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Identification of Ureolytic Bacteria for Concrete Formation and Antifungal Activity from the Soil with Long-Term Application of Urea Fertilizer

Haiyue Pang^{1, a}, Gueyhorng Wang^{1, 2, b}, Hueian Yang^{3, c}, Hongtan Wu^{1, 2, d}, Xinhui Wang^{3, e}, Yupei Chen^{1, 2, 3, *}

¹Application Technique Engineering Center of Natural Cosmeceuticals College of Fujian Province, Xiamen Medical College, Xiamen 361023, Fujian, China.

²Department of Medical Technology, Xiamen Medical College, Xiamen 361023, Fujian, China

³Department of Biological Science and Technology, China University of Science and Technology, Taipei 115, Taiwan

*Corresponding author e-mail: 201600080006@xmmc.edu.cn, ^a1004788518@qq.com, ^b2659294971@qq.com, ^cdiane50233@yahoo.com.tw, ^d470043915@qq.com, ^ecindywang1206@yahoo.com.tw

Abstract. Ureolytic bacteria in 12 isolates, namely TCU1-TCU12, obtained from the soil with long-term application of urea fertilizer were screened and characterized. Urea degrading activity of all isolates was ranging from 2.4 to 22.9 mM. The ureolysis profiles of the isolates corresponded to fluctuations in pH value. Urease inhibitors, including acetohydroxamic acid and phenyl phosphorodiamidate, were applied to prove urea degrading activity. Moreover, the antifungal activity was verified by the TCU4 and TCU5 strains. The 16S rRNA gene sequence analysis revealed that among the 12 isolates, eight were *Bacillus aryabhattai*, three were *Bacillus megaterium*, and one was *Bacillus subtilis*. The random amplified polymorphic DNA was used to detect the genetic relationship among the 12 isolates. In addition, next-generation sequencing indicated that Proteobacteria was the most abundant phylum at 47.1% in the soil with long-term application of urea fertilizer. *Bacillus* species occupied 0.12%. Consequently, these ureolytic bacteria had the potential to be applied in the fields of concrete structure and medicine.

1. Introduction

Urease, a metalloenzyme, is widely found in various organisms, including bacteria, fungi, and plants. One mole of urea and two mole water can be hydrolyzed into one mole of ammonia carbonate by urease. This process further leads to an increase in pH because of ammonification [1]. Much attention has been focused on the calcium carbonate precipitation using ureolytic bacteria due to ureolytic activity [2-5]. They are used to prevent brick deterioration through water absorption by biocalcification, which is also known as microbiologically induced calcite precipitation (MICP) [6]. In addition to ureolytic activity, ureases have various biological properties, such as insecticidal activity and the activation of blood platelets [7]. The antifungal activity of urease is also demonstrated in recombinant *Helicobacter pylori* urease, embryo-specific soybean urease, and jackbean major urease [8].



The National Center for Biotechnology Information (NCBI) GenBank contains information on various bacteria with urease enzymes; however, few studies have been conducted to identify bacteria with a high ureolytic activity from agricultural soil. In the present study, we evaluated the biochemical and genetic characteristics of indigenous ureolytic bacteria in soil with long-term application of urea fertilizer. The genetic relationship of ureolytic bacteria with urea degrading activity, pH value, and urease inhibitor effects was established. Subsequently, the unculturable method of NGS was employed to estimate the isolates in the microbial community structure of the soil.

2. Materials and methods

2.1. Screening and cultivation of ureolytic bacteria

The serially diluted soil sample was plated on the modified M9 minimal agar medium (w/v, 0.6% Na₂HPO₄, 0.3% KH₂PO₄, 0.05% NaCl, 0.1% Urea, 0.2% Glucose, 0.002% phenol red, 2 mM MgSO₄, 0.1 mM CaCl₂, and pH value was adjusted to 6.2). Urea was used as the sole nitrogen source for selectively screening ureolytic bacteria, which are capable of degrading urea. Phenol red was used as a pH indicator to confirm the ureolytic bacteria. Colonies were purified and inoculated into the modified M9 minimal medium broth for further analyzing urea degrading activity and DNA.

2.2. Analyses of urea degrading and antifungal activity of ureolytic bacteria

Ureolytic bacteria were inoculated into the modified M9 minimal medium broth at 37°C for the time course analysis. To measure the urea degrading activity of ureolytic bacteria, 1 mL of assay broth was harvested and centrifuged. The optical density of the suspension was measured using a spectrophotometer at 570 nm [9]. To explore the effect of urease inhibitors on ureolytic bacteria, acetohydroxamic acid, and phenyl phosphorodiamidate were used to estimate urea degrading activity. To detect the antifungal activity of ureolytic bacteria, *Trichoderma harzianum* BCRC31867 and *Penicillium varians* BCRC33128 were used to perform a dual culture assay. Paper discs 6 mm in diameter with the culture broth of ureolytic bacteria were placed surrounding the fungal block of pathogenic fungi. The plates were cultured at 28°C and the growth inhibitors of the pathogenic fungi were observed.

2.3. Identification of ureolytic bacteria

Ureolytic bacteria were cultured in a broth medium for the chromosomal DNA extraction. To identify isolates, a set of primers for amplifying 16S rRNA gene (27F, 5'-AGAGTTTGGATCMTGGCTCAG-3', and 907R, 5'-CCGTCAATTCCTTTTRAGTTT-3') was designed to be used in polymerase chain reaction (PCR). The PCR products were extracted and purified for DNA sequencing. The sequence data were aligned and compared with the data of the NCBI GenBank (<http://www.ncbi.nlm.nih.gov/>), using a BLAST tool. The phylogenetic tree was constructed using the neighbor-joining method by using MEGA software with 1000 bootstrap replicates [10].

2.4. Random amplified polymorphic DNA

The genetic relationship among ureolytic bacteria was further determined using a random amplified polymorphic DNA (RAPD) amplification technique. The RAPD amplification was conducted with 10 different 10-mer random primers. The PCR included the following steps: an initial denaturation of 5 min at 94°C, 40 cycles for 1 min at 94°C, 30 s at 37°C, and 3 min at 72°C with a final extension of 5 min at 72°C. The DNA bands were recorded as binary data and analyzed using NTSYS-PC software by using the Unweighted Pair Group Method with Arithmetic Mean method.

2.5. Metagenomic analysis of 16S rRNA gene

To comprehensively examine the soil microbial community structure, metagenomics sequencing was conducted using the next-generation sequencing (NGS) method. The procedure included the amplification of the 16S rRNA gene of the microbial soil DNA. The primer set, 517F: 5'-

TTGCCAGCAGCCGCGGTAA-3' and 926R: 5'-CCCCGTCAATTYYTTTTRAGTTT-3', was designed for targeting the hypervariable V3–V4 region of the 16S rRNA gene. The triplicate PCR amplicons were pooled and purified through gel extraction for metagenomic sequencing. The library was constructed using a TruSeq DNA Sample Preparation kit (Illumina, San Diego, CA, USA) and quantified using a GeneRead Library Quant Kit (Qiagen, Valencia, CA, USA). The sequences of 2×300 bp paired-end reads were generated using a Miseq Reagent kit V3 (Illumina) for the Miseq sequencer (Illumina).

3. Results and discussion

To characterize ureolytic bacteria, the details of the urea degrading activity, cell growth, and pH value were monitored during 96 h of incubation, and the results are shown in Fig. 1. The ammonia released during urea degradation in the liquid medium ranged between 2.4 and 22.9 mM with variations in all isolates. The maximal ammonia release was recorded in the TCU2 isolate followed by the TC11 isolate. However, the minimal urea degrading activity was observed in TCU1 and TCU4–TCU8 isolates. During ureolysis, an increase in pH value was accompanied by urea degrading activity because of ammonification [1, 11]. Therefore, the time courses of pH value were also determined from an initial pH value of 6.0–6.3 after 96 h incubation. As expected, the pH change observed in all the isolates were in agreement with the degree of ammonia released. The maximal pH increase was observed in the TCU2 strain from 6.3 to 7.4 after 96 h of incubation and associated with the high urea degrading activity. In addition, the cell density of the TCU2 strain was higher than that of the others. The minimal ammonia release was consistent with the unchanged pH value in the TCU1 and TCU4–TCU8 isolates. Furthermore, the growth of these strains was slightly increased.

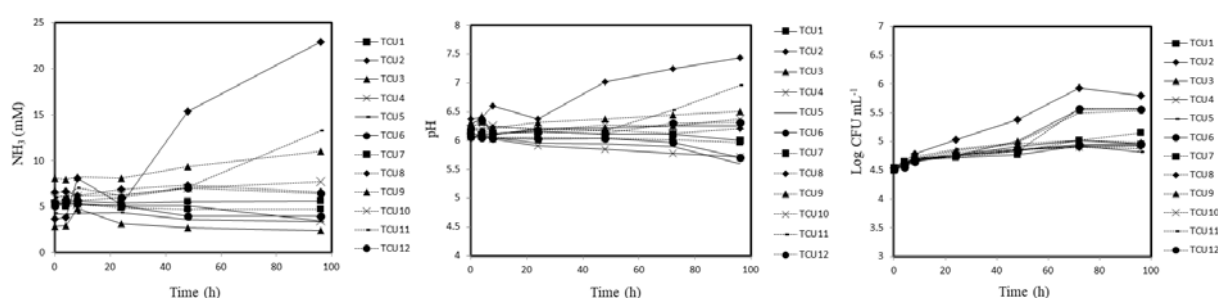


Figure 1. Physiological properties of ureolytic bacteria isolated from soil with long-term application of urea fertilizer. Submerged cultures of 12 isolates were incubated at 37°C on a 150 rpm rotary shaker for 4 days. The ammonia release, pH value, and cell density in a broth medium were determined.

The application of urease inhibitors can reduce the ammonia loss and confirm urease activity. The effect of two urease inhibitors, acetohydroxamic acid and phenyl phosphorodiamidate acting as a metal chelator and competitive inhibitor, respectively, on the maximal urea degrading activity of the TCU2 isolate was explored during 96 h of incubation (Fig. 2). Ureolysis of TCU2 strain was markedly suppressed by both acetohydroxamic acid and phenyl phosphorodiamidate. A high concentration of urea inhibitors did not cause pH fluctuations during the ureolysis process. However, after 72 h of cultivation, ammonia release was slightly increased by adding 0.01% urea inhibitors. The pH profiles also corresponded to urea degrading activity.

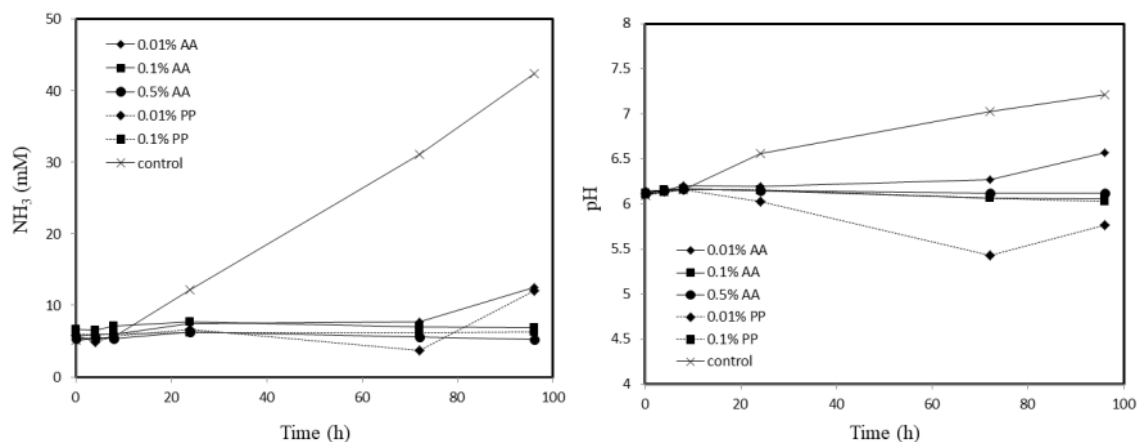


Figure 2. Urea degrading activity and pH value of the TCU2 isolate in a broth medium without a urease inhibitor as a control and with the addition of urease inhibitors, acetohydroxamic acid (AA) and phenyl phosphorodiamidate (PP), were measured. The submerged culture of the TCU2 isolate was incubated at 37°C on a 150 rpm rotary shaker for 4 days.

H. pylori urease showed antifungal activity [12]. To detect the antifungal activity of ureolytic bacteria, a dual culture assay was performed using two pathogenic fungi, *T. harzianum* BCRC31867 and *P. varians* BCRC33128 (Fig. 3). Consequently, only TCU4 and TCU5 isolates were effectively antagonistic against the *T. harzianum* BCRC31867 and *P. varians* BCRC33128 fungi. Furthermore, other isolates had no effect on the growth inhibition of the two fungi.

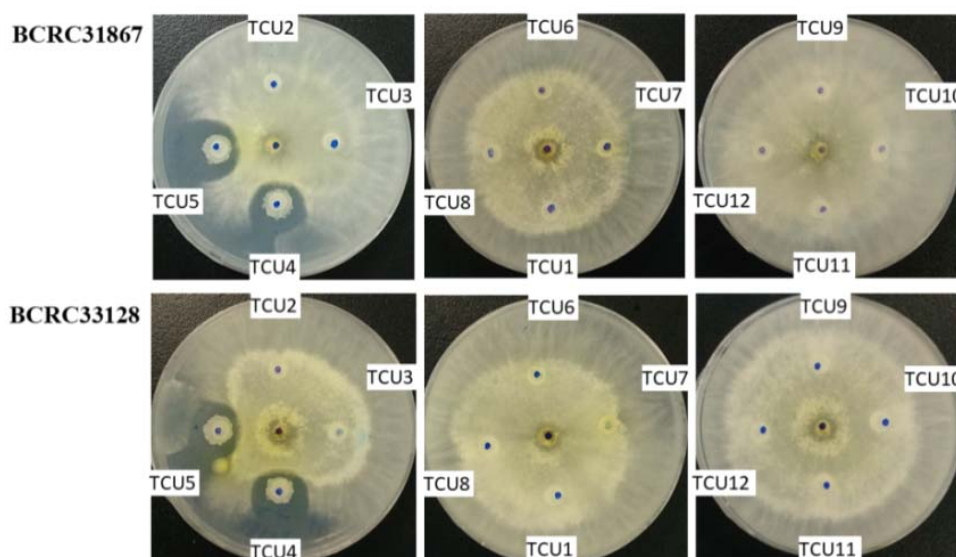


Figure 3. The analysis of the antifungal activity of ureolytic bacteria against *Trichoderma harzianum* BCRC31867 and *Penicillium varians* BCRC33128. Paper discs 6 mm in diameter with the addition of 12 isolates cultured for 4 days were placed surrounding the fungal block of the pathogenic fungi. The growth inhibitions of pathogenic fungi were observed at 28°C.

To identify ureolytic bacteria, 16S rRNA gene sequence and phylogeny analyses were performed (Fig. 4A). These ureolytic bacteria belonged to the *Bacillus* genus using the BLAST program, which were consistent with previous studies [2-5, 7]. Phylogenetic analysis identified eight isolates of *B.*

aryabhatai, three isolates of *B. megaterium*, and one isolate of *B. subtilis*. This study is the first to propose *B. aryabhatai* with various degrees of urea degrading activity. Because all of these ureolytic bacteria belonged to the *Bacillus* genus, RAPD amplification conducted with 10 different 10-mer random primers was further used to study the DNA polymorphism (Fig. 4B). A total of 127 RAPD fragments were scored and the genetic relationship was revealed. The similarity coefficient of 12 isolates ranged between 0.63 and 0.98 and two distinct clusters were observed. To estimate the distribution of *Bacillus* genus in the soil, genetic fingerprinting was performed to examine the total eubacterial community structure by using NGS (Fig. 4C). After raw reads were trimmed and chimeric reads were removed, the average reads were 287 bp in length, and 161,559 reads were obtained. The results indicated that Proteobacteria, Chloroflexi, and Acidobacteria were the predominant phyla with the relative abundance of 47.1%, 17.9%, and 11.8%, respectively. Taxonomic division revealed that *Geobacillus* species, *B. flexus*, *B. coagulans*, and *Bacillus* species existed at the family level of Bacillaceae, constituting only 0.13%. At Bacillaceae level, *Bacillus* sp. occupied 68.5% followed by *B. flexus* with 22.7%.

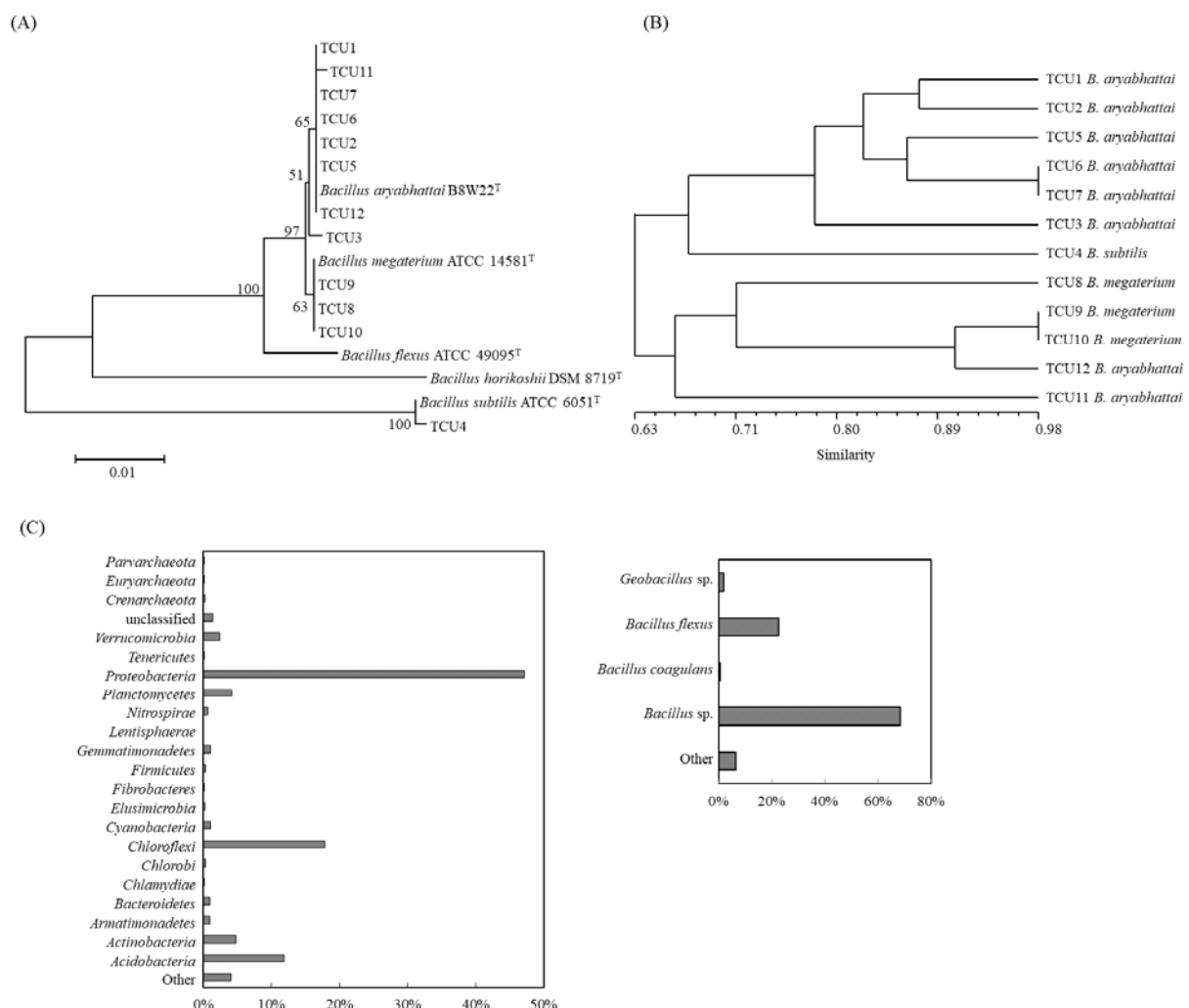


Figure 4. Identification of the ureolytic bacteria. (A) Phylogenetic tree of 16S rRNA gene from isolates and various bacteria. The tree was constructed using the neighbor-joining method by using the MEGA software. Bootstrap values were shown in nodes according to 1000 replications. (B) The dendrogram of random amplified polymorphic DNA from isolates was constructed using the

Unweighted Pair Group Method with Arithmetic Mean method by using the NTSYS-PC software. (C) The abundance of microbial community structure in soil with long-term application of urea fertilizer. The microbial distribution was interpreted using QIIME software using the Greengenes database for the phylum and Bacillaceae family taxonomy.

4. Conclusion

Ureolytic bacteria from the soil with long-term application of urea fertilizer were identified as *Bacillus* species. The high urea degrading activity of isolates was in accordance with an increase in pH value of the culture medium based on the ureolysis process. Furthermore, antifungal susceptibility was demonstrated in two isolates, *B. subtilis* (TCU4) and *B. aryabhattai* (TCU5), which was independent of ureolytic activity. Our results indicated that these strains had potential and applicable for practical respects in concrete structure and medicine.

Acknowledgments

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