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Isolation and characterization of some gut microbial symbionts from fungus-cultivating termites (*Macrotermes* and *Odontotermes* spp.)

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Microbiota of termites is crucial for nitrogen cycle activities and degradation of recalcitrant components of plant biomass that influence soil structure and carbon mineralization in tropical and subtropical regions. The aim of this study was to isolate and characterize gut bacteria that may be potentially associated with nitrogen metabolism from two fungus-feeding termites (*Macrotermes* and *Odontotermes* spp.). Twenty termites from the intact colony of each termite species were aseptically degutted. Gut homogenate was inoculated and cultured on selective media for the isolation of pure bacteria. Pure bacterial isolates were characterized using their morphological, biochemical and molecular characters. DNA was extracted from the isolates, polymerase chain reaction (PCR) amplified and sequenced. The 16S rDNA gene sequences were blastn analyzed against the Genbank database and phylogenetic analysis was performed using MEGA 5 software. All forty-six isolates reacted positive for the ammonifying, nitrate, catalase and nitrogenase tests. Phylogenetic analysis grouped the isolates into three phyla: *Firmicutes, Proteobacteria* and *Actinobacteria*. Isolates were affiliated with the genera *Pseudomonas*, *Citrobacter, Enterobacter, Proteus, Klebssiella, Bacillus, Staphylococcus, Rhodococcus* and *Micrococcus*. The results confirm that termites harbor diverse gut bacterial groups that have different physiological/enzymatic activities and might have functional implications in the termite-microbe symbiotic association.

Key words: Termites, symbiosis, mutualistic interactions, termite gut bacterial diversity, nitrogen fixation

INTRODUCTION

Termites (Order: *Blattodea*, Family: *Termitidae*) are a large and diverse group of soil macrofauna (Ahmed et al., 2011). They are thought to have originated about 150 million years ago and have since successfully colonized the temperate and tropical ecological zones (Thorne et al., 2000). Nearly 3,000 species of termites have been described and they are conventionally classified into lower and higher termites (Zhu et al., 2012). They are

important decomposers due to their ability to biodegrade complex substances like cellulose and hemicellulose found in plant materials (Sugimoto et al., 2000).

Termites harbor diverse microbial populations most of which are unique to the termite gut ecosystem (Otani et al., 2014). The microbiota associated with termites are crucial for degradation of recalcitrant components of plant biomass (Brune and Ohkuma, 2011) and this has a major influence on soil structure and carbon mineralization (Ohkuma, 2003). Termites' gut microbiota are exchanged between colony members and transferred to the next generation through trophallaxis (Brune and Ohkuma, 2011). The isolation and cultivation of bacterial strains from termite guts has partially contributed to their classification as decomposers of lignocellulose, uric acid and/or other aromatic compounds, as nitrogen-fixers and/or as H_2/CO_2-acetogens (Breznak, 2000). Nonetheless, the majority of the microbial species (~99%) are difficult or currently uncultivable, thus limiting understanding of their function in the gut ecosystem (Breznak, 2000).

The macrotermitinae comprises of the economically important termite species (Ahmed et al., 2011) that have been comprehensively studied (Mathew et al., 2012; Makonde et al., 2015; Otani et al., 2014, 2015). Isolation and characterization of termite gut bacterial species faces challenges since majority of the bacterial species are difficulty to culture (Breznak, 2000). Considering the plant-biomass degradation and nitrogen metabolism in termites, the presence of diverse bacterial lineages in termites (Makonde et al., 2015; Otani et al., 2015;) and the evolutionarily diverse termite species, the termite gut microbial populations are of significant interest and need to be isolated and characterized in order to better understand the termite symbiotic systems. In this study, the authors isolated and partially characterized some termite gut bacterial species that have different physiological/enzymatic activities. The results indicate some functional roles of the termite gut bacterial isolates.

MATERIALS AND METHODS

Sample collection and processing

Termite samples were collected in July, 2012 at the Jomo Kenyatta University of Agriculture and Technology (JKUAT) in Juja (latitude 10° 0' 5'' S, Longitude 37° 0' 00" E; at an altitude of 1525 m above the sea level), Kiambu County, Kenya. Termite mounds (approximately 1 to 3 km apart), which were colonized by *Odontotermes* and *Macrotermes* species were excavated to a depth of ≈1.0 m. Termite samples were collected and put into sterile plastic boxes. Worker-caste termites were used in the experiments due to their foraging activities during the establishment and maintenance of the fungus gardens. Twenty (20) worker termites (for each termite sample from different mounds) were degutted separately using sterile fine tipped forceps; subsequently, the guts were homogenized in 10 ml of sterile normal saline. The resultant gut homogenate was then serially diluted using sterile normal saline into 10^{-1}, 10^{-2} and 10^{-3} that were subsequently used for the isolation of the gut microbial symbionts.

Isolation and purification of gut bacteria

Isolation of the different groups of gut bacteria was performed using selective media. Nitrifying bacteria were isolated using a mineral medium for nitrifying bacteria with the following composition: Na_2HOP_4, 13.5 g; KH_2PO_4, 0.7 g; $MgSO_47H_2O$, 0.1 g; $NaHCO_3$, 0.5 g; $(NH_4)_2SO_4$, 2.5 g; $FeCl_3$ $6H_2O$, 14.4 mg, $CaCl_2$ $7H_2O$, 18.4 mg; agar, 15 g. All the components were mixed and brought up to a 1 L volume of distilled water at pH 8.0. The isolation of denitrifying bacteria was done using the recommended nitrate agar medium, while the ammonifying bacteria were isolated using peptone agar medium. The nitrogen fixing bacteria were isolated using nitrogen free media containing: K_2HPO_4, 1 g; $MgSO_4.7H_2O$, 0.2 g; $CaCO_3$, 1 g; NaCl, 0.2 g; $FeSO_4.7H_2O$, 5 mg; glucose, 10 g; $NaMoO_4$, 5 mg; agar, 15 g; distilled water, 1 L; pH 7.0. The spread plate technique was used as described by Holt et al. (1994). In each case, plates were incubated and monitored daily at 30°C for up to 72 h. Bacterial colonies that formed on the plates were subsequently subcultured on the corresponding media previously described until axenic cultures were obtained.

Morphological and physiological characterization of the isolates

Colony morphology of the pure isolates was described using standard microbiological criteria with special emphasis on pigmentation, shape, form, elevation and margin formation. Preliminary characterization by Gram staining of the isolates was done as described by Holt et al. (1994). Temperature and pH ranges and optima for growth were determined in LB broth. Growth of isolates was measured using the Shimadzu model UV240 spectrophotometer at 660 nm in cuvettes with a 1-cm light path. The ability to tolerate sodium chloride and ammonia was tested by inoculating the isolates in LB broth supplemented with different concentrations of sodium chloride (0, 3, 5, 7 and 10%) and ammonium sulphate (0, 5, 10, 15 and 20%) as described by Muller et al. (2006).

Biochemical and enzymatic characterization

Catalase test was conducted by growing bacteria in nutrient broth overnight at 37°C. Catalase activity was observed by adding few drops of 3% H_2O_2 to the broth cultures, kept on the glass slides. The production of effervescence due to catalase catalyzed breakdown of H_2O_2 to molecular oxygen indicated a positive reaction. The ability of the isolates to attack nitrogen and carbon bonds in amide compounds was determined using urea brot media containing a phenol red indicator. Bacterial cultures were inoculated

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in the medium and incubated at 37°C for 48 h. A positive reaction was indicated by development of deep pink color. For nitrate reduction test, the isolates were incubated at 37°C overnight followed by addition of 0.5 ml each of sulphanilic acid (0.8% in 5 N acetic acid) and α-naphthylamine (0.5% in 5 N acetic acid). The appearance of red or pink color indicated the positive test for nitrate reduction. Hydrogen sulfide production test was performed using SIM agar (Peptone 30 g/l, beef extract 3 g/l, ferrous ammonium sulphate 0.20 g/l, sodium thiosulphate 0.025 g/l, agar 3 g/l (pH 6.0) mixtures) that was inoculated with actively growing cultures. Blackening along the line of inoculation shows a positive test after 3 - 5 days at 30°C.

For methyl red (MR) and Voges–Proskauer (VP) tests, the MR-VP broth was inoculated with actively growing bacterial cultures and incubated at 30 C for 48 h. An uninoculated tube with the medium served as a negative control. MR indicator (Barrit's reagent) was added and positive cultures for the MR test appeared red while positive cultures for the VP test gave a rose coloration. An Indole test detects the production of indole from the amino acid tryptophan. Tryptone broth was inoculated with the isolates and incubated at 30°C for 48 h. Kovac's reagent (3 drops) was added into the tubes. A red colored layer on the surface of the tube indicated a positive test for indole. The citrate utilization was performed by inoculating the bacterial cultures on Simmon's citrate agar plates and incubating at 30°C for 48 h. A blue colourization of the medium indicated a positive reaction. The ammonifying and nitrifying test were determined by inoculating bacterial cultures in test tubes containing 5 ml peptone broth and ammonium sulfate broth media, respectively. The cultures were incubated at 30°C for 5 days and the presence of ammonia, an indicative of ammonification was detected by a yellow color when 3 drops of Nessler's reagent were added to the test cultures. A positive reaction for the nitrifying test was indicated by the development of a blue-black color upon addition of a few drops of Trommsdorfs reagent and sulfuric acid to the test cultures.

Qualitative enzymatic screening

Qualitatively screening of the isolates for production of enzymes such as cellulases, xylanases, amylases, proteases, gelatinases, lipases and nitrogenases was performed. Screening for cellulases and xylanasese was performed according to the procedures described by Ruijassenaars and Hartmans (2001). Briefly, the freshly growing bacterial cultures were spot inoculated on nutrient agar plates supplemented with 0.2% carboxymethyl cellulose (CMC) and xylan substrates for cellulases and xylanases, respectively. The plates were incubated at 30°C for 5 days and were overlaid with Congo-red (1 g/ml) solution for 15 min. After washing the plate surface with 1 M NaCl, clear zone around colony indicated the enzyme activity. Gelatin hydrolysis was performed in nutrient gelatin medium, which was inoculated with a loopful of actively growing bacterial cultures and incubated for 3 days at 30°C. Control tubes solidified when placed in ice whereas medium in inoculated tubes remained unsolidified, showing positive gelatin hydrolysis test.

To detect amylase activity, nutrient agar plates supplemented with 0.3% soluble starch were aseptically inoculated with actively growing bacterial cultures and incubated for 3 days at 30°C. The plates were then flooded with Gram's iodine, a clear yellow zone around the inoculation spots indicated starch hydrolysis. The protease activity was detected using casein hydrolysis where skim milk agar (Skim milk powder 100 g/l, agar 15 g/l) plates were aseptically spot inoculated with actively growing cultures and incubated for 3 days at 30°C. Clear zones around the inoculation spots indicated casein hydrolysis. The production of lipases by the isolates was determined by culturing the isolates on basal media (1% KH_2PO4, 0.01% $MgSO_4.7H_2O$, 0.005% $CaCl_2.2H_2O$, 1% NaCl and 1% Na_2CO_2) supplemented with 1% olive oil (domestic grade) as the sole carbon source. The plated medium was then inoculated by spotting bacterial cultures and incubated for 3 days at 30°C. Lipase production was indicated by the precipitation of calcium crystals around the colonies. The nitrogenase activity of the bacterial cultures was estimated by conducting the acetylene reduction assay. The isolates were grown in 5 ml of nitrogen free semi-solid media in 15 ml serum bottles sealed by rubber stoppers. The air (10%) was removed and replaced with acetylene. The head space of the cultural tube was sampled (1 ml of air) to determine ethylene production after twelve hours on a Shimadzu Gas Chromatograph (GC-9A, Japan). A standard ethylene gas was used as a positive control. An un-inoculated tube was used as a negative control as described by Eckert et al. (2001).

Molecular characterization of the isolates

Genomic DNA (gDNA) extraction and PCR amplification

Pure isolates were separately inoculated into sterile falcon tubes containing 15 ml Luria Bertani (LB) medium and incubated for 48 h in a shaking incubator (at 150 rpm) at 37°C. The isolate cultures were then centrifuged at 6000 rpm for 10 min. The resultant pellets were separately re-suspended in sterile micro tubes containing 0.2 ml of tris-ethylenediaminetetraacetic acid (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The cell suspensions were used for gDNA extraction using the UltraClean® Mega soil DNA isolation kit (MO BIO Laboratories, Inc.) according to the manufacturer's protocol. Purified DNA was quantified photometrically (NanoDrop; Thermo Fisher Scientific, Germany) and used as a template for amplification of 16S rRNA genes using the universal bacterial primers (8F 5'-AG (A/G) GTTTGATCCTGGCT-3' forward primer and 1492R 5'-CGGCTACCTTGTTACGACTT-3' reverse primer) according to their position in relation to the *Escherichia coli* gene sequence (Lane, 1991). For each PCR, 1 µl (25 ng/ µl) of the template was mixed with TaKaRa Ex Taq™ HS (5 units/µl) according to the manufacturer's protocol. The PCR conditions were as described by Mackenzie et al. (2007) except the final extension was at 72°C for 8 min. PCR product size was checked using a 1% agarose gel stained with ethidium bromide. The amplicons were gel purified using Macherey-Nagel NucleoSpin extract II kit as per the manufacturer's protocol and eluted in 30 µl of TE Buffer (5 mM, pH 8.0).

DNA Sequencing and phylogenetic analysis

Sequencing of the purified PCR products was done using a commercial service provider (Macrogene, South Korea). Sequences of the isolates were manually edited in chromas and checked for presence of artifacts or chimeric structures using the Mallard software (Ashelford et al., 2006). A search for similar sequences using BLASTN program was performed, and sequence alignment was performed using the CLUSTAL Omega program (http://www.clustal.org). A neighbor-joining tree of the aligned sequences was constructed using MEGA V5.10 (Tamura, 2011). Evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004). To obtain support values for the branches, bootstrapping (Felsenstein, 1985) was conducted with 1000 replicates. All sites, including gaps in the sequence alignment, were excluded pairwise in the phylogenetic analysis. Using the resultant neighbor-joining tree, each isolate was assigned to the proper taxonomic group. The taxonomic

assignment was confirmed at a 90% confidence level using the naïve Bayesian rRNA classifier on the RDP website (Cole et al., 2005). All sequences were deposited in GenBank nucleotide database with the accession numbers KF872743 to KF872766.

RESULTS

Characterization of the isolates

A total of 46 bacterial isolates were isolated from guts of *Odontotermes* and *Macrotermes* using selective media. Morphologically (Table 2), the isolates exhibited diverse colony characteristics differing in their form, color, margin, cell arrangement and the Gram reaction. The cell shape was either rod or cocci with a positive or negative Gram reaction (Table 1). Biochemical tests revealed that all the isolates were positive for the ammonifying, nitrifying and catalase tests (Table 1 and Figure 1A). About 85% of the isolates were positive for the citrate test and this indicated their ability to use citrate as a source of carbon. Fifty two percent of the isolates were positive for the urease test that demonstrates the ability of the isolates to attack nitrogen and carbon bonds in amide compounds. Forty-one percent and thirty percent of the isolates were positive for the MR and VP tests (Table 1 and Figure 1A). For the nitrate reduction test, only seventeen isolates (35%) were positive. This showed that these isolates could reduce nitrates to nitrites or beyond. Only 15% of the isolates could produce indole from the amino acid tryptophan as revealed by the positive indole production test. Twenty-eight percent of the isolates had the ability to produce hydrogen sulfide from substrates such as sulfur containing amino acids and organic sulfur as they were positive for the hydrogen sulfide production test (Table 1 and Figure 1A).

The growth pH of the isolates ranged from pH 4.0 to 8.0. Isolates (Bacto15, 24, 25, 26, 28 and 41) grew well at pH 4 and 6 (Figure 1B), while isolate Bacto31 had an optimum growth at pH 6.0 and Bacto40 had the least growth at all the pH ranges. The isolates also exhibited growth at different temperatures; however the optimum growth temperatures ranged from 30 to 37°C. Isolates (Bacto31 and 40) had the least growth in all temperature ranges (Figure 1B). They also showed varied tolerance to different concentrations of sodium chloride up to 7% with a generally poor growth at 10% sodium chloride concentration. The isolates exhibited varied tolerance to different concentrations of ammonium sulfate up to 15% and a generally poor at 20% of ammonium sulfate demonstrating that the isolates could not tolerate very high levels of ammonia (Figure 1B). Isolates (Bacto21, 31 and 40) had the least growth in almost all ammonium sulfate concentrations. All the isolates tested positive for the acetylene reduction test (ARA) meaning they all produced the nitrogenase enzyme (Figure 1A). Forty-six percent of the isolates were positive for casein hydrolysis while 28% of the isolates were able to hydrolyze starch and gelatin (Table 1 and Figure 1A). 30% of the isolates were positive for cellulase activity while 20% of the isolates were positive for the xylanase activity. This showed that these isolates were able to degrade cellulose and xylan, respectively, suggesting their potential role in the degradation of plant biomass in the environment. Twenty-four percent of the isolates were positive for lipolytic activity (Table 1 and Figure 1A).

Affiliation of 16S rRNA gene sequences of the isolates

A total of 26 representative isolates from guts of *Macrotermes* and *Odontotermes* spp., were selected based on the morphological (Table 2) and biochemical characteristics (Gram, catalase, urease, methyl red and Voges Proskauer tests) for further characterization using molecular approach. The isolates (prefixed as Bacto with their accession numbers in parenthesis) in the inferred phylogenetic tree were phylogenetically diverse and affiliated with known members from different phyla including *Firmicutes, Proteobacteria* and *Actinobacteria* (Figure 2A and B). Comparison of the newly isolated 16S rRNA gene sequences to known sequences in the Genbank database using Blastn analysis indicated sequences similarities of >90% with known sequences (Table 2). Most of the isolates (27%) were closely affiliated with members of the genus *Enterobacter* with >95 sequence identity. Four isolates (Bacto19, 24, 26 and 35) had >98% sequence identities with known members of the genus *Pseudomonas* that together formed a single sub-cluster supported with a bootstrap value of 99% (Table 2 and Figure 2A). Bacto26 [KF872751] and Bacto35 [KF872760] were obtained from *Macrotermes* spp. and were phylogenetically identical and had 100% sequence affiliation with *Pseudomonas libanensis* [KC789764] and *Pseudomonas rhodesiae* [KF923822]. Bacto19 [KF872744] was also obtained from *Macrotermes* sp. and was 100% affiliated with *Pseudomonas monteilii* [KF475842] while Bacto24 [KF872749] was isolated from *Odontotermes* sp. and on the same branch together with other *Pseudomonas* species (Table 2; Figure 2A). This was supported by a bootstrap value of 99%. Four isolates (Bacto32, 34, 39 and 40) were affiliated with members of the genus *Serratia* [with 97–100% sequence identities] (Table 2). These isolates formed a single sub-cluster with several other members of the genus *Serratia* as indicated in the inferred phylogenetic tree (Figure 2A). Bacto32 and 34 were isolated from *Odontotermes* spp. while Bacto39 and 40 were from *Macrotermes* spp. Isolate Bacto37 [KF872762] was 100% affiliated with *Klebsiella pneumonia,* while Bacto41 [KF872766] was 100% related to *Citrobacter farmer* (JX393004) and clustered with other *Citrobacter* species. Bacto28 [KF872753] had 100%

Table 1. Biochemical and enzymatic properties of bacterial isolates obtained from the guts of *Odontotermes* and *Macrotermes* species collected from JKUAT in July 2012.

Isolate code	Gram test	Catalase test	Urease test	Nitrate reduction	Citrate utilization	HS production	Ammonifying test	MR Test	VP test	Nitrite test	Indole test	Casein hydrolysis	Starch/ gelatin hydrolysis	Cellulase	Xylanase	Lipase
Bacto1	+	+	-	-	+	-	+	-	-	+	-	-	+	-	-	-
Bacto2	+	+	-	+	-	-	+	-	-	+	-	+	-	-	-	+
Bacto3	+	+	-	-	+	-	+	-	-	+	-	-	-	-	-	+
Bacto4	+	+	-	-	+	-	+	-	-	+	-	-	+	-	-	+
Bacto5	-	+	+	-	+	-	+	-	-	+	-	+	+	-	+	+
Bacto6	+	+	+	-	-	-	+	-	-	+	-	-	-	-	-	-
Bacto7	+	+	-	-	+	-	-	+	-	+	-	-	-	-	-	-
Bacto8	-	+	-	+	+	+	+	+	+	+	-	-	-	-	-	-
Bacto9	-	+	+	-	-	+	+	-	-	+	-	-	-	-	-	+
Bacto10	-	+	+	-	+	+	+	+	-	+	-	-	-	-	-	+
Bacto11	+	+	-	+	+	+	-	-	-	+	+	+	-	-	-	+
Bacto12	+	+	-	-	+	+	+	-	-	+	+	+	-	-	-	-
Bacto13	-	+	+	-	+	+	+	-	+	+	-	+	+	-	-	-
Bacto14	+	+	-	+	+	+	+	-	-	+	+	+	+	-	-	-
Bacto15	+	+	+	+	+	+	+	-	-	+	+	+	-	+	-	-
Bacto16	+	+	+	+	+	+	+	-	-	+	+	-	-	+	-	-
Bacto17	-	+	+	+	+	-	+	-	+	+	-	+	-	-	-	-
Bacto18	-	+	+	+	+	-	+	-	+	+	-	+	-	-	-	-
Bacto19	+	+	+	-	+	-	+	-	-	+	-	-	-	-	-	-
Bacto20	-	+	-	+	+	-	+	-	+	+	-	-	+	+	-	+
Bacto21	+	+	-	-	+	-	+	-	-	+	-	+	-	-	-	-
Bacto22	-	+	+	+	+	-	+	-	+	+	-	-	+	+	-	-
Bacto23	+	+	-	+	-	-	+	+	+	+	-	-	+	-	-	-
Bacto 24	-	+	-	-	+	-	+	-	-	-	+	-	-	+	-	-
Bacto 25	-	+	-	-	+	-	+	+	-	+	-	-	-	-	+	-
Bacto 26	-	+	+	+	+	-	+	+	-	+	+	+	-	+	-	-
Bacto 27	+	+	+	-	+	-	+	+	+	+	-	+	-	+	-	-
Bacto 28	-	+	+	+	+	-	+	+	-	+	-	+	-	-	-	-
Bacto 29	-	+	-	-	+	-	+	+	+	+	-	-	-	-	-	-
Bacto 30	+	+	-	-	+	-	+	+	+	+	-	-	-	-	-	-
Bacto 31	+	+	-	+	+	+	-	-	+	+	-	-	-	-	-	-
Bacto 32	-	+	+	-	+	-	+	-	-	+	+	-	-	-	+	-
Bacto 33	-	+	-	-	+	-	+	+	-	+	-	-	-	-	+	-
Bacto 34	-	+	-	+	-	-	+	-	-	+	-	-	-	-	+	+
Bacto 35	+	+	-	-	+	-	+	+	-	+	-	-	-	-	-	+
Bacto 36	-	+	-	-	+	-	+	+	+	+	-	-	+	-	-	-
Bacto 37	+	+	+	-	+	-	+	-	+	+	-	-	-	-	+	-
Bacto 38	+	+	-	-	-	-	+	-	+	+	-	-	-	+	-	+
Bacto 39	-	+	+	-	+	-	+	+	-	+	-	-	+	+	+	-
Bacto 40	-	+	+	-	+	-	+	-	-	+	-	+	-	+	+	-
Bacto 41	-	+	+	-	+	-	+	-	-	+	+	+	-	+	-	-
Bacto 42	+	+	-	-	+	-	+	-	-	+	-	+	-	+	-	-
Bacto 43	-	+	+	-	+	-	+	-	-	+	-	+	-	-	-	-
Bacto 44	+	+	-	-	+	-	+	+	-	+	-	+	-	-	+	-
Bacto 45	+	+	-	-	+	-	+	+	-	+	-	+	-	+	-	-
Bacto 46	+	+	-	-	+	-	+	-	-	+	-	+	-	-	-	-

+ Denotes a positive result and - denotes a negative result for the test. MR– Methyl Red, VP – Voges-Proskauer.

Table 2. Taxonomic affiliation and percentage sequence similarities of bacterial isolates with closest relatives from the Genbank database.

Sample ID	Accession No.	Representative isolates ID	Host/Termite sp.	Closest taxonomic affiliation	Isolation Source	ID (%)
Bacto27	KF872752	-	*Odontotermes* sp.	*Enterobacter cancerogenus* strain Lpq4 (HQ154134)	rice paddy soil	99
Bacto33	KF872752	-	*Macrotermes* sp.	*Enterobacter cancerogenus* strain Lpq4 (HQ154134)	rice paddy soil	99
Bacto29	KF872754	-	*Odontotermes/ Macrotermes* spp.	*Enterobacter* sp. (AY596467)	N/A	100
Bacto36	KF872761	-	*Odontotermes* sp.	*Enterobacter* sp. (AY596467)	*N/A*	100
Bacto38	KF872763	-	*Macrotermes* sp.	*Enterobacter* sp. LCd2 (KF411753)	Alfalfa root nodules	99
Bacto22	KF872747	Bacto20	*Macrotermes* sp.	*Enterobacter* sp. RsN-1 (AB673456)	*R. speretus*	98
Bacto31	KF872756	Bacto11, 12, 14	*Odontotermes/ Macrotermes* spp.	*Enterobacter cancerogenus* strain Lpq4 (HQ154134)	rice paddy soil	96
Bacto34	KF872759	-	*Odontotermes* sp.	*Serratia* sp. Cd22 (AB673460)	*C. domesticus*	97
Bacto32	KF872757	Bacto5	*Odontotermes* sp.	*Serratia marcescens* strain Z1085 (KC212068)	N/A	100
Bacto39	KF872764	-	*Macrotermes* sp.	*Serratia nematodiphila* strain Z65 (KC212076)	Milk	100
Bacto40	KF872765	Bacto43	*Macrotermes* sp.	*Serratia nematodiphila* strain Z65 (KC212076)	Milk	100
Bacto24	KF872749	-	*Odontotermes* sp.	*Pseudomonas aeruginosa* strain GH2T (KC864775)	Water	99
Bacto26	KF872751	Bacto18, 17	*Macrotermes* sp.	*Pseudomonas libanensis* strain BGR5 (KC789764)	Soil	99
Bacto35	KF872760	Bacto44, 45	*Odontotermes/ Macrotermes* spp.	*Pseudomonas libanensis* strain BGR5 (KC789764)	Soil	99
Bacto19	KF872744	Bacto6, 7	*Macrotermes* sp.	*Pseudomonas monteilii* strain IHB B 2329 (KF475842)	Rhizosphere soil	100
Bacto15	KF872745	Bacto16	*Odontotermes* sp.	*Bacillus licheniformis* strain DGB (KF840408)	Sugarcane bagasse	98
Bacto30	KF872755	-	*Odontotermes* sp.	*Bacillus cereus* (JX155762)	Rhizosphere soil	100
Bacto37	KF872762	-	*Macrotermes* sp.	*Klebsiella pneumonia* (JX390619)	Ethnomedicinal	100
Bacto25	KF872750	Bacto33	*Odontotermes* sp.	*Staphylococcus* sp. MEF7 (JN660060)	Fermented food	100
Bacto13	KF872743	-	*Marotermes* sp.	*Bacillus simplex* (KJ161416)	Sediment	99
Bacto41	KF872766	-	*Odontotermes* sp.	*Citrobacter farmeri* strain W17-1 (JX393004)	Frog gut	100
Bacto28	KF872753	-	*Odontotermes/ Macrotermes* spp.	*Proteus mirabilis* strain ZK1 (KF471515)	Waste water	100
Bacto23	KF872748	-	*Macrotermes* sp.	*Micrococcus terreus* strain DL20 (HQ009859)	River water	99
Bacto21	KF872746	Bacto2, 3, 4	*Macrotermes/ Odontotermes* spp.	*Rhodococcus equi* strain SW9 (KF873018)	Soil	99

N/A denotes not applicable; ID denotes identity

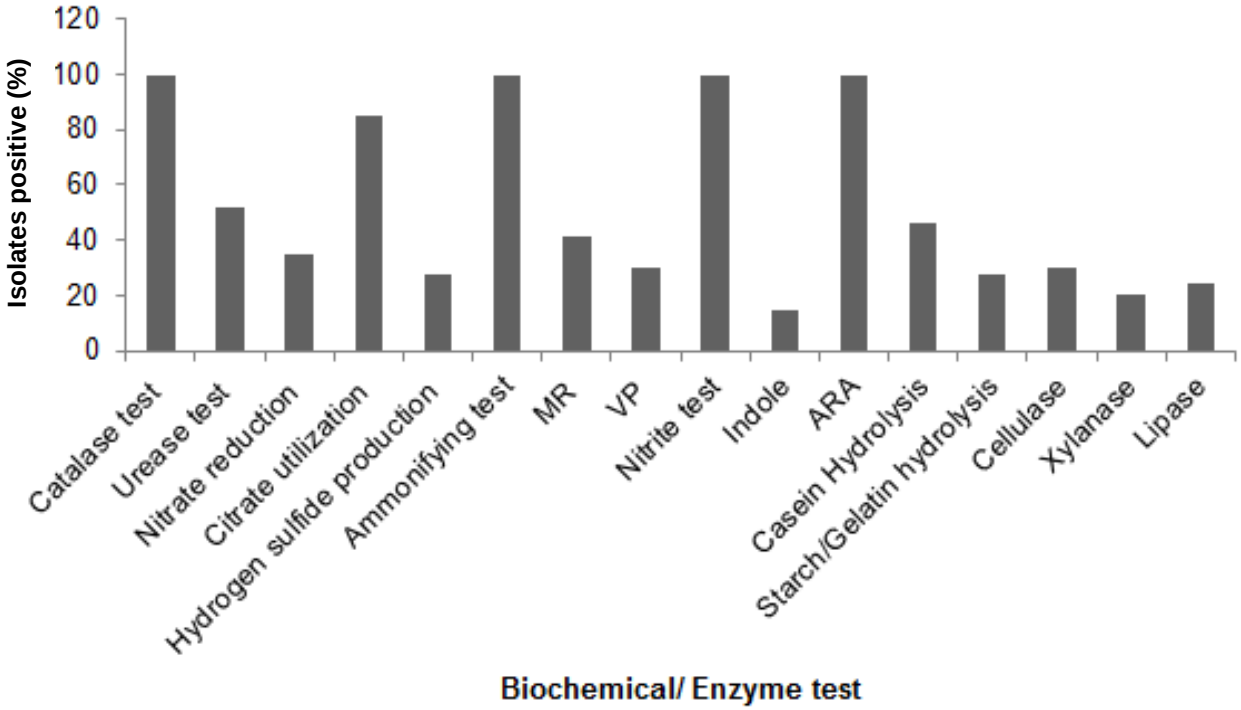

Figure 1A. Biochemical and enzymatic properties of bacterial isolates obtained from termite gut. MR 'denotes' Methyl Red; VP 'denotes' Voges–Proskauer; ARA 'denotes' acetylene reduction assay.

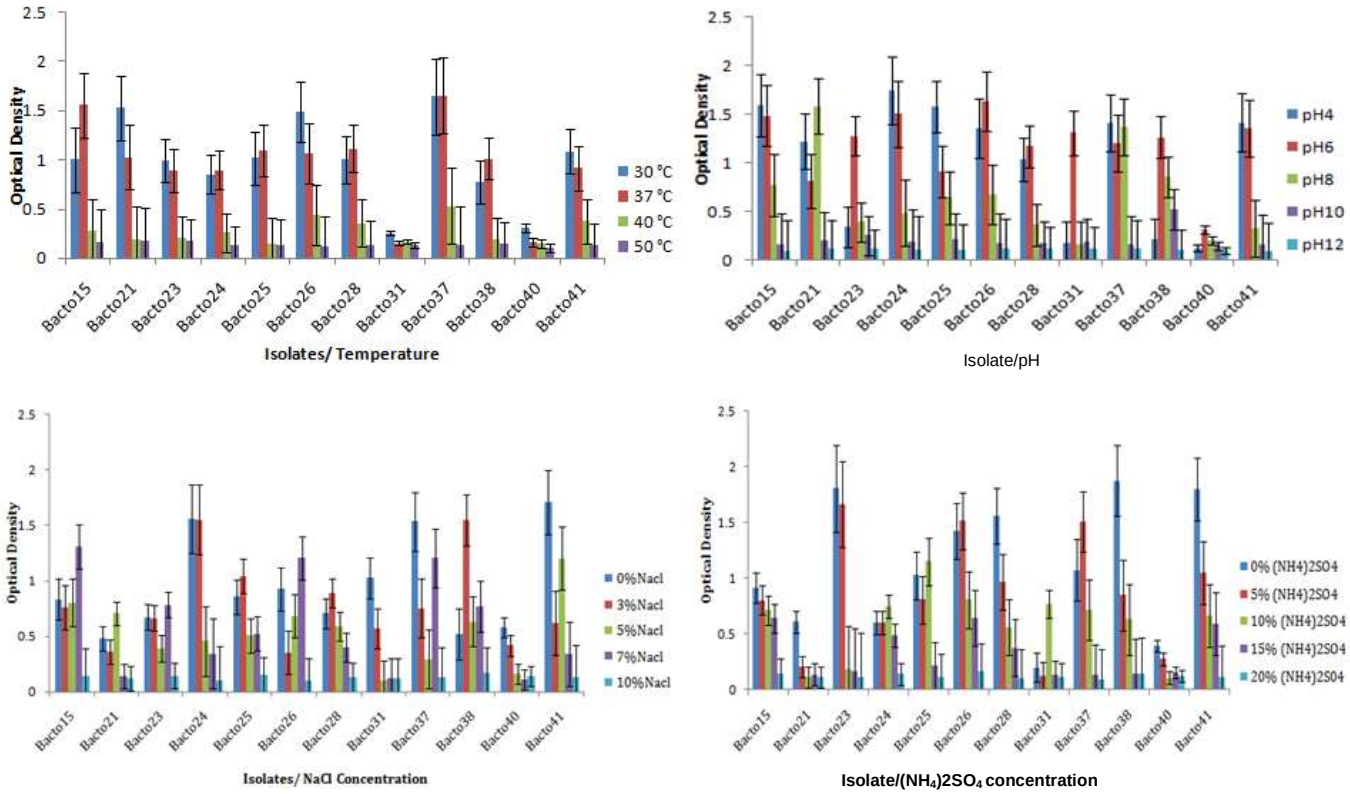

Figure 1B. Physiological properties of bacterial isolates.

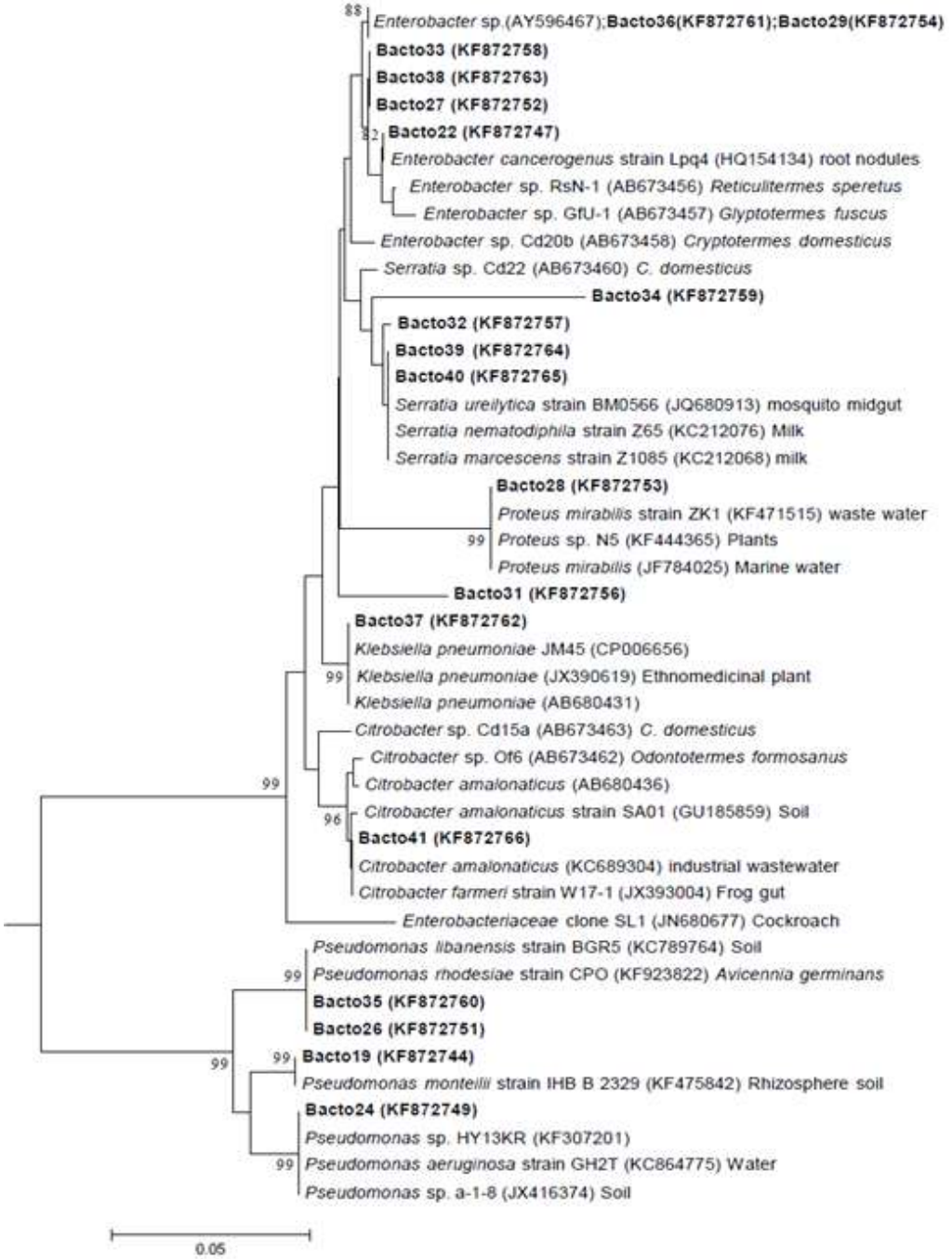

Figure 2A. Evolutionary relationships between partial 16S rRNA gene sequences of the isolates and some selected known bacterial species.

sequence similarity with other *Proteus* species that together formed a separate branch supported by a bootstrap value of 99% (Figure 2A). Isolates Bacto13, Bacto15 and Bacto30 were closely affiliated with members from the genus *Bacillus* (with between 98-100% sequence identities) and were from different termite species (Table 2). The three isolates formed a separate minor sub-cluster together with other known *Bacillus* species (Figure 2B). Bacto25 clustered with members of the genus *Staphylococcus*. Two isolates Bacto23 [KF872748] and Bacto21 [KF872746] formed a separate sub-cluster with some members belonging to the phylum *Actinobacteria.* Bacto23 had 99% sequence similarity with *Micrococcus terreus* (HQ009859) while

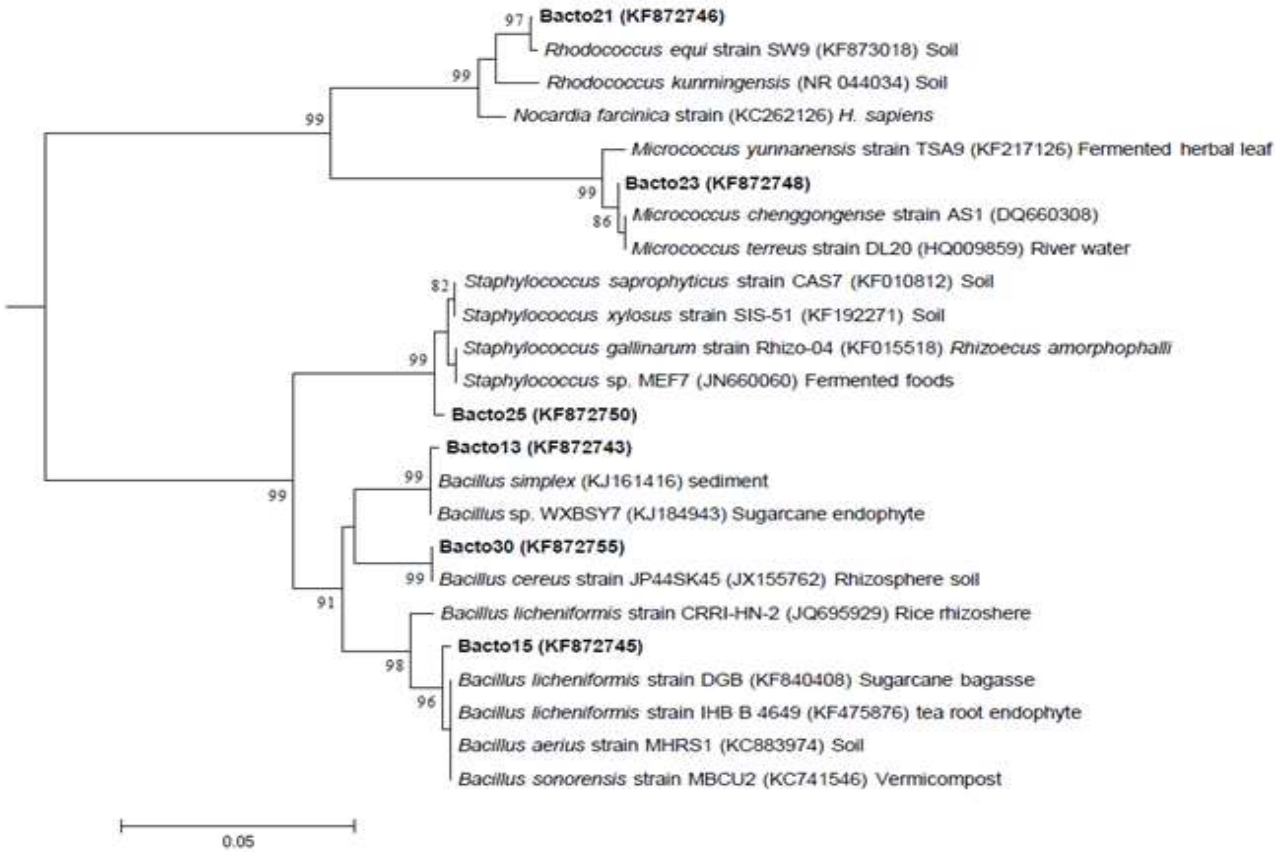

Figure 2B. Evolutionary relationships between partial 16S rRNA gene sequences of the isolates and some selected known bacterial species. The scale bar indicates approximately 5% sequence difference. Isolates are prefixed as Bacto and in bold font with accession numbers in parenthesis. Numbers at nodes indicate bootstrap values of each node out of 100 bootstrap re-samplings. The 16S rRNA sequence of *Methanoculleus thermophiles* (accession number, AB065297) was used to root the tree.

Bacto21 was 99% related to *Rhodococcus equ i* (KF873018) (Table 2 and Figure 2B). Four poorly resolved sequences (Bacto1, 5, 9 and 46) were excluded from the phylogenetic tree analysis.

DISCUSSION

In this study, the authors successfully isolated and partially identified termite gut bacterial microsymbionts that could be potentially involved in nitrogen cycle activities and degradation of plant biomass. The findings of this study support previous data that termites harbor gut bacterial symbionts, which aid in nitrogen fixation (Majeed et al., 2012) and lignocellulose digestion (Brune and Ohkuma, 2011). The isolates had the ability to degrade nitrogenous biopolymers and subsequently release ammonia, which is enzymatically converted to nitrites and nitrates as well as produce the enzyme catalase that converts hydrogen peroxide to water as demonstrated by the ammonifying, nitrifying and catalase tests, respectively. Majority of the isolates also had the ability to use citrate as the sole source of carbon. The abilities of some isolates to oxidize glucose and reduce nitrates to nitrites (Table 1 and Figure 1A) indicate their possible roles in the nitrogen metabolism activities (Lengeler et al., 1999). Notably, all the isolates demonstrated nitrogenase activity, which possible implicate them in nitrogen fixation activities. Brauman et al. (2015) demonstrated that termites' gut microbiota are responsible for nitrogen fixation that underscores nitrogen fixation activities within the guts of the fungus cultivating termites (Majeed et al., 2012).

Molecular characterization and phylogenetic analysis showed that all the isolates were affiliated with three bacterial phyla (*Proteobacteria*, *Actinobacteria* and *Firmicutes*) and that they belonged to ten different general (Figure 2A and B). The isolates Bacto22, 27, 29, 31, 33, 36, and 38 were obtained from different termite species (Table 2) phylogenetically related to the genus

Enterobacter (with >95% sequence similarity) and this was further supported by their ability to reduce nitrate to nitrite and being catalase positive. Members of *Enterobacter* that were previously isolated from termite guts were shown to play a role as nitrogen fixers (Adams and Boopathy, 2005). The detection of isolates (such as Bacto41, affiliated with the genus *Citrobacter*) demonstrates the presence of termite gut symbionts that can ferment glucose, reduce nitrates to nitrites and use citrate as a carbon source. Some members of this group have previously been isolated from the gut of termites (Adams and Boothy, 2005) and may play an important role in the nitrogen metabolism. The detection of gut isolates (Bacto19, 24, 26 and 35) belonging to genus *Pseudomonas* may suggest their contribution in assisting termites to degrade low-molecular-weight compounds derived from the breakdown of plant- and animal-biomass (Madigan and Martinko, 2005). Though Bacto35 and 26 formed a monophyletic sub-cluster, they exhibited different biochemical characters associated with members of the genus *Pseudomonas.* Bacto35 was Gram positive, MR positive, and Indole negative while Bacto26 had opposite reactions to these tests. Such contradiction in the biochemical tests may be due to the complications associated with this genus on its taxonomy that is better resolved by the analysis of the ribosomal RNA as depicted in the phylogenetic tree (Figure 2A).

The inferred phylogenetic tree positioned isolates Bacto37 and Bacto28 together with the genera *Klebsiella* and *Proteus,* respectively. Members of the genus *Klebsiella* are Gram negative, rod shaped and facultative anaerobic bacteria as observed from the morphological and biochemical results of isolate Bacto37. Some *Klebsiella* species including *Klebsiella pneumonia*, *Klebsiella oxytoca* and *Klebsiella planticola* are capable of fixing nitrogen and are classified as associative nitrogen fixers (Chelius and Triplett, 2000). Isolate Bacto23 was phylogenetically related to members of the genus *Micrococcus* with 99% sequence similarity.

Micrococcus has the ability to aerobically produce acid from glucose, glycerol and aesculin hydrolysis, major pigment production, motility, and conversion of nitrate to nitrite. It has been reported that members of this genus play a key role in the nitrogen cycle as denitrifying bacteria (Smith et al., 1999). Indeed, termites form a model system for studying host-symbiont relationship and bioreactor systems. The isolation of different bacterial strains from the guts of termites demonstrates that termites harbor diverse bacterial lineages, some of which perform different activities associated with plant biomass degradation and nitrogen metabolism.

Conflict of Interests

The authors have not declared any conflict of interests.

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