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Identification of a Nitrogen Fixation Endophyte from *Odontotermes formosanus*

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Termites are worldwide pests causing considerable damage to agriculture, forestry and buildings. Although physical and chemical methods have been tried to eliminate termite populations, they have the limitations such as low effectiveness, high-toxicity residue, environmentally harmful and high cost. Therefore, it has attracted much attention to develop highly effective, low-toxicity, long residual period, environmental friendly and low-cost termiticides. Here, we report the characterization andnitrogen fixation activities of a nitrogen fixation endophytic bacterium, strain HDZK-BYTG4, isolated from *O. formosanus*. The morphology and physiochemical characteristics of HDZK-BYTG4were very similar to that of *Klebsiella pneumoniae*. Analysis of 16S rDNA gene sequence showed that strain HDZK-BYTG4 exhibited >99 % sequence similarity to *K. pneumoniae*. Combining above phenotypic and phylogenetic properties, strain HDZK-BYTG4 was identified as *K. pneumoniae*. Strain HDZK-BYTG4 had nitrogen ûxation activities to apparently complement the termite's nitrogen deficient diet and HDZK-BYTG4 produced signiûcant amounts of ammonia when it was grown on nitrogen deûcient medium under anaerobic condition. These results provided the basis for studying the nitrogen-fixing mechanisms in the termite gut and facilitated the development of nitrogen-fixing microorganism resources.The objective of this study was to isolate, identify, and characterize facultative anaerobic bacteria in the gut of *O. formosanus* and their possible role of facilitating the development of nitrogen-fixing microorganism resources.

Key words: *Odontotermes formosanus*; biocontrol; *Klebsiella pneumoniae*; Endophytic bacteria; Nitrogen fixation.

Termites are infamous economic pests on a global scale. In fact, termites are the most damaging class of pests in the tropics and can cause considerable problems in agriculture, forestry and housing. In addition, they can damage many other types of materials, such as paper, fabrics, wood structures, and non-cellulose materials such as asphalt, asbestos, bitumen, lead and metal foils (Bultman *et al*., 1979). The cost for damaged houses, trees, dams and buried cables is estimated to be $250-300 million in China(Zhang *et al*., 2008).

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The termite gut contains endosymbiotic protozoa and bacteria (Ding *et al*., 2013). The prokaryotic microorganisms found in the termite gut perform a wide range of physiological functions such as digestion of cellulose and hemicellulose, acetogenesis, hydrogenesis, methanogenesis, sulfate reduction, and nitrogen fixation (Ohkuma *et al*., 1996; Mark *et al*., 2008). The process of cellulose digestion begins with the flagellated protozoans of the gut. The protozoa degrade cellulose into hydrogen, acetate and carbon dioxide (Nakashima *et al*., 2002). Acetogenic bacteria of the gut take the hydrogen and carbon dioxide produced by the protozoa and metabolizes them into acetate(Mark *et al*., 2008).

A termite's diet includes nitrogen poor wood, typically having nitrogen content as low as

0.3-1.0 g/kg. Therefore, the nitrogen fixing bacteria in the hindgut of the termites are considered important because the termite's diet is nitrogen poor (Breznak *et al*., 1973). A symbiotic relationship between termites and microorganisms inhabiting their guts contributes to termites being able to live by xylophagy (Ohkuma *et al*., 1996). Although their diet is usually low in nitrogen source, they thrive in great abundance, particularly in tropical regions. Nitrogen fixation in termites has been demonstrated by using the acetylene reduction assay.As early as 1973, Breznak*et al*.(1973) used acetylene reduction and nitrogen fixation reagent combination to demonstrate parts of the bacteria in the termites considered as associative nitrogen fixers.Having now been confirmed,the nitrogen-fixing microorganisms are important (Leadbetter *et al*., 1996).The level of nitrogen fixation activity depends on the species and composition of symbiotic microorganism in the gut of termites. Only a few nitrogen fixing bacteria, however, have been isolated from termite guts. The purpose of this study was to isolate, identify, and characterize facultative anaerobic bacteria in the gut of *O. formosanus* and their possible role in the microbial ecosystem.We have isolatednitrogen fixation activity endophytic bacteriaHDZK-BYTG4 from the gut of*O. formosanus*, and strain HDZK-BYTG4 belonged to the species *Klebsiella pneumoniae*. The results showed the role of nitrogen fixing bacteria in the microbial ecology of termite gut ecosystem, and provided the basis for studying the nitrogen-fixing mechanism in the termite gut, and facilitated the development of nitrogen-fixing microorganism resources.

MATERIALS AND METHODS

Bacterial strains, medium and culture conditions

Strain HDZK-BYTG4 was separated from the gut of *O. formosanus*. The storage location of strain HDZK-BYTG4 was the Key Laboratory of Microbiology, College of Life Science and China center for type culture collection, Heilongjiang University. In the meantime, the reference *K. pneumoniae*s train was provided by China General Microbiological Culture Collection Center.

Morphological examination

According toShinzato*et al* (2007), strain HDZK-BYTG4 was cultured at 37°C on beef extract and peptone medium for24 h.After inoculating at three distinct spots, the bacterium would be cultivated at 37°C for another 24h.Furthermore, as Shinzato*et al* (2007) stated, the strain HDZK-BYTG4 was tested and confirmed to the races and species standards based upon the morphological character. Both light microscopy and transmission electron microscope have been applied to test the morphology of the strain HDZK-BYTG4.

Physiological and biochemical characteristics

The physiological and biochemical characteristics of the strain HDZK-BYTG4 and reference strains was examined by Manual of systermatic determinative bacteriology(Dong *et al*., 2001) and Microbiology experiment(Shen *et al*., 1999).

16S rDNA gene sequencing and phylogenetic analysis

Strain HDZK-BYTG4 was cultured as previously described(Bassam *et al*., 1991). Extract the total DNA as gene amplification template. Primer pairs were 5'-AGAGTTTGATCATGGC TCAG-3' and 5'-ACGGTTACCTTGTTACGACTT-3'. The reactions were followed through PCR amplification kit of shanghai biological engineering company. The PCR was performed under conditions of 95°C denaturation for 30s, running 30 cycles of 55°C for 1min and 72°Cfor 2 min. The analysis of sequence splicing and sequence similarity was completed by DNA Star software; the genetic comparisons were carried out by the NationalCenter for Biotechnology Information NCBI database (http://www.ncbi.nlm.nih.gov).

Fatty acid dyeing and SDS-PAGE analysis

Fatty acid dyeing was implemented as discussed above (Lu *et al*., 1997). In addition, the result of GC operating conditions was tested according to the method described of Igor *et al*. (2005).

For SDS-PAGE analysis,concentration of separation gel and stacking gel is 12.5% and 4%. 15 g Tris, 72 g of glycine and5g SDS were dissolved in 1 L of deionized water and diluted 5-fold with time. All samples were electrophoresed with electric current of 25 mA for 2-2.5 h. Silver staining was color developed and 2 repetitions per treatment (Görg *et al*., 2004). The gel was visualized by UMAX Powerlook 2100 XL (Japan) scanning.

Nitrogen fixation assay

The method was accordance with the

approaches described by Ma *et al.* (2007) and had been improved. To be specific, 5 ml improved nitrogen medium was made into agar slant in a 15 mm×150 mm tube at first. After inoculating nitrogen fixing bacteria, the agar slant was cultured for 72 h at 28°C. Before the injection of acetylene gas, the rubber seal must be changed. After promoting the acetylene to a final concentration of 10%, continue to cultivate for another 72 h. Next, 100µL reactant gas will be detected by gas chromatograph for production of ethylene.Then, the results of nitrogenous activity of strain was calculated with the formula, enzyme activity ($nmol \cdot mg^{-1} \cdot h^{-1}$) = C_2H_4(nmol) / [bacterial protein (mg) × reaction time (h)]. The blank agar slant which had not been inoculated was negative control. Moreover, strain HDZK-BYTG4was positive control, 3 repetitions per treatment. Data was analyzed by SPSS software.

Statistical analysis

Statistical and graphical analysis was employed with SPSS software to evaluate differences in nitrogenase activity test. Data was expressed as X±SD.

RESULTS

Morphological characterization

Strain HDZK-BYTG4 in the nitrogen free medium formed with round,translucent,milky white andregular edges, which was moist on the surface and not easy to pick up. Strain HDZK-BYTG4 was gram-negative, spore staining negative, capsule staining positive and semisolid puncture negative. Compared with the reference strain(the cell size was 0.3-1.5µm × 0.6-6.0µm),the cell size of strain HDZK-BYTG4 was 0.6-0.9 µm × 2-2.5µm (Fig.1). Strain HDZK-BYTG4 contained a large polysaccharide capsule. They usually occurred singly or in pairs.Sometimes they existed in aggregates of several cells (Fig. 1).

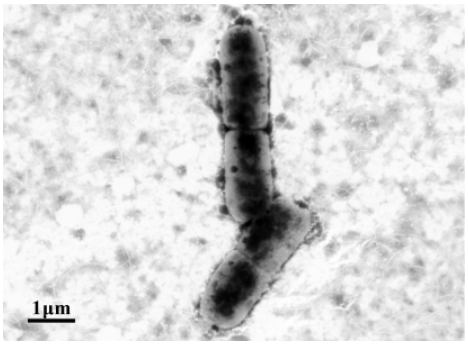

Fig. 1. Morphology of the strainHDZK-BYTG4 with nitrogen fixation activity under a transmission electron microscope (magnification, 80×)

Physiological and biochemical characteristics of strain HUB- IV-004

As shown in Table 1, the physiological and biochemical characteristics of strain HDZK-BYTG4 were in accordance with those of the reference *K. pneumoniae*strain. Strain HDZK-BYTG4 was gelatin hydrolysis negative, starch hydrolysis test positive, urea use test positive,fat hydrolysis negative, indole production test negative, V-P reaction test positive, methyl red test positive, citrate use test positive, and urinary enzyme test positive. The isolate which confirmed the isolate as *K. pneumoniae* produced gas, fermented glucose, lactose, sucrose, maltose and acid and was negative for hydrogen sulfide.

Table 1. Physiological and biochemical properties of the strain HDZK-BYTG4

Characteristics	Strain HDZK-BYBN004	Reference strain
Starch hydrolysis	+	+
Fat hydrolysis	-	-
Gelatin hydrolysis	-	-
Hydrogen sulfide	+	+
Urea utilization	+	+
Indole production	-	-
Methyl red	+	+
V-P reaction	+	+
Citrate utilization	+	+
Urinary enzyme	+	+

+ indicates positive reaction; - indicates negative reaction.

Molecular analysis

The 16S rDNA of strain HDZK-BYTG4 was amplified by PCR with size of about 1,600 bp. The newly identified sequence was submitted and deposited into GenBank (Accession number JN848784). It was found that the 16S rDNA sequence of the strain HDZK-BYTG4 were similar to genus *Klebsiella* through BLAST, After exporting identified 16S rDNA of strains, ClustalW is adopted for multiple sequence alignment and phylogenetic tree construction. As shown in Fig. 2, strain HDZK-BYTG4 was the closet to the *K. pneumoniae*. Specifically, their homology was above 99%.

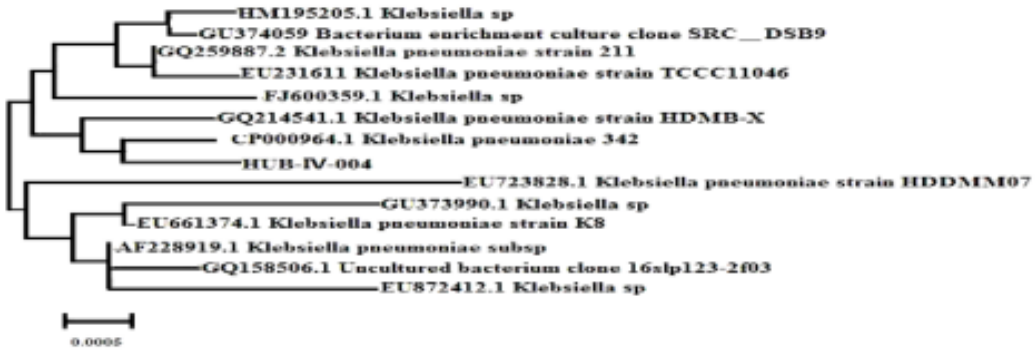

Fig. 2. Phylogenetic tree showing the relationship of the strain HDZK-BYTG4 with other related bacterial species from GenBank based on their homologous sequences of 16S rDNA

Fatty acid dyeing and SDS-PAGE analysis

Fatty acid dyeing analysis showed that a peak was phenol, 2.4-bis (l, 1-dimethylethyl) with a chemical formula of $C_{14}H_{22}O$, b peak was 1-Decanol, 2-hexyl- with a chemical formula of $C_{16}H_{34}O$, and c peak was tetratriacontyl heptafluorobutyrate with a chemical formula of $C_{38}H_{69}F_7O_2$ (seen in Fig.3). SDS-PAGE analysis showed that the protein banding of strain HDZK-BYTG4 was in accordance with those of the reference *K. pneumoniae* strain (Fig. 4). Due to the morphological characterization, physiological and biochemical characteristics, Molecular analysis, phylogenetic analysis, fatty acid dyeing and SDS-PAGE analysis, strain HDZK-BYTG4 was categorizedthe genus *Klebsiella* as a species of *Klebsiella pneumoniae*.

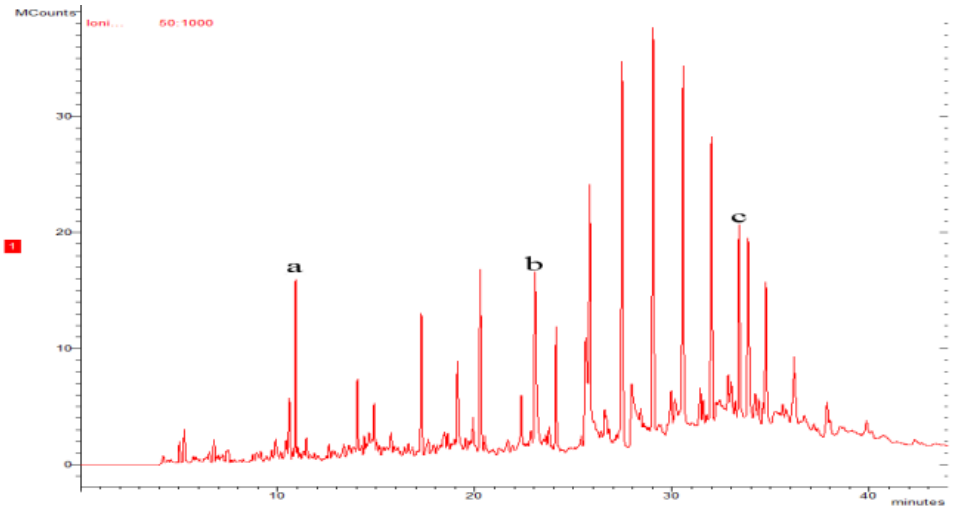

Fig. 3. GC-MS spectrum of the extract from the strain HDZK-BYTG4 using fatty acid dyeing analysis. a peak was phenol, 2.4-bis (l, 1-dimethylethyl) with a chemical formula of $C_{14}H_{22}O$; b peak was 1-decanol, 2-hexyl- with a chemical formula of $C_{16}H_{34}O$; and c peak was tetratriacontyl heptafluorobutyrate with a chemical formula of $C_{38}H_{69}F_7O_2$

Nitrogenase activity detection

The strain HDZK-BYTG4could grow on the nitrogen free medium, which showed a high level of nitrogenase activity of 213.46 ± 12.43 nmol C_2H_4/ (h·ml). Under anaerobic or microaerobic conditions, *K. pneumoniae* was considered as an associative nitrogen fixer. Isolates of *Klebsiella* have been found in living or decaying wood, bark,

and composted wood (Descamps *et al*., 1983). Therefore, it is reasonable to consider that the *K. pneumoniae* may play an important role in the hindgut microbial ecology.

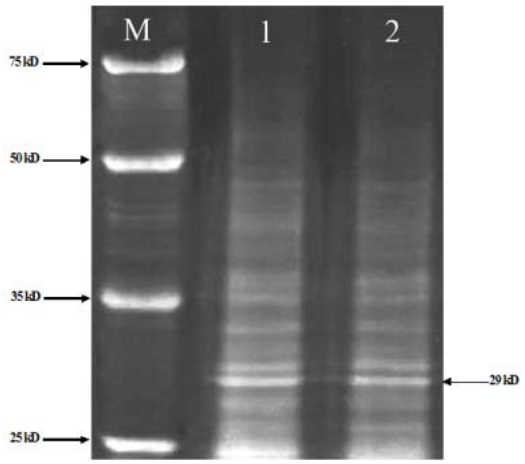

Fig. 4. Protein banding analysis of proteins extracted from the strain HDZK-BYTG4 using SDS-PAGE. Lane 1: protein banding from the strain HDZK-BYTG4; Lane 2: protein banding from the reference *K. pneumoniae* strain; M: protein marker including 25 kD, 35 kD, 50 kD and 75 kD

DISCUSSION

Having now been confirmed, the nitrogen fixing bacteria in the hindgut of the termites are important because the termite's diet is nitrogen deficient. We have isolatedendophytic bacteria with nitrogen fixation activity from the gut of*O. formosanus;* one of them is the strain HDZK-BYTG4. In addition, the morphological and physiological, biochemical characteristics, 16S rDNA sequences showed a close relationship of strain HDZK-BYTG4 with the genus *Klebsiella*. Molecular identification has been increasingly used as a powerful tool supplementary to the traditional systematic classification. In our study, the classical morphological identification was consistent with the molecular biological analysis, indicating that the strain HDZK-BYTG4 represents a nitrogen fixation bacterium from *O. formosanus*. Here we named it *K. pneumoniae*.

SDS-PAGE of protein extracts is a routine method to group and identify novel isolates based on protein patterns. Mark *et a*l. (2008) studied the diversity of *Klebsiella* using SDS-PAGE pattern clustering and the results were supported by 16S rDNA gene sequence analysis. Our results indicated that strain HDZK-BYTG4 harbored diverse nitrogen-fixing bacteria. The cellular fatty acid compositions of bacteria have been studied extensively and the fatty acid profiles of the whole bacterial cells have been proved useful for taxonomy studies. Lu *et al*. (1997)and Zhang *et al*. (2000) identified the *B. anthraci* and the related species and the pathogen bacteria using gas chromatograph. The finding of high nitrogenase activity in some endophytic diazotrophs suggests that these isolates might have a potential use in agriculture (Tan *et al*., 2009). The profiles of fatty acid of representative strains have been compared, and shown to be different from each other. According to the species and genus definition of the 16S rDNA sequence, HDZK-BYTG4 may represent a group of *K. pneumoniae.*

According to previous studies, nitrogen-fixing bacteria are capable of nitrogen recovery. To be specific, nitrogen-fixing bacteria can provide vitamins for improvement of activities of other microbial life; however cellulose decomposing bacteria decompose cellulose to provide a carbon source for nitrogen-fixing bacteria. Accordingly, azotobacter and cellulose decomposing bacteria can work mutually (Hethene *et al*., 1992). It's difficult to complete the biological degradation of cellulose alone; but it can work well with two or more microorganisms.Cheng *et al*. (2007) made azotobacter and cellulose-decomposing bacteria a mixed culture. In addition, the number of bacteria increased when the cellulose-decomposing bacteria and nitrogen-fixing bacteria were cultured together (Zhou *et al*., 2007). To further characterize the extracted product from strain HDZK-BYTG4, the characterizationof nitrogen fixation is necessary. It is critical to understand the mechanisms of how this bacterium was evolved, which is currently under investigation. Further research is needed to understand the ecology of this microbe. The isolation of strain HDZK-BYTG4 provides a new resource of important nitrogen fixation.

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