Introduction

Nitrogen is a key building element of protein and nucleic acid molecules upon which all life is based on, thus it is an indispensable component of simple organism such as bacteria as well as complex organism like higher plants. All of vegetational cover of the earth including rice is dependent on inorganic nitrogen for growth, so it requires continual conservation and maintenace of this form of nitrogen. The available form of nitrogen for plants are ammonium-nitrogen (NH_4^+) and nitrate-nitrogen (NO_3^-). Thus, the application of nitrate, ammonia or urea which can be converted to ammonium fertilizer, directly increase crop yield. However the fertilizer production by industrial process is dependent on fossil fuel. It was estimated that about 1.5 kg of fuel oil is needed to manufacture and deliver 1 kg of nitrogen fertilizer to the farm (1). So it is necessary to investigate an alternative method for the production of nitrogen fertilizer from renewable source such as biological nitrogen fixation in the paddy fields.

Biological nitrogen fixation is an interesting energy-saving potential process, because nitrogen-fixing organisms utilized light energy, directly or indirectly to produce ammonia from atmospheric nitrogen (78 % of the air) and since manufacture occurs on the site, delivery costs are not neccessary. To approach this aim, we must know which N_2-fixing bacteria that account for N_2-fixation in our rice ecosystem.

In the paddy fields, dinitrogen fixation associated with many rice varieties were reported, including N_2-fixation contributed by cyanobacteria that occurs on or in the leaf sheath, and basal portion of shoot (2), and a large number of N_2-fixing heterotrophic bacteria in the root zone (rhizosphere soil, rhizoplane and histosphere). Particularly the heterotrophic bacteria strongly associated with the rhizoplane of rice roots should account for significant amount of nitrogenase activity in the paddy fields (3-9). Watanabe and Barraquio had confirmed the N_2-fixing activity of some rhizosphere isolates, comparing with Azospirillum lipoferum SpBr 17 by using $^{15}N_2$-incorporation method and reported that two isolates could incorporate 0.18-0.25 atom % excess into cellular nitrogen, which was lower than A. lipoferum SpBr 17 (6). However, when N_2-fixing Pseudomonas sp. H8 and A. lipoferum 34H were inoculated seperately into wetland rice in pot-experiment, the inoculation promoted only reproductive growth of wetland rice and increased acetylene reduction activity and the number of N_2-fixing bacteria associated with roots, whereas neither an increase of total dry weight nor N content was observed. There was little difference in the ^{15}N content in the whole plant inoculated with P. H8 and A. 34H This results suggested that, even when bacterial colonization in the root is succesful and promotes N_2-fixation associated wetland rice, such stimulation and absorption of the N-fixed by rice could be too low to be detected significantly in the ^{15}N contents of rice plants (10).

Using the acetylene reduction assay of washed and surface sterilized roots, 259 bacterial cultures were isolated from acid soil and semi-acid soil of Thailand (4). All of them were Gram-negative rod. Only three representative bacterial cultures were selected from

the top 8 % according to their acetylene reduction activity namely R17, R15 and R25. The original information of these rhizospheric N_2-fixing bacteria are shown in Table 1.

Table 1 The original information of unknown bacterial cultures (4)

Bacterial code	Rice experimental site	Rice variety	Isolation site	Incubating medium	Optimum temperature (^{o}C)
17R1a2 (R17)	Tapra	RD.6	Root (non-sterile)	NF+YE	37°
15RR12 (R15)	Rangsit	RD.7	Root (non-sterile)	NF	37°
25Sa12 (R25)	Tapra	RD.6	Soil	NF+YE	$42-46^{o}$

The aim of this thesis is to identify the genus of these 3 unknown N_2-fixing bacteria isolated from the rhizosphere of local cultivars of rice by comparing their biochemical characters with reference bacterial isolates elsewhere.

Current studies showed that in general, Pseudomonas, Azospirillum, Enterobacter and Klebsiella are the commonly encountered nitrogen-fixing bacteria associated with many rice varieties (2,11,12). Pseudomonas was found to be the most predominant in wetland rice root (2,6) and dominant in the histosphere samples (13). The histosphere isolates were identified as Pseudomonas by gel immunodiffusion and fluorescent antibody techniques in combination with their biochemical characteristics (14). Azospirillum was also found to associated with wetland rice roots and stems (6,9). This genus has recently attracted

the attention of microbiologists, and ecologists. On the basis of
DNA homology studies, genus Azospirillum has been reclassified into
species : A. lipoferum and A. brasilense (15). Other methods for the
identification these species is based on immunological and biochemical
techniques (16). Reports on the presence of Enterobacter and Klebsiella
in the rhizosphere of rice are scarce (17) and there appears to be no
published information available on the enumeration of these enterobac-
teria. The physiology of N_2-fixation by these organisms has been
studied mainly to determine the oxygen tolerance of their nitrogenase
activity (18) but so far very little is known about the utilization
of carbon and energy sources for N_2-fixation. Most examinations have
been carried out with glucose as the carbon source (19).

Because of this group diversity, different isolation media
and conditions are required to obtain these bacteria from the same
source. It is most interesting to compare associative N_2-fixer such
as Klebsiella oxytoca with their relative which are free-living N_2-
fixer (Klebsiella pneumoniae) or associative N_2-fixer in different
geography i.e. temperate region (Japan) and tropical region (Philippines,
Thailand); especially their specific difference in biochemical characters.

Thus, chemotaxonomy is the principle used in this thesis to
classify our N_2-fixing organisms (Table 1.) according to their mutual
affinities or similarities with other reference genera previously
mentioned.

Since our reference bacteria are classified in three genera
i.e. Pseudomonas, Azospirillum and Klebsiella, minimum suitable bioche-
mical character were selected to distinguish one group of organisms
from the other. From the information of these reference strains (Table 2.)

Table 2   Information fo reference strains (14,16)

Family	Genus	Species and Strain No.	Rice variety	Isolation site (country)	Incubating medium	Optimum temperature (^{o}C)	G+C content (moles %)
Enterobacteriaceae	Klebsiella	K. oxytoca 1301 (K. 1301)	not known	Rice rhizosphere (Japan)	NF	35-37°	54.7
Pseudomonadaceae	Pseudomonas	P. H8	IR 26	Histosphere	GYE	30°	64.2
		P. KLH76	Khao-lo	Histosphere (Philippines)			65.2
Spirillaceae	Azospirillum	A. lipoferum FS (A. FS)	–	Field soil	NFb	35°	70.8
		A. lipoferum 34H (A. 34H)	IR 34	Histosphere (Philippines)			69.4

the mol % G + C content is one important parameter to distinguish
Klebsiella sp. from Azospirillum spp. and Pseudomonas spp. but not
crucial for the last two from each other.

Determination of the base composition of deoxyribonucleic acid (DNA)

There are three widely used methods of G+C content determination.
The oldest method is chemical analysis of DNA bases (20). The G+C
content of DNA has also been shown to be directly proportional to its
buoyant density (ρ) in cesium chloride. Schildkraut et al. (21) reported
that the GC content of a large variety of DNAs was correctly determined
using the buoyant density method. The relation used for calculation
is %G+C = 1038.47 (ρ - 1.6616). The constants in this equation are
slightly modified when data on nonbacterial DNA are included. However,
problem of this method is the effect on the density of odd bases or of
glucose. A good example is the DNA from T-even bacteriophage, whose
densities unpredictable from their base composition reflect their
hydroxymethylcytosine and varied glucose contents. Even without odd
bases, by using the density method, all predict a base composition value
that is rather high comparing to others. The advantage of this method
is that, the analysis can be made on a lysate without having to purify
the DNA.

Another method of G+C content determination commonly used is
based on the thermal melting of DNA. Marmur & Doty (22) have shown
that the DNA bases composition is related to its thermal denaturation
temperature. From the data on 96 strains of bacteria, it was calculated
that % G+C = 2.44 (Tm - 69.4°C). Tm appear to be unaffected by the
substitution of cytosine by hydroxymethylcytosine. This equation is
also valid for nonbacterial DNA. The linear relation between Tm and

buoyant density on identical samples shows that the base compositions deduced from the linear relations are generally within the expected probable error. A few deviations occur in the very low G+C region (25 to 33 %). However in no case was the difference greater from that corresponding to either $2.5^{\circ}C$ or 0.006 g/c.cm. Thus, although some further work remains to be done when samples of very low G+C content become available, the agreement can be taken as satisfactory over the range of 30 to 75 % and compositions estimated from either Tm or buoyant density taken as interchangeable.

Because of the ease of melting temperature method, it was selected for measurement of G+C content of references and unknown bacteria in this research.

When DNA is heated in solution, a sharp increase in its extinction coefficient occurs at the temperature where the transition takes place from the native, double-stranded structure to the denatured state. The temperature corresponding to the midpoint of the absorbance rise, the Tm, is linearly related to the average DNA base composition; a higher G+C content confers a higher thermal stability. It can be summarized thus: for a solvent containing 0.2 M Na^{+}, Tm = 69.3 + 0.41 (G+C) where Tm is in degree celcius and G+C refers to the mole percentage of guanine plus cytosine. The deviation of experimental points from this relation are no more than that expected from the uncertainties of base analysis and the variations of a half degree in the reproducibility of determining the Tm. Consequently it appears that the measurement of the Tm is a satisfactory means of determining base composition in DNA. The Tm values are most simply measured by following the absorbance at 260 nm as a function of temperature of the DNA solution and noting the midpoint of the hyperchromic rise. Only 10 to 50 μg of

DNA are required. The method proved to be highly reliable and repro-
ducible. Finally, by determining the Tm value several times on the
preparation, the uncertainty arising from the measurement itself can
be kept within the equivalent of 1 mole percent.

Recently, the determination of bacterial DNA base composition
by spectrofluorimetric method has been developed (23). The DNA was
stained in fluorescence dye and then its G+C content was determined
using fluorescence spectroscopy. The advantages of this technique
were firstly, a very short time was required to perform the assay,
secondly the determination could be done on whole cells, thereby
circumventing the need for DNA extraction, and thirdly only small
cultures were needed. However, several technical problems emerged.
First, one must avoid the use of glass when exposing the bacterial
cells to the fluorescence dye. It was found that if glass test tubes
were used for the assay or if glass Pasteur pipettes were used for
transferring samples, inconsistent results were obtained. The other
point which became apparent was that at periods longer than 10 min,
the samples began to lose their fluorescence. Finally it was found
that this set of experimental conditions is not ideal for isolated
DNA. Therefore, this fluorescence technique is inappropiate method
for this research.

DNA sequence homology

The second biochemical character used to identify unknown N_2-
fixers is the analysis of DNA homology by reassociation experiment.

Reassociation is the process of formation of double-stranded
molecules between two strands which are separated and at least one of

which is free to diffuse, logically should be called association,
since the two strands usually come from difference original molecules.
The rate of reassociation is affected when the pairing sequences are
short or have noncomplementary bases. On the average, it appears
that the presence of enough unmatched bases to reduce the melting
temperature by $10^{o}C$, will increase the reassociation time by a factor
of two (24). Thus, we used this process for analysis of DNA homology
between unknown and refernce bacterial DNAs.

To determine that two bacteria probable belong to the same
genus, the DNA from them were checked for Tm value and reassociation
rate compared to each other. The DNA from these different bacteria
were mixed and determined for the reassociation time of the hybrid
DNA. If the reassociation time of the hybrid DNA were close to the
reassociation time of homologous DNA, it is likely that the two
bacteria are related species because they have similar base sequence
in their DNAs, so they can form new double-stranded DNA molecules.

Thus, it is seen that when the melting-temperature method is
used for G-C content determination, by using the same sample, analysis
of DNA base sequences by reassociation process can be easily performed
by this optical method.

Plasmid pattern and restriction cut

Another criteria that concerned in this study is the plasmid
pattern and the enzyme restriction cut pattern of the bacterial
chromosomal DNA. It is assumed that related bacteria should result
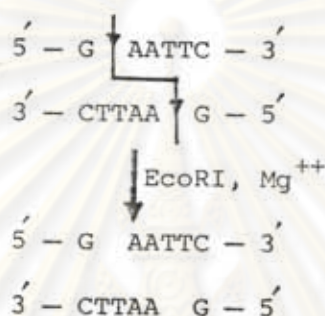in similar plasmid pattern.

Plasmid are small circular extrachromosomal DNA founded in some bacterial cell. In general, they carry some genes that controlled the synthesis of specific enzyme(s) essentially for bacteria or some genes conferring resistance to antibiotics or drugs. In some cases, they also have some genes controlling toxins production. By conjugation, plasmid can be transfferred into new bacteria and then transfer their properties to that host. These genetic elements are sometimes nonessential for growth so that under many conditions they can be lost or gained without harm to the cell (25).

To determine whether a given bacterium harbors plasmids, it is necessary to isolate DNA by plasmid isolation procedures. Chromosomal and plasmid DNA are often obtained from cells that are treated with lysozyme and lysed with a detergent. The DNAs are freed of RNA and proteins by RNase and protease treatments that are followed by extraction with phenol. Just before phenol extraction, the plasmid DNA is released from the folded chromosomal complex by a shearing step or by SDS treatment. The plasmid DNA can be resolved as covalently closed circular molecules by isopycnic centrifugation in cesium chloride containing ethidium bromide (26).

To avoid such time-consuming steps, the rapid procedure for detection and isolation of plasmid developed by Kado and Liu (27) was used in this research. This method utilized the molecular characteristics of covalently closed circular deoxyribonucleic acid (DNA) that is released from cells under conditions that denature chromosomal DNA by using alkaline sodium dodecyl sulfate (pH 12.6) at elevated temperatures. Proteins and cell debris were removed by extraction with phenol-chloroform. Under these conditions chromosomal DNA concentrations were

reduced or eliminated. The clarified extract was used directly for electrophoretic analysis.

As for DNA cutting by restriction endonuclease, EcoRI extracted from Escherichia coli RY 13 was used. It has specific hexa-nucleotide recognition sequences with the cleavage site on double-stranded DNA. Target nucleotide sequence $(5'-3')$ for EcoRI is G↓AATTC. After cutting DNA fragments with $5'$ cohesive end are obtained (28).

$$5' - G \downarrow AATTC - 3'$$
$$3' - CTTAA \uparrow G - 5'$$

$$\downarrow EcoRI, Mg^{++}$$

$$5' - G \quad AATTC - 3'$$
$$3' - CTTAA \quad G - 5'$$

These DNA fragments are then subjected to agarose gel electrophoresis. By comparison of the restriction pattern of DNA fragments, identical bacterial genus should give similar pattern.

Finally to support the results obtained form these genotypic characteristics, other biochemical and physiological properties as shown in Table 3 were selected as additional tests. Positive test for Voges-Proskauer reaction, indole production and malonate utilization serve to distinguish Klebsiella spp. from other bacteria. As for Azospirillum spp., they seemed to be different from the other two genera with respect to cultural characters i.e. growth on rich or nutrient agar plates, and physiological characters especially their requirement to biotin for growth which can also distinguish the two species of this genus; A. lipoferum and A. brasilense from each other.

Table 3   Distinction between reference strains on the basis of pheno-
          typic characters.

Character or test	Klebsiella spp.	Azospirillum spp.	Pseudomonas spp.
Voges-Proskauer	+	−	−
Indole production	+	−	−
Malonate utilization	+	−	−
Colonies on rich agar plate	−	yellow-colony	−
Colonies on nutrientagar plate	−	pink-colony	−
Growth in presence of 3 % NaCl	+	+	−
Growth in presence of 5 % NaCl	+	−	−
Biotin requirement	−	+	−

The results obtained form this study indicated that R15 and
R17 were Klebsiella like strains while R25 seemed to belong to
Azpspirillum spp. Because of the requirement of biotin for growth
and it could grow in NFb semisolid medium supplied with 0.5 % glucose
in place of malate, R25 was identified as A. lipoferum strain.

In order to confirm the conclusions, R15 and R17 were cultured
in specific defined medium of Klebsiella spp. and were tested for N_2-
fixing activity (ARA) whereas R25 was cultured in NFb medium of
Azospirillum spp. In addition, R25 was tested for ARA under microaerobic

conditions because Azospirillum spp. can only fix N_2 under this condition not aerobic or anaerobic conditions.

After the identification and characterization of these N_2-fixing isolates form the rhizosphere of rice, it is hoped that their fixing potential can be improved, by genetic manipulation, so that when they were applied back into the paddies, nitrogen fixation in that areas should be promoted more efficiently.