

IDENTIFICATION OF NOVEL *BACILLUS* SPECIES ISOLATED FROM COW BEZOAR IN TAIWAN

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Introduction

Calculus Bovis is a valuable Chinese medical material commonly known as cow bezoar. It is valued as an effective Chinese medicine, although most cow bezoar is now imported from other countries. The demand for cow bezoar remains high but due to the small amounts which are produced, the cost is high in Taiwan. Many commercial systems are able to recognize microorganisms by phenotypic and genotypic characterization. Microbiology laboratories need rapid methods for the identification of unknown microorganisms. The purpose of this study is to identify the isolated bacterium by appropriate methods recommended for the taxonomic description of bacterial species.

Materials and Methods

Morphological features. All assays were performed in duplicate. Colony morphology was visualized by growth on tryptic soy agar. The purity of the isolate was checked microscopically. Gram staining and catalase tests were carried out using standard protocol (Murray et al., 1994; Smibert and Krieg 1994).

Cellular fatty acid analysis of bacteria. Cellular fatty acid analysis of bacteria was analyzed according to the instructions of manufacture (MIDI, Newark, DE, USA). The isolate bacteria were quadrant streaked onto tryptic soy agar and grown for 24 h at 28°C. Fatty acid methyl esters were obtained from wet biomass by saponification, methylation and extraction. Individual fatty acid methyl esters were compared using the Sherlock Microbial Identification System database version 5.0.

Determination of physiological characteristics. The determination of physiological characteristics was performed according to the instructions of the manufacturer. The oxidation of 95 different carbon sources was tested using Biolog GN Micro Plate with an inoculum grown on tryptic soy agar plates. A Biolog plate was designed to test the ability of a gram-negative organism to oxidize different carbon sources. Oxidation of individual carbon sources was detected indirectly by observing reduction of tetrazolium dye with Biolog computer software after 4 and 24 h incubation at 28°C.

Isolation of 16S rDNA and sequencing. The isolation of genomic DNA was modified using the method of Wintetzingero et al. (1997). The amplification of 16S rDNA was described by Kim et al. (2000). The 16S rDNA was enzymically amplified using two pairs of oligonucleotide primers, 27F & 1492R. Sequence analysis was carried out with Perkin-Elmer Gene Amp 9600 PCR system and the ABI 3730 DNA Analyzer.

Results and Discussion

Morphological properties. Samples were inoculated on the plates and broths from cow bezoar. Representative colonies were subcultured for further study. One strain was isolated from samples. The vegetative cells of novel *Bacillus* species stained with Gram negative were rod-shaped, single or short chain. The colonies were small (up to 2 mm in diameter), yellow-grey and with a glossy surface. The novel strain showed positive for catalase.

Cellular fatty acid analysis of bacteria. The dominant fatty acids in the strain were anteiso C_{17:0}, C_{18:1ω9c}, iso C_{15:0} and anteiso C_{15:0} components (data not shown). Fatty acid profiles of this bacterium were not detected in the database of microbial identical system. The bacterium was therefore subjected to Biolog system for further testing.

Determination of physiological characteristics. This bacterium oxidized 35 and weakly responded 49 of the 95 different carbon sources analyzed with the Biolog identification system but the profiles of Biolog data of the isolate were not recognized in this microbial identical system. The strain was therefore subjected to 16S rDNA sequencing.

Isolation of 16S rDNA and sequencing. A 16S rDNA sequence of the strain was determined. A PCR product was generated for the isolate bacterium. Phylogenetic analysis based on 16S rDNA sequences and chemotaxonomic data indicated that this bacterium belonged to the genus *Bacillus*. A rooted phylogenetic tree is given in Fig. 3. Comparison of 16S rDNA sequence between the isolated bacterium and *B. shackletonii* showed that this species was very homogeneous (99%), with very few nucleotide differences. This finding suggests that

colonization of these two sites is from a common source. This is most likely to have occurred from the air. The 16S rDNA gene sequence of strain was submitted to the GenBank database of NCBI and has been assigned an accession number of EF506944.



Figure 1: Photomicrograph of vegetative cells of the type *Bacillus* sp. nov.

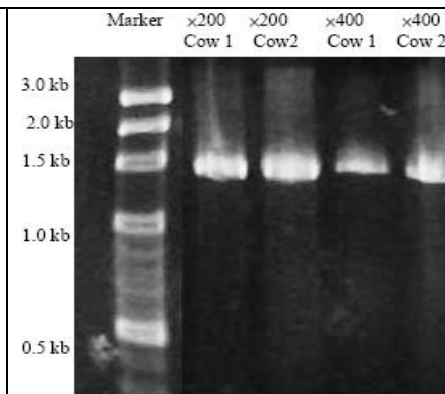


Figure 2: PCR products of 16S rDNA of *Bacillus* sp. nov.

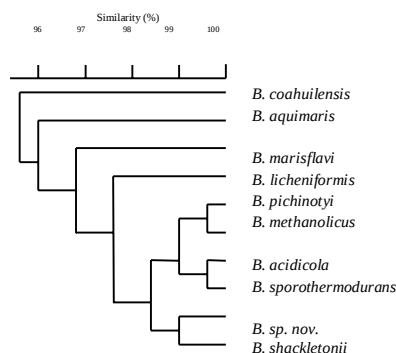


Figure 3: Phylogenetic tree showing the evolutionary position of *Bacillus* sp. nov. and the related genus *Bacillus*.

Conclusions

The microbial profile from cow bezoar was isolated and identified in Taiwan. This study demonstrated the applicability of a strategy that led to the unambiguous taxonomic assignment of cow bezoar isolates to the genus *Bacillus*, irrespective of phenotypic differences previously considered of taxonomic importance, colony properties and morphological traits. The strategy included the use of several methods. Prescreening of isolates by rapid fatty acid analysis and physiological characteristics was not matched to the database. Sequence analysis of 16S rDNA from a selection of this strain confirmed this taxonomic identification. The finding of 16S rDNA was rather high (99%) 16S rDNA similarity values between the novel isolated bacterium and *B. shackletonii*. This species exhibited more genomic and phenotypic diversity. The role of minor components of cow bezoar is a subject for further studies.

References

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