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Klebsiella variicola, a nitrogen fixing activity endophytic bacterium isolated from the gut of *Odontotermes formosanus*

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Termites play an important role in promoting agricultural ecosystem balance and the degradation of lignocelluloses, but also have caused considerable damage to agriculture, forestry and buildings. Previous studies showed that there were a lot of nitrogen-fixing bacteria in the termite gut, and up to 60% of the nitrogen in termite organs came from the nitrogen fixation of symbiotic microorganisms which lived in the hindgut of termites. We obtained a endophytic bacterium from *Odontotermes formosanus*, and the morphology, physiochemical characteristics, 16S rDNA sequence, fatty acid dyeing and SDS-PAGE analysis of strain HUB-IV-005 were very similar to those of *Klebsiella variicola*. Strain HUB-IV-005 had nitrogen fixation activities because it could grow on nitrogen deficient medium under anaerobic condition with nitrogen gas in the headspace. In addition, the nitrogenase gene from HUB-IV-005 was cloned and transformed into *E. coli*, and we identified that the expressed protein was a nitrogenase (iron protein) by mass spectrometry. Strain HUB-IV-005 had nitrogen fixation activities and belonged to the species *K. variicola*. The results provided the basis for studying the nitrogen-fixing mechanism in the termite gut, and nitrogen fixation also provided useful information for further explaining the mechanism of the termite's biological nitrogen fixation.

Key words: *Odontotermes formosanus*, *Klebsiella variicola*, endophytic bacteria, nitrogen fixation, protein separation and purification.

INTRODUCTION

Termites not only attack living trees, but also degrade wood and other celluloses in the terrestrial environment (Long et al., 2010). They can damage many other kinds of materials, from paper, fabrics and wood structures to non-cellulose materials such as asphalt, asbestos, bitumen, lead and metal foils (Lax and Osbrink, 2003; König, 2006; Monica et al., 2009). Biodegradation of wood caused by termites is recognized as one of the most serious problems for wood utilization. The annual damage to trees and wooden structures is estimated as $300 million dollars in New Orleans, LA, USA, and the cost for damaging houses, trees, dam, and buried cables is estimated at $250-300 million dollars in China (Zhong and Liu, 2002; Zhang et al., 2008). The total global damage by termites has been estimated to exceed $3 billion annually worldwide (Cheng et al., 2007). Therefore, there is a huge potential market for effective termiticidals to control the propagation of termites.

On the other hand, termites are important social creatures; they play important roles in the material cycling in nature and carbonization process. They are typical organisms that usually thrive on nitrogen-poor food

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(Liu et al., 2010), such as wood materials rich in cellulose, hemi-cellulose and lignin (Yamin, 1981; Oppert et al., 2010). Previous research has shown that the nitrogen content of termites' organs are similar to that of other animals, nitrogen-fixing process happened in the termite gut, and up to 60% of the nitrogen in termites' organs comes from the nitrogen fixation of symbiotic microorganisms living in the hindgut (Sarcinelli and Schaefer, 2009; Shi et al., 2010). The termite's hindgut has been recognized as the world's smallest bioreactor, and in decades it has attracted the attention of microbial researchers (Mei et al., 2002).

There are a lot of protozoans in the lower area of the termites' guts, symbiotic protozoans play very important roles in the digestion of lignocelluloses and there is some sort of symbiosis between the lower gut area of termites and their symbiotic animal relationships. Studies have shown that the basis of this symbiotic relationship is protozoan degradation of lignocelluloses material. Nitrogen fixation has been demonstrated in all termite families. Given their global distribution, termites may have widespread importance in the biogeochemical cycling of nitrogen in terrestrial ecosystems. Termites may contribute significant amounts of nitrogen to terrestrial ecosystems with their microbial flora of nitrogen-fixing bacteria (Curtis and Waller, 1998). As early as in 1973, Brenak adopted a combination of experimental methods with acetylene reduction and nitrogen fixation indicators; it confirmed that bacteria could fix nitrogen in the lower area of the termite's body (Breznak et al., 1973). In 1996, Ohkuma successfully cloned a *nifH* gene fragment encoding nitrogenase iron protein from the *Reticulitermes speratus* mixed intestinal microbial flora, which further confirmed that nitrogen-fixing microbes exist in the termite's intestine (Ohkuma et al., 1996). The nif*H* gene encodes nitrogenase and its main structure is conservative. The *nifH* nitrogenase activity contributes largely to the termite nitrogenase activity, and there is a high similarity of amino acid sequences derived from other wood-feeders' nitrogenase and termite's nitrogenase *nifH*. It explains why in termites *in vivo* nitrogenase diversity is related to the type of host life and its phylogenetic position (Inward et al., 2007).

We have isolated more than 183 endophytic bacterial strains from *Juniperus virginiana* grown in Louisiana and *Chamaecyparis lawsoniana* grown in Oregon, USA and the intestine of *Odontotermes formosanus* that lived in Logia Hill of Wuhan, China. One strain HUB-Ⅳ-005 with strong nitrogenase fixation activity was selected for this study. Through the analysis of morphological, physiological, biochemical characteristics, 16S rDNA sequences, fatty acid dyeing and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the expressed protein of nitrogenase (*nifH*) gene from HUB-IV-005 was a nitrogenase (iron protein) by mass spectrometry, we demonstrated that the strain HUB-IV-005 had nitrogen fixation activities and belonged to the species *Klebsiella variicola*. These results showed the role of nitrogen fixing bacterium 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

Strains for bioassay

The strain HUB-IV-005 with nitrogen fixation activity, which was isolated from the gut of *O. formosanus*, was stored in the Key Laboratory of Microbiology, College of Life Science and China center for type culture collection (CCTCC NO：M 20113132), Heilongjiang University. The reference *K. pneumoniae* strain was obtained from China General Microbiological Culture Collection Center. *Escherichia coli* DH5α and BL21 were kindly provided by the Laboratory of Molecular Biology, Heilongjiang University.

Morphological examination of the strain HUB-IV-005

Strain HUB-IV-005 was activated at 37°C on a 9 cm plate of beef extract and peptone medium containing 3 g/L beef extract, 10 g/L peptone and 5 g/L NaCl at 37°C for 10-24 h (Shen and Chen, 2007). The bacterium was inoculated at three different spots on the plate and then cultured at 37°C for 12-24 h. As described previously, the strain HUB-IV-005 was detected and identified to the genus and species levels based on the morphological characteristics (John et al., 1994; Shen and Chen, 2007). The morphology of the strain HUB-IV-005 was examined with both light microscope and transmission electron microscope. Somatic diameters were measured after Gram staining, spore staining, negative staining, and silver staining of the flagella. Digital micrographs of colonies were taken with a Coolpix 995 camera (Nikon, Tokyo, Japan).

Physiological and biochemical characteristics of the strain HUB-IV-005

Physiological and biochemical characteristics of the strain HUB-IV-005 and reference strains were examined as previously described (Ladha et al., 1983; Tan et al., 2009).

Molecular analysis of the strain HUB-IV-005

Culture and collection of mycelia were carried out as previously described (Liu et al., 2008). The 16S rDNA sequences were amplified using polymerase chain reaction (PCR) with primer pairs 5'-AGAGTTTGATCATGGCTCAG-3' and 5'-ACGGTTACCTTGTTACGACTT-3'. The PCR reactions were carried out as previously described (Zhao et al., 2011). The obtained sequences were submitted to GenBank for homology search with BLAST (http://rdp.cme.msu.edu and http//ncbi.nim.nih.gov). A phylogenetic tree was constructed from the evolutionary distances by PHYLIP (version 3.57c).

Fatty acid dyeing and SDS-PAGE analysis of the strain HUB-IV-005

Fatty acid dyeing was carried out as previously described (Lu et al., 1997). 40 mg wet bacterium were collected in a clean culture tube, then 1.0 ml of 15% (w/v) NaOH-methanol was added into the culture tube. The tubes were securely sealed with Teflon lined caps,

caps, vortexed briefly and heated in a boiling water bath for 30 min. The cooled tubes were uncapped, and 2.0 ml of 25% (v/v) HCl-methanol was added. The tubes were capped, vortexed briefly, and heated for 10 min at 80°C. One millilitre of hexane-methytert butyl ether (1:1, v/v) was added to the cooled tubes, followed by recapping and gentle tumbling the tubes for about 10 min. The tubes were uncapped and the aqueous (lower) phases were pipetted out and discarded. About 3 ml of 12% NaOH was added to the organic phase remaining in the tubes. Then the tubes were recapped, and tumbled for another 5 min. About 2/3 of the organic phase was pipetted into a GC vial, which was capped and ready for analysis. GC operating conditions was in accordance with the method described of Igor et al. (2005).

For SDS-PAGE analysis, 8% acrylamide gel was used and protein extraction of the *K. variicola* isolates was carried out as previously described (Alavandi et al., 2001). Discontinuous buffer system with 10% resolving gels and 5% stack gels was used. All samples were heated for 5 min in boiling water and electrophoresed with constant voltage of 120 V until the dye-front reached the bottom of the gel. The protein bands were visualized by Coomassie Brilliant blue-R250 staining.

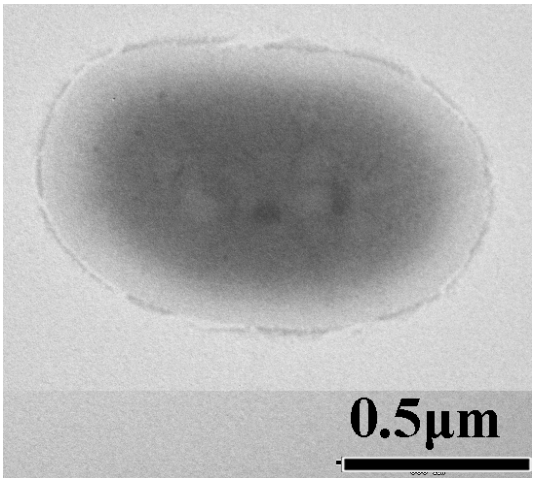

Figure 1. Morphology and capsular of the endophytic bacteria HUB-IV-005 under a transmission electron microscope (magnification, 50000×).

Nitrogen fixation assay of the strain HUB-IV-005

The strain HUB-IV-005 was activated, and cultured for different times at 37°C on a plate of nitrogen free medium composed of sucrose 10 g/L, malic acid 5.0 g/L, $K_2HPO_4{\cdot}H_2O$ 0.1 g/L, $KH_2PO_4{\cdot}H_2O$ 0.4 g/L, $MgSO_4{\cdot}7H_2O$ 0.2 g/L, NaCl 0.1 g/L, $CaC1_2{\cdot}2H_2O$ 0.02 g/L, $FeC1_3$ 0.01 g/L, $Na_2MoO_4{\cdot}H_2O$ 0.002 g/L and agar 7.5 g/L, pH 7.2 (Zhang et al., 2000).

Bacterial suspension 100 µl of strain HUB-IV-005 and 2 ml of liquid medium composed of sucrose 10 g/L, malic acid 5.0 g/L, $K_2HPO_4{\cdot}H_2O$ 0.1 g/L, $KH_2PO_4{\cdot}H_2O$ 0.4 g/L, $MgSO_4{\cdot}7H_2O$ 0.2 g/L, NaCl 0.1 g/L, $CaC1_2{\cdot}2H_2O$ 0.02 g/L, $FeC1_3$ 0.01 g/L, $Na_2MoO_4{\cdot}H_2O$ 0.002 g/L and peptone 0.2 g, pH 7.2 (Zhang et al., 2000) was added to culture bottles, and then cultured at 37°C for 24 h. Rubber stoppers were then replaced with cotton stoppers so that 0.5 ml air was exhausted from the sample bottles using 1 ml syringe, and 0.5 ml ethylene was reinjected. The holes produced by syringes were sealed by parafilm; the bottles were cultured at 30°C for another 24 h. Then 5.0 µl gas as aforementioned was exhausted from the bottle with 10.0 µl trace syringe. The ethylene quantity was tested by GC. Then nitrogen fixation activity was calculated according to ethylene quantity. Enzyme activity was demonstrated by gram-molecule of produced ethylene per hour per milliliter of the broth [nmol ethylene/ (h·ml)]. Three replicates were used for each test. The result was the average of three replicates.

Cloning and expression of nifH gene from the strain HUB-IV-005

The *nifH* gene sequences were amplified by PCR with primer pairs P3 (5'-GAATTCTCAGGCCGCGTTTTCTTCAG-3') and P4 (5'-CATATGATGACCATGCGTCAATGCG-3'). The plasmid of pET28a was extracted using TIANprep Mini Plasmid Kit (DP103-02, TIANGEN). The recombinant plasmids were identified by *EcoR*Iand *Nde*Idigestion and PCR amplification. Then the *nifH* gene was ligated overnight with pET28a vector by using T_4 DNA ligase. The protein expression was induced by the addition of IPTG, which was inoculated into 20 ml of Luria-Bertani (LB) medium that contained 50 µg/ml kanamycin. The affinity purified proteins were analyzed by SDS-PAGE (Wu and Liu, 2012) and stained by Coomassie blue; then it was bleached using a bleaching solution. Gel-spot at the theoretical pI and molecular weight was analyzed using mass spectrometry (Li et al., 2004). Tryptic digest was analyzed on a nanoESI-MS/MS (QSTAR XL hybrid quadrupole (Q) TOF). The peptides were separated on a PepMap100 C_{18} column using acetonetrile and water. The MS/MS data were submitted to Mascot software for searching the MS/MS spectra against a local version of NCBInr.

Statistical analysis

The Scheffe multiple comparison procedure from the SAS statistical program was employed to evaluate differences in nitrogenase activity test. Results with $p < 0.05$ were considered statistically significant. All the experiments were repeated three times and each measured in triplicate. All results were expressed as mean ± standard deviation (SD).

RESULTS

Morphological characterization

The colony of strain HUB-IV-005 was white without gloss, translucent, round, regular edges, which was moist on the surface and not easy to pick up with loops in the nitrogen free medium. Strain HUB-IV-005 was Gram-negative, and the cells were short rod-shaped, arranged single or short chain-like arrangements. The cell size of the strain were in accordance with the reference strain (the cell size was 0.2-1.2 × 0.4-5.0 µm), with a cell size of 0.5-0.8 × 1.0-2.0 µm, and there were no spores or movable flagella around the cell but thicker capsular (Figure 1).

Physiological and biochemical characteristics of strain HUB- IV-005

The basic biochemical tests confirmed the isolate as *K. variicola.* As shown in Table 1, the physiological and biochemical characteristics of strain HUB-IV-005 were the same as those of the reference *K. variicola* strain. Strain HUB-IV-005 was Gram-negative, citrate utilization positive,

Table 1. Physiological and biochemical characteristics of strain HUB-IV-005.

Characteristic	Strain HUB-IV-005	Reference strain
Gram staining	-	-
Citrate utilization	+	+
Spore staining	-	-
Capsule stain	+	+
Semisolid puncture	-	-
Starch hydrolysis	+	+
Fat hydrolysis	-	-
Gelatin hydrolysis	-	-
Urea utilization	+	+
H_2S	-	-
Indole production	-	-
V-P reaction	+	+
Methyl red	-	-
Urinary enzyme	+	+
Sucrose fermentation	A^+B^+	A^+B^+
Maltose fermentation	A^+B^+	A^+B^+
D-sorbitol fermentation	A^+B^+	A^+B^+
Rhamnose fermentation test	A^+B^+	A^+B^+
Mannitol fermentation	A^+B^+	A^+B^+
Lactose fermentation	A^+B^+	A^+B^+
Trehalose fermentation	A^+B^+	A^+B^+

+indicates positive reaction;indicates negative reaction; A^+ indicates acid production; B^+ indicates gas production.

spore staining negative, capsule staining posi-tive, semi-solid puncture negative, starch hydrolysis posi-tive, fat hydrolysis negative, gelatin hydrolysis negative, H_2S test positive, urea utilization positive, indole produc-tion negative, V-P reaction positive, methyl red positive and urinary enzyme positive. The isolate fermented glu-cose, lactose, sucrose, maltose, acid and gas.

Molecular analysis

The 16S rDNA of strain HUB-IV-005 was successfully amplified using PCR with an expected size of 1,579 bp. After sequencing, this newly identified sequence have been submitted and deposited into GenBank (Accession number GQ892930). After homology searching against GenBank or the proprietary bacteria DNA database, the sequence of strain HUB-IV-005 were found to share 99% similarity with those of *K. variicola*. A phylogenetic relationship was established through the alignment and analysis of homologous nucleotide sequences among these bacteria species (Figure 2). We found that strain HUB-IV-005 was the closest to the genus *Klebsiella*.

Fatty acid dyeing and SDS-PAGE analysis

Fatty acid dyeing analysis showed that a peak was phenol, 2, 4, 6-tris (1-methylethyl) - with a chemical formula of $C_{15}H_{24}O$, b peak was dibutyl phthalate with a chemical formula of $C_{16}H_{22}O_4$, c peak was 1-decanol, 2-hexyl- with a chemical formula of $C_{16}H_{34}O$, and d peak was hexacosyl acetate with a chemical formula of $C_{28}H_{56}O_2$ (Figure 3). SDS-PAGE analysis showed that the protein banding of strain HUB-IV-005 was consistent with those of the reference *K. variicola* strain (Figure 4).

According to phylogenetic analysis, fatty acid dyeing and SDS-PAGE analysis, strain HUB-IV-005 was classified to the genus *Klebsiella* as a species of *K. variicola*.

Nitrogenase activity detection

The strain HUB-IV-005 was able to grow on the nitrogen free medium. The colony of the strain was white and translucent with round and regular edges, which were moist on the surface and not easy to pick up with loops. The strain HUB-IV-005, an azotobacter in the gut of termite, showed a high level of nitrogenase activity of 167.46±4.40 nmol C_2H_4/ (h·ml).

Cloning and sequence analysis of nifH gene

The *nifH* gene of the strain HUB-IV-005 was successfully amplified by PCR with an expected size of 882 bp (Figure 5). To confirm the PCR product was *nifH* gene, the PCR product was inserted into pMD18-T vector and transformed

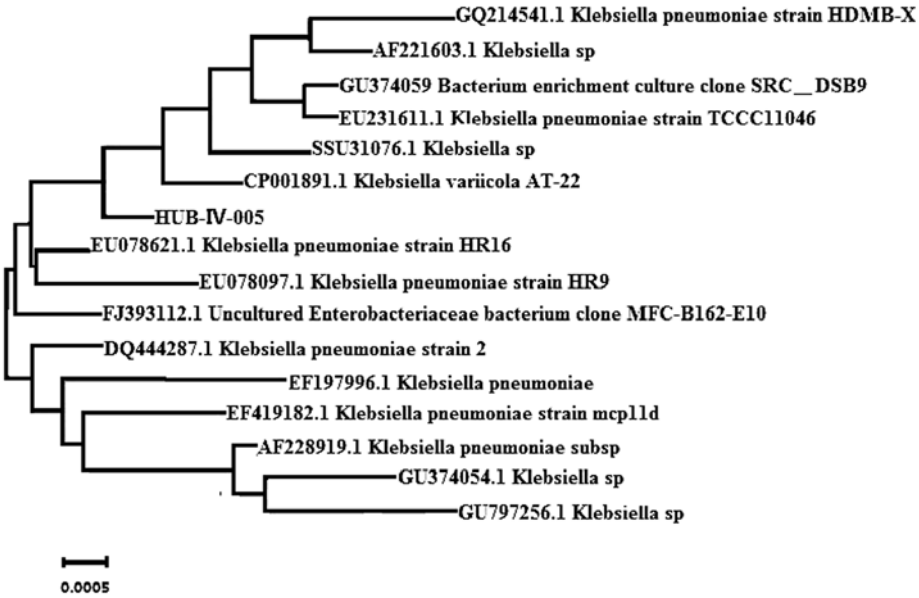

Figure 2. Phylogenetic tree showing the relationship of the strain HUB-IV-005 with other related bacterial species from GenBank based on their homologous sequences of 16S rDNA.

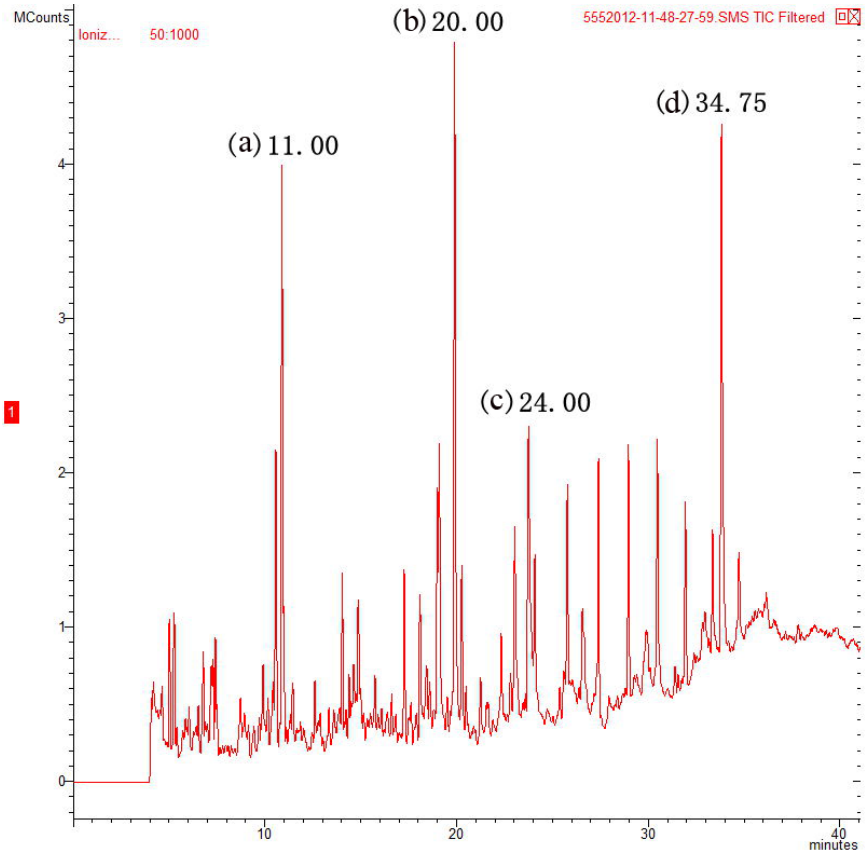

Figure 3. GC-MS spectrum of the extract from the strain HUB-IV-005 by fatty acid dyeing analysis. a peak was phenol, 2, 4, 6-tris (1-methylethyl) - with a chemical formula of $C_{15}H_{24}O$; b peak was dibutyl phthalate with a chemical formula of $C_{16}H_{22}O_4$; c peak was 1-decanol, 2-hexyl- with a chemical formula of $C_{16}H_{34}O$; d peak was Hexacosyl acetate with a chemical formula of $C_{28}H_{56}O_2$.

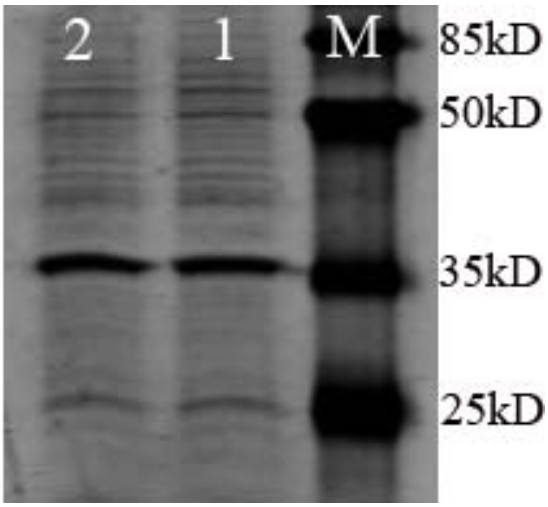

Figure 4. Protein banding analysis of proteins extracted from the strain HUB-IV-005 using SDS-PAGE. Lane 1: protein banding from the strain HUB-IV-005; Lane 2: protein banding from the reference *K. variicola* strain; M: protein marker including 80 kDa, 50 kDa, 35 kDa and 25 kDa.

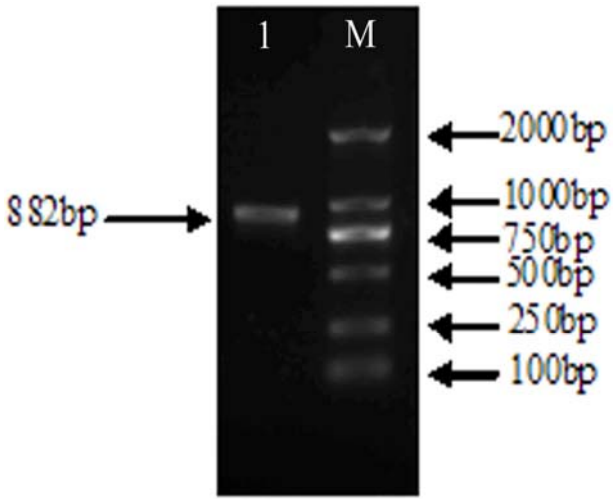

Figure 5. Analysis of PCR product by agarose gel electrophesis. Lane M: DNA marker DL2000; Lane 1: PCR product of the *nifH* gene.

into *E. coli* DH5α. The recombinant plasmid pMD18-T-*nifH* were extracted from the positive clones and analyzed by electrophoresis (Figure 6a). The recombinant plasmid pMD18-T-*nifH* was identified by *EcoR* I □and *Nde* I enzyme digestion (Figure 6b) and PCR amplification (Figure 6c), and the positive clones were sequenced, and analyzed by BLAST. It was 882 bp in length and 99% homology with the *nifH* gene sequences in GenBank. This sequence was submitted to GenBank, and the accession number was JX 570695.

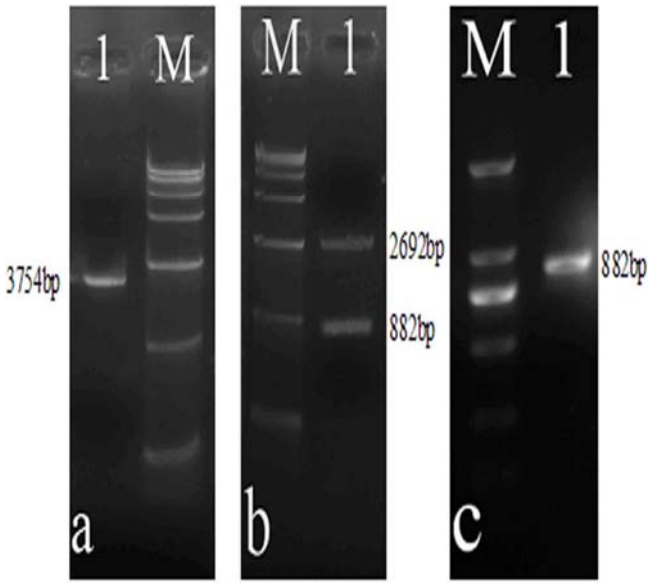

Figure 6. The extraction and identification of recombinant plasmid pMD18-T-*nifH*. a: the extraction of recombinant plasmid pMD18-T-*nifH* (Lane M: DNA marker DL15000; Lane 1: the extracted recombinant plasmid pMD18-T-*nifH*); b: identification of the extracted recombinant plasmid pMD18-T-*nifH* by *EcoR* I and *Nde* I enzyme digestion (Lane M: DNA marker DL15000; Lane 1: enzyme digestion of the plasmids); c: identification of the extracted recombinant plasmid pMD18-T-*nifH* by PCR (Lane M: DNA marker DL15000; Lane 1: PCR amplification of the plasmids).

Analysis and expression protein identification of the nifH gene expression

The plasmid pET28a was extracted and analyzed by electrophoresis (Figure 7), and then the *nifH* gene was ligated with pET28a and identified by *EcoR* I □and *Nde* I enzyme digestion (Figure 7), and then the recombinant plasmid pET28a-*nifH* was transformed into the *E. coli* BL21. The SDS-PAGE analysis showed that the expression products of the *nifH* gene could be efficiently recovered to a high degree of purity by single-step affinity chromatography. The protein was furthermore found to be recovered as predominantly full- length products of expected size. The protein of *nifH* gene in *E. coli* BL21 containing pET28a-*nifH* was expressed after IPTG induction at 4 and 5 h, while there was no specific proteins in the control containing the plasmid pET28a (Figures 8 and 9).

After mass spectrometric identification of the target protein, two credible identification results were gained (Table 2). It was *K. variicola* nitrogenase iron protein, and its molecular weight was 32.463 kDa, an isoelectric point of 4.72, a score of 731, a total of 39 peptides, and coverage of 46%. The other one was *Delftia tsuruhatensis* nitrogenase iron protein. The molecular weight was 31.690 kDa, an isoelectric point of 4.68, a score of 224, and a total of 14 peptides and 21% coverage. Therefore,

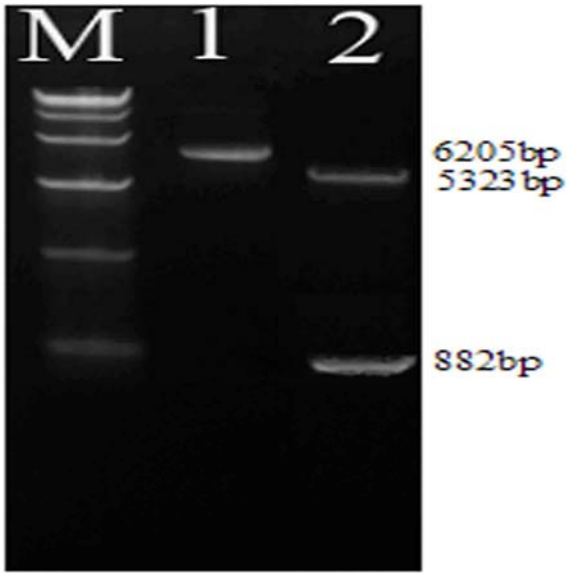

Figure 7. The extraction and identification of recombinant plasmid pET28a-*nifH*. Lane M: DNA marker DL15000; Lane 1: the extracted recombinant plasmid pET28a-*nifH*; Lane 2: identification of the extracted recombinant plasmid pET28a-*nifH* by *EcoR* I and *Nde* I enzyme digestion.

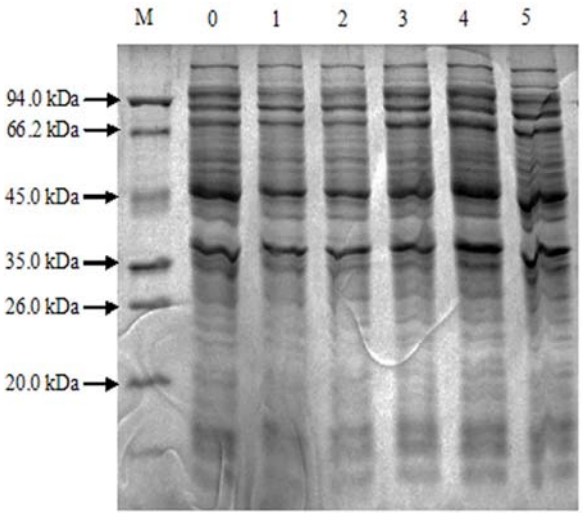

Figure 8. SDS-PAGE analysis of pET28a (without the *nifH* gene) transformed into the *E. coli* BL21.
Lane M: protein molecular weight marker; Lane 1-6: IPTG induction for 0, 1, 2, 3, 4, 5 h, respectively.

based on the mass spectrometry results, *nifH* genes could be properly expressed as the nitrogenase iron protein in *E. coli* BL21 (Hou et al., 2007).

DISCUSSION

Termite gut has become one object of study in microbial diversity on the earth, because there are more than a

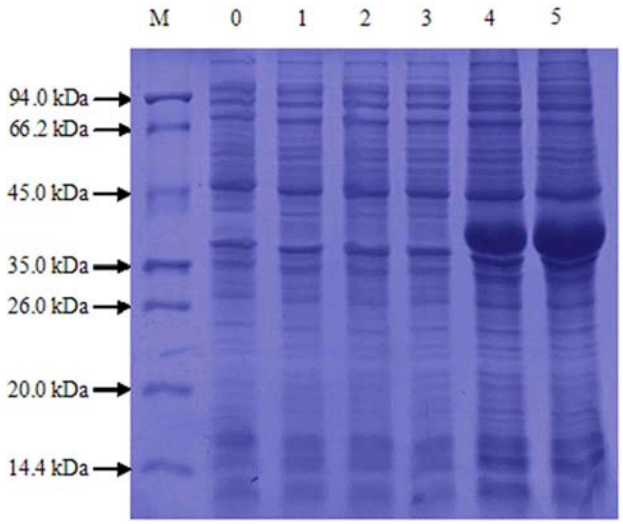

Figure 9. SDS-PAGE analysis of expressed proteins from the recombinant plasmid pET-28a-*nifH* in the *E. coli* BL21. Lane M: protein molecular weight marker; Lane 1-6: IPTG induction for 0, 1, 2, 3, 4, 5 h, respectively.

thousand species of termites, their feeding habits and structures of the intestine are different. Nitrogen limits plant growth in many ecosystems (Fiore et al., 2010). Previous studies showed that there are a lot of nitrogen-fixing bacteria in the termite gut, and up to 60% of the nitrogen in termite organs came from the nitrogen fixation of symbiotic microorganisms which lived in the hindgut of termites. It was been confirmed that the nitrogen fixing bacteria in the hindgut of the termites are important because the termite's diet is nitrogen deficient. Termite's gut symbiotic microbial diversity is extremely rich. The digestion of cellulose and hemicellulose by low termites mainly relies on hindgut symbiotic microorganisms (including protozoan). The high termites' digestion of lignin relies on digestive juices secreted by the salivary glands and midgut. The capability of nitrogen fixation may meet the termites' metabolic needs. The level of nitrogen fixation depends on the species and composition of symbiotic microorganism in the gut of termites. We have isolated endophytic bacteria with nitrogen fixation activity from the gut of *O. formosanus;* one of them is the strain HUB-IV-005. In addition, the morphological and physiological, biochemical characteristics, 16S rDNA sequences, fatty acid dyeing and SDS-PAGE analysis showed a close relationship of strain HUB-IV-005 with the genus *Klebsiella*. Molecular identification has been increasingly used as a powerful tool supplementary to the traditional systematic classification. In our study, the *nifH* gene from HUB-IV-005 was successfully cloned, and the expressed protein of the *nifH* gene was a nitrogenase (iron protein), indicating that the strain HUB-IV-005 represents a nitrogen fixation bacterium from *O. formosanus*. Based on these results, we named strain HUB-IV-005 as *K. variicola*.

According to previous studies, nitrogen-fixing bacteria

Table 2. Protein identification from MS/MS.

Accession no.	Protein name	Organism	pI/MW exp (kDa)	MW Theo (kDa)	Mascot score	Sequence coverage (%)	Pep
gi|206580625	Nitrogenase iron protein	*Klebsiella pneumoniae* 342	4.72/ 32.463	32	731	46	39
gi|45269096	*nifH*	*Delftia tsuruhatensis*	4.68/ 31.690	32	224	21	14

is capable of nitrogen fixation or utilization. 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 is 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 HUB-IV-005, the characterization of nitrogen fixation is necessary. In our study, we investigated the nitrogenase activity of strain HUB-IV-005, that was able to grow on the nitrogen free medium. At the same time, we also detected nitrogenase activity of strain HUB-IV-005 by acetylene reduction method. Strain HUB-IV-005, an azotobacter in the gut of termite, showed a high level of nitrogenase activity of 167.46±4.40 nmol C_2H_4 $(h \cdot ml)^{-1}$. Under anaerobic or microaerobic conditions, *K. variicola* was considered as an associative nitrogen fixer (Ladha et al., 1983). 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. variicola* may play an important role in the hindgut microbial ecology. The presence of a nitrogen fixing spirochete has also been identified in the termite gut (Lilburn et al., 2001).

Although researchers have shown that the termite gut has a large number of nitrogen-fixing bacteria, pure cultures of nitrogen-fixing bacteria were rarely reported. In the nitrogenase discovered to date, one was the *nifH* gene encoding the molybdenum and ferritin protein. It has been recognized as the best nitrogen-fixing capacity, the complete set of structure and function in nitrogenase system. *nifH* gene cluster was found in *Klebsiella* nitrogen-fixing bacteria. Nitrogenases were expressed by a relatively conservative *nifH* gene and the expression levels of *nifH* gene contribute largely to the organism nitrogenase activity. For this reason, it was appropriate to study the bacterial molecular diversity. However, only a small amount of the *nifH* gene could be preferentially transcribed and expressed, most of the *nifH* gene could not be successfully expressed. The *nifH* gene in our study was cloned and the *nifH* gene was expressed in order to understand the nitrogen-fixing mechanism in termites; at the same time, we separated and purified the nitrogenase, which was produced by the *nifH* gene, and the sequence alignment with *K. variicola* was 99% sequence identity. Our results provided the basis for studying a nitrogen-fixing mechanism in the termite gut, and facilitated the development of nitrogen-fixing micro-organism resources and developing new termite control methods with important theoretical and practical potential.

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