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Pilibacter termitis gen. nov., sp. nov., a lactic acid bacterium from the hindgut of the Formosan subterranean termite (*Coptotermes formosanus*)

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A previously undescribed anaerobic, heterofermentative, non-spore-forming, Gram-positive rod was isolated from the hindgut of the Formosan subterranean termite *Coptotermes formosanus* Shiraki. The DNA G+C content of this bacterium was 37·8 mol%. Sequence analysis of the 16S rRNA gene revealed that this organism is related to, but distinct from, several genera of lactic acid bacteria, principally several species of the genus *Enterococcus*. Phenotypic traits that serve to separate this organism from related genera include high levels of the cellular fatty acid C18 : 1ω9c and the production of ethanol along with lactic acid as fermentation products. Based on the collected phylogenetic and phenotypic evidence, it is proposed that the unknown organism represents a novel species in a new genus, *Pilibacter termitis* gen. nov., sp. nov. The type strain is TI-1^T (=ATCC BAA-1030^T=CCUG 49613^T).

It has been demonstrated that the guts of termites house a diverse collection of bacteria encompassing several disparate phyla and even a novel phylum (Ohkuma & Kudo, 1996). However, the majority of the microbial population is thought to be recalcitrant to isolation in the laboratory. Despite this problem, there remains a considerable population of bacteria in termites which may be isolated and characterized using conventional bacteriological techniques. An example includes bacteria of the order '*Lactobacillales*', which have been recovered from several different species of termites, including *Mastotermes darwiniensis*, *Cryptotermes primus*, *Heterotermes ferox*, *Coptotermes lacteus*, *Schedorhinotermes intermedius*, *Nasutitermes exitiosus* (Eutick *et al.*, 1978), *Reticulitermes flavipes* (Schultz & Breznak, 1978; Bauer *et al.*, 2000), *Nasutitermes arborum*, *Thoracotermes macrothorax* and *Anoplotermes pacificus* (Bauer *et al.*, 2000). Many of the endemic bacteria associated with the termite hindgut, such as species of the genus *Bacteroides*, *Desulfovibrio termitidis* and *Acetonema longum*, readily utilize lactic acid as a carbon source (Schultz & Breznak, 1979; Trinkerl *et al.*, 1990; Kane & Breznak, 1991). Therefore, it is feasible that the lactic acid bacteria play an important role in maintaining the homeostasis of the bacterial community associated with the termite hindgut. In this paper, we report the isolation of a member of a new genus of lactic acid bacteria isolated from the gut of *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae).

Workers of the termite species *Coptotermes formosanus* were obtained from three separate colonies on the campus of the University of Hawaii at Manoa. The termites were divided into three treatment groups for study. The first group was dissected within 24 h of collection. The second group was maintained for 1 week on a diet of Douglas fir wafers in arenas of sand moistened with distilled water. The third group was maintained in Petri dish arenas and fed a diet of Whatman No. 3 filter paper for 1 week. Fifteen termites from each of the three treatment groups were selected for dissection, cultivation and enumeration of hind-gut bacteria.

Termites were surface sterilized with 80 % ethanol. Using an aseptic technique, the hindgut was dissected and homogenized and the homogenate was subjected to serial dilutions and grown on Todd–Hewitt agar at 30 °C under anaerobic conditions using H_2/CO_2 Gas-Paks (BBL Becton-Dickinson). After 3 days of incubation, several strains of a Gram-positive rod with distinct irregular rod morphology were recovered. It was found that bacteria presenting the distinct irregular rod morphology comprised a large fraction of the recovered lactic acid bacteria: 31 % of enumerated

Abbreviations: DMA, dimethyl aldehyde; PYR, L-pyrrolidonyl β-naphthylamide.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain TI-1^T is AY533171.

colonies from the immediately dissected termites, 12 % of enumerated colonies from the laboratory-reared termites maintained on a wood diet and 19 % of enumerated colonies from the laboratory-reared termites maintained on a filter-paper diet. Each of the irregular rod-shaped bacteria tested from the different termite treatment groups demonstrated similar profiles when subjected to standard bacteriological tests (i.e. acid from a variety of carbohydrates, aesculin hydrolysis, urea hydrolysis and nitrate reduction). Three of the irregular rod-shaped bacteria were selected on the basis of 16S rRNA gene sequence analysis and subjected to further characterization. The first isolated bacterium from the immediately dissected group was designated termite isolate 1 (TI-1^T). Strain TI-2 was isolated from the filter-paper-fed treatment group and strain TI-3 was isolated from the wood-fed treatment group. Based on the results of this study we propose that TI-1^T is the type strain of a new genus and species, *Pilibacter termitis* gen. nov., sp. nov.

Hydrolysis of bile aesculin, gelatin and urea, and production of indole from tryptophan, were determined using API 20AN systems (API bioMérieux). Production of acid from various carbon sources was determined using API 50CH systems in conjunction with API 50CHL medium (API bioMérieux). The cupules were overlaid with sterile mineral oil to maintain an anoxic environment. Results are given below.

Hydrolysis of hippurate was determined by using the method of Hwang & Ederer (1975). Hydrolysis of DNA was determined by using DNase agar (Difco). Hydrolysis of starch was determined by the addition of soluble starch (Merck) to trypticase soy agar (TSA; Difco). Hydrolysis of L-pyrrolidonyl β-naphthylamide (PYR) was determined by using commercial PYR discs and reagents (Hardy). Susceptibility to bacitracin and optochin was determined using commercial discs (BBL Becton-Dickinson). Reduction of nitrates was determined by the use of nitrate reduction broth (Difco) supplemented with 0·1 % agar. Voges–Proskauer and methyl red tests were conducted in Voges–Proskauer broth supplemented with 0·1 % agar. Production of ammonia from arginine was determined using purple broth base (Difco) supplemented with 0·5 % arginine (Fisher). Long-chain cellular fatty acid analysis was performed using the MIDI system (Sasser, 1997). Fermentation products were assayed using enzymic test kits for the detection of acetic acid, ethanol, formic acid, lactic acid and succinic acid (Boehringer Mannheim).

Determination of the DNA G + C content was performed by the Identification Services of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) using the methods of Mesbah *et al.* (1989). DNA–DNA hybridization was also performed by the DSMZ; DNA obtained from $\sim$ 3 g (wet weight) of bacterial cell mass from strain TI-1^T was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite (Cashion *et al.*, 1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970), with the modifications described by Huß *et al.* (1983), using a Cary 100 Bio UV/Vis spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in-situ* temperature probe (Varian).

Scanning electron microscopy was performed by the Socolofsky Microscopy Facility at Louisiana State University, using cultures of strain TI-1^T fixed with 2 % glutaraldehyde and 1 % formaldehyde, post-fixed with 2 % osmium tetroxide, rinsed, applied to graphite-coated specimen mounts, air-dried, sputter coated and imaged with a Cambridge 260 scanning electron microscope.

DNA from bacterial colonies was isolated using a QIAGEN DNeasy Tissue kit. The 16S rRNA gene was amplified using the primer sets 8F and 926R and 533F and 1492R (Lane, 1991). Cycling conditions were as described by Hugenholtz *et al.* (1998). Sequencing was performed using a Beckman CEQ 2000 DNA analyser. The closest known relatives were identified by performing database searches using the National Center for Biotechnology Information (NCBI). Sequences were uploaded from NCBI, aligned using the CLUSTAL X program (Thompson *et al.*, 1997) and edited manually using the BIOEDIT program (Hall, 1999). A phylogenetic tree was constructed with the neighbour-joining method of Felsenstein using the program NEIGHBOR (Felsenstein, 1989). Stability of the grouping was estimated using the programs DNABOOT, DNADIST, NEIGHBOR and CONSENSE with 1000 evaluations for bootstrapping (Felsenstein, 1989).

Strain TI-1^T grew readily on standard nutrient-rich laboratory media, including TSA, Todd–Hewitt agar and 5 % sheep's blood TSA. When grown on Todd–Hewitt agar for 3 days under anaerobic conditions at 30 °C, cells of strain TI-1^T were found to be irregular Gram-positive rods. The rods typically occurred alone or in pairs and were often observed in palisade formations. They were variable in morphology with both regular rods and tapered, spindle-shaped forms occurring. A scanning electron micrograph of cells of strain TI-1^T is shown in Fig. 1. The rods had a tendency to decolorize and often produced mixed Gram stains. In older cultures (i.e. more than 3 days) occasional swellings were observed in cells, particularly when grown on blood agar. Subsequent staining using the Schaeffer–Fulton method revealed that these swollen forms were not spores. The organism grew poorly under aerobic conditions both on solid media and in broth. Colonies grown aerobically on agar plates demonstrated marked reduction in colony size and were opaque, as compared with cream coloured when grown under anaerobic conditions. On 5 % sheep's blood agar, alpha haemolysis was observed. Motility was not observed by using hanging drop suspensions and through inoculation into semi-solid thioglycollate media (BBL Becton-Dickinson).

The organism did not hydrolyse DNA, gelatin, hippurate, starch or PYR, or produce ammonia from arginine. The organism was positive for methyl red, negative for the Voges–Proskauer reaction and it was resistant to optochin at a concentration of 5 μg per disc. Resistance to bacitracin

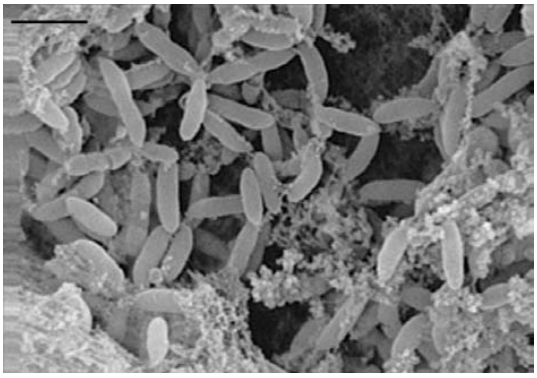

Fig. 1. Scanning electron micrograph of cells of *Pilibacter termitis* TI-1^T. Note that some cells display a curved morphology. Bar, 2 μm.

discs at a concentration of 0·05 IU was variable. The organism failed to grow at 42 °C and did not grow in trypticase soy broth with NaCl concentrations of 6·5 %. The organism was catalase- and oxidase-negative. The major fermentation product of the organism was lactic acid (8·03 mM) and the minor fermentation product was ethanol (1·15 mM). Acetic acid, succinic acid and formic acid were not detected as fermentation products. The DNA G+C content of the organism was 37·8 mol%.

Using API 20AN strips, aesculin was found to be hydrolysed but urea was not, and indole was not produced from tryptophan. Using API 50CH strips, acid was found to be produced from D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, D-sorbitol, *N*-acetylglucosamine, amygdalin, arbutin, salicin, D-cellobiose, D-maltose, D-lactose, D-trehalose and gentiobiose, but not from glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, L-xylose, D-adonitol, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, D-melibiose, sucrose, inulin, D-melezitose, D-raffinose, starch, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate or potassium 5-ketogluconate. The production of acid from methyl *β*-D-xylopyranoside was variable, with strain TI-1^T testing negative and strains TI-2 and TI-3 testing positive. Using API 50CH, aesculin was found to be hydrolysed.

The predominant long-chain cellular fatty acids of the three strains were C18 : 1ω9*c*, C18 : 1ω9*c* dimethyl aldehyde (DMA), C16 : 0, C14 : 0, a summed feature consisting of C18 : 1ω7*c* and the unknown fatty acid C17·834, a summed feature consisting of C17 : 2 and C17 : 1ω9*c*, and C16 : 1ω7*c*. Complete fatty acid profiles are given in Table 1.

A phylogenetic tree was constructed using the neighbour-joining method with *Bacillus subtilis* selected as the outgroup (Fig. 2). The results of this tree demonstrate that, whereas strain TI-1^T is related to other organisms within the order '*Lactobacillales*', it forms a distinct branch from the

Table 1. Fatty acid compositions of *Pilibacter termites* strains

Fatty acid	TI-1^T	TI-2	TI-3
C12 : 0	0·58	–	0·30
C14 : 0	9·70	8·11	8·44
C14 : 1ω5*c*	–	0·41	0·45
C15 : 0	–	0·73	0·67
C16 : 0	12·57	13·71	14·72
C16 : 0 aldehyde	1·17	1·23	1·31
C16 : 0 DMA	3·03	2·89	3·21
C16 : 1ω5*c*	0·72	0·65	0·71
C16 : 1ω7*c*	5·48	5·31	5·13
C16 : 1ω7*c* DMA	–	–	0·51
C18 : 0	1·55	1·88	1·99
C18 : 1ω7*c* DMA	1·75	1·50	1·57
C18 : 1ω9*c*	32·25	33·91	31·43
C18 : 1ω9*c* DMA	15·37	12·59	12·12
C18 : 2ω9*c* DMA	–	0·89	1·99
Summed feature 7*	6·53	5·66	5·31
Summed feature 10†	9·30	10·01	10·15

–, Not detected.

*C17 : 2/C17 : 1ω9*c*.

†C18 : 1ω9*c*/unknown C17·834.

enterococci and the streptococci–lactococci branches. Strain TI-1^T shared greatest 16S rRNA gene sequence similarity (99 %) with a culture-independent sequence of an undescribed bacterium cloned from gut samples of *Coptotermes formosanus*, clone BCf6-17 (GenBank accession no. AB062833). With recognized organisms, strain TI-1^T was found to share greatest similarity with members of the genus *Enterococcus* (i.e. 92·8 % with *Enterococcus saccharolyticus*, 92·5 % with *Enterococcus gallinarum*, 92·2 % with *Enterococcus hirae* and 92·0 % with *Enterococcus faecalis*). Data obtained from DNA–DNA hybridization indicates that strain TI-1^T had a DNA–DNA relatedness level of 31·35 % with *E. faecalis* DSM 20478^T, 36·65 % with *E. gallinarum* DSM 2068^T, 48·3 % with *E. hirae* DSM 20160^T and 61·2 % with *E. saccharolyticus* DSM 20726^T. It has been postulated that members of the same genus share a sequence similarity of >94 % (Collins *et al.*, 1994) or >97 % (Drancourt *et al.*, 2000); these data, along with the low hybridization values and the distinct branching present in the phylogenetic tree, indicate that strain TI-1^T represents a member of a new genus.

Strain TI-1^T exhibits significantly different physiological characteristics from those associated with the genus *Enterococcus*. TI-1^T was found to have a cellular fatty acid profile different from those associated with the enterococci. A common fatty acid found in the enterococci is C18 : 1ω7*c*, comprising 23 % of the total fatty acids (Bosley *et al.*, 1990). By contrast, C18 : 1ω7*c* formed only a minor component of the cellular fatty acids of strain TI-1^T (1·75 % of the total). TI-1^T was also found to have high levels of the fatty acid C18 : 1ω9*c* (32·25 %), whereas this fatty acid occurs at low

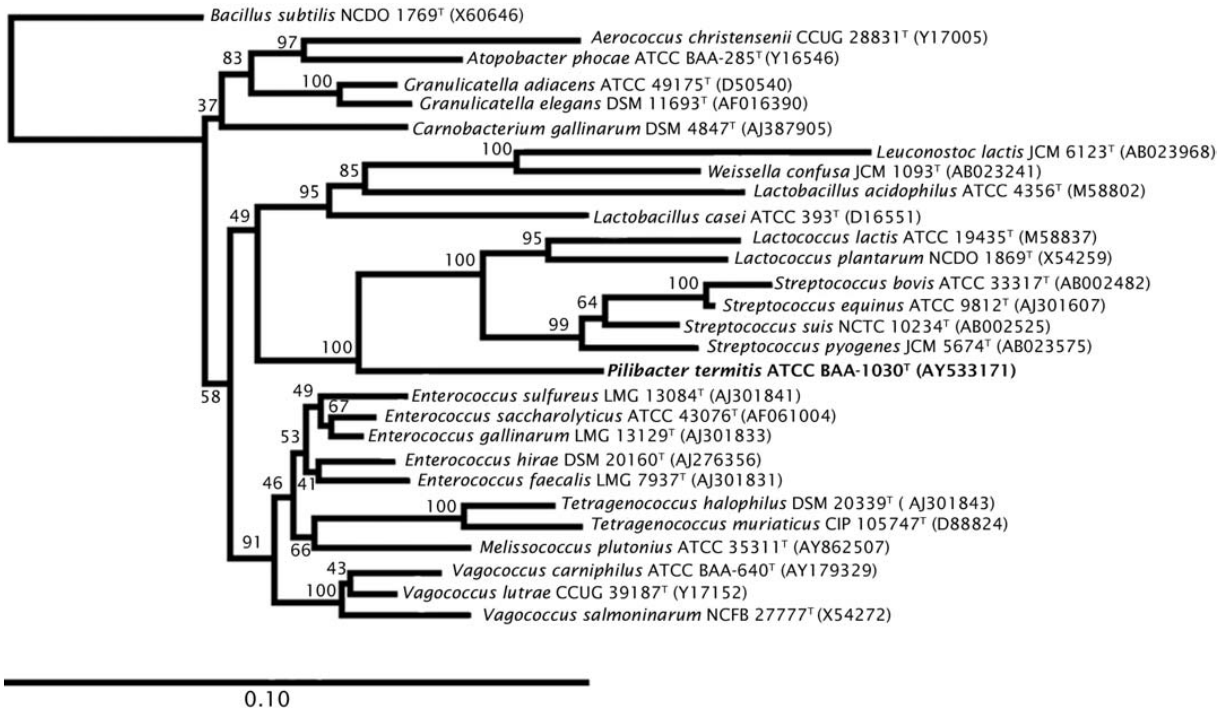

Fig. 2. Phylogenetic tree showing the relationship of *Pilibacter termitis* TI-1^T with representative bacteria of the order '*Lactobacillales*'. The tree was constructed by using the neighbour-joining method with *Bacillus subtilis* serving as the outgroup and is based on 1320 nt aligned using CLUSTAL X. Bootstrap values are expressed as a percentage of 1000 replications and are indicated at branching points. Bar, 0·10 nucleotide substitutions per site.

levels in the cell membranes of the enterococci (1 %; Bosley *et al.*, 1990). Production of ethanol in the fermentation of glucose by TI-1^T is another key feature separating TI-1^T from the homofermentative *Enterococci* (Stiles & Holzapfel, 1997). Finally, TI-1^T tolerates oxygen poorly compared with most members of the genus *Enterococcus*. Several phenotypic characteristics may be used to separate TI-1^T from related species of *Enterococcus*. Specifically, these are negative results for hydrolysis of L-PYR, the Voges–Proskauer reaction and production of acid from L-arabinose, glycerol, mannitol, melibiose and sorbitol (Table 2). Based on the collected data, we propose that strain TI-1^T represents a novel species of a new genus, *Pilibacter termitis* gen. nov., sp. nov.

Description of *Pilibacter* gen. nov.

Pilibacter (Pi'li.bac.ter. L. neut. n. *pilum* a heavy javelin; N.L. masc. n. *bacter* from Gr. n. *bakteron* rod; N.L. masc. n. *Pilibacter* a rod that appears tapered and pointed, like the head of a spear).

Cells consist of non-motile, non-spore-forming irregular rods occurring alone, in pairs and in palisade formations. Rods are usually straight, but curved forms do occur and the rods typically appear tapered at the ends. Cells stain Gram-positive, but some have a tendency to lose colour, particularly when taken from older (over 3 days) colonies. Anaerobic growth is poor under ambient atmospheric conditions. Growth is observed at 20 °C but not at 42 °C. The DNA G+C content of the type strain of the type species is 37·8 mol%, which indicates that this genus is member of the low-G+C-content Gram-positive bacteria. The major fermentation product is lactic acid with ethanol as a minor fermentation product. *Pilibacter* is a member of the order '*Lactobacillales*' of the Gram-positive bacteria. The type species is *Pilibacter termitis*.

Table 2. Phenotypic characteristics that differentiate *Pilibacter termitis* gen. nov., sp. nov. from closely related species of *Enterococcus*

Species: 1, *P. termitis*; 2. *E. faecalis*; 3, *E. gallinarum*; 4, *E. hirae*; 5, *E. saccharolyticus*. Data for *Enterococcus* species were obtained from Devriese *et al.* (1993). Characteristics are scored as: +, positive; D, different or variable; D+, usually positive; −, negative.

Characteristic	1	2	3	4	5
Pyrrolidonyl arylamidase	−	+	+	+	−
Voges–Proskauer reaction	−	+	+	+	−
Acid from:					
L-Arabinose	−	−	+	−	−
Glycerol	−	+	−	−	−
Mannitol	−	+	+	−	+
Melibiose	−	−	+	+	+
Sorbitol	−	D+	D	−	+

Description of *Pilibacter termitis* sp. nov.

Pilibacter termitis (ter′mi.tis. L. n. *termes* a worm that eats wood, a woodworm, and in zoology the scientific name of a genus of termite; N.L. gen. n. *termitis* of a termite).

Has the following characteristics in addition to those given above for the genus. Colonies are cream coloured and alpha-haemolytic when grown on blood agar. Anaerobic. Catalase- and oxidase-negative. No growth is observed in 6·5 % NaCl. Nitrates are not reduced. Aesculin is hydrolysed but not DNA, gelatin, hippurate, starch, urea or L-PYR. Positive for methyl red but negative for the Voges–Proskauer reaction. Indole is not produced from tryptophan. Resistant to optochin. Susceptibility to bacitracin varies. Acid is produced from D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, D-sorbitol, *N*-acetylglucosamine, amygdalin, arbutin, salicin, D-cellobiose, D-maltose, D-lactose, D-trehalose and gentiobiose, but not from glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, L-xylose, D-adonitol, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, D-melibiose, sucrose, inulin, D-melezitose, D-raffinose, starch, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate or potassium 5-ketogluconate. The production of acid from methyl β-D-xylopyranoside is variable. The major long-chain fatty acids are C16 : 0, C18 : 1ω9*c* and C18 : 1ω9*c* DMA.

The type strain is TI-1^T (=ATCC BAA-1030^T=CCUG 49613^T), isolated from the hindgut of the Formosan subterranean termite, *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae).

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Extent:
Price:
Invoice: IR/

PAYMENT BY CREDIT CARD *(Note: we cannot accept American Express)*

Please charge the sum of £_____ to my credit card account.

My Access/Eurocard/Mastercard/Visa number is (*circle appropriate card; no others acceptable*):

Expiry date ☐☐

Signature: _____ Date: _____

Cardholder's name and address*: _____

_____

**Address to which your credit card statement is sent. Your offprints will be sent to the address shown at the top of the form.*

April 2003