Discussion

Taxonomy of N_2-Fixing bacteria associated with non-Leguminous plants such as rice is a new area of study, although their importance may not be less than the legume-symbiotic rhizobia. Numerous species of these associative nitrogen-fixing bacteria appear to have potential as fertilizer (39), so it is interesting to know about the relativity of these bacteria especially in the aspect of chemotaxonomy. The problem of which genera that our local N_2-fixing strains belong to, could be investigated by several means namely, by numerical taxonomy which is the method for identification covering a spectrum of characteristics i.e., morphology, physiology and biochemistry, including nutritional screening, temperature range of growth etc., correlation with the genotypic characteristics. Similar or identical characters above 75 % of the test characters indicate close relativity between the two strains. For chemotaxonomy, emphasis is on the similarity of basic chemical composition between unknown and standard reference strains i.e. the mol % gaunine plus cytosine content (G +C) in the bacterial DNA and their sequence homology.

The mole percent G + C value is a constant characteristic of a particular organism. A careful examination of this value will show that, in general, those organism which are genetically and/or taxonomically related have similar base compositions (22). A large difference in the mole percent G + C values from DNAs of two organisms indicates the lack of a close genetic similarity. The mole percent GC of the DNA preparation was determined by their thermal melting point (Tm) by using an automatic recording spectrophotometer. Although the DNA was

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fragmented to the extent that it was difficult to spool, the Tm was similar to that of DNA obtained from cells lysed by enzymes or detergent. For example, passage of a propionibacterium DNA preparation through a Frence pressure cell at 0.9 to 1.0 kbar lowered the Tm by the same amount as did shearing a preparation of E. Coli B DNA prepared from detergent-lysed cells (40). However, a similarity in the two mole percent G + C values does not necessarily mean that the organisms are similar with regard to the sequence in their DNAs. In the present research, we have analyzed the DNA sequence homology by reassociation experiment (optical method). By comparing the reassociation time (t_r) and the remelting temperature (T_r) of hybrid molecules to the original molecules, qualitative conclusion about the relatedness of the two original molecules could be obtained (24).

Our studies have indicated that R15 and R17 seemed to be closer to the Klebsiella spp. They are indistinguishable at the genus level by their close G + C content (52-56 mol %) and would be excluded from the genera Azospirillum and Pseudomonas because they have approximately 10 % difference in mean G + C content which indicate the unrelated genera among the tested bacterial strains (41). Besides these reference genera, a strain of Alcaligenes was recently reported as associative N_2-fixer in the rhizosphere of rice (33) but the mol % G + C of this genus is exceptionally broad, 57.9-70 % (44).

On the basis of DNA base composition, R25 could be expected in the N_2-fixing genera either Azospirillum or Pseudomonas (G + C content range from 64.2 to 70.8 mol %). If any significant DNA sequence homology exists between R25 and some established members of a particular genus, this would constitute strong evidence for as assigning R25 to that genus.

Based on the reassociation experiments as shown in Table 5,6 and Figure 13, DNAs from R15, R17 and K.oxytoca 1301 exhibited high homology sequence with each other, whereas R25 was found to exhibit high sequence homology with only standard reference strains from Azospirillum spp. Nonetheless difference in G + C values between R25 and P.H8/P.KLH76 was not greater than 5 %, assignment of R25 to the genus Pseudomonas does not preclude (41). Johnson & Francis (42) had studied about rRNA-DNA homology and suggested that genes governing ribosomal RNA are very highly conserved and are useful for indicating broader relationships between strains than are evident form DNA-DNA homology experiment that seemed to be useful at the species level not the genus level of taxonomy. In the absence of such studies, however, assignment of R25 to an appropriate genus must be done on the basis of phenotypic characteristics.

Comparison of plasmid pattern by rapid method developed by Kado and Liu (27) of all N_2-fixing bacteria showed that all strains except K.oxytoca 1301 contained at least one plasmid. The plasmid of R15, R17, P.H8 and P.KLH76 were detected after 30 min of incubation at 65°C whereas the plasmids of R25, A.FS and A.34H were detected after 60 min of incubation. These results correlated well with the the difference in G + C content among these bacteria because R25, A.FS and A.34H contained the highest G + C content (68.8-70.8 mol %) and should need longer heating time to degrade their chromosomal DNA. In this aspect R15 and R17 were different from K.oxytoca 1301 and other K.oxytoca varients x-3-d, NSG 3 and NG 13 reported by Barraquio et al and Uozumi et al (33,34) as non-plasmid harboring strains. Besides, they also reported that Alcaligenes sp. OSG 47 harbored a 90 Mdal plasmid, but in this experiment we could not estimate the plasmid

molecular weight of R15 and R17, so from these information we could not conclude about the similarity or difference in plasmid size between R15, R17 and Alcaligenes sp. As reviewed by Elmerich (39) plasmid of various molecular weight have been discovered in many diazotroph e.g. Azospirillum and Enterobacter, although plasmid content may be varied among strains within the same genus. As for R25, its plasmid pattern was similar to that of A.34H and A.FS which potentially confirmed their genotypic homology. In case of P.H8, Barraquio et al (33) report the presence of two plasmids in this bacterial strain namely pTAH8a (35 Mdal) and pTAH8b (64 Mdal). In this research only one plasmid was detected in P.H8. This discrepancy may have arisen from the difference in methodologies used. Since Barraquio et al. isolated plasmid by alkali lysis method of Casse et al. (43) and purified plasmids by CsCl-EtBr density gradient centrifugation before separation on agarose gel electrophoresis.

From the preliminary reports (34), we know that nif genes of all reference bacterial genera were located on chromosomal DNA, thus we tried to determine the location of nif genes in R15, R17 and R25 by using Southern hybridization technique. The first step was the chromosomal DNA or plasmid DNA cutting by selected restriction endonuclease such as EcoRI etc., then hybridized with nif probes from K.pneumoniae M5al. By comparison of the restriction pattern of nif genes, of each strain, the genetic similarity between strains may be obtained, Although this method seemed to be unappropiate for species identification, because several enzymes were usually required and it was uncertain to observe significant difference. In this research, we did not succeed in finding the proper condition for restriction

cut of all bacterial DNA, therefore incomplete data of this experiment was not shown.

Besides their genotypic similarities R15/R17 and K.1301 showed uniform results for several biochemical reactions namely; positive Voges-Proskauer (V.P.) reaction test, that involves the detection of diacetyl or its precursors acetoin and 2,3-butanediol formed in the butanediol fermentation of glucose, and indole production test that determines the ability of an organism to produce indole from tryptophan. They exhibited no requirement of biotin for growth and can use malonate as sole carbon source. In addition, these bacteria are able to grow in the presence of 5-10 % SDS. From the preliminary report (32) about extreme detergent resistance among the Enterobacteriaceae demonstrated that the bacteria appeared to tolerate the SDS rather than metabolized it. Growth was energy dependent and cell lysis commonly occured during stationary phase. SDS resistance appears to be a fundamental characteristic of the genus Enterobacter and of the tribe Klebsielleae and not a plasmid-coded acquired characteristic. Plasmid analyses were conducted on 72 Klebsielleae strains isolated from the chesapeake Bay (N.V. Hamlett, unpublished data) by method of Kado and Liu. They contained from 0 to 8 plasmids, with and average of 2.4 plasmids. Significantly, there were no plasmids common to all isolates and nine SDS-resistant strains contained no detecatable plasmids (32). Organisms able to grow in the presence of 5 % SDS (154 mM) or any other charged detergent, be termed saponotolerant or saponophilic whereas the detergen-sensitive strains would be saponophobic. Accordingly R15, R17 and K. 1301 are saponophilic whereas R25 is saponophobic, which reflect their difference in the chemical composition of cell wall and cell membrane.

The other interesting characteristic of R15, R17 and K.1301 is that, they could tolerate to salt (NaCl) at 3 and 5 % concentration. This seems to be the advantage property because they can survive even in saline soil in the North-Eastern part of Thailand while some other N_2-fixing bacterial strains could not survive. The saline soil was shown as one of the important ecological factor that reduced the efficiency of symbiotic-nitrogen fixation (44).

For antibiotic resistance tests among these bacteria, R15, R17 and K.1301 were sensitive to chloramphenicol, and high concentrations of ampicillin (>25 ug/ml), streptomycin (>6.25 ug/ml) and kanamycin (>6.25 ug/ml). This property seemed to be common for Klebsiellae bacteria. In this aspect R15 and R17 should differ from Alcaligenes which is sensitive to kanamycin at 6.25 ug/ml (33). Extensive studies showed that the proportion of resistant strains is increasing steadily, which may be due to mutations but in many cases is probable caused by transfer of plasmids carrying resistance determinants to a varying number of durgs (45).

Some essential differences, exist between the local varients R15, R17 and K.1301 based on colonial morphology on rich agar plate. K.oxytoca 1301 produced brown area diffusible in an agar but R15, R17 resembled K.pneumoniae M5al did not. This result suggested that R15 and R17 are not the 'oxytoca' variants of Klebsiella.

On the basis of purely phenotypic characteristic, such as the preferential for the salts of organic acid (malate) as carbon source, colonial character on RM and nutrient agar plates, and also ability to resist to ampicillin drug (50 ug/ml), the genus Azospirillum seems to be the most suituable for R25. Moreover, R25 could grow in NFb

semisolid medium supplied with 0.5 % glucose in place of malate and they unfared without biotin addition, these properties render R25 to Azospirillum lipoferum species.

Some interesting similarities exist between R25, A.34H/A.FS and P.H8/P.KLH76. For example, these organisms require microaerophilic condition for the expression of nitrogenase activity (Table 12,13) and N_2 were fixed only in media supplemented with small amount of combined nitrogen (from yeast extract) and also supported by various organic compounds which include sugars (glucose) and organic acids (malate). Anyhow, biotin was not required for growth in case of Pseudomonas spp. and they did not resist to ampicillin as Azospirillum spp.

The results of antibiotic resistance tests suggested that there is no direct correlation between plasmid presence and antibiotic resistance in these N_2-fixing strains.

For acetylene reduction assay, it is noticeable that both aerobic and microaerophilic conditions are optimum for nitrogenase activity of R15, R17 and K.1301, thus it is conceivable that they might have respiratory protection or other means of protection for nitrogenase whereas the other five strains lack such protection because N_2 was fixed by them only under microaerophilic condition. From preliminary report by Harinasut (4), R15 and R17 had higher ARA than the values obtained here (75 and 52 n mol/tube/h respectively), it is probable that N_2-fixing potential decreased along with several passages in free-living form. Harinasut did not succeed in finding the relationship between ARA and growth of R25 and suggested that, it might be because of the lack of some neccessary factors required for

the pure bacteria to express the N_2-fixing activity after being isolated from the rhizosphere ecosystem for a period of time. Since chemotaxonomy indicated that R25 is an S.lipoferum, it seemed likely that the necessary factor was biotin, however, when biotin was added under aerobic condition, R25 also failed to express nitrogenase activity. This result is strong evidence that N_2 is fixed by R25 only under microaerophilic condition.

From all the results, R15 and R17 are Klebsiella-like strains but not the oxytoca varient based on their mol % G + C content, DNA sequence homology, and many specific physiological tests. They are saline and SDS tolerant strains and show various degree of resistance to several antibiotics. Glucose is the preferential carbon-source for N_2-fixation under both aerobic and microaerobic conditions.

As for R25, it is identified as an A.lipoferum strain, because of its requirement of biotin and low levels of yeast extract (0.005 %) for growth in mineral media. N_2-fixation in NFb medium is obligative to microaerophilic condition because of its lack of oxygen-protection mechanisms for its nitrogenase (46,47). When R25 was supplied with combined nitrogen (2.5 g/l of NH_4Cl) it grew as an aerobe but did not fix N_2 (data not shown). Salts of organic acids such as malate and succinate were found to be satisfactory oxidizable carbon and energy sources. All these properties distinguished R25 from aerobic N_2-fixing bacteria Derxia gummosa which has a closely related mol % G + C of 70.4, and does not grow on malate or succinate, catalase negative and acid tolerant (45).

Further species and strain identification of these bacteria should be conducted in the area of immunological properties of each strains and then cross-reaction between strains. At present, this method is widely used to identify strains of N_2-fixing bacteria from rice rhizosphere (12,13,15). As indicated from all the results R15 and R17 may be belong to the same species so the cross-reaction among R15 and R17 should be conducted and specific fluorescence antibody should be produced. If they have high degree of cross-reaction, they might be belong to the same species. Their immunological properties might be compare to K.oxytoca 1301, low degree of cross reaction would suggest for difference in species.

The characterization of surface antigen among these associative N_2-fixing strains isolated from the rhizosphere of rice might disclose some common surface antigen required for the association between these bacteria and rice root.

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