CHAPTER II

MATERIALS AND METHODS

A. MATERIALS

1. Biological materials

1.1 Legume seeds

Seeds of Phaseolus vulgaris were obtained from Rhizobium Section, Department of Agriculture, Ministry of Agriculture and co-operatives.

1.2 Bacteria

Rhizobium phaseoli TAL 113 was obtained from Rhizobium Section, Department of Agriculture, Ministry of Agriculture and co-operatives.

2. Chemical materials

Chemical and solvents used, were of analytical or reagent grades.

3. Radioactive materials.

$^{22}NaCl$ (carrier free) was purchased from Amersham International plc. U.K.

4. Bacterial media

4.1 Yeast mannitol medium (YM.) (Vincent, 1970)

Dipotassium hydrogen phosphate	0.5 g per 1.
Magnesium sulfate	0.2 g per 1.
D-mannitol	10.0 g per 1.
Yeast extract	0.4 g per 1.
Sodium chloride	0.1 g per 1.

The medium was adjusted to pH 6.8 with 1 N HCl, autoclaved at 120°C for 15 min. To make a solid medium, an addition of bacto agar, 15 g. per 1., was performed.

4.2 Yeast mannitol medium with 0.3 M. NaCl. (YM + 0.3 M. NaCl.)

It was prepared as in 3.1, with an addition of sodium chloride to a final concentration of 0.3 M.

4.3 Selection media for rhizobia. (Vincent, 1970)

4.3.1 Yeast mannitol medium with congo red.

The rhizobial indicator, congo red, was added to a final concentration of 0.0025% in yeast mannitol medium.

4.3.2 Glucose peptone medium

Glucose	5 g. per 1.
Bacto peptone	10 g. per 1.
Brom cresol purple (1.0% in ethanol)	10 ml per 1.

5. N-free solution for plant. (Jensen, 1942)

Solution I	Potassium dihydrogen phosphate	34 g. per 1.
Solution II	Magnesium sulfate	123 g. per 1.
Solution III	Potassium sulfate	65 g. per 1.
Solution IV	Ferric chloride	1.4 g. per 1.
	Ethylene diaminetetraacetic acid	1.7 g. per 1.
Solution V	Potassium chloride	0.75g. per 1.
	Boric acid	124 mg. per 1.
	Manganese sulfate	67 mg. per 1.
	Zince sulfate	46 mg. per 1.
	Cupric sulfate	10 mg. per 1.
	Molybdenum trioxide	2 mg. per 1.

1 ml of each solution (I-V) was diluted with 1 litre of distilled water, 0.1 g. calcium sulfate was added and adjusted to pH 6.5 with 1 N KOH. The medium was autoclaved at 120°C for 15 min.

6. Instruments.

Analytical balance model H 10 Tw. (Mettler Instrument, Switzerland).

Autoclave model HA-3D (Hirayama Manufacturing Corporation, Japan).

Beckman centrifuge model J-21C (Beckman Instruments Inc., California, U.S.A.).

Colony counter model 3326 (American Optical Corporation, New York, U.S.A.).

Electron microscope model JEM-200 CX. (JEOL Ltd. Japan).

Gamma Scintillation counter model BPGD. (Packard Instrument Company, U.S.A.).

Gas chromatography model 3700 (Varian, California, U.S.A.).

Hot air oven (Memmert, Western Germany).

Hydrogen generator model 15 EHG (General Electric Company, Willington, U.S.A.).

Laminar Flow (International Scientific Supply Co. Ltd, Thailand).

Oxygen monitor model 53 (Yellow Spring Instrument, Co. Ltd., Yellow Spring, Ohio, U.S.A.).

Oil rotary vacuum pump. model RP. S 100 H (Makashi Seisakusho Co. Ltd. Japan).

pH meter. model PHM 83 (Radiometer Ltd., Denmark).

Recorder model 613 (Instrumentation Specialities Company, Lincoln, Nebraska, U.S.A.).

Shaking water bath (Heto Lab Equipment, Denmark).

Spectronic 20 (Bausch and Lomb Inc., Ltd., U.S.A.).

Ultratome V. 2208 (LKB., Sweden).

UV-Visible Recording Spectrophotometer model UV.-240; Graphic printer, model PR-I. (Shimadzu Corporation, Japan).

B. METHODS

1. A selection of salt-tolerant mutants.

Ten isolated colonies of R. phaseoli clone grown on YM plate were used as the cell starters. Each isolate was then inoculated and grown in 5 ml of YM at 30°C with aeration. When a cell density of each culture reached approximately 0.6 unit of OD_{500}, a series of 0.5 ml portion from that inoculum were used to reinoculate into a 5 ml of YM supplemented with NaCl to a final concentration of 0.01, 0.05, 0.1, 0.2 and 0.3 M respectively. Performing like this, 50 samples were, therefore, available for the next step of selection. At this stage the incubation was prolonged for several days until a visualization of the cloudy cell suspension was noticable. Then an appropriate number of cells from each inoculum were used to spread on YM solid media supplemented with NaCl at the final concentrations as mentioned previously. Any outgrowth colonies, formed on YM plate of the highest concentration of NaCl were picked up and purified and used as the second starting colonies. The similar treatment was repeated thrice thereafter, no observable growth was obtained even after the incubation was extended for several days.

2. Growth of bacteria.

A single colony, being kept on YM congo red plate was used to prepare a starter inoculum. Whenever, growth of bacteria was required, 10^7 cells per ml from each starter inoculum was transferred into a 125 ml capacity of an Erlenmeyer flask containing 20 ml of cultivating medium. The culture was incubated on the rotary shaker with a speed of 100 rpm at 30°C and its cell density was observed in OD_{500} with a Spectronic 20 or in KU with a Klett-Summerson colorimeter. In certain cases, viable cell count was also performed by dilution plating method in accordance with the measurement of the cell density.

3. A maintenance and a preservation of bacterial strains.

3.1 For short-term storage.

Bacteria were grown on the surface of YM agar, and maintained at 4°C for at least 6 weeks without observable loss of viability.

3.2 For long-term storage.

In case of long-term storage, 0.5 ml of bacterial culture was mixed with 0.5 ml of autoclaved glycerol. The bacterial suspension in 50% glycerol was kept at -20°C for years.

4. A detection of the bacterial symbiotic activity.

4.1 Preparation of a Leonard jar (Leonard, 1944)

The bottom part of a Leonard jar was made of a wide mouthed-bottle and was served as a nutrient reservoir. The upper part was a bottom free, long necked-bottle which when placed upside down to the bottom part would made a Leonard jar assembly. After filling with steriled river sand, the upper part was used for plantation whereby a property of nodulation could easily be checked. For example, at each time interval of the inspectation of bacterial nodulation, young plant was pulled off and attached sand were eliminated by soaking into water. Steriled nutrient solution was supplied to plant twice a week.

4.2 A preparation of planting seed.

Seeds of a healthy look were subjected to surface sterization by soaking in 20% chlorox or 70% ethanol for 5 min. The seeds were then soaked in sterile distilled water for several times in order to remove the sterilizing agent. Seed germination was performed by an incubation of the steriled seeds on a moisted cotton wool in a steriled petri-dish for 2 days at room temperature.

4.3 Plantation and Inoculation. (Gibson, 1980)

The pre-germinated seeds of uniformed appearance were selected and sown in Leonard jar. 10^9 cells of rhizobial culture were promptly inoculated into each jar except the control. All samples were cultivated either in a glass house or in a growth cabinet.

4.4 A measurement of acetylene reduction activity. (A.R.A.) (Bergersen, 1980)

A plant sample of 4 weeks old, free of sand, was placed in 50 ml capacity of an Erlenmeyer flask. Each flask was sealed with a sleeved-stopper. Evacuation and devacuation was successively repeated several times and finally the atmosphere was replaced with argon. Acetylene and oxygen were subsequently substitued for argon to a final concentration of 10% and 20% respectively. Incubation was performed at $30^{\circ}C$ for an hour. The amount of the evolved C_2H_4 was detected by an injection of 100 µl of the gaseous phase into a Gas Chromatograph. An actual concentration of C_2H_4 was quantitated by comparing of the peak height with that of standard C_2H_4. A specific activity of the acetylene reduction was expressed in ηmol C_2H_4/hr/g. dry wt. of nodules. In addition, nodule number, height and wet weight of each plant were also recorded.

5. A study of bacterial morphology

Samples of WT and its mutants were sent to the Scientific and Technological Research Equipment Center, Chulalongkorn University for a visualization of bacterial morphology by scanning electron microscope (SEM) and transmission electron microscope. (TEM).

6. A detection of cell surface antigen by a fluorescent antibody technique. (Bohlool and Schmidt, 1970)

A fluorescent antibody technique, employed as a mean for a visualization of an antigen-antibody complex was stepwisely devided as follows:

6.1 An immunization of a rabbit.

6.1.1 A preparation of antigen for rabbit injection.

Cells of R. phaseoli (wild type) grown in YM were harvested and washed twice with 0.85% NaCl. Cells were resuspended to give a final concentration of 10^9 cells per ml. The cell suspension was heated for an hour in a boiling water bath to inactivate flagella and other protein antigen. Merthiolate, a chemical preservative was added to obtain a final concentration of 0.01%. The inactivated cells were kept at $4^{\circ}C$ until used.

6.1.2 A preparation of antibody.

Young-adult female rabbits were used to immunize by the following timetable:

Day of an injection	ml. of antigen injected
1^{st}	1.0
2^{nd}	1.5
3^{rd}	2.0
$4\text{-}6^{th}$	rest
7^{th}	1.5
8^{th}	2.0
9^{th}	2.0
$10\text{-}15^{th}$	rest

A week after the final injection, rabbits were bled through the marginal ear yein for checking of an agglutination titer of antibody. Whenever a titer showed primary detection of at equal or higher than 1,280, a portion (30-40 ml) of blood was collected via a cardiac puncture. Serum were pooled and preserved by merthiolate and kept frozen at-20°C.

6.2 A fractionation of serum globulin.

To a known volume of serum (usually 15 ml), an aqual volume of cold 3.9 M ammonium sulfate was slowly added at 4°C. The cloudy serum mixture was kept cool at least for an hour and the serum globulin was separated by centrifugation at 10,000 rpm for 30 min. The pellet was partially purified by performing a two successive cycles of an addition of 3.9 M ammonium sulfate to an equal volume of the globulin dissolved in 13 ml of 0.85% NaCl. The final pellet was dissolved in a minimum volume of 0.85% NaCl and dialysed against 0.85% NaCl until the dialysate showed no detectable amount of sulfate ion. Dialysate of the serum globulin was kept at 4°C until used.

6.3 Preparation of fluorescent antibody (F.A.)

After an appropriate adjustment of the serum globulin, approximately 20 mg protein per ml, the fluorescein isothiocyanate (FITC) was added to obtain a final ratio between the fluorescein dye and the globulin protein of 0.01. A bicarbonate buffer of 0.1 M pH 9.0 was then added to obtain the final concentration of 10% v/v. The mixture was allowed to conjugate by a gentle stirring for an hour and standing overnight at 4°C. The conjugated fluorescent antibody was separated from the unconjugated and unreacted FITC by using gel filtration. The conjugated mixture (about 300 mg of total protein) was applied to a 2.2 x 35 cm. and eluted with 0.1 M phosphase buffer saline pH 7.2. The fractions of conjugated fluorescent antibody were

pooled and kept in freezer (-20°C) until used.

6.4 An initial testation of fluorescent antibody.

An optimum working dilution of the fluorescent antibody was accomplished by making a serie of 2-fold dilution. The diluted antibody was tested for the antigen-antibody reaction with the smear of WT as further described in Methods section 6.5. The smear of R. japonicum was used to approve the specificity of the fluorescant antibody by a double staining with ethidium bromide. After the cell of R. japonicum was stained with the fluorescent antibody, the washed cells were re-stained with the solution of ethidium bromide and viewing under the fluorescent microscope.

6.5 A detection of the antigen-antibody reaction.

The cell surface antigen of all mutants was compared with their WT as follows:

A drop of bacterial culture was smeared on the glass slide and flooded with a drop of an appropriate dilution of F.A. Staining was achieved by an incubation of the sample in a moist-chamber for 20 min. The excess FA. was rinsed off by a saline phosphate buffer pH 7.2 (2.9 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCL, 0.85% NaCl and 0.02% NaN_3). Each slide was subsequently dipped into distilled water. The air-dried slides were mounted with mounting fluid and the antigen-antibody complex was observed under a fluorescent microscope.

7. A measurement of GC content.

7.1 An isolation of the chromosomal DNA. (Rodriguez and Tait, 1983).

A 80 ml of bacterial culture at stationary phase was harvested by a centrifugation at 10,000 rpm for 15 min and washed once with 10 ml of SET buffer (20% sucrose, 50 mM Tris-HCl, pH 7.6, 50 mM EDTA).

The cell pellet was subsequently freezed in a dry ice-ethanol bath for 2 min. and immediately thawed in warm water. After the step of freezing and thawing , cells were resuspended in 2 ml of SET buffer and kept on ice. The cell suspension was lysed by an addition of 0.2 ml of Lysozyme solution (5 mg/ml in TEN buffer, 10 mM Tris HCl pH 7.6, 1 mM EDTA, 10 mM NaCl) and of 0.1 ml of RNase buffer (10 mg/ml in 0.1 M sodium acetate pH 4.8, 0.3 mM EDTA, preheated at 80°C for 10 min.). The cell mixture was incubated in ice for 15 min. After the incubation, the cell were transfered into a centrifuge tube and 0.5 ml portion of 25% SDS was added, gentle shaking was employed at 37°C for 3-6 hrs. To the mixture, 0.3 ml of Pronase (2 mg/ml in TEN buffer) and 1.5 ml of chloroform/ n-amyl alcohol (24 :1) were added. The incubation was continued overnight with a gentle shaking at 37°C. A chloroform extraction was performed in the following day by an addition of 1 ml steriled water and 2 volumes (10 ml) of chloroform/ n-amyl alcohol. After 5 min of a gentle mixing, the phases were separated by a centrifugation at 9,000 rpm for 5 min. A wide mouth pipette was used to remove the upper phase into a clean tube. The chloroform extraction was repeated twice. Precipitation of the chromosomal DNA was achieved by an addition of 0.2 ml of 5 M NaCl and of 2 volumes of ice-cold 95% ethanol. The solution was gently mixed and stand in ice bath for 5 min. The DNA thread with a fibrous look was removed by a glass rod. The remaining of low molecular weight contaminants was eliminated by dipping the DNA to fresh 95% alcohol. The purified DNA precipitation was dissolved in TEN buffer and stored at 4°C until used.

7.2 A measurement of GC content from a DNA melting curve. (Adams, et. al., 1971)

A DNA solution at a concentration of 1 µg/ml (OD_{260} ~ 1.0) was heated under FE-2 thermo-bath (HAAKE, Germany). The hyperchomicity was relatively recorded with the resputive temperature raised from 25-100°C. A percentage of the GC content of the chromosomal DNA was calculated from the scanned melting curve using the equation as follow:

$$\%GC = (Tm - 69.3) \times 2.44 \qquad (Tm = \text{Melting temperature})$$

8. A measurement of an O_2 consumption. (Thipayathasana, 1975)

The cell culture at a mid-log phase cultivated either in YM or YM + 0.3 M NaCl, was used for the measurement of the O_2 consumption. The oxygen uptake of cell culture was monitored with an oxygen electrode (Model 53, Yellow Spring Instrument. Co.) at 30°C in a 5 ml vessel. An O_2 consumption was expressed as µmole O_2 per mg protein per min.

9. The measurement of Ca^{2+}, Mg^{2+} ATPase activity.

The lysozyme-millipore assay for the activity of ATPase described previously (Thipayathasana, 1975) was modified as follows: Samples of WT and mutants cells (0-250 µg total protein) were incubated in 10 ml of lysozyme solution (20 µg/ml lysozyme in 0.1 M Tris-HCl pH 6.8, 20% sucrose, 1 mM EDTA) at 37°C for 15 min. Lysozyme-treated cells were instantly transfered into a bank of millipore filters (0.45 µm pore size). Cells were collected and washed with 10 ml of 0.01 M Tris buffer pH 8.0 and the filters were transfered to assay tubes containing ATPase assay mixture (7.5 µmoles ATP, 2.5 µmoles $MgCl_2$ in 60 µmoles Tris buffer pH 9.0). The filter pads containing ATPase were immersed in the assay tubes using glass rod and incubated at 37°C for 30 min. the releasing of inorganic phosphate was measured as previously

described in Methods section 11.2. Specific activity was expressed as μ moles of inorganic phosphate released per mg protein per min.

10. A measurement of sodium efflux.

10.1 Preparation of Starved cells (Zilberstein, et. al.,1980)

Cells of a mid-log phase were harvested by a centrifugation at 10,000 rpm for 15 min. Cells were resuspended in 0.1 M phosphate buffer pH 7.2 containing 0.01% $MgSO_4$ to achieve a final concentration of 2 mg cell protein per ml. Starving of cells was performed by shaking this cell mixture at $30^{\circ}C$ for at least 12 hrs.

10.2 A loading of $^{22}NaCl$ and an assay of sodium efflux.

(Schuldiner and Fishkes, 1978).

To an appropriate portion of the starved cells, $^{22}NaCl$ was added to a final concentration of 10 mM which having the specific activity of 2.5×10^5 cpm/μmol. The cell mixture was incubated at $4^{\circ}C$ for 2 hrs. Before the assay of sodium efflux was performed, the cell mixture was standed at room temperature for a few min. Sodium succinate was immediately added to a final concentration of 10 mM. An aliquot of 0.5 ml of the cell sample was rapidly transferred into a pad of millipore filter (0.45 μm per size) and the reaction was terminated by an addition of 2 ml of 0.1 M LiCl. The cells were rapidly filtered and washed once with 2 ml of 0.1 M LiCl. The filter-pad was removed and used for ^{22}Na counting by a γ-counter (model BPGD, Packard Instrument, U.S.A.). The efflux of sodium was expressed in ηmoles/min/mg protein.

11. General methods.

11.1 Protein determination. (Lowry, et. al., 1951)

After washing cells with 0.85% NaCl, protein was extracted by mixing an equal volume of cell suspension with 1 N NaOH and hydrolysed in boiling water bath for 5 minutes. After cooling, 1.0 ml of sample was mixed with 2.5 ml of carbonate-copper reagent (1 ml of 0.5% $CuSO_4$, 1 ml of 1% sodium potassium tartrate in 50 ml of 5% sodium carbonate) and let stand for 10 min at room temperature. Subsequently 0.5 ml of phenol reagent (dilute 1 : 1) was instantly added, mixed and held at least for 30 min at room temperature. The absorbance was measured at 650 nm. Calibration curve was prepared by using bovine serum albumin as standard.

11.2 Determination of inorganic phosphate. (Shapiro, B.M. and Stadman, E.R., 1970)

Inorganic phosphate was determined as followed 1 ml of sample was mixed with 3.5 ml of Iron reagent (4 g $FeSO_4$, 1.25 ml of 7.5 N H_2SO_4 in 500 ml of distilled water) and 0.3 ml of molybdate reagent (6.6% of ammonium molybdate in 7.5 N H_2SO_4). After mixing, promptly read an absorbance at 660 nm. KH_2PO_4 was used for making the calibration curve.