論文の内容の要旨

応用生命工学専攻
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論文題目

Taxonomic studies on bacterial strains isolated from root nodules of leguminous plants in the Philippines

(フィリピン産マメ科植物根粒由来細菌分離株の分類学的研究)

The Philippines is an agricultural country that provides a rich source of microbial diversity. One agriculturally important group of microorganisms that exists in the country is the nitrogen-fixing bacteria or rhizobia which are beneficial in enhancing soil fertility and plant production. However, many of these indigenous rhizobial isolates have not been fully characterized and described. The great diversity of bacteria and leguminous plants, environmental differences and sometimes the exclusive interaction between plants and rhizobia provide a window of opportunity that new or novel species of bacteria can still be isolated. In this regard, taxonomic works on these indigenous isolates are deemed important, not only to orderly place these microorganisms in appropriate taxa, but also as basis of comparison for new microbes. Thus, the primary objective of this study is to isolate, identify and determine the taxonomic position of the novel species of rhizobia based on polyphasic taxonomy.

The symbiotic relationship of certain bacteria with the root nodules of leguminous plants (pod-bearing) paved the way to the identification and classification of several nitrogen fixing-nodulating and non-nitrogen fixing bacteria under the order *Rhizobiales* (class *Alphaproteobacteria*). To date, this order comprised of 12 families namely, *Rhizobiaceae, 'Aurantimonadaceae', Bartonellaceae, Brucellaceae, Phyllobacteriaceae, Methylocystaceae, Beijerinkiaceae, Bradyrhizobiaceae, Hyphomicrobiaceae, Methylobacteriaceae, Rhodobacteriaceae and Xanthobacteraceae*[1].

The bacterial strains used in this study were obtained mostly from the root nodules of several leguminous plants locally found in the Philippines for the three year sampling periods (2004, 2006, & 2007), lyophilized rhizobial isolates were provided by the Microbial Culture Collection of BIOTECH-Philippines and from the root nodules of leguminous plants in the coastlines of Japan. Root nodules were superficially disinfected with 10% sodium hyphochlorite and 70% ethyl alcohol. The nodules were aseptically crushed and the aliquots were serially diluted and plated on the rhizobium medium supplemented with antifungal agent (Kabicidin) and congo red. Colonies that grew on the plates were randomly selected, purified and maintained in agar slants. The universal primer for the

amplification of the 16S rRNA gene was used to partially identify all the axenic cultures. Isolates that showed 98% or lower BLAST sequence similarities were further investigated to clarify their taxonomic position. The polyphasic approach used in this study involved molecular techniques (like the 16S rRNA sequencing, DNA-DNA hybridization, amplification of the nitrogen fixing gene (*nif*H) and nodulation gene (*nod*D)) in conjunction with *in silico* analyses (Bioedit, Mega4 and ClustalX programs), chemical and physiological assays namely fatty acid profiles, G+C content, respiratory quinone type, API 50 CH (bioMérieux), API ZYM™ (bioMérieux) strips, Biolog GN2 Microplate (Biolog), antibiotic sensitivity tests and infection/nodulation test. Light microscopy was also employed to observe the motility and to determine the size and shape of the isolates.

A total of 411 bacterial isolates were obtained from 48 different species of leguminous plants. Among these leguminous plants, *Leucaena leucocephala* and *Desmodium scorpiurus* were found to be the promiscuous plants since several species of rhizobia (*Bradyrhizobium, Rhizobium, Mesorhizobium* and *Ensifer*) were isolated from their root nodules sampled from different localities. On the other hand, leguminous plants like *Sesbania sesban* and *Mimosa* species prefer only the species of *Rhizobium.* Among the species of rhizobia, the *Rhizobium* and *Bradyrhizobium* species were isolated from several species of plants suggesting their wide host range. Other groups of bacteria were isolated as contaminants because the medium used in this study is not highly selective to rhizobia.

The almost complete sequences of the 16S rRNA gene of the isolates were used to construct the phylogenetic tree (Fig.1) to reveal that there were 7 probable new species of rhizobia that intermingled within the members of family *Rhizobiaceae* and *Hypomicrobiaceae*. Strain Yak96B isolated from the root nodule of kudzu plant (*Pueraria lobata*) growing from the coastline of Yakushima Island, Kagoshima, Japan was identified as *Devosia yakushimensis*[2] (family *Hyphomicrobiaceae*). The said strain is closely related to *Devosia neptuniae* J1^T, the type strain of the genus *Devosia*. However, DNA-DNA hybridization and the significant differences in the physiological tests and the fatty acid contents proved that strain Yak96B is a novel species of *Devosia*.

Strains P5b, M30a, T25a, M9cR1, 56b, P-Ab and ELS-4 isolated from the root nodules of indigenous leguminous plants in the Philippines were clustered under the heterogeneous family of *Rhizobiaceae*. All of the aforementioned Philippine isolates have high G+C contents (mol%) and possessed ubiquinone 10 (Q_{10}) as their major respiratory quinone. Strain P5b was isolated from the national tree of the Philippines, the narra tree (*Pterocarpus indicus*). This sodium chloride sensitive strain was identified as *Shinella philippinensis*[3] which is closely related to *Shinella granulli* ChO6^T. Strain ELS-4 isolated from *Desmodium styracifolium* is closely related with several *Rhizobium* species but DNA-DNA reassociation values proved that it is a novel strain and proposed as *Rhizobium orientalis.*

Strains T25a and M9cR1 isolated from *Vigna radiata* and *Cajanus cajan* belong to the same species and proposed as *Rhizobium luzonensis* which is closely related to *Rhizobium undicola* LMG 11875^T. Strain 56b isolated from *Desmodium scorpiurus* is also a close neighbor of *Rhizobium*

undicola LMG 11875^{T} and proposed as *Rhizobium mindanawensis.* Strains M30a and P-Ab isolated from *Aeschynomene indica* and *Desmodium triflorum*, respectively. Strain M30a was proposed as *Rhizobium*

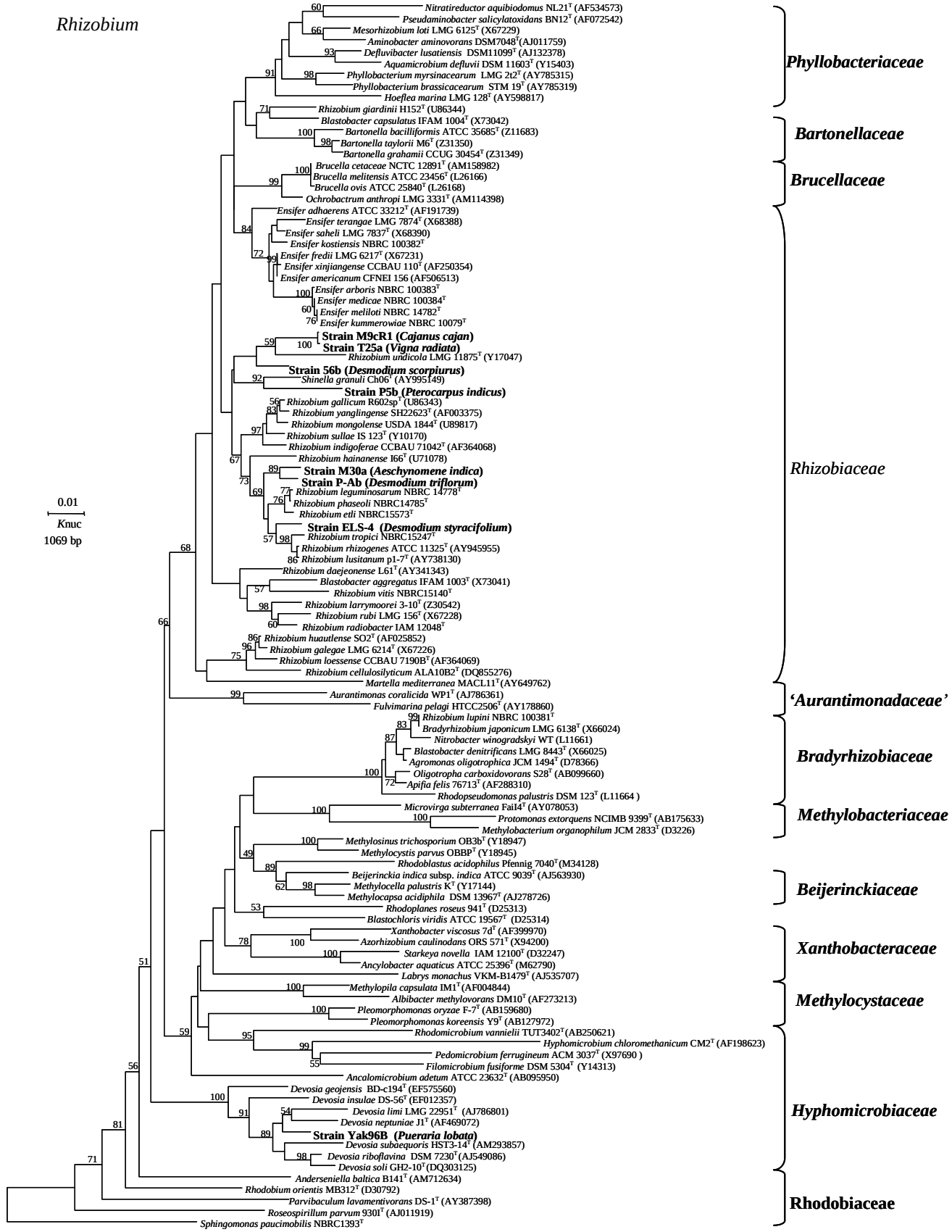

Fig.1. Neighbor-joining phylogenetic tree based on the 16S rRNA gene sequences showing the taxonomic position of the novel strains from the Philippines and Japan with respect to some of the members of the families belonging to order *Rhizobiales.* Bootstrap values greater than 50% are shown as percentages of 1000 replications. GenBank accession numbers are given in parentheses. Bar, 1 substitution per 100 nucleotide.

mindoronensis. Whereas, strain P-Ab as *Rhizobium isabelanensis*.

With regards to the presence of the symbiotic genes, the nitrogen fixing (*nif*H) and nodulating (*nod*D) genes were not detected in all the aforementioned novel species by PCR amplification using several primer sets. Moreover, all strains failed to infect the roots of the promiscuous *Macroptilium atropurpureum* (siratro) as host plant in the nodulation/infection assay. This was also observed in the recognized species of *Mesorhizobium thiogangeticum* and *Bradyrhizobium betae i*solated from the roots of *Clitoria ternatea* and *Beta vulgaris*, respectively, both devoid of symbiotic genes.

In conclusion, our study successfully isolated and classified 7 novel species of rhizobia belonging to family *Rhizobiaceae* and *Hyphomicrobiaceae*. The use of 16S rRNA sequences in conjunction with DNA-DNA hybridization and physico-chemical tests are important in delineating closely related species. The newly described strains will further add to the ever growing members of the Order *Rhizobiales*. Moreover, the rhizobia group is biological diverse not only in terms of their heterogeneous characteristics and taxonomy but also with their interaction/association with leguminous plants (host range).

1. Lee, K-B, C.T. Liu, Y. Ansai, H. Kim, T. Aono and H. Oyaisu. (2005). The hierachical system of the '*Aplhaproteobacteria*': description of *Hyphomonadaceae* fam.nov., *Xanthobactereceae* fam. nov. and *Erythrobacteraceae* fam. nov. *Int J Syst Evol Microbiol* 55:1907-1919
2. Bautista *et al*. (2007). *Int J Syst Evol Microbiol*. : Submitted
3. Bautista *et al*. (2007). *Int J Syst Evol Microbiol*. : In preparation