CHAPTER I

INTRODUCTION

1. The Significant of BNF and *Rhizobium*.

The discovery that microorganism, not the plant itself, that caused the biological nitrogen fixation (BNF.) which was detected in leguminous plant, had now entered its last decade of a century. It took approximately 50 years just to reveal this established fact, if counting after an establishment of a method to assay the BNF in 1841. For that long duration of time, merely no appropriate method being known to cultivate the N_2 fixing bacterium out from root nodule. Furthermore no one had succeeded to detect the BNF of the bacterium after being isolated and cultivated outside the root plant.

At present, it has been established that BNF occurs exclusively in prokaryotes. Not only bacteria, isolated from plant, but those that are isolated from animals, soil or sea could also fix nitrogen. There has been an ecological esmitation that 70% of the N composition presented in organic N-containting compounds was originated from BNF. (Burns and Hardy, 1975).

Development in the basic knowledge of BNF has been enormously extended since 1973, being remembered as the energy crisis year. Two main reasons were used to recall for an admittance in BNF as the utmost significant biological process of the highest potential to alliviate the energy crisis. First, to its direct contribution of N-source to various kinds of economical crops, either in the form of obligatory or associative symbiosis with those plants. Second, to its indirect contribution to the N-protein in legumes especially soy bean protein which has been accepted as the cheapest source of the best protein known to human being.

Rhizobium has been adapted as the group of bacteria which live obligatory symbiosis with leguminous plants. This genus of N_2 fixing bacterium plays the top most cruciality in N-protein contribution to Leguminosae. General characteristics of Rhizobium are as follows.

- Gram negative rods (0.5-0.9 μm x 1.2-3.0 μm)
- Generally motile, having peritrichous, polar or subpolar flagella.
- Contained with prominent granules of poly β-hydroxybutyrate.
- No endospores
- Aerobic chemorganotrophs with best growth at 25^{o} - 30^{o}C.

2. Classification of Rhizobium (Elkan, 1981)

Among nitrogen fixing bacterium, Rhizobium was the first genus to be studied in detail. Many types of its properties including physiological, biochemical, genetical or even ecological natures have been used for classification and identification. Some popular classifications including the defined properties are summarized as follows.

2.1 Based on Growth Characteristics.

According to Allen and Allen (1950), the genus Rhizobium has traditionally been divided into two groups.

i) Fast-growing rhizobia, likely to have a mean generation time of 2-4 hrs, give little detectable growth on agar media in 24 hrs, but generally form relatively large (2-4 mm in diameter), gummy, colorless or white colony in 3-5 days.

ii) Slow-growing rhizobia, likely to have a mean generation time of 6-8 hrs, yielding small colonies ($\leq$ 1 mm) after 7-10 days; generally colorless, white, gum less abundant than the fast grower, dense and sticky.

2.2 Based on Cross-inoculation.

In Bergey's Manual, 8th ed. (Buchanan and Gibbons, 1974) concluded a classification of Rhizobium based on the cross-inoculation (or plant affinity) properties as follows.

i) R. trifolii : nodulated with clover groups or Trifolium spp.

ii) R. leguminosarum : nodulated with Pisum, Lens, Lathyrus and Vicia.

iii) R. phaseoli : nodulated with Phaseolus vulgaris

iv) R. meliloti : nodulated with Melilotus, Medicago and Trigonella

v) R. japonicum : nodulated with Glycine max

vi) R. lupini : nodulate with Lupinus and Ornithopus

vii) Miscellaneous groups of Rhizobium : nodulated with Cowpea group of Vigna, Desmodium, Arachis, Centrosema, Stylosanthes etc.

2.3 Based on Serological Property.

According to Graham's review in 1969, there are 3 broad serological groups as follows.

i) R. trifolii, R. leguminosarum and R. phaseoli

ii) R. meliloti

iii) R. japonicum and R. lupini

Other alternative methods being used to classify Rhizobium species are the ability to use various carbon sources, the % GC content, the DNA hybridization and lately the analysis of protein fingerprints of crude extract of Rhizobium.

3. Techniques for Estimation of BNF in Rhizobia.

As research in N_2 fixation and analytical technique advanced, a variety of assay method for N_2 fixation were developed. The observation of growth and morphology of Legume root nodule development is one of the most simple indirect methods. In addition, analysis of increasing N-content refering uptake of N by the test-legume is also commonly used. However, to acheive higher sensitivity, another two techniques are availabel to use. (Herridge, 1982)

3.1 ^{15}N methods

There are three techniques for assaying N_2 fixation with stable isotope ^{15}N, are used. The first and most popular one involved the exposure of the N_2-fixation system under study to $^{15}N_2$ in a gas-tight container. The second technique, generally referred to as isotope dilution, involved labelling of soil N with ^{15}N, the degree of N_2 fixation was analysed by measuring the total N and ^{15}N. In the third isotope technique, variation in natural abundance caused by discrimination during N_2 fixation in favour of the lighter isotope (^{14}N) is measured. The very small abundance can be detected on sensitive mass spectrometers. This ^{15}N method has the disadvantage that methods for detection and determination of ^{15}N was complicated and time-consuming and required expensive instruments which are difficult to operate and maintain.

3.2 Acetylene reduction assay

The acetylene reduction assay arose from the independent observations of Dilworth (1966), and Schöllhorn and Burris (1967) that the nitrogen-fixing enzyme, nitrogenase, reduced acethylene (C_2H_2) to ethylene (C_2H_4). Since that time the reliability of acetylene reduction as an indicator of nitrogenase activity has been established for a wide range of biological systems and the technique has assumed a vital role in N_2 fixation studies. In brief nodules, soil containing nodules or whole plant system were enclosed in gas-tight container, filled with C_2H_2 which contained a partial pressure of C_2H_2. The atmosphere was sampled after a suitable incubation period and analysed for C_2H_4 using gas chromatography with a flame ionization detector.

The theoretical relationship between acetylene reduction and N_2 fixation dictated that 3 moles of C_2H_2 are reduced for each mole of N_2 fixed. This is based upon the fact that 2 electrons are needed to reduce C_2H_2 to C_2H_4 but 6 electrons required to reduce N_2 to $2NH_3$. The variation of this factor was discussed by Hardy et. al. (1973). The sensitivity of this method was claimed to be about 10^3 times greater than the ^{15}N method (Burns and Hardy, 1975).

4. Environmental Effects on Legume Nitrogen-Fixation. (Sprent, 1979)

Success or failure in nodule establishment is dependent on chemical, physical and biotic influence which act upon three different biological entities: the rhizobia, the host plant and the symbiotic system itself. Various important factors affected the BNF are summarized as follows:

4.1 Temperature

In various parts of the world, temperature may be a major factor limiting the extent of N_2 Fixation. It also affects the formation and activity of nodules. Generally, the symbiotic system is more temperature sensitive at both extremes of low and high temperature. It has been established, lacking of sufficient ATP, produced by plant to nodules, might be the major cause of low activity of nodule when plant is exposed to high temperature. (Halliday, 1976). The effect of low temperature has been shown pronounced on the infection process and be the cause of the delay in nodule formation. (Roughley, et. al, 1970).

4.2 Water relation

Water supply has a mojor effect on nodulation and N_2 fixation. Lower water potential in the nodules may directly reduce N_2-fixation activity with an accompanying reduction in nodule respiration, and the transport of fixed nitrogen out of nodules may be depressed. (Pankhurtst and Sprent, 1975). In addition, nodules developed under water-logging, showed markedly a reduction in N_2-fixing ability (Minchin and Pate, 1975)

4.3 pH

With respected to legume N_2-fixation, pH is often considered in conjunction with calcium, molybdenum, aluminium and manganese. At low pH value, the first two tend to be deficient, but the last two tend towards toxic concentrations. All these factors influenced both rhizobia and infection process of nodulation (Sprent, 1979)

4.4 Salinity and associated effects

Generally, high salt concentration will affect both the survival and growth of rhizobium in soil. (Steinborn and Roughley, 1975)

In addition, a reduction of rhizobial colonization around root surface is observed when its host plant is grown in salinized culture medium. (Singleton and Bohlool, 1984). It has been established that the early process involved in nodule formation is extremely sensitive to high concentration of sodium chloride, in which a significant reduction in nodule number and weight is generally its consequent result. (Singleton and Bohlool, 1984)

Furthermore, high concentration of solute in soil also affects the nodule activity in a variety of ways. For example, water can be withdrawn osmotically from host shoot and induces disturbance in the host metabolism causes an abnormal root system which is not appropriate for rhizobial infection. (Lakshimi Kumari, et. al, 1974). In short, high concentration of salt causes a detrimental effect on both plants and microorganisms.

4.5 Nutrient effects

Legumes need the same nutrients as other green plants. Nitrogenase activity and synthesis of Leghaemoglobin required additional Mo and Fe. Probably, the most common limiting nutrient in many areas is phosphorus which act on nodulation, N_2-fixation and plant growth. In addition, combined nitrogen also reduced nodulation and N_2 fixation ability.

4.6 Survival of rhizobia

It is one thing to establish an efficient host-rhizobium combination under laboratory conditions, and quite another to establish it in the field. In soil, a particular bacterial strain has to survive fluctuation in temperature, moisture and ion content, to be able to compete successfully for nutrients with a wide variety of microorganisms including other rhizobia. It also has to survive any diseases and the effect of natural and artificial bacteriocides, herbicides and other assorted chemicals. Having survival all of these conditions and multiplied, it then has to infect the host.

5. Problems Associated with Legume-Use.

Saline soil is one of the ecological factors that effect symbiotic N_2-fixation. Agronomists have failed to use the rhizobium for increasing crop-productivity of Legumes in saline soil condition. There are two types of saline soils in Thailand, namely : coastal and in-land saline soils. The dominant salt in both areas are mainly sodium chloride. The marine salinization of the coastal saline soil covered more than 200,000 hectares of the cultivated land. The in-land saline soil in Northeast Thailand are results of geological salinization and covered more than one million hectares consisting of slightly to severe and potentially salt affected area. (Sinanuwong and Takaya, 1974).

Unsuccesful symbiosis might be explained by the failure of the infection process due to salt affecting the establishment of Rhizobium. The success of a crop in such soil condition could possibly be acheived by a salt-tolerant legume-Rhizobium combination.

6. Known Mechanisms for Salt Tolerance in Bacteria.

In living cell, water serves as a medium of interactions of small and large molecules. The solutes dissolved in cell water and the structure of this water control all the vital process; enzyme action and regulation, assembly and disassembly of organelles, membrane structure and function. Adaptation of microorganisms in the presence of high salt concentration may associated with their ability to serve cell water. The mechanisms which they used to adapt in such environments are summarized as follows:

6.1 Proline and its roles for enhancement of osmotolerance.

In 1955 Christian made the interesting observation that proline, added exogenously at low concentration, specifically stimulated the growth rate and respiration rate of some bacteria in the media of high osmolarity (Christian and Waltho, 1966). The isolated mutant of Salmonella typhimurium which over-produce proline was found to grow faster than WT in media of elevated osmolarity (Rudulier, et. al., 1980). Studies in Klebsiella pneumoniae, the stimulatory effect of additional proline on the growth rate was manifested in the condition of extreme osmotic inhibition. But proline exerted a much greater stimulatory effect on nitrogenase activity at high osmolarity. The effect of the mutation resulting in proline-over production in K. pneumoniae was similar to the effect seen when proline was supplied exogenously (Rudulier, et. al, 1980).

There are two explanations which suggest this osmoregulation of proline. Firstly, proline might be an osmotic balancer which, when presented at high intracellular concentration might act to prevent osmotic dehydration of the cytoplasm. (Measures, 1975). Secondly, proline might exert as a stimulatory effect for growth in a medium of high osmolarity, for it could interact with molecule of protein and keep the biological function of that protein stable in the presence of salt. (Schobert and Tschesche, 1978).

6.2 Porin proteins : an alteration to osmotic stress

The outer membrane of E. coli contains a set of abundant proteins, the porins, that serve as channels for the passive diffusion of small hydrophilic molecules. In E. coli K - 12 strains, the two major porins have been established as OmpF and OmpC. The relative amounts of these proteins have been shown to vary as a function of growth condition and to be influenced by such factors as the ionic strength and osmolarity of the growth medium (Garrett, et. al., 1983). The amounts of OmpF and OmpC varied in a reciprocal manner, high osmolarity, favoring OmpC transcription and depressing OmpF expression (Villarejo and Case, 1984). Regulation of porin genes are being the studying subject in various lab. (Hall and Silhavy, 1981)

6.3 Na^+/H^+ Antiporters

An Na^+/H^+ antiporters is a carrier protein that mediates the exchange of Na^+ and H^+, ie., translocates Na^+ and H^+ in opposite direction accross a membrane.

Harold and Papineau (1972) were the first who reported the presence of Na^+/H^+ antiport activity in Streptococcus faecales. Subsequent report was from West and Mitchell in 1974 who reported the presence of this system in the membrane of E. coli including showing that transportation of sodium ion either in (influx) or out (efflux) was coupled to the electron transport in the aerobic respiration. In fact, proton pump has been shown to be the mahor electrochemical energy to drive the transport of solutes such as lactose, proline, etc., including moving bacterial flagella (Hinkel and McCarty, 1978). Furthermore, Na^+/H^+ antiporters are commonly found in the membrane of most bacteria, such as halophiles, nonhalophiles, alkalophiles, and nonalkopheles (Krulwich, 1983).

It was realized that most of halotolerant micro-Organisms never do acheive osmotic balance with their external environment. (Kushner, 1978). Experiments in halophilic bacteria have been shown that, the intracellular Na^+ concentration was lower than the outside.

As one of the inner membrane protein, Na^+/H^+ antiporter helps the influx of proton and the efflux of Na^+ in the exchange reaction, in which the osmoregulation of the bacterial cell depends upon. It is likely that bacterium will extrude sodium ion until it reaches an optimal state which survives them from the toxicity of Na^+. The biological roles altributed to the Na^+/H^+ antiporter, are numerous and important. These roles include the exclusion of cytotoxic Na^+ and regulation of cell volume at high osmolarity, and also pH homeostasis (Krulwich, 1983).

7. Selection of Salt Tolerant Rhizobium

It is generally believed that Rhizobium is one type of bacteria which is readily being a revertable strain. Infact, strain diversity could be easily found eventhough within one root-nodule. It is, therefore, possible to select strains which are tolerate in salt from those existing spontaneous mutants. In addition, using of mutagens such as NTG (N-methyl-N´-nitro-N-nitrosoquanidine) might cause unpredictable side effects on nodulation or on the efficiency of BNF. We therefore applied a principle of sibling selection as the method used to isolate-tolerant strain of R. phaseoli. The sibling principle for spontaneous mutation first explained by Cohen-Bazire and Jolit (1953). It was hoped that if strains of salt tolerance could be successively obtained, it might be a useful strain for certain purposes. For example, it could be used as the recipient strain for performing a protoplast fusion or else it may be proved as a useful strain to promote an effectiveness of BNF in plant cultivation in saline soil of Thailand.

8. The Aim of This Research.

Objectives of the study were as follows:

i) To select salt-tolerant strains of R. phaseoli by an application of sibling selection.

ii) To study some roles of osmoregulation in the salt-tolerant mutants isolated.