, lactose, maltose, xylose and mannose but not mannitol or sorbitol.

Characteristic	<i>Thermobacteroides leptospartum</i> *	<i>C. stercorarium</i> †	<i>C. thermolacticum</i> ‡
Culture collection number	ATCC 35414 ^T (strain C17-70)	NCIMB 11754 ^T	DSM 2910 ^T (strain TX41)
Revised taxonomic designation§	<i>C. stercorarium</i> subsp. <i>leptospartum</i> subsp. nov., comb. nov.	<i>C. stercorarium</i> subsp. <i>stercorarium</i> subsp. nov., comb. nov.	<i>C. stercorarium</i> subsp. <i>thermolacticum</i> subsp. nov., comb. nov.
Habitat	Compost	Compost	Compost
Morphology and size	Rods (4.5–15 × 0.25–0.45 µm)	Rods (2–4 × 0.3–0.4 µm)	Rods (2.7–7.7 × 0.7–0.8 µm)
Spores	—	+	+
Motility	—	+	+
Flagella	Absent	Peritrichous	Single lateral
Temperature optimum	60 °C	65 °C	60–65 °C
pH optimum	7.5	7.3	7.0
G + C mol %	43 (±2)	39	41
End products from glucose§	Acetate, lactate, ethanol	Acetate, lactate, ethanol	Acetate, lactate, ethanol
H ₂ S production	No	No	No
Substrate spectrum:			
Cellulose§	+	+	+
Melibiose	±	+	+
Raffinose	+	+	+
Arabinose	—	—	—
Ribose	—	Not reported	+
Fructose	—	+	+
Sucrose	—	+	+
Gelatin§	—	—	—
Casein§	—	—	—

* Data from Toda *et al.* (1988).

† Data from Madden (1983).

‡ Data from Le Ruyet *et al.* (1985).

§ This study.

lose (Merck) and Whatman filter paper. Thus all the three strains share the phenotypic trait of cellulose degradation, which is common to members of cluster III. The major end products from cellulose degradation were ethanol and acetate, with lactate being produced as a minor product. When all the three strains were grown on glucose as a fermentable carbon source at 60 °C, a similar end product profile of ethanol and acetate as the major end products and lactate as a minor product was obtained for all the strains. Our results are slightly different to those reported previously for *C. thermolacticum*, in which lactate was found to be a major end product (Le Ruyet *et al.*, 1985), and for *Thermobacteroides leptospartum*, in which no lactate was detected from glucose fermentation (Toda *et al.*, 1988). It is widely accepted that the end product profile type and the concentrations of the end products produced are culture condition dependent (e.g. temperature, growth medium, pH), so these differences were not unexpected (Wang *et al.*, 1983).

We also observed that small amounts of L-alanine (< 0.1 mM per mol glucose consumed) were produced from glucose fermentation by all the three strains. The trait of L-alanine production from glucose has been reported previously for some members of the order *Thermotogales* (Ravot *et al.*, 1996). *Thermobacteroides leptospartum* was also reported to be a proteolytic bacterium (Toda *et al.*, 1988). However, in our hands no sustainable growth was obtained on casein or gelatin by *Thermobacteroides leptospartum*, *C. thermolacticum* or *C. stercorarium*.

Based on the results presented above, it is clear that *Thermobacteroides leptospartum*, *C. thermolacticum* and *C. stercorarium* are subspecies of the same species. Because *C. stercorarium* was validated first, it has priority, and therefore *Thermobacteroides leptospartum* and *C. thermolacticum* should be assigned to this species as *C. stercorarium* subsp. *leptospartum* subsp. nov., comb. nov. and *C. stercorarium* subsp.

thermolacticum subsp. nov., comb. nov. (Lapage *et al.*, 1992). This automatically creates *C. stercorarium* subsp. *stercorarium* subsp. nov., comb. nov. The transfer of *Thermobacteroides leptospartum*, the sole member of the genus *Thermobacteroides*, also invalidates this genus. The three strains, *C. stercorarium* subsp. *leptospartum*, *C. stercorarium* subsp. *thermolacticum* and *C. stercorarium* subsp. *stercorarium*, can be differentiated on the basis of a number of characteristics including the range of substrates used (Table 1).

Description of *Clostridium stercorarium* emend. Fardeau, Ollivier, Garcia and Patel

Clostridium stercorarium (ster.cor.ar.i'um. L. adj. *stercorarium* pertaining to dung, referring to the source of the isolate, a compost heap).

The following description is based on the data of Madden (1983), Le Ruyet *et al.* (1985), Toda *et al.* (1988) and data presented in this paper. Cells are rods (0.25–0.8 × 2–15 µm), and may be motile by peritrichous or single laterally inserted flagella. Spores may be present. Chemo-organotrophic and obligate anaerobes. The optimum temperature for growth is between 60 and 65 °C at pH 7.0–7.5. Ferments cellulose, cellobiose, lactose, maltose, xylose and mannose but not mannitol, sorbitol, gelatin or casein. Acetate, lactate, ethanol, H₂, CO₂ and small amounts of L-alanine are produced during glucose fermentation. Does not produce H₂S. The G + C content range of the DNA is 39–43 mol%. Belongs to cluster III of the order *Clostridiales*, domain *Bacteria*. The species is divided into three subspecies, *Clostridium stercorarium* subsp. *stercorarium* subsp. nov., comb. nov., *Clostridium stercorarium* subsp. *thermolacticum* subsp. nov., comb. nov. and *Clostridium stercorarium* subsp. *leptospartum* subsp. nov., comb. nov. All three subspecies are isolated from compost. The type species is *Clostridium stercorarium* and the type strain is NCIB 11754^T.

Description of *Clostridium stercorarium* subsp. *stercorarium* subsp. nov., comb. nov.

Clostridium stercorarium subsp. *stercorarium* (ster.cor.ar.i'um. L. adj. *stercorarium* pertaining to dung, referring to the source of the isolate, a compost heap).

For characteristics, refer to Madden (1983).

Description of *Clostridium stercorarium* subsp. *thermolacticum* subsp. nov., comb. nov.

Clostridium stercorarium subsp. *thermolacticum* (ther.mo.lac.ti'cum. Gr. n. *thermus* heat; L. gen. n. *lacteus* of milk; N.L. n. *thermolacticum* pertaining to the high growth temperature and a growth substrate).

For characteristics, refer to Le Ruyet *et al.* (1985).

Description of *Clostridium stercorarium* subsp. *leptospartum* subsp. nov., comb. nov.

Clostridium stercorarium subsp. *leptospartum* (lep.to.spar'tum. Gr. n. *leptos* thin; Gr. n. *sparton* rope; L. n. *leptospartum* thin rope).

For characteristics, refer to Toda *et al.* (1988).

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