CHAPTER II

MATERIALS AND METHODS
1. **Organisms**

*Rhizobium japonicum* D211 was obtained from Dr. H. Mareckova, Research Institute of Crop Production; Ruzyné, Prague; Czechoslovakia. Various mutants were isolated of this particular strain and those that were used in this study have been listed in Table 1. *Rhizobium japonicum* 61A76 was kindly supplied by Dr. W.J. Brill, Department of Bacteriology, University of Wisconsin, Madison, Wisconsin, U.S.A.

Phage M-1 that forms plaques on *R. japonicum* D211 was isolated earlier in our lab (299).

2. **Maintenance of the Cultures**

The stock rhizobial cultures were maintained on yeast mannitol (YM) medium solidified with 1.5% Davis agar. The culture was stored as slants after sealing with wax for long periods (3 to 6 months) at 0-4°C.

Lysates of phage M-1 prepared in normal saline 0.15 M were preserved over chloroform for 3 to 6 months at 0-4°C in sealed glass vials.

3. **Media**

a. Yeast mannitol medium (YM) described by Vincent (300) contained K₂HPO₄ 0.5g;
Table 1: List of *R. japonicum* D211 mutants used in this study

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Mutant strains</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>azi 1 to 9</td>
<td>azi</td>
</tr>
<tr>
<td>2</td>
<td>ara azi 1 to 9</td>
<td>ara azi</td>
</tr>
<tr>
<td>3</td>
<td>str azi 1 to 4</td>
<td>str azi</td>
</tr>
<tr>
<td>4</td>
<td>ilv</td>
<td>ilv</td>
</tr>
<tr>
<td>5</td>
<td>ilv azi 1 to 4</td>
<td>ilv azi</td>
</tr>
<tr>
<td>6</td>
<td>ilv rif 1 to 4</td>
<td>ilv rif</td>
</tr>
<tr>
<td>7</td>
<td>str rif 1 to 4</td>
<td>str rif</td>
</tr>
<tr>
<td>8</td>
<td>rif amp 1 to 4</td>
<td>rif amp</td>
</tr>
</tbody>
</table>

azi - resistance to sodium azide; 
ara - arabinose utilization defective; 
str - streptomycin resistance; 
ilv - auxotroph for isoleucine and valine; 
rif - rifampicin resistance; 
amp - ampicillin resistance.

MgSO₄·7H₂O, 0.2g; NaCl, 0.1g; Mannitol, 10.0g; 
Yeast extract (Difco), 1.0g dissolved in one litre distilled water. The pH of the medium being adjusted to 7.3.

b. Complex medium (CM) was identical to that described by Balassa (162) and contained the following in one litre distilled water, 
K₂HPO₄, 3.4g; NaCl, 0.5g; MgSO₄·7H₂O, 0.05g;
KH$_2$PO$_4$, 0.4g; Na$_3$ citrate.2H$_2$O, 0.5g; Yeast extract (Difco), 1.0g; Caseamino acids vitamin free (Difco), 1.0g; D-glucose, 1.0g; (NH$_4$)$_2$SO$_4$, 1.0g; pH adjusted to 7.4. Glucose and ammonium sulphate were added aseptically after sterilization of the media.

c. Minimal medium (MM), contained per litre distilled water, K$_2$HPO$_4$, 7.0g; NaCl, 0.5 g; MgSO$_4$.7H$_2$O, 0.05g; KH$_2$PO$_4$, 2.0g; D-glucose, 1.0g; (NH$_4$)$_2$SO$_4$, 1.0g; pH was adjusted to 7.4. Glucose and ammonium sulphate were autoclaved separately and then mixed with the medium before use.

d. Competence medium (CMM) was similar to CM and contained, K$_2$HPO$_4$, 3.4 g; NaCl, 0.5g; MgSO$_4$.7H$_2$O, 0.05; KH$_2$PO$_4$, 0.4g; Na$_3$ citrate 2H$_2$O, 0.5g; Yeast extract (Difco), 1.0g; caseamino acids vitamin free (Difco), 2.0g; D-glucose, 1.0g; (NH$_4$)$_2$SO$_4$, 1.0g. This was dissolved in 1 litre of distilled water and then the pH adjusted to 7.4. The glucose and ammonium sulphate were autoclaved separately and were mixed aseptically with the medium before use.
4. **Conditions of growth**

Liquid cultures were grown in YM broth in 100 ml conical flasks with side arm on a shaker (150 rpm) at 30°C. Growth was followed turbidometrically by measuring the absorbance at 550 nm in a Bausch and Lomb Spectronic-20 colorimeter. A culture in logarithmic phase of growth (1 x 10^8 cells/ml) showed an absorbance of 0.2.

5. **Procedure for use of N-methyl-N-nitro-N-nitroso-guanidine (NTG)**

Stock solutions were prepared directly before use by dissolving NTG in the appropriate buffer (usually 0.05 M phosphate buffer, pH 5.0) to give a solution of 1 mg/ml. Cells were grown in YM medium to the exponential log phase, centrifuged and washed twice in saline (0.15 M) and resuspended (1 x 10^8 cells/ml) in 0.05 M phosphate buffer (pH 5.0). NTG was added to a final concentration of 100 µg/ml to the cells suspended in phosphate buffer. When NTG conc. of 1 mg/ml was used then the cells after centrifugation were directly resuspended in 0.05 M phosphate buffer (pH 5.0) containing 1 mg/ml NTG. Incubation in both cases was carried out at 30°C for 30 min.

After 30 min. the cells were centrifuged (5000 x g for 5 min.) washed twice in saline (0.15 M). The cells
were then transferred to broth (YM) and grown for 6 to 18 hrs depending upon the marker to be selected for, and then plated out.

6. **NTG Survival curves**

Survival curves were done with constant time of exposure and varying doses of NTG, or with a constant concentration of NTG and different times of exposure. In both cases the procedure for use of NTG was identical to that already explained. Only after centrifugation for the elimination of NTG (see above) the cells were directly plated out after serial dilution to determine the viable count at each point. In each case a control was used where no NTG was added. The viable count from the titer plates for each time interval/concentration were determined.

7. **Characterization of Biochemical Mutants**

The method used here was the replica plating technique introduced by Lederberg and Lederberg (301). The initial screening involved picking suspected auxotrophs from YM plates after NTG mutagenesis by comparing them with their replicas on MM medium. Master grid plates were prepared on YM medium with sterile wooden toothpicks. 50 colonies were accommodated per plate as described by Miller (302, p.59). The auxotrophs were then identified
by replica plating these grid plates on the various biochemical pools as described by Holiday (303).

8. Lysis of *Rhizobium japonicum* for studies on enrichment of mutants

Lysis of *R. japonicum* D211 was studied by measuring the decrease in turbidity at 550 nm. For this the culture was grown in YM medium to give an absorbance of 0.2 at 550 nm (1 x 10^8 cells/ml) by incubating on a shaker (150 rpm) at 30°C. The cells were then distributed in 10 ml lots into 100 ml conical flasks with side arms. Then various antibiotics were added and the culture incubated on the shaker at 30°C. Absorbance was measured at regular intervals of time.

9. NTG mutagenesis for the study of closely linked double mutations

A culture of *R. japonicum* in exponential phase of growth in YM medium was centrifuged (5000 x g for 5 min) and cells resuspended (1 x 10^8 cells/ml) in 0.05 M phosphate buffer (pH 5.0) containing 100 µg/ml NTG. This suspension was incubated for 30 min at 30°C, after which the cells were chilled and washed free of NTG with ice cold saline (0.15 M) by repeated centrifugation (5000 x g for 5 min) in cold. The cells were then resuspended in pre-warmed YM medium (100 ml). 20 ml were withdrawn and kept aside. Antibiotic was added to the
rest (80 ml) after allowing to segregate (6 hr). Then both cultures were grown over night (18 hr) and plated out, one for single (unselected) and the other for double (selected) mutants respectively.

10. Sequential Mutagenesis using NTG

Logarithmically growing cells of *R. japonicum* D211 were synchronized as described by Dogra and Vyas (304) by resuspending these cells (1 x 10^8 cells/ml) into fresh YM medium with 0.25% phenethyl alcohol (PEA) incubating on a shaker (150 rpm) for 4 hrs at 30°C. The cells were then washed free of PEA by centrifugation and resuspended in fresh YM medium, incubated on the shaker at 30°C. 5 ml portions of this synchronously growing culture were then removed every 10 min, centrifuged, resuspended in 0.05 M phosphate buffer (pH 5.0) containing 100 μg/ml NTG and incubated for 30 min. The cells were then washed free of NTG as described above, resuspended in fresh YM medium (5 ml) and allowed to segregate for 6 hrs on the shaker at 30°C. Each sample was scored for mutants resistant to various antibiotics by plating out on YM plates containing the individual antibiotics.

11. Preparation of Phage M-1 lysates

High titre phage lysates were prepared as described by Adams (46, p.456). YM agar plates were overlayered with mixture of broth culture of *R. japonicum* D211 and
enough phage to give barely confluent lysis as a result of the overlapping of plaques. For this usually $10^5$ phages were applied to each plate. The soft agar was then scraped into a test tube, crushed thoroughly with a glass rod. Then saline (0.15 M) and chloroform were added, mixed and centrifuged (10,000 x g for 20 min) to remove cell debris and agar. The supernatant (lysate) thus obtained was preserved over chloroform at 0-4°C. Lysates prepared in this manner were stable at 0-4°C till 2 years.

12. Assay of Phage by agar layer method

Titre of phage lysate was determined by agar layer method of Gratia (305).

The phage lysate was diluted in saline (0.15 M) using standard dilution blanks of 4.5 and 5 ml. for 1:10 and 1:100 dilutions respectively. Tubes containing 3 ml of soft agar (0.75% Difco Agar, 0.85% NaCl in distilled water) were kept at 45°C in a water bath. To each tube 0.1 ml of a log phase culture of *R. japonicum* D211 (1 x $10^8$ cells/ml) and 0.1 ml of the respective diluted lysate were added, mixed and overlayered over previously prepared YM agar plates (YM medium solidified with 1.5% Davis agar). The plates were gently rocked to evenly spread the melted soft agar. The plates were incubated
at 30°C until the development of plaques. The titre was calculated by multiplying the number of plaques by the dilution factor and expressed as pfu/ml.

13. Phage adsorption

For studying adsorption and the effect of various effectors on the adsorption of phage M-1, _R. japonicum_ D 211 cells (1-5 x 10⁴ cells/ml) were suspended in distilled water with various effectors in a 2 ml system. Phage M-1, diluted in 0.15 M saline was then added to give an MOI of 1. After incubating for 10 min at 30°C, the system was centrifuged at 5000 x g for 10 min. The unadsorbed phages in the supernatant fluid were titrated by plating out in triplicate 0.02, 0.05 and 0.1 ml with the indicator bacteria (_R. japonicum_ D211).

14. Phage inactivation test with various sugars and EPS

The phage M-1 (1-5 x 10⁴ pfu/ml) was incubated with different concentrations of each sugar/EPS in distilled water. The final volume was 2 ml. After incubation for 30 min at 30°C the surviving phages were titrated by plating out in triplicate 0.02, 0.05 and 0.1 ml with the indicator bacteria (_R. japonicum_ D 211).

15. Extraction of exopolysaccharide (EPS)

The EPS was extracted from the culture free supernatant fluid of 8 to 10 day old cultures of _R. japonicum_ D 211 grown
In YM medium. The culture broth was separated from the bacterial cells by diluting with 3 to 5 volumes of saline (0.15 M) and centrifuging down the cells at 15000 x g for 30 min. The supernatant was passed through a membrane filter (0.45 μ) to eliminate any remaining bacteria. The resulting filtrate was extracted with phenol as described by Westphal and Jann (306). The crude EPS thus obtained in 0.02 M phosphate buffer (pH 7.2) was treated with DNase I and RNase A (50 μg/ml) at 37°C for 1 hr. This was followed by deproteinization with an equal volume of chloroform:isoamylalcohol (24:1). After centrifugation at 15,000 x g for 40 min, the aqueous layer was removed and precipitated with 3 volumes of ethanol. The precipitate was lyophilised and dissolved in either distilled water or 0.02 M phosphate buffer (pH 7.0) and used for receptor studies.

16. Chromatography of EPS

The EPS preparation was hydrolyzed in 3N HCl for 12 hr, at 100°C under a nitrogen atmosphere. The hydrolysates after evaporating to dryness were chromatographed on Whatman No.1 paper using the solvent system butanol-1/pyridine/water (9:5:4 V/V) for 24 hr. Identification was carried out by comparing with standard sugars after staining with the alkaline silver nitrate (307).
Separation was also carried out on thin layer chromatography plates, Silica Gel-G impregnated with 0.5 M NaH₂PO₄ using the solvent system acetone/isopropanol/0.1 M lactic acid (40:40:20 V/v). The plates were developed by spraying with a DPA reagent (aniline/diphenylamine/aceton/80% H₃PO₄, 4 ml/4g/200 ml/15 ml) and heating at 100°C for 20 min (308).

17. Detection of Phage EPS-depolymerase

The culture of *R. japonicum* D 211 at logarithmic phase in CM media was inoculated with the phage M-1 at an MOI of 0.01 and incubated on a rotary shaker (150 rpm) at 30°C. When the turbidity cleared, the bacterial debris was removed by centrifugation. The protein was precipitated with 0.6 saturation of ammonium sulphate and was dissolved in 0.02 M phosphate buffer (pH 7.2) and dialysed overnight at 4°C against the same buffer. This dialysed extract was taken as the crude enzyme.

18. EPS depolymerase assay

The reaction mixture (final volume of 1 ml) contained: 25 μ moles phosphate buffer (pH 7.2); 2000 μg EPS (in 0.02 M phosphate buffer pH 7.2); 100 μ moles Ca²⁺ (as CaCl₂·2H₂O) and an appropriate aliquot of the enzyme solution. After incubating at 30°C for 10 min the reducing sugar was determined by the method of Park and Johnson (309).
using D-glucose as standard. One unit of enzyme activity is defined as the release of 1 nmol of reducing sugar (glucose equivalents) per min.

19. One step growth curve of Phage M-1

The method used was adapted from that of Ellis and Delbruck (310). An overnight (18 hr) grown culture of *Rhizobium japonicum* D 211 was centrifuged and resuspended in fresh medium. After 4 to 6 hrs growth on the shaker (150 rpm) at 30°C, the culture was centrifuged and mixed (1 x 10^8 cells/ml) with phage M-1 to give an MOI of 5.0 in 0.05 M phosphate buffer pH 6.5 containing 1 x 10^-2 M KCN and 1 x 10^-3 M CaCl_2·2H_2O.

After 8-10 minutes of incubation at 30°C required for adsorption, the cells were collected by centrifuging at 5000 x g for 10 min, washed with saline (0.15 M), and diluted 10^4 fold the last dilution being made in YM medium.

The suspension of infected bacteria was sampled at regular intervals until lysis was completed and each sample was assayed for the phage count.

20. Intracellular growth of Phage M-1

The procedure used here was adapted from Doerman (311) and is identical to that described above for the one step
growth curve. The difference was that the intracellular phage was titrated by inducing lysis of the cells with chloroform. Therefore, the aliquots that were removed at regular intervals were added into chloroform first to lyse the cells and then were subsequently titrated for the phage count with the indicator bacteria.

21. Lysogeny in Phage M-1

Lysogeny was tested by picking the turbid centre of the plaque and making single colony isolates. These isolates were given at least three serial transfers before exposure to ultraviolet. Ultraviolet irradiation of the cells (10^7/ml) in 0.05 M phosphate buffer (pH 7.2) was carried out for 60 sec at a distance of 30 cm from a 15 watt Philips germicidal lamp. The cells were centrifuged (5000 x g for 10 min) and resuspended in YM medium. They were allowed to grow for 4 hr. at 30°C and then plated out with the indicator bacteria (strain D 211) to count the infective centers.

22. Isolation of mutants of R. japonicum D 211 lysogenic for Phage M-1

Mutagenesis was carried out with 100 µg/ml NTG in 0.5 M phosphate buffer (pH 5.0) for 30 min at 30°C. The mutagenized cells were chilled by the addition of equal volume of ice-cold saline (0.15 M), washed free of NTG with 0.15 M saline and centrifuged down (5000 x g for
10 min). The culture was then allowed to segregate for 8 hr in YM medium at 30°C. Sodium azide was then added (2 x 10^{-3} M) to this culture which was allowed to grow for 2 days at 30°C to enrich the azide resistant mutants. The culture was then plated out on YM plates containing 2 x 10^{-3} M azide and azide resistant mutants were isolated. To obtain phenethyl alcohol resistant (pea) mutants, the azide resistant (azi) mutants were streaked on YM plates containing 0.25% (w/v) PEA (phenethyl alcohol) and cross streaked with phage M-1 for host-controlled-lytic function deficient mutants (hcl). The str azi and ilv azi mutants were obtained when single str and ilv mutants of strain D211 were mutagenized as above with NTG and selected for azide resistance. The ara azi double mutants, however, appeared after one step treatment with NTG and were found among some of the azide resistant clones. This was due to the fact that ara and azi were closely linked.

23. Characterization of azi mutation

The rate of respiration was determined by the standard Warburg manometric technique.

For the measurement of K⁺ efflux the cells were grown in MM medium to cell density of 5 x 10^8 cells/ml. After centrifugation (5000 x g for 10 min) they were transferred
into an equal volume of 0.1% peptone. Samples (5 ml) were removed every 2 min up to 10 min and were filtered using membrane filters (0.45 μ) to separate the cells. K⁺ was measured from the filtrate by flame photometry.

24. Extraction of Transforming DNA

An 18 hr culture of *R. japonicum* D 211 R (streptomycin resistant) in CM was inoculated into fresh CM medium to give a cell density of 1 x 10⁷ cells/ml, and incubated on a rotary shaker (150 rpm) for 8 hr at 30°C. The cells were collected by centrifugation at 5000 x g for 10 min at 4°C and after washing, suspended in a saline ethylenediaminetetra acetate (EDTA) solution, pH 8.4 (0.15 M NaCl + 0.1 M EDTA). The DNA was extracted by following the method of Marmur (312) with a slight modification. Lysis was effected by the addition of 0.2% sodium dodecyl sulfate and incubated at 45°C for 20 min and then cooled. Lysis of the culture resulted in a dramatic increase in viscosity accompanying the release of the nucleic acid components. Pronase (Calbiochem) at 20 μg/ml concentration was added to the lysate and incubated at 40°C for 2 hr. The lysate was then deproteinised twice with an equal volume of chloroform-isoamylalcohol (24:1 v/v) at 40°C for 2 hr, by gentle occasional shaking and centrifuged (20,000 x g for 40 min). The aqueous layer containing
the nucleic acids was pipetted into twice its volume of ice cold ethanol. The fibrous material was taken in 0.1% SSC (0.15 M NaCl + 0.015 M trisodium citrate, pH 7) and treated with 100 µg/ml of RNase for 30 min at 37°C, deproteinised with chloroform-isoamylalcohol and centrifuged (20,000 x g for 40 min). The upper layer was directly loaded on a Sephadex G-50 column 1 x 20 cm, previously equilibrated with 1 x SSC. Fractions of 3 ml. were collected and DNA was estimated by diphenylamine reaction of Burton (313). UV absorption ratio at 260 and 280 nm was determined to check the purity of all the preparations, which usually ranged between 1.8 - 2.0. This method was preferred to obtain RNA and protein free DNA having homogeneous high molecular weight.

25. Determination of competence and transformation

Competence in R. japonicum D 211 was determined as described by Raina and Modi (178).

An overnight culture (18 hr) grown in CM was centrifuged and washed with 0.05 M phosphate buffer (pH 7.0). The cells were resuspended in fresh CMM (competence medium) medium to a density of 1 x 10⁶ cells/ml and incubated on a rotary shaker (150 rpm) at 30°C. Every 30 min the culture was removed for studying competence and DNA (10 µg/ml) was added to make the final volume to 1 ml. Incubation was carried out at 30°C for 30 min and
the reaction terminated by adding DNase (50 μg/ml) containing 5 mM MgCl₂. The culture was then plated out on YM agar plates and incubated at 30°C for 4 hr and then overlaid with 2 ml of soft agar containing 5 mg of streptomycin sulphate to score for str transformants. The plates were incubated for 72 hrs and the colonies counted. The controls included (1) the culture plated without DNA treatment (2) plating of donor DNA (3) addition of DNase before adding DNA.

26. Isolation of competence factor (CF)

For the isolation of CF, an 18 hr culture of Rhizobium japonicum D 211 was taken, washed by centrifugation (10,000 x g for 10 min) with saline (0.15 M) and transferred (1 x 10⁶ cells/ml) into competence medium (CMM). After growth on a rotary shaker (150 rpm) at 30°C for 4 hr the cells were separated by centrifugation (10,000 x g for 15 min). The culture free supernatant was collected and cooled (0-4°C) and the protein was precipitated by the addition of solid sulfate to 80% saturation. The precipitates were collected by centrifugation (20,000 x g for 30 min), dissolved in 0.05 M phosphate buffer (pH 7.2) and dialysed overnight against the same buffer. This preparation was designated as crude CF.
27. Assay of biological activity of CF

The biological activity of CF was assayed by testing its ability to render non-competent cells competent. Logarithmically grown cells of R. japonicum D 211 washed by repeated centrifugation with saline (0.15 M) were taken as the non-competent cells. These cells were incapable of genetic transformation and were used in the assay as non-competent cells. The transformation system contained 20 μg donor DNA, 2 x 10^6 non-competent cells, an appropriate concentration of CF and the total volume was made to 2 ml, with fresh competence medium. This was incubated on a rotary shaker (150 rpm) for 30 min at 30°C, then 0.2 ml of DNase (1 mg/ml in 0.05 M phosphate buffer pH 7.0 with 5 mM Mg^{2+}) was added and let incubate for 10 min. The contents were plated out on YM agar plates and allowed to segregate by incubating the plates for 4 hr at 30°C. The plates were then overlaid with 2 ml soft agar containing 2.5 mg/ml streptomycin sulfate. The transformants were scored after 72 hr of further incubation at 30°C.

28. Sephadex G-100 chromatography of CF

The crude CF was concentrated 10 fold with polyethylene glycol (20 M) as described by Kohn (314), and about 35 mg. of this material was applied to a column of Sephadex G-100. The column was previously equilibrated with 0.05 M
phosphate buffer (pH 7.2), and elution was carried out with the same buffer. 5 ml fractions were collected. All fractions were tested for CF activity by adding 0.5 ml into the transformation system and scored for the number of transformants. The active fractions were pooled and used for the studying the properties of the CF. Protein was measured by the method of Lowry et al (315).

29. Molecular weight determination of CF

Molecular weight determination was carried out by comparing the elution volume of the CF with that of other reference proteins by gel filtration on Sephadex G-100. The reference proteins used were lipoxidase (Soyabean) 102,000; bovine serum albumin (monomer) (BSA) 68,000; lysozyme (egg white) 14,000 and RNase A (bovine pancreas) 12,700. 10 mg of each protein was loaded individually and the profile of each was determined separately by measuring the protein in each of the fractions by the method of Lowry et al (315). Elution was carried out using 0.05 M phosphate buffer (pH 7.2).

30. Precipitation of CF from crude extract by changing pH

The crude extract of CF was adjusted to pH 9.0 with 1 M NaOH and the centrifuged (20,000 x g for 30 min) and
precipitates were preserved. The supernatant was then adjusted to 7.0, 6.0, 5.0, 4.5, 4.0, 3.0 and 2.0 pH in a stepwise manner with the addition of 1 N HCl and centrifuged (20,000 x g for 30 min). The precipitates at each step were collected and dissolved in 0.05 M phosphate buffer (pH 7.2). 0.5 ml of each fraction was used in the transformation system described above for the detection of CF activity.