論文の内容の要旨

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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 Xanthobacteraceae¹.

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 (*nifH*) and nodulation gene (*nodD*)) 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 ZYMTM (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 and Desmodium triflorum, respectively. St

Nitratireductor aquibiodomus NL21^T (AF534573)

Pseudaminobacter saticylatoxidams BN12^T (AF634573)

Aminobacter aminovorans DSM7048^T (A011759)

32 Defluvibacter lusatiensis DSM11097^T (A1532378)

Aquamicrobium defluvii DSM 11603^T (Y15403)

98 Phyllobacterium myrsinacearum LMG 212^T (AY785315)

Phyllobacterium brassicacearum STM 19^T (AY785319)

Hoeflea marina LMG 128^T (AY598817)

Rhizobium giardniii H152^T (U86344)

Blastobacter capsulatus IFAM 1004^T (X73042)

Bartonella bacilifjorniis ATCC 35685^T (Z11683)

98 Bartonella taylorii M6^T (Z31350)

Bartonella prahamii CCUG 30454^T (Z31349)

100] Brucella ceticace NCTC 12891^T (AM158982)

Brucella avis ATCC 25840^T (L26168)

Ochrobactum anthropi LMG 3331^T (AM114398)

Ensifer adhaerens ATCC 33212^T (AF191739)

Ensifer terangae LMG 7874^T (X68388)

Ensifer satheii LMG 7837^T (X68390)

Ensifer fredii LMG 6217^T (X67231)

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Ensifer fredii LMG 6217^T (X67231) Rhizobium Phyllobacteriaceae Bartonellaceae Brucellaceae Ensifer kostiensis NBRC 100382¹
nsifer fredit LMG 6217⁷ (X67231)
Ensifer xinjungense CCBAU 110⁷ (AF250354)
nsifer americanum CFNEI 156 (AF506513)
100 [Ensifer arboris NBRC 100383⁷
60 [Ensifer medicae NBRC 100384⁷
60 [Ensifer medicae NBRC 100387
76 Ensifer kummeroviae NBRC 10079⁷
Strain M9cR1 (Cajanus cajan)
100 Strain TZ5a (Vigna radiata)
Rhizobium undicola LMG 11875⁷ (Y17047) Strain 56b (Desmodium scorpiurus)

Shinella granuli Ch06¹ (AY995149) Shinella gramuli Ch06' (AY995[49)

Shinella gramuli Ch06' (AY995[49)

Rhizobium gallicum R602-p² (U86343)

Rhizobium yanglingense SH22.623' (AF003375)

Rhizobium mongolense USDA 1844' (U89817)

Rhizobium mongolense USDA 1844' (U89817)

Rhizobium midigoferne CEBAU 71042' (AF364068)

Rhizobium hainanense 166[†] (U71078)

Strain M30a (Aeschynomene indica)

Strain P-Ab (Desmodium triflorum)

74 Rhizobium leguminosarum NBRC 14778'

105 Rhizobium phaseoli NBRC 14785'

105 Rhizobium phaseoli NBRC 14785' Rhizobiaceae Rhizobium leguminosarum NBRC Rhizobium phaseoli NBRC14785¹ Rhizobium etli NBRC15573^T 0.01 Rhizobium edi NBRC15573^T

Strain ELS-4 (Desmodium styracifolium)
Rhizobium tropici NBRC15247^T

[Rhizobium tricogenes ATCC 11325^T (AY945955)
86 Rhizobium taitanum pl.-7^T (AY738130)
Rhizobium daejeonense L61^T (AY341343)

Blastobacter aggregatus IFAM 1003^T (X73041)
Rhizobium viis NBRC15140^T

Rhizobium larrymoorei 3-10^T (Z30542)

Rhizobium viis LMG 156^T (X67228)

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Rhizobium radiobacter LAM 12048^T Knuc 1069 bp Khizobium rubi LMG 156' (x6/228)
60 Rhizobium rubi LMG 156' (x6/228)
Rhizobium huaultense SO2^T (AF025852)
Rhizobium galegae LMG 6214^T (X67226)
Rhizobium loessense CCBAU 7190B^T (AF364069) obium loessense CCBAU 7190B* (AF364069)

Rhizobium celludosilyiicum ALA10B2* (DQ855276)

Martella mediterranea MACLL11* (AY649762)

Aurantimonas coralicida WP1* (AJ786361)

Fulvimarina pelagi HTCC2506* (AY178860)

92 Rhizobium lupini NBRC 100381*

87 Bradynizobium japonicum LMG 6138* (X66024)

Nitrobacter vinogradskyi WY (L11661)

Blastobacter denitrificans LMG 8443* (X66025)

Agromonas oligotrophica ICM 1494* (D78366)

Oligotropha carboxidovorans S28* (AB099660)

72 Apifia felis 76713* (AF288310)

Rhodosvedomonas palustris DSM 123* (L116*) 'Aurantimonadaceae' Bradyrhizobiaceae Rhodopseudomonas palustris DSM 123^T (L11664) Microvirga subterranea FaiI4^T (AY078053) Methylobacteriaceae Protomonas extorquens NCIMB 9399^T (AB175633)

Methylobacterium organophilum JCM 2833^T (D3226) Methylosinus trichosporium OB3b² (Y18947)
Methylosyaits parvus OBBP² (Y18947)
Methylocyaits parvus OBBP² (Y18947)
Rhodoblastus acidophilus Piennig 7040⁷ (M34128)
Beijerincki antidca subsp. indica ATCC 9039² (A1563930)
Methylocella palustris K² (Y17144)
Methylocepa acidiphilu DSM 13967² (A278726)
Rhodoplanes roseus 941² (D25313)
Blastochloris viridis ATCC 19567² (D25314)
Xanthobacter viscosus 7d² (A4799970)
Azorhizobium caulinodans ORS 571² (X94200)
Starkeva novella IAM 12100² (D32247) Beijerinckiaceae Xanthobacteraceae **Starkeya novella IAM 12100** (D32247)

Ancylobacter aquaticus ATCC 25396 (M62790)

Labrys monachus VKM-B1479 (AJ535707) Methylopila capsulata IMI (AF004844)

Methylopila capsulata IMI (AF004844)

Albibacter methylovaras DM10[†] (AF273213)

100 Pleomorphomonas oryzae F-7[‡] (AB159680)

Pleomorphomonas koreensis Y9[‡] (AB12972)

Rhodomicrobium vannielii TUT3403[‡] (AB250621)

Hyphomicrobium chlorom

Pedomicrobium ferrugineum ACM 3037[‡] (X97690)

Filomicrobium fissiforme DSM 5304[‡] (Y14313)

Ancalomicrobium adetum ATCC 23632[‡] (AB095950)

Devosia insulae DS-56[‡] (EP012357)

Devosia insulae DS-56[‡] (EP012357)

Devosia limi LMG 22951[‡] (AJ786801) 100 Methylocystaceae Hyphomicrobiaceae Devosia limi LMG 22951^T (AJ Devosia neptuniae J1^T (AF469072) (AJ786801) Strain Yak96B (Pueraria lobata)

Devosia subaequoris HST3-14^T (AM293857) Devosia subaequoris HST3-14 (AM

98 Devosia riboflavina DSM 7230 (AJS490
Devosia soli GH2-10' (DQ303125)

Anderseniella baltica B141^T (AM712634)

Rhodobium orientis MB312' (D30792)

Parvibaculum lavamentivorans DS-1^T (AY387398)

Roseospirillum parvum 9301^T (AJ011919)

onas paucimobilis NBRC1393^T Rhodobiaceae

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 (nifH) and nodulating (nodD) 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 isolated 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).

- Lee, K-B, C.T. Liu, Y. Ansai, H. Kim, T. Aono and H. Oyaisu. (2005). The hierarchical 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