INTERACTION BETWEEN THE NITROGEN FIXING BACTERIUM RHIZOBIUM JAPONICUM AND SOYBEAN (GLYCINE MAX L.) INVOLVING SPECIFICITY AND RECOGNITION

SIVRAMIAH SHANTHARAM

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INTERACTION BETWEEN THE NITROGEN FIXING BACTERIUM *RHIZOBIUM JAPONICUM* AND SOYBEAN (*Glycine max* L.) INVOLVING SPECIFICITY AND RECOGNITION

by

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A thesis submitted in partial fulfillment
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Doctor of Philosophy

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TO MY BELOVED PARENTS

ABSTRACT

The initial cell surface interaction between legumes and rhizobia during infection, is believed to be mediated by legume seed lectins. The specificity and host-recognition are the two important prerequisites for the successful infection of legumes by rhizobia. The present study deals with the involvement of soybean lectin in the interaction between soybean and Rhizobium japonicum.

Using biochemical and cytological techniques, soybean lectin binding properties of a non-mucoid strain of R. japonicum under two different laboratory culture conditions were studied. The primary lectin binding sites were localized in the capsular extracellular polysaccharide (EPS) of the bacteria using ferritin and colloidal gold labeled soybean lectin at the ultrastructural level. It was also possible to show the secondary soybean lectin binding sites to be the outer cell wall of the bacterial cells. Further, the lectin specific cells were characterized as coccoid forms embedded in a capsular matrix and rod forms having a polar capsule. In experimental studies both capsulated coccoid and rod forms were found to attach to the root hair surface with the rod forms showing distinct polar attachment.

Differential centrifugation was used to fractionate the rod and coccoid forms which were then further

characterized. Both the forms were clearly distinguishable at the light and electron microscope levels. The rods were often found aggregated by their polar ends to form 'stars'. The coccoid forms were found to be either capsulated or non-capsulated. The coccoid and rod cells when allowed to grow separately in fresh nutrient medium were shown to undergo interconversion. The lectin binding kinetics of both the types of cells were studied. The nodulation capacity of these two types against soybean were carried out.

Gas chromatographic analyses of EPS and LPS fractions of the bacteria grown in yeast extract mannitol medium did not reveal any detectable quantity of lectin specific receptors (galactose or N-acetylgalactosamine). However, when the bacteria were grown in soil extract medium, galactose, a soybean lectin specific sugar, was found to be one of the components in both EPS and LPS. This induction of galactose synthesis was accompanied by increases in cell size, percentage of coccoid forms and in the amount of lectin bound to the bacterial cells. These results indicate that more lectin binding receptors can be induced in rhizobia grown in soil extract medium.

The study leads to the following conclusions: (1) the lectin binding sites are located in the capsular extracellular polysaccharide (primary lectin binding site) and in the lipopolysaccharide of the outer cell wall envelope (secondary binding site) of the bacteria; (2) there are distinct rods and coccoid forms of bacteria in the broth

culture reflecting the different phases of growth; and (3) that the lectin receptor sites are induced in Rhizobium when culture conditions simulate the natural environment (soil) of the bacteria.

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INTRODUCTION

Nitrogen is an important element of cells, being incorporated into protein molecules which are responsible for cell structure and function. Atmosphere is composed of 78.1% of nitrogen on molar basis. Most of the organisms cannot take in free elemental nitrogen and therefore "fixation" of nitrogen, a process by which the nitrogen is converted into a combined form for uptake, is of fundamental importance to life. Such a process has been going on since the existence of life forms (Yung and McElroy, 1979). Reactions between nitrogen and oxygen in the air takes place due to lightning discharges and ultraviolet radiation. The oxides of nitrogen formed, are subsequently converted into nitrites and nitrates. It has been estimated that lightning and subsequent atmospheric chemistry can provide as much as 10^6 tons of nitrogen per year, sufficient to fill the primitive ocean to its present level of nitrate in less than 10^6 years (Yung and McElroy, 1979).

The nitrogen is cycled through plants, animals and microbes. The combined nitrogen is either cycled through animals or returned directly to the soil (Fig. 1) where it is 'mineralized' and further combined for uptake by the living forms. As the flow of nitrogen within the terrestrial and aquatic systems is governed by biological activities, the

Fig. 1. A simplified diagram of nitrogen cycle depicting the pathways through which the element is cycled between the atmosphere and the biosphere.

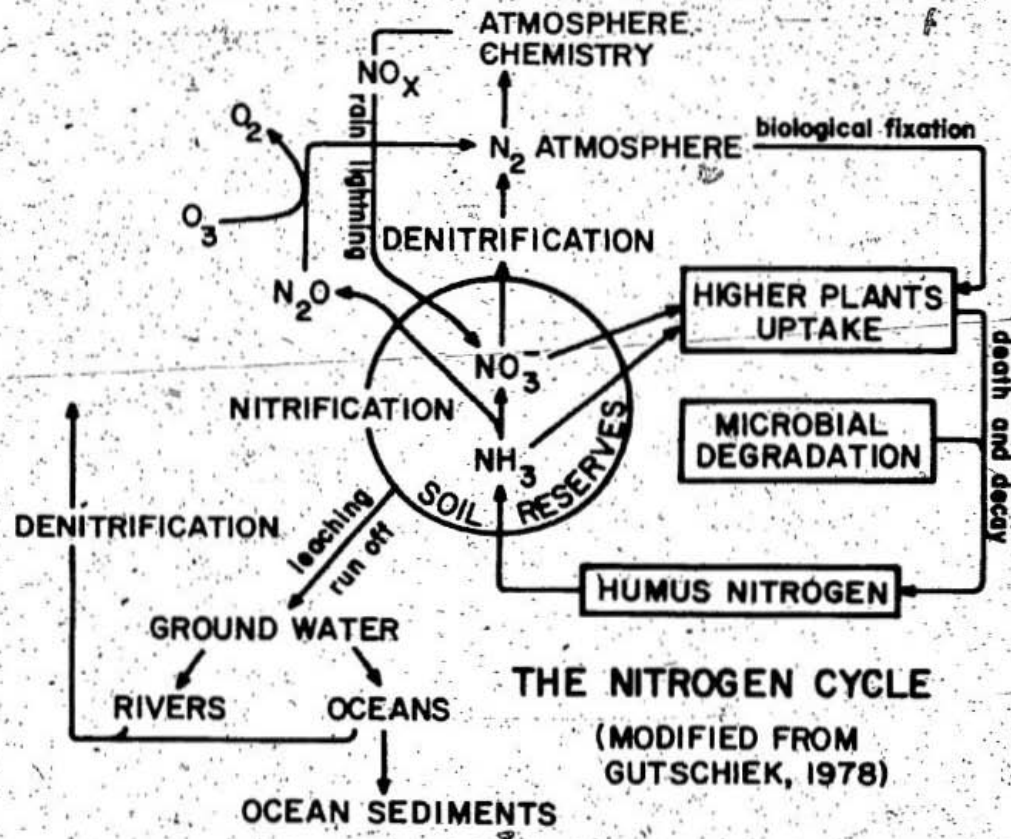

ATMOSPHERE CHEMISTRY
NO_x
rain
lightning
O_2
O_3
N_2 ATMOSPHERE
biological fixation
DENITRIFICATION
N_2O
HIGHER PLANTS UPTAKE
death and decay
NO_3^-
NITRIFICATION
MICROBIAL DEGRADATION
NH_3
SOIL RESERVES
DENITRIFICATION
leaching
run off
HUMUS NITROGEN
GROUND WATER
RIVERS
OCEANS
OCEAN SEDIMENTS
THE NITROGEN CYCLE
(MODIFIED FROM GUTSCHIEK, 1978)

Fig. 1

nitrogen cycle is closely linked to the cycles of geobiochemicals. Estimates show that 122 x 10^6 metric tons of N per year is fixed by biological activity when compared to industrial process which is 40 x 10^6 metric tons per year (Burris, 1980).

Industrial fixation of nitrogen is energy demanding and is becoming increasingly expensive as world's natural oil reserves are becoming scarce. About 35% input into world's agricultural productivity is made up of chemical nitrogenous fertilizer alone. The global consumption of chemical fertilizer for 1978 was 50,000,000 tons whereas annual biological nitrogen fixation of 150-175,000,000 tons has not changed (Delwiche, 1977). It takes 30 cubic feet of natural gas to produce one pound of nitrogen fertilizer. Because oil and gas are non-renewable natural resources, a concerted and intensive search for alternative fertilizer technology is warranted. The answer seems to lie in rhizobial technology. Research into farming systems, like making more extensive use of legumes, green manure and winter cover crops, forage legumes and possible inter-cropping or mixed cropping of legumes with non-legumes may provide a suitable alternative. Possible application of genetic engineering techniques for the improvement of both the host and the microorganism in legume-Rhizobium symbiosis, actinomycete nodulated angiosperms and blue-green algal technology for rice cultivation needs to be explored for beneficial exploitation.

Table 1: The Nitrogen fixing plants and their associative microorganisms.

1. Free-living organisms.
   (a) Heterotrophic bacteria, eg: Azotobacter, Clostridium, Spirillum, Beijerinckia, Klebsiella.
   (b) Autotrophic bacteria, eg: Rhodopseudomonas, Rhodospirillum, Thiobacillus.
   (c) Blue-green algae, eg.: Anabaena, Calothrix, Nostoc, Plectonema, Mastigocladus, Gloeotrichia.

2. Associative symbiosis:
   eg. Paspalum notatum - Azotobacter paspali
   Digitaria decumbens - Spirillum liploferum

3. Root nodule forming symbioses:
   (a) Rhizobium-legume associations, eg: Glycine max, Phaseolus vulgaris, Vicia faba, Trifolium repens, etc.
   (b) Rhizobium-non-legume associations, eg: Trema Cannabina
   (c) Actinomycete-non-leguminous angiosperm associations, eg: Alnus glutinosa, Myrica gale, Hippophae rhamnoides, Casaurina equisetifolia, etc.
   (d) Cycad-blue green algae associations, eg: Bowenia, Cycas, Encephalartos, etc.

What is of important concern is, to use biological nitrogen fixation as a means of providing nitrogenous fertilizer. Although biological nitrogen fixation is a highly energy dependent process, when coupled with photosynthesis it can limit the use of fossil fuels to other agricultural needs.

The ability to fix nitrogen is found only in procaryotes such as blue-green algae, actinomycetes, clostridium, Azotobacter and Rhizobium. These microorganisms can fix nitrogen either independently or in association with higher plants (see Table 1) (Stewart, 1977). Of these, nodulating members of the family Leguminosae (Fabaceae) have been recognized as of agronomic importance since the beginning of agriculture. The nodulated legumes fix atmospheric nitrogen symbiotically in their root nodules. The legume root nodules are small, rounded, peripheral growths on the sides of legume roots and are caused by the infection of a soil bacterium of the genus Rhizobium.

I. Mechanism of Symbiotic Nitrogen Fixation

Biological nitrogen fixation has two major requirements, a continuous supply of adenosine triphosphate (ATP) and reduced oxygen tension for nitrogenase activity. Oxidative phosphorylation of the photosynthetic carbohydrates, and leghaemoglobin in conjunction with the membrane envelope provides these conditions (Yocum, 1964; Bergersen, 1971; Appleby, 1974; Wittengerg et al., 1974). Poly-β-hydroxybutyrate which constitutes about 50% (Klucas and Evans, 1968) of the dry

Fig. 2. A schematic representation of the biochemical components of the root nodule, involved in the symbiotic nitrogen fixation.

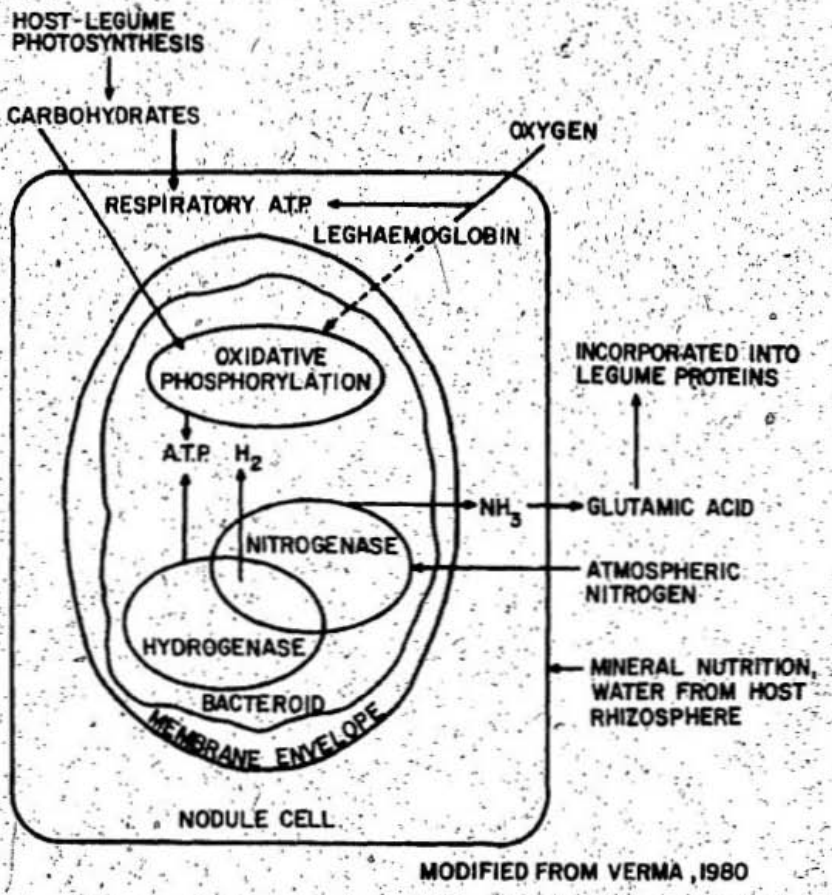

HOST-LEGUME PHOTOSYNTHESIS
CARBOHYDRATES
OXYGEN
RESPIRATORY ATP
LEGHAEMOGLOBIN
OXIDATIVE PHOSPHORYLATION
INCORPORATED INTO LEGUME PROTEINS
ATP
H_2
NH_3
GLUTAMIC ACID
NITROGENASE
ATMOSPHERIC NITROGEN
HYDROGENASE
MINERAL NUTRITION, WATER FROM HOST RHIZOSPHERE
BACTEROID
MEMBRANE ENVELOPE
NODULE CELL
MODIFIED FROM VERMA, 1980

Fig. 2

Fig. 3. A schematic representation of nodular symbiotic nitrogen assimilation. A and B represent aspartate aminotransferase and asparagine synthetase, respectively.

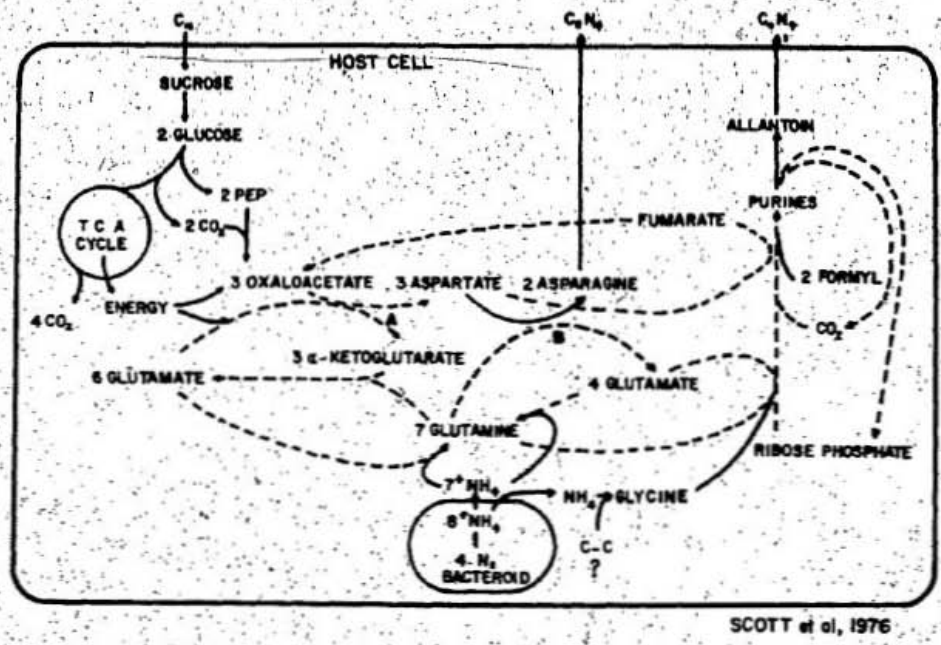

HOST CELL
SUCROSE
2 GLUCOSE
2 PEP
2 CO2
TCA CYCLE
4 CO2
ENERGY
3 OXALOACETATE
3 ASPARTATE
2 ASPARAGINE
FUMARATE
ALLANTOIN
PURINES
2 FORMYL
CO2
3 α-KETOGLUTARATE
6 GLUTAMATE
4 GLUTAMATE
7 GLUTAMINE
RIBOSE PHOSPHATE
7 NH4
NH4
GLYCINE
8 NH4
4 N2
BACTEROID
C-C
?
SCOTT et al, 1976

Fig. 3

weight of bacteroids, supports the process of nitrogen fixation under limited photosynthetic conditions (Bergersen, 1977). Bacteroids are morphologically and functionally distinct cells of Rhizobium in their nitrogen fixing state within the root nodule.

The overall mechanism of nitrogen fixation in the legume root nodule is shown in a simplified diagram (Fig. 2). The primary product of nitrogen fixation is ammonia and its further assimilation is carried out as shown in the diagram (Fig. 3) (Scott et al., 1976). The reduction of nitrogen to ammonia in Rhizobium bacteroids can be summarized as follows:

$$N_2 + n\text{ATP} + 6\ \text{NADPH} + 2H^+ \rightarrow 2NH_4^+ + n\text{ADP} + nP_i + 6\ \text{NADP}^+ + 6e^-$$

where $n = 6.0 - 6.9$ or $6.5\ \text{ATP}/NH_4^+$ (Rawsthorne et al., 1980).

High concentration of hydrogen is known to competitively inhibit the reduction of nitrogen (Bergersen, 1977). During nitrogen fixation 30% of the electron flux through the nitrogenase enzyme is expended in the reduction of protons to hydrogen (Evans et al., 1978).

Some strains of Rhizobium oxidize hydrogen with the help of hydrogenase and utilize the energy from this reaction (Maier et al., 1978). In such rhizobia, there is a high efficiency of nitrogen fixation (Schubert and Evans, 1976).

Fig. 4. A schematic flow chart of the events following the cell surface interaction between Rhizobium and legume leading to the internalization of the bacteria and establishment of a symbiotic root nodule. Items in dark letters denote the contribution to knowledge presented in this thesis.

ESTABLISHMENT OF HOMOLOGOUS RHIZOBIA IN THE HOST - LEGUME RHIZOSPHERE

↓

BETTER EXPRESSION OF MUTUAL INTERACTION PROPERTIES IN THE RHIZOSPHERE-INCREASED LECTIN BINDING (INDUCTION OF LECTIN BINDING SITES) IN RHIZOBIA; INCREASE IN THE PERCENTAGE OF COCCOID FORMS

↓

INCREASE IN INFECTIBILITY OF HOST-LEGUME ROOT HAIRS

↓

ATTACHMENT OF RHIZOBIA TO HOST ROOT HAIR BY EXTRACELLULAR POLYSACCHARIDE (CAPSULE)

↓

MUTUAL RECOGNITION BY LIPOPOLYSACCHARIDE ?

↓

INTAKE OF RHIZOBIA BY INVAGINATION OF ROOT HAIR AND FORMATION OF INFECTION THREAD

↓

RELEASE OF RHIZOBIA INTO THE NODULE CELL

↓

MODIFICATION OF RHIZOBIA INTO BACTEROIDS

↓

COMPARTMENTALIZATION OF BACTEROIDS BY MEMBRANE ENVELOPE

↓

FUNCTIONAL DIFFERENTIATION OF BACTEROIDS-INDUCTION OF NITROGEN FIXING ENZYMES

↓

SYMBIOTIC NITROGEN FIXATION

Fig. 4

II. The Symbiotic-Legume Root Nodule

The sequence of events that lead to the formation of a functional root nodule is presented in the table 4. The nodules are broadly classified into round or oval, elongate or club-shaped, branched to coralloid, and collar shaped. The shape is determined by the host's pattern of meristematic activity. In soybean, the initial meristematic activity is spread throughout the nodule and after 10 - 20 days the meristematic activity is shifted to localized pockets in the periphery. The effective nodule is filled mainly with the central bacteroid zone surrounded by leghemoglobin which is outside the membrane envelopes and which in turn is surrounded by cortex supplied with conducting elements of the vascular system (Verma and Bal, 1976). The meristematic activity is preceded by cell expansion in both the host cell and the rhizobia.

Bacteria within the nodule undergo changes in shape and size and become 'bacteroids'. While these morphological changes occur in the bacteria, their cytochrome system is altered and the outer cell wall of the bacteria undergoes a change (Van Brussel, 1973, 1977; Sutton and Paterson, 1979; Bal et al., 1980 manuscript accepted for publication). Reviews have been published (Dart, 1977; Newcomb, 1980) that describe the cytological changes during root nodule morphogenesis. Functional aspects have been described by Bergersen (1974, 1977), Boland et al. (1980) and Dilworth (1980).

III. The Legume and its Rhizosphere

The term 'legume' is used somewhat non-specifically to describe the seed pod itself and to describe the members of the family Leguminosae. Members of this family evolved probably in the late Jurassic and expanded and diversified during the Cretacious (Delwiche, 1978). The importance of legumes in agronomy was realized by Aryans and Greeks but it was only in the 19th century that Hellriegel and Wilfarth (1888) scientifically demonstrated the biological fixation of nitrogen by the association of legumes and microorganisms.

Generally the legumes, particularly in the seeds, are rich in protein content (soybean, peas, beans, peanuts, etc.) due to the symbiotic fixation of nitrogen in their root nodules. However, only a few cultivated legume species have been used as a source of 'protein' because of the presence of toxic substances in the large majority of species (Aykroyd and Doughty, 1964; Milner, 1975).

Hiltner (1904) was the first to coin the term 'rhizosphere'. It generally refers to the thin layer of soil adhering to the root surface (Katznelson, 1965). The thickness of the rhizosphere varies from species to species depending upon the nature of root exudates and the moisture content. In contrast, the rhizoplane is the area on the root surface. The rhizosphere harbours various microbes like protozoa, algae, fungi, nematodes and bacteria. Apart from the well established legume-Rhizobium and mycorrhizal associations, there is no evidence that a plant species

supports a specific microbial population (Baker and Snyder, 1965).

Some of the early studies on the ultrastructure of the rhizosphere have shown that root surface is coated with an amorphous mucilage in which the bacteria are embedded (Campbell and Rovira, 1973). The methods employed were not suitable for the identification of the bacteria (Foster and Marks, 1967; Foster and Rovira, 1973; Old and Nicholson, 1975). Recently Foster and Rovira (1978) have described the ultrastructure of the rhizosphere of Trifolium subterraneum L. The mucilage matrix (mucigel) measures about 20 µm. There is a clear zone demarcating the capsule of the bacteria. They have also identified more than eight types of bacteria based on their ultrastructural cytology. There is a concentration gradient of microbes in the rhizosphere. Numbers are greater nearest the root and gradually decrease farther away from the root surface (Rovira and Davey, 1965a). Roots generally stimulate fast-growing gram-negative bacteria by providing the growth substances like glucose, amino acids and other organic root exudates (Rovira, 1965b). It is well known that colonization of a particular rhizosphere is dominated by certain types of bacteria. It is difficult to envisage a nutrient in the root exudate which will not be utilized by the general soil microflora and yet will stimulate Rhizobium (Rovira, 1961). Some of the components of root exudates are vitamins, amino acids, amides, sugars and nucleotides (Fahraeus and Ljunggren, 1968). It

is believed that some simple amino compounds could be involved in inciting infection and nodulation in the legume rhizosphere (Valera and Alexander, 1964). Such "nodulation factors" which are diffused into the rhizosphere have been shown to exist indirectly (Thornton, 1929; Lie, 1964). However, an antagonist factor is also believed to be present in the rhizosphere (Elkan, 1961). This substance perhaps could be a gibberellin-like substance (Darbyshire, 1964). In the model systems of Krasilinikov (1958) it was shown that different species of plants can stimulate different types of bacteria.

The leaching of the root exudates is greatly influenced by the microorganisms in the following ways. The permeability of root cells is affected and the root metabolism and the nutrient availability to plants is altered (Rovira and Davey, 1965b). It is rather difficult to assess the effects of any single group of microorganisms or factor and pinpoint their role in the rhizosphere. The role of bacteria, fungi and nematodes have been previously reviewed (Baker and Synder, 1965; Rovira and Davey, 1965b; Fahraeus and Ljunggren, 1968; Loutit and Miles, 1978).

IV. Rhizobium - the Nitrogen Fixing Bacteria

The bacteria belonging to the genus Rhizobium are those that can induce root nodules in legumes after infection. They are gram negative rods (0.5 - 0.9 µm x 1.2 - 3.0 µm) occurring singly or in pairs and they are generally

motile. Those that are motile have peritrichous, polar or sub-polar flagella (Buchanan and Gibbons, 1974). Glycogen and poly-β-hydroxybutyric acid are present as storage granules. They do not produce endospores, they are aerobic diazotrophs, and their optimum temperature for growth is 25° - 30°C in yeast extract medium. Some are fast growers (mean generation time of 2 - 4 hr) while others are slow growers (mean generation time of 6 - 8 hr) (Vincent, 1977). Unlike the fast growers, slow growers do not produce a lot of mucoid extracellular polysaccharide on agar.

A mature Rhizobium cell is rod shaped and shows the typical cell-wall envelope of a gram negative bacteria (Thorne, 1977; DieRienzo et al., 1978; Nikaido and Nakae, 1979). The intracellular inclusions include a central nucleoid (DNA) in a homogeneous cytoplasmic matrix interspersed with poly-β-hydroxybutyric acid, glycogen and ribosomes. Flagella are not visible all the time. The young and the motile cells have characteristic flagella. R. japonicum has a polysaccharide capsule which is firmly adhering to the cells, in addition to a soluble exopolysaccharide. Another important feature of free-living Rhizobium is its ability to infect specific legume hosts and convert into bacteroids within the host root nodule and to fix atmospheric nitrogen symbiotically. Such a bacteroid state can be induced in cultures (Jordan, 1962). The bacteroids are characterized by the highly irregular shape resulting from changes in the outer cell wall

envelope (Van Brussel, 1973; Sutton and Paterson, 1979; Bal et al. 1980; manuscript accepted for publication). The terminal respiratory chain in bacteroids and certain cytochromes are either altered or lost (Bergerson, 1977, 1978; Yates, 1978). However, rhizobia have also been shown to fix nitrogen in a free living state (Artishevskaya, 1969; Pagan et al., 1975; McComb et al., 1975; Kurz and LaRue, 1975; Wilcockson and Werner, 1976; Kaneshiro et al., 1978; Skotnicki et al., 1979).

Rhizobia in general produce various indole compounds (Vincent, 1977). Tetracycline antibiotics are generally active against rhizobia although they are susceptible to a wide spectrum of antibiotics. A compound from the seeds of white clover identified as 'myrecetin' has been shown to be toxic to Rhizobium (Fottrell et al., 1964).

Antagonism is exhibited towards rhizobia by fungi, actinomycetes, eubacteria and indeed by rhizobia itself. Other soil microorganisms such as protozoans, myxobacteria and Bdellovibrio are the predators of rhizobia. Lysogeny caused by rhizophages has been recorded (Vincent, 1977). Data related to agglutination, precipition reactions, complement fixation and antibody absorption have helped a great deal in understanding the chemical structure, taxonomy and identity of rhizobia (Vincent, 1977). Rhizobia are usually absent in highly acidic soils and in soils where legumes are not cultivated (Nutman et al., 1978).

A comprehensive chapter on the ecology of Rhizobium has been written by Parker et al. (1977).

V. The Concept of Cell to Cell Interaction

In order to understand the cell surface interaction between Rhizobium and the legume during the infection process, it is relevant to introduce the existing concepts regarding cell-cell interaction, cell recognition and specificity in embryonic morphogenesis and differentiation. A theoretical model involving specificity for the adhesion of cell to cell has been proposed by Bell (1978). The mechanism is operative at the cell surface involving ligand molecules such as lectins.

Investigations into cellular adhesion, both of a biochemical and biophysical nature, have not yet produced an established theory or a widely accepted hypothesis to explain the mechanics of this fundamental biological process although much information concerning the structure and function of the mammalian cell surface has been gained. At the present time there is increasing evidence to suggest that cellular adhesion is mediated by specific cell surface macromolecules which are capable of forming protein-carbohydrate complexes possibly resembling those found between plant lectins and their carbohydrate substrates (Greig and Jones, 1977; Frazier and Glaser, 1979).

Specificity is hard to define in a multicellular organism. Whether specificity reflects the cytoarchitecture at the

time of cell disaggregation, or should the cellular affinities indicate the earlier embryonic hierarchy remains a big unanswered question. The terms cell recognition and adhesion which are often used interchangeably may in some instances refer to reversible and irreversible associations respectively, and in others to a combination of these events. It is apparent that even defining specificity is difficult and it varies from one biological system to another, and from one type of assay to another (Frazier and Glaser, 1979). This fact is very true in legume-Rhizobium symbiotic associations.

The simplest model for cell-cell adhesion assumes that specific cell-cell recognition is brought about by the interaction of at least two mutually complementary ligands, much in the same manner as an antibody, an enzyme or a lectin binds a ligand. This first step is reversible followed by one or more apparently irreversible events, denoted schematically by the formation of multiple cell-cell adhesion and a change in cell shape (Frazier and Glaser, 1979). These events are strikingly temperature dependent. It is implicit in the model that one of the interacting molecules is a protein which exhibit the required specificity. The model completely ignores any other second ligand like another protein or a carbohydrate, as has been assumed in other model systems (Roseman, S., 1970; Shur, B.D., and S. Roth, 1975). It is clear that other

non-specific surface forces can alter cell to cell adhesion, but cannot by themselves account for the specificity observed in many cell to cell adhesion assay. The specificity for reversal may be as important as the specificity of binding in accounting for the ultimate position of a cell within an organized structure (Frazier and Glaser, 1979).

VI. Properties of Lectins

The word lectin has been derived from the Latin 'legere', meaning to choose. Legume seed lectins (Horrisberger and Vonlanthen, 1980) have been used to study the cell surface carbohydrates in a variety of biological systems (Schrevel et al., 1979; Horrisberger and Vonlanthen, 1980). These proteins are of non-immune origin and are capable of agglutinating erythrocytes and precipitating carbohydrates.

Some of the exciting features of lectins are their ability to distinguish between human blood groups, normal and malignant cells, to act as antitumor agents, mitogens, and to stimulate histamine from the mast cells. The role of lectins in plants is largely unknown. They may be involved in sugar transport, storage, specificity in host-parasite/symbiont interaction and they may act as enzymes such as L-galactosidase (Hankins and Shannon, 1978). Recently, Talbot and Etzler (1978), Hankins et al. (1979) isolated prolectins from the leaves of Dolichos biflorus which might be considered a starting point for the better understanding of lectin's metabolism. Some of the other properties of

lectins include agglutination of lymphocytes, tumor cells, microorganisms, viruses and vesicles; stimulation of cell division in lymphocytes, inhibition of fungal growth, insulin like activity on fat cells, degranulation of mast cells, cytotoxic activity toward mammalian cells and modulation of the immune response.

Some of the above properties have been very useful in blood typing, characterization of complex carbohydrate containing molecules, fractionation of viruses, cellular organelles and vesicles, identification of cell surface antigens, models for carbohydrate-protein and antigen antibody interactions and to generate lectin-resistant variants of eucaryotic cells for studies of glycoprotein structure and metabolism.

Of particular interest in this study is the soybean lectin, also known as soybean agglutinin. Soybean lectin is routinely isolated and purified on immobilized derivatives of galactose (Gordon et al., 1972) or galactosamine (Allen and Neuberger, 1975). It has a molecular weight of 120,000 (Lis and Sharon, 1977). Its polymeric form has a molecular weight of 240,000 and is mitogenic (Schechter et al., 1976). It is made up of four subunits with a molecular weight of 30,000 each.

Soybean lectin is a glycoprotein containing 4.5% mannose and 1.5% N-acetylglucosamine. The carbohydrate integrity is not essential for its biological activity (Lis and Sharon, 1977). Based on hapten inhibition studies it

has been established that soybean lectin is specific for N-acetylgalactosamine and galactose (Lis and Sharon, 1977).

The diverse and dynamic ligand patterns specify the nature of cell surfaces and decode the information necessary for cell recognition and specific cell association (Moscona, 1975). The nature and function of cell surface receptors reveal not only their presence but also their dynamics in the plane of surface membranes (Rutishauser et al., 1975; Siu et al., 1975).

Multicellular organisms exhibit a well regulated and coordinated cell division, cell movement and specific cell to cell interaction in their development. The cell surface membrane components like glycoproteins, appear to play a major role in acting as receptors for hormones, growth factors and antigens. They also mediate cellular events such as mitogenesis, morphogenetic movement or specific cell adhesion (Edelman, 1976). After the anchoring of such molecules on the cell surface, modulation of the cell surface components take place which possibly signals recognition as exemplified in T-lymphocytes (Nussenzweig, 1974; Schreiner and Unanue, 1976; Dickler, 1976). The morphological changes in lymphocytes by mitogenic lectins is accompanied by increases in protein, RNA and DNA synthesis (Lis and Sharon, 1973). The exact mechanism of lymphocyte stimulation by lectins is not yet clear.

Both glycolipids (Surolia et al., 1975) and glycoproteins (Dulaney et al., 1979) serve as lectin receptors

on cell surfaces. The lectin receptors are not affected by proteases which suggests that similar carbohydrate groups may exist on different protein species and also the same lectin receptor may contain receptor sites for more than one lectin (Marchesi et al., 1972; Brown and Hunt, 1978). The lectin receptors on lymphocytes have been identified as glycoproteins and glycopeptides (Goldstein and Hayes, 1978). Acidic glycoproteins and glycoproteins have been isolated as lectin receptors from neuronal cells (Goldstein and Hayes, 1978).

Exogenous lectins have been detected in cell surface events in membranes of lymphocytes (Kieda et al., 1978). There seems to be a cooperativity of lectin binding to lymphocytes where receptors are clustered and conformational changes in membrane structure take place a priori to mitogenic stimulation (Prujansky et al., 1978). Endogenous lectins have been implicated in the functional role of the recognition process during internal tissue differentiation in vertebrate embryos (Simpson et al., 1978). Precipitins, agglutinins, lysins, opsonins and neutralizing antibodies, all evoke the same kind of T-cell mediated immunoresponse causing hypersensitivity and immunity (Waksman, 1979). Such phenomena have tremendous implications in the understanding of cell surface components and their functions.

The importance of membrane glycoproteins in recognition phenomena is well established (Barondes and Rosen, 1976; Frazier et al., 1976; McMahon and Hoffman, 1978;

Singer and Morrison, 1978; Frazier and Glaser, 1979). Ashwell and Morrell (1977) have described the information encoded in specific sugar residues of membrane glycoproteins which exert a tremendous effect on cellular activity. Lectins and agglutinating proteins (Sharon, 1972; Dulaney, 1979) have been used to gain an understanding of the cell surface biochemistry of animals and plants (Schatten and Mazia, 1976; Goldstein et al., 1977; Greig and Jones, 1977; Gonatas, 1977; Ofek et al., 1978; Kabat, 1978; Clarke and Knox, 1978; Damsky et al., 1979; Pfenninger and Jamieson, 1979a, 1979b; Clarke and Knox, 1979; Sharon and Lis, 1979).

VII. Specificity in Plant-Microbe Interactions

In nature, plants are exposed to a multitude of microorganisms, yet only a few of them cause infection. Some plants resist the microorganisms and others are susceptible. There is a certain degree of specificity between plants and microorganisms whether they are pathogens or symbionts. The specificity and recognition is mutual and these properties reside in the host plant or in the microorganism.

The resistance by the plants also operates on the basis of specificity, with the attacking microbe triggering or inducing the formation of a group of antimicrobial compounds known as 'phytoalexins'. These compounds have similar properties to that of antibiotics and are accumulated when exposed to cell surface oligosaccharides of microbial origin (Albersheim and Valent, 1978).

Such a phenomenon does not occur in host-symbiont interactions or in disease affected host-plants. A low degree of host-pathogen specificity is seen in the genus Agrobacterium (Anderson and Moore, 1979). Enrichment of hydroxyproline-rich glycoproteins takes place in the cell wall as a result of developing defence mechanism for the anthracnose disease in muskmelon (Esquerre-Tugaye, 1979) which is again related to specificity.

Hence, plants have developed a capacity to recognize 'self' and 'nonself' at the cellular level, through a process of coevolution of the host and its related microbe. The mechanism of such a process is not thoroughly understood at the biochemical level. The basic tenets of specificity in the host-pathogen interaction lies in their mutual recognition and in eliciting a host-response (Sequeira, 1978).

Several specific steps are essential before the soil bacterium Rhizobium can enter the homologous host and fix atmospheric nitrogen symbiotically. The most important of them is the mutual recognition on cell surface contact between the Rhizobium and legume. The legume-Rhizobium symbiosis is characterized by a high degree of host specificity which has formed the basis for the classification of Rhizobium. Baldwin et al. (1927) asked "why should the organism responsible for nodule formation on the roots of lima bean (Phaseolus lunatus) be able to infect the roots of cowpea (Vigna ungiculata), and not those of the garden pea (Phaseolus vulgaris)?" In reply, they themselves wrote: "The

answer must be found in differences among the various species of the host plant, and the various races of root nodule organisms." Subsequent research has found it to be true.

The ubiquitous presence of lectins in plants and their well known properties of differentiating animal cells with minor differences in cell surface components has prompted many people to look for the possibility of lectin mediated host-specificity and recognition in Rhizobium-legume interaction.

Ever since the implication of lectins in legume-Rhizobium specificity (Bohlool and Schmidt, 1974), a considerable amount of work has been done with respect to lectin mediated specificity in Rhizobium-legume symbiosis (Schmidt, 1979).

Brian (1976) has suggested that specificity in plant-parasite interactions exists at five different levels as follows: (a) pathogens versus non-pathogens, (b) broad host-range pathogens, (c) narrow host-range pathogens, (d) single species specific pathogens, and (e) race-specific pathogens. Whatever be the level of interaction and specificity, the net result is either the entry or rejection of the pathogen by the host. Perhaps, susceptibility defines recognition and specificity much better than resistance. Whatever the host response is, it has to be mediated by certain cell surface molecules and lectins seem to be a favourite choice.

The recognition system in plants involves the complementary interaction of a saccharide containing macromolecule from one of the interacting organisms and a lectin like molecule on the other. Although lectins are not able to differentiate protoplasts of different species (Larkin, 1978) there are, however, well studied recognition phenomena in yeasts (Brock, 1979), Chlamydomonas (Snell, 1976) and slime molds (Raper, 1960; Simpson et al., 1974; Newell, 1977). Of particular interest is the cell recognition and immunity in flowering plants (Clarke and Knox, 1978, 1979). Also, lectins have been found in the plant cell walls and in organelles of plant cells (Clarke et al., 1975; Allen and Neuberger, 1975; Chrispeels, 1976; Sequeira and Graham, 1977).

VIII. Lectins and Recognition in legume-Rhizobium Interaction

It is common knowledge that there is a high degree of specificity between the two partners in Rhizobium-legume symbiosis. The general pattern of the infection process leading to the successful establishment of a symbiotic root nodule is well known but knowledge concerning the specific interaction and host-recognition by the bacteria is rather poor.

A very attractive hypothesis implicating legume seed lectins acting as cell surface recognizing molecules has been proposed by Bohlool and Schmidt (1974). Since then there have been many publications in which a precise biological role for lectins is proposed in this process.

In spite of evidence against the involvement of lectin (Dazzo and Hubbell, 1975; Rouge and Labroue, 1977; Law and Strijdom, 1977; Pull et al., 1978) there seems to be an overwhelming interest to assign lectins as the possible candidates for recognition and specificity following the report of Bohlool and Schmidt (1974). The lectin binding in soybean-R. japonicum interaction is reversibly inhibited by N-acetylgalactosamine (Bhuvaneswari et al., 1977; Bal et al., 1978; Shantharam et al., 1980). Certain strains of R. japonicum which did not bind to soybean lectin in laboratory media were found to bind when cultured in rhizosphere/rhizoplane conditions (Bhuvaneswari and Bauer, 1978). Interestingly enough, when R. japonicum was cultured in soil extract medium there was increased lectin binding (Shantharam et al. 1980 submitted for publication). Subsequently, many other reports strongly suggested that lectins play an important role in cell-surface interactions a priori to the infection process (Broughton, 1978; Dazzo, 1979a; Schmidt, 1979).

There are many reports of lectin binding to lipopolysaccharide (LPS) (Dazzo and Hubbell, 1975; Wolpert and Albersheim, 1976; Planque and Kine, 1977; Maier and Brill, 1978; Kato et al., 1979). On the other hand lectins have also been shown to bind to the extracellular polysaccharide (EPS) of rhizobia (Bohlool and Schmidt, 1976; Bal et al., 1978; Calvert et al., 1978; Kamberger, 1979a, 1979b; Shantharam et al., 1980). There are some reports of

lectin activity in the root system (Dazzo et al., 1978; Pueppke et al., 1978; Kato et al., 1979).

There are also considerable variations in the appearance of lectin receptors during the different growth phases of Rhizobium (Bhuvaneswari et al., 1977; Dazzo et al., 1979b). The fact that many legumes have multiple lectins and with different sugar specificities (Kauss and Glaser, 1974; Bowles and Kauss, 1975) and also some lectins are either modified or exist in different forms as a cell wall constituent in the roots (Kijne et al., 1980) has prompted many workers to look for not only erthyocyte agglutinating proteins but other lectin like proteins to be involved in legume-Rhizobium specificity and recognition.

The specificity has been defined at the very early stages of Rhizobium-legume interaction. It has been shown that the very act of bacterial docking on the root hair surface is a selective but specific process operated by the rhizobial capsular polysaccharide (Reporter et al., 1975; Bohlool and Schmidt, 1976; Dazzo et al., 1976; Bal et al., 1978; Sanders et al., 1978; Dazzo and Brill, 1977, 1979; Kamberger, 1979; Hughes et al., 1979). The bacteria have been shown to produce 'glycocalyx' fibres that help in adhering to the surfaces and to other cells (Costerton et al., 1978). However, Chen and Phillips (1976) have suggested that simple attachment of Rhizobium to the root hair surface is non-specific in nature.

The present study deals with fractionation and characterization of R. japonicum cells on the basis of morphological characteristics and their soybean lectin binding patterns.

MATERIALS AND METHODS

I. Organisms and Their Culture Conditions

Rhizobium japonicum Nitragin 61A76, *R. leguminosarum* Nitragin 92A1, *R. meliloti* 102F28 (Nitragin Co., Wisconsin), *R. trifolii* ATCC 10140, were used. Rhizobial strains were maintained on yeast-extract-mannitol agar slants at 26°C and subcultured every three months.

The rhizobial strains were broth cultured in yeast-extract-mannitol medium (mannitol, 10g; K_2HPO_4, 0.5g; $MgSO_4$ $7.H_2O$, 0.2g; NaCl, 0.1g; yeast extract, 1.0g; distilled water, 1 liter, pH 6.8-7.00) and soil extract medium coil extract, 200 ml; yeast extract, 1.0g; mannitol, 10.0g; distilled water, 800 ml, pH 7.2) and shake cultures in 125 ml culture flasks with 30 ml media at 220 rpm on a Gyrotory shaker model GZ (New Brunswick Scientific Co.) at 30°C.

The soil extract was prepared by mixing one volume of soil (obtained from Agriculture Canada, Research Station, St. John's West, Nfld.) with two volumes of distilled water and steaming for one hour or autoclaving for a few minutes; after cooling the liquid was decanted, allowed to stand overnight or centrifuged clear (McLachlan, 1973).

II. Growth Pattern of R. japonicum 61A76

The growth curve of *R. japonicum* 61A76 was plotted by measuring the turbidity of growth using a Shimadzu digital

double-beam spectrophotometer UV-210 at a wavelength of 600 nm spectrophotometrically both in yeast extract mannitol and soil extract media with or without 350 µg of soybean lectin. Starting with 1 ml and 1 loopful of the inoculum separately, the growth of R. japonicum 61A76 was compared for their generation time in yeast extract mannitol medium. The source of inocula was a stationary phase broth culture of R. japonicum 61A76.

III. Electron Microscopy

Bacterial culture (heterogeneous, rods and coccoid forms) at stationary phase was pelleted by centrifugation and fixed (a) in a mixture of 4% paraformaldehyde and 2.5% glutaraldehyde (Karnovsky, 1965) in 0.1M Sorensen's buffer, pH 7.2 for 1 hr at 23°C and (b) in 2.5% glutaraldehyde in the same buffer under the same conditions. After thorough washing in buffer, the pellet clumps were treated with 1% osmium tetroxide in 0.1M Sorensen's phosphate buffer, pH 7.2 for 1 hr at 4°C. This was followed by dehydration in ethanol series and subsequent embedding in Spurr's medium (Spurr, 1969). Ultrathin sections were cut with a diamond knife and stained with uranyl acetate and lead citrate. Some of the sections were also stained with phosphotungstic acid (PTA) and chromic acid mixture (Roland et al., 1972), and others were stained with periodic acid-lead specific stain for glycogen (Perry, 1967). For staining extracellular polysaccharides, ruthenium red (1 mg/ml) was mixed with

aldehyde fixative and osmium tetroxide during fixation (Pate and Ordal, 1967). The whole mount preparations of coccoid and rod forms of bacteria treated with colloidal gold labeled soybean lectin were made on carbon stabilized Formvar-coated grids without chemical fixation and staining. Observations were made using a Zeiss 9S electron microscope.

IV. Soybean Lectin (SBL)

Soybean lectin labeled with ^{125}I, ferritin and colloidal gold was employed. SBL-ferritin conjugate was a gift from D.P.S. Verma (Dept. of Biology, McGill University, Montreal). Soybean-lectin type VI was purchased from Sigma Chemical Company. The purity of soybean lectin was confirmed by zone electrophoresis in polyacrylamide gels (Davis, 1964). The phytohaemagglutination property of soybean lectin was tested against human erthrocytes 'A' in physiological saline with galactose (50mM), N-acetygalactosamine (5mM) as controls (Lis et al., 1970; Lis and Sharon, 1973).

(a) Preparation of ^{125}I-Soybean Lectin

Soybean lectin (Sigma Chemical Co.) was radioiodinated with ^{125}I (New England Nuclear) by the lactoperoxidase catalyzed method (Rittenhouse et al., 1973) and dialyzed to remove unbound ^{125}I (Gow et al., 1976); the lectin concentration was determined by the method of Herbert et al. (1971). Radioactivity was measured with a Beckman 7000 gamma counter. The labeled soybean lectin had a final specific activity of 1140 cpm/µg of soybean lectin.

(b) ^{125}I-Soybean Lectin Binding Studies

Bacterial suspensions were standardized to 100 µg dry weight of cells per ml using the method for correcting inherent errors in optical density measurements (Lawrence and Maier, 1977).

Varying amounts of ^{125}I-SBL (50 µg/ml to 500 µg/ml) were added to the reaction mixtures containing 1 ml of bacterial suspension in 0.2M phosphate buffer and sufficient distilled water was added to give a total reaction volume of 2 ml. The reaction mixtures were incubated for 30 mins. The binding saturation concentration of ^{125}I was determined.

Control reaction mixtures were included by treating the bacterial cells with galactose (50mM) and N-acetyl-galactosamine (5mM) for 30 mins at room temperature prior to incubation with ^{125}I-SBL (Lis et al., 1970; Lis and Sharon, 1973). The reaction mixtures were washed three times at 115,000 x g for 15 mins each in 0.2M phosphate buffer.

The results were calculated according to the method of Bhuvaneswari et al. (1977) and Steck and Wallach (1965). The plots obtained were a result of linear regression done by computer.

(c) Ferritin-Soybean Lectin Binding Studies

Pellets prefixed in paraformaldehyde-glutaraldehyde mixture (a) at 0°C were washed thoroughly in cold buffer for 18 hrs and then treated with ferritin-lectin conjugate for

1 hr at 25°C. This was followed by repeated (5x) washings in buffer for 18 hrs at 35°C with continuous shaking (Bal et al., 1976). After treatment with osmium tetroxide, the pellet clumps were processed for electron microscopy.

For in vivo binding, stationary phase bacterial cultures were washed in 0.1M phosphate buffer, pH 7, to remove the nutrient media and were then treated with soybean lectin (Miles Laboratories) conjugated with ferritin (Andres et al., 1973). After five washes in buffer (repeated centrifugation), the final pellet was fixed in aldehyde fixatives and processed for transmission electron microscopy.

(d) Preparation of Colloidal Gold

The most common method of preparing gold hydrosol consists of reducing chlorauric acid with a suitable agent (Weiser, 1933). A 2.5 ml of 0.6% potassium carbonate solution was added to 120 ml of glass distilled water followed by the addition of 1 ml of pure ether-saturated phosphorous solution. The solution was boiled for 15 mins in an open flame in a hood when the wine red colloid gold solution is formed (Au I). The boiling removes the excess ether and excess phosphorous by atmospheric oxidation.

(e) Preparation of Colloidal Gold-SBL Conjugate

Soybean lectin which is a water soluble protein was used to coat gold at pH 7.0 by centrifugation at 18,000 x g for 30 mins, 35,000 x g for 45 mins, 78,000 x g for 45 mins (Faulk and Taylor, 1971; Garland, 1973). Soybean lectin

saturating point for colloidal gold was determined according to Horrisberger et al. (1975) and was found to be 500 µg of lectin/ml. Usually a 10% excess of soybean lectin was added to saturate the gold (Horrisberger and Rosset, 1977).

(f) Colloidal Gold-Soybean Lectin Binding Studies

For in vivo binding, stationary phase bacterial cultures were washed in 0.1M phosphate buffer, pH 7, to remove the nutrient media and treated with colloidal gold conjugated soybean lectin for 1 hr at 25°C (Shantharam et al., 1980). After five washes in buffer (repeated centrifugation), the final pellet was fixed in aldehyde fixatives and processed for transmission electron microscopy. Whole mounts of the above bacteria were prepared on Formvar coated and carbon stabilized grids without chemical fixation or staining.

V. Sucrose Density Gradient Centrifugation

Both linear and step gradients of various concentrations of sucrose (10% - 40% w/v) were employed. The centrifugation was performed using a SW-27 rotor in a Beckman ultracentrifuge for 3 hrs.

VI. Differential Centrifugation

Bacterial cells were harvested from 24 hr stationary phase broth cultures and two fractions were separated by differential centrifugation at 12,000 x g for 10 mins, contained the rod form and the supernatant the coccoid form. The coccoid form was then sedimented by centrifugation at

115,000 x g for 60 mins (Shantharam et al., 1980).

VII. Measurement of Bacterial Growth, Cell-Size and Number

The bacterial growth in broth culture was monitored spectrophotometrically at 600 nm in each of the media and the generation time was calculated graphically. Fractionation of rods and cocci was done by differential centrifugation as described above and the fractions were examined by using a Zeiss microscope fitted with phase contrast optics.

VIII. Incorporation of ^{3}H-Thymidine in Coccoid and Rod Forms

The fractionated coccoid and rod forms of cells were adjusted to 1 x 10^{9} cells per ml concentration in 0.1M phosphate buffer and were separated into 3 ml aliquots in duplicates. The bacterial suspensions were continuously labeled with ^{3}H-Thymidine for 10 mins. The labeling was washed by 5% trichloroacetic acid at 1 min, 5 min and 10 min intervals using a millipore filtration apparatus and washed three times. The samples were counted in a Beckman liquid scintillation counter.

IX. Interconversion of Coccoid and Rod Forms

(a) Conversion of Coccoid Forms to Rod Forms

Under asceptic conditions, pellets obtained at 115,000 x g (coccoid fraction) were inoculated into yeast extract mannitol medium and incubated on a rotary shaker at

220 rpm at 30°C. Samples of bacteria were prepared on glass slides for phase contrast microscopic observation at 8 hr intervals and random counts of coccoid and rod forms were made (Shantharam et al., 1980).

The coccoid fraction of cells were streaked onto sterile yeast extract agar plates and incubated at 30°C. After 5 days small isolated colonies appeared and these colonies were examined by light microscopy to determine if the bacterial population was heterogeneous with respect to rod and coccoid forms.

(b) Conversion of Rod Forms to Coccoid Forms

The rod fraction was obtained by centrifugation at 12,000 x g for 10 mins using sterile centrifuge tubes. A loopful of bacteria was inoculated into a fresh yeast extract mannitol medium and incubated on a rotary shaker at 220 rpm at 30°C. Samples of bacteria were examined by phase contrast microscopy smeared on glass slides at 8 hr intervals and random counts of coccoid and rod forms were made as described above.

X. Root Hair Attachment Studies

Roots of 5 day old soybean seedlings grown in sterile vermiculite were placed in a bacterial suspension of stationary phase R. japonicum 61A76. The final concentration of bacterial cells was adjusted to 100 µg dry weight of

cells per ml.

The roots were thoroughly washed by rinsing repeatedly in a series of 500 ml beakers containing sterile distilled water. After washing, free hand sections were cut and stained with 1% Rose Bengal (Rose Bengal, 1g; $CaCl_2$, 0.01g in 5% phenol). Root hairs showing attached bacteria were counted. Some R. japonicum attached root hairs were processed for transmission electron microscopy.

XI. Nodulation Studies

The nodulation tests of coccoid and rod fractions were carried out using soybean in an environment chamber. A modified Leonard's jar assembly was used (Vincent, 1970). Soybean seeds were surface sterilized with 0.5% Clorox, washed with sterile distilled water and were allowed to germinate in sterile Petri dishes. The bacterial cells were fractionated and grown in yeast extract mannitol nutrient medium for 24 hrs (Shantharam et al., 1980) and suspended again in the nutrient medium without the yeast extract. The cells were adjusted to 100 µg dry weight of cells per ml (Lawrence and Maier, 1977) and 5 ml of this suspension per seed were used to inoculate 5 day old 25 soybean seedlings. The heterogeneous broth culture and seedlings without inoculum were used as controls. The plants were harvested after 25 days and the nodules were counted.

XII. Carbohydrate Analyses

(a) Large-Scale Cultivation of R. japonicum

The inoculum for large-scale culture was grown in 30 ml of medium for 24 hrs and transferred to 300 ml of the same medium. The 300 ml culture (grown for 48 hrs at 30°C with aeration) was then transferred to 2.5 liters of the same medium and grown for 48 hrs). This was used as the inoculum for a 25 liter batch grown in a commercial fermenter (New Brunswick Scientific Co. Model No. MF-1285) for 7 days at 30°C with an air flow of 5 - 6 liters per minute. The cells were harvested by continuous centrifugation and then lyophilized.

(b) Isolation of Lipopolysaccharide (LPS)

The LPS from the freeze dried cells was extracted by the phenol-water method of Westphal et al. (1952) with two wash cycles of the phenol layer. The combined aqueous layers were dialyzed against tap-water. The retentate was centrifuged at 5000 x g for 20 mins to remove cellular and other precipitated debris prior to ultracentrifugation 105,000 x g for 3 hrs. The resulting gel was resuspended and recentrifuged twice, before lyophilization to give the purified LPS.

(c) Isolation of Extracellular Polysaccharide (EPS)

The EPS was isolated from the supernatant of the broth culture by mixing the supernatant with DEAE-Cellulose, eluting the DEAE-Cellulose with N-NaCl and precipitating the polysaccharide with 95% ethanol overnight at 4°C

(Humphrey et al., 1974). The precipitated polysaccharide was centrifuged and lyophilized.

(d) Analysis of Extracellular and Lipopolysaccharides

The polysaccharides were hydrolyzed in N-H_2SO_4 (1 ml) for 4 hrs at 100°C for analysis of neutral sugars. The hydrolysate was neutralized with $BaCo_3$, filtered and divided into equal parts. One part was concentrated and run against standards on paper chromatography; the other was reduced with sodium borohydride (for 1 hr) and subsequently acidified with acetic acid. Borate ion was removed by co-evaporation three times with acidified methanol. The reduced monosaccharides were acetylated with a 1:1 mixture of pyridine and acetic anhydride (1 ml) for 15 mins at 100°C (Lehnhardt and Winzler, 1968).

Gas-liquid chromatography of acetylated alditols was performed using a Perkin-Elmer Model 3920 gas chromatograph, using the column of 1.5% ECNSS-M on Gas Chrom Q (Applied Science Laboratories) at 190°C, with a helium flow rate of 40 ml per minute. Use of 1.5% ECNSS-M rather than the usual 3% ECNSS-M column allowed the detection of acetylated amino sugar alditols in a reasonable time (approximately 60 mins).

Paper chromatography was carried out by descending irrigation with ethyl acetate: pyridine: water: (10:4:3) and the monosaccharides were visualized with p-anisidine hydrochloride or with the alkaline silver nitrate reagent of Trevelyan et al. (1950).

Amino sugars were qualitatively analyzed by paper chromatography after hydrolysis of the polysaccharides with 3.9 N-HCl (100°C, 10 hrs) and detection with silver nitrate reagent or ninhydrin (2% in acetone).

RESULTS

I. Culture Conditions and Growth Pattern

R. japonicum 61A76 grown in yeast extract mannitol medium showed a typical sigmoidal growth pattern (Fig. 5). The mean generation time was 5 - 6 hrs when 1 ml of inoculum was used to generate the broth culture in 30 ml medium. But, when one loopful of inoculum was used to start the culture, the mean generation time appeared to be 10 hrs. This strain of bacteria attains stationary phase in less than 14 hrs or in about 70 hrs, depending on the size of the inoculum.

II. Morphology of R. japonicum as Revealed by Light and Electron Microscopy

The morphology of a typical cell of *R. japonicum*, as obtained from the extensive electron microscopic observations, is diagrammatically represented in Fig. 6. It has a typical gram-negative bacterial cell wall envelope. The outer membrane composed of lipids, lipopolysaccharides and protein, the dense middle layer composed mainly of peptidoglycan and the inner plasma membrane. The cytoplasm is homogeneous enclosing glycogen, poly-β-hydroxybutyric acid and ribosomes. There is a central core of DNA. The cell has a single polar or sub-polar flagellum, as clearly demonstrated from the

Fig. 5. Growth curve of R. japonicum 61A76 in yeast extract mannitol broth culture (0.1% yeast extract) at 30°C at 220 rpm with varying amounts of starter inoculum.

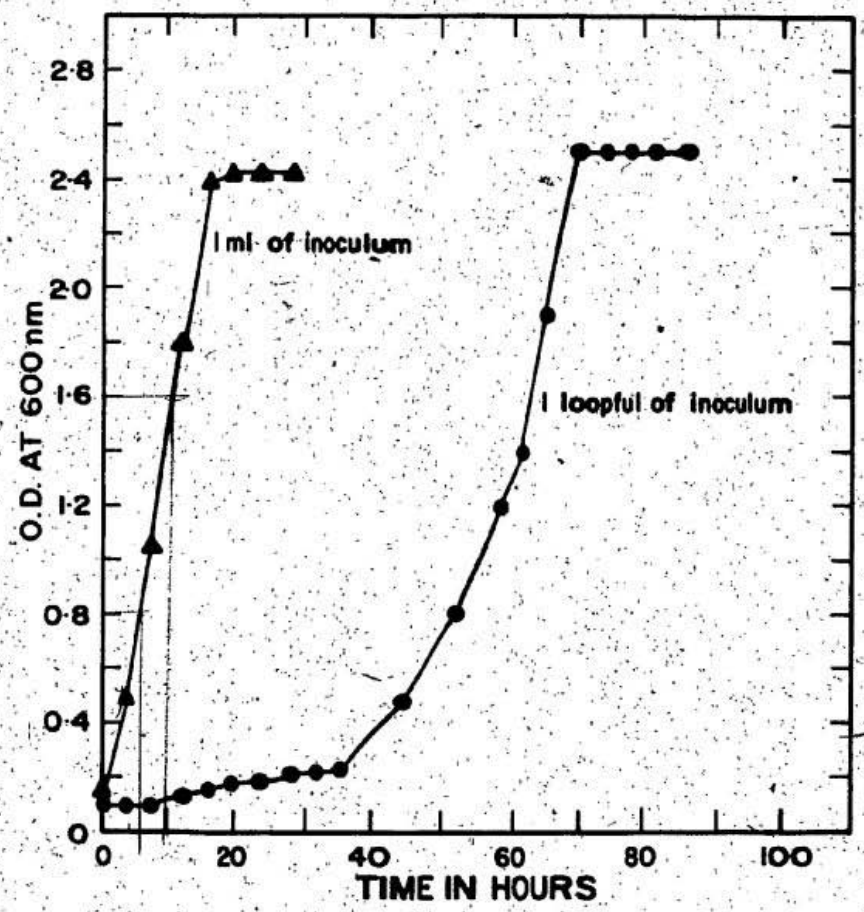

2·8
2·4
2·0
1·6
1·2
0·8
0·4
0
O.D. AT 600nm
1 ml of inoculum
1 loopful of inoculum
0
20
40
60
80
100
TIME IN HOURS

Fig. 5

Fig. 6. Schematic diagram of a typical cell of R. japonicum as revealed by electron microscopic study.

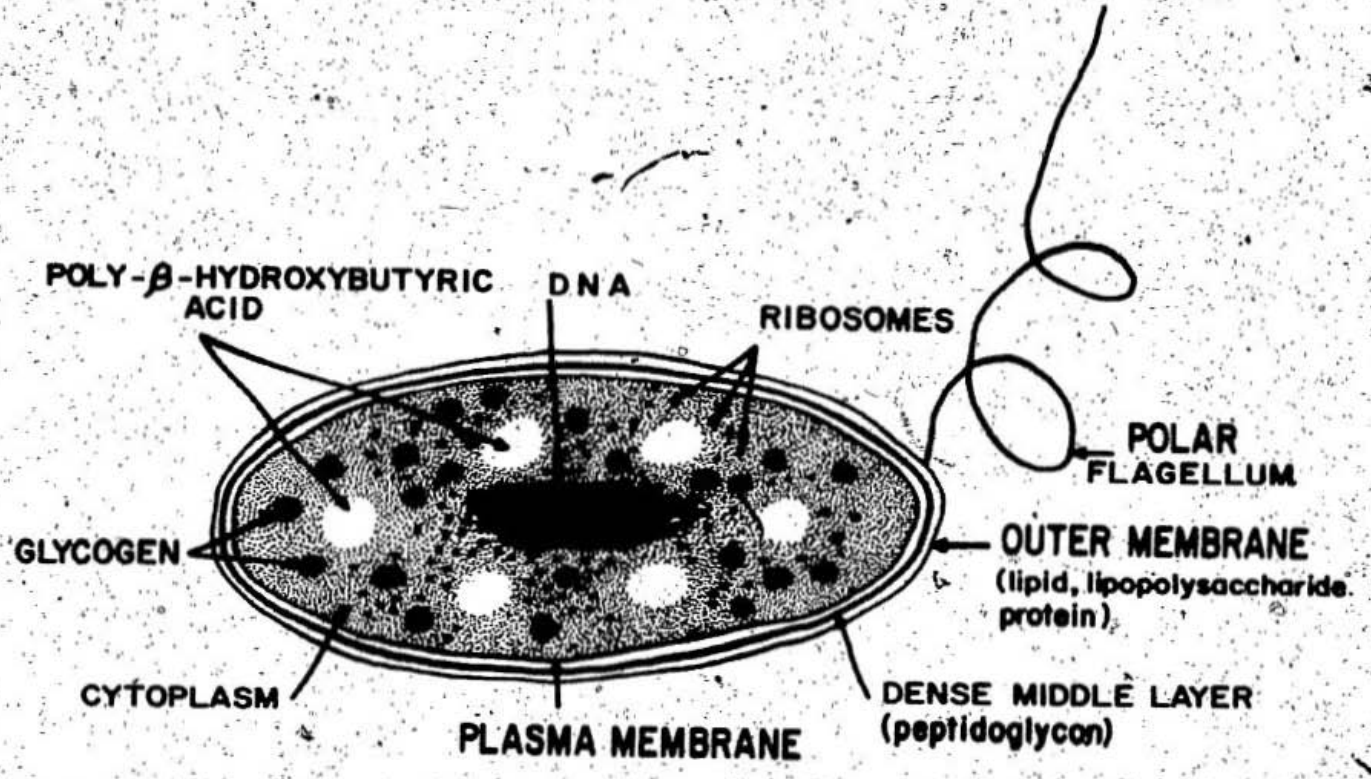

POLY-β-HYDROXYBUTYRIC ACID
DNA
RIBOSOMES
POLAR FLAGELLUM
GLYCOGEN
OUTER MEMBRANE
(lipid, lipopolysaccharide, protein)
CYTOPLASM
PLASMA MEMBRANE
DENSE MIDDLE LAYER
(peptidoglycan)

Fig. 6

Fig. 7. Whole mounts of R. japonicum 61A76 cells showing the polar and sub-polar flagella. The preparation has not been stained or fixed with any chemical.

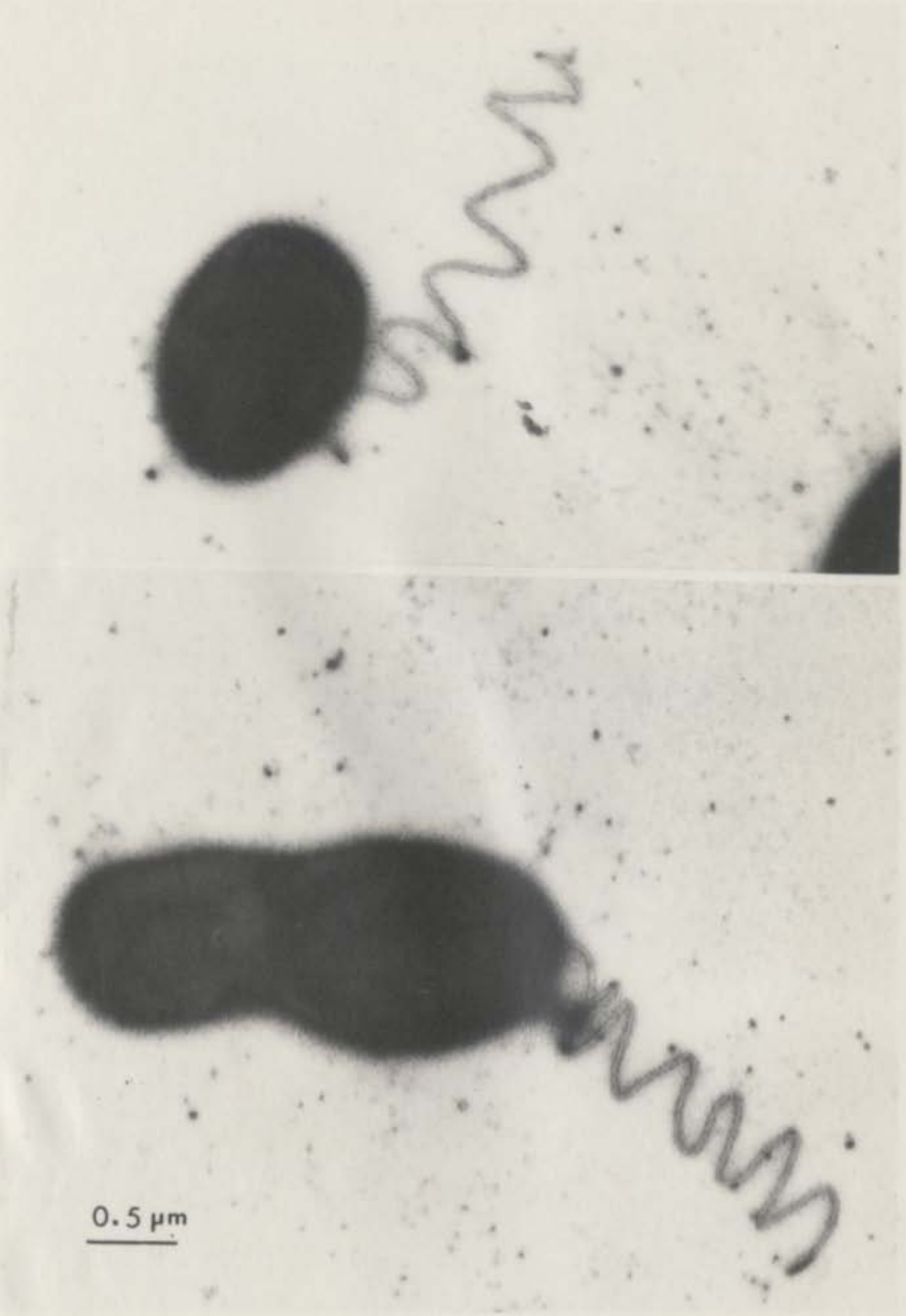


Fig. 7

Fig. 8. Chemically unfixed and unstained whole mount of capsulated coccoid fraction of cells; (A) clustered coccoid forms within a common capsular matrix, (B) Beaded or linear row of coccoid forms of cells within a common capsule.

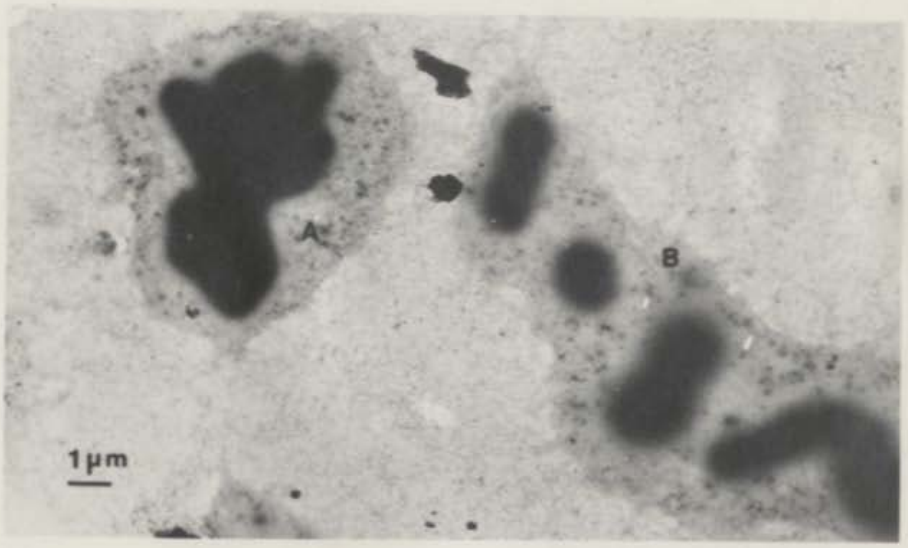


Fig. 8

Fig. 9. Chemically unfixed and unstained whole mount of 'star' of R. japonicum 61A76.

Fig. 9

Fig. 10. Whole mount electron micrograph of a 'star'. Note the single sub-polar flagellum attached to one of the bacteria. Insert: Photomicrograph of the rod fraction (12,000 x g pellet) showing the 'stars' as well as free rods.

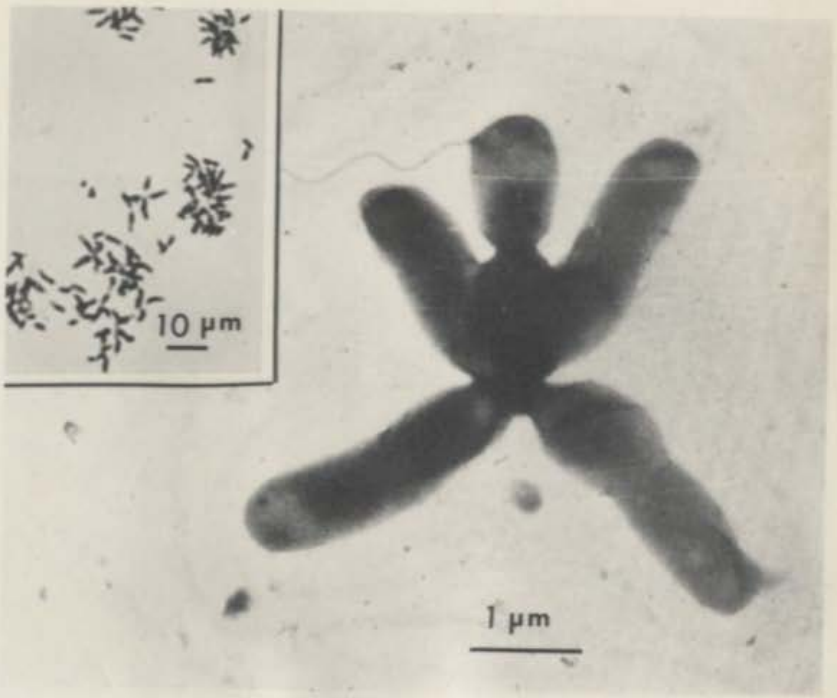


Fig. 10

Fig. 11. Electron micrograph of a whole mount preparation of capsulated coccoid fraction treated with colloidal gold labeled soybean lectin. Note the thick and massive capsule immediately surrounding the cell and the diffusion of capsular material into the surrounding medium. Insert: Photomicrograph of the coccoid fraction (105,000 x g pellet); free and clustered bacteria as well as the ones which appear as beaded chains or in linear rows.

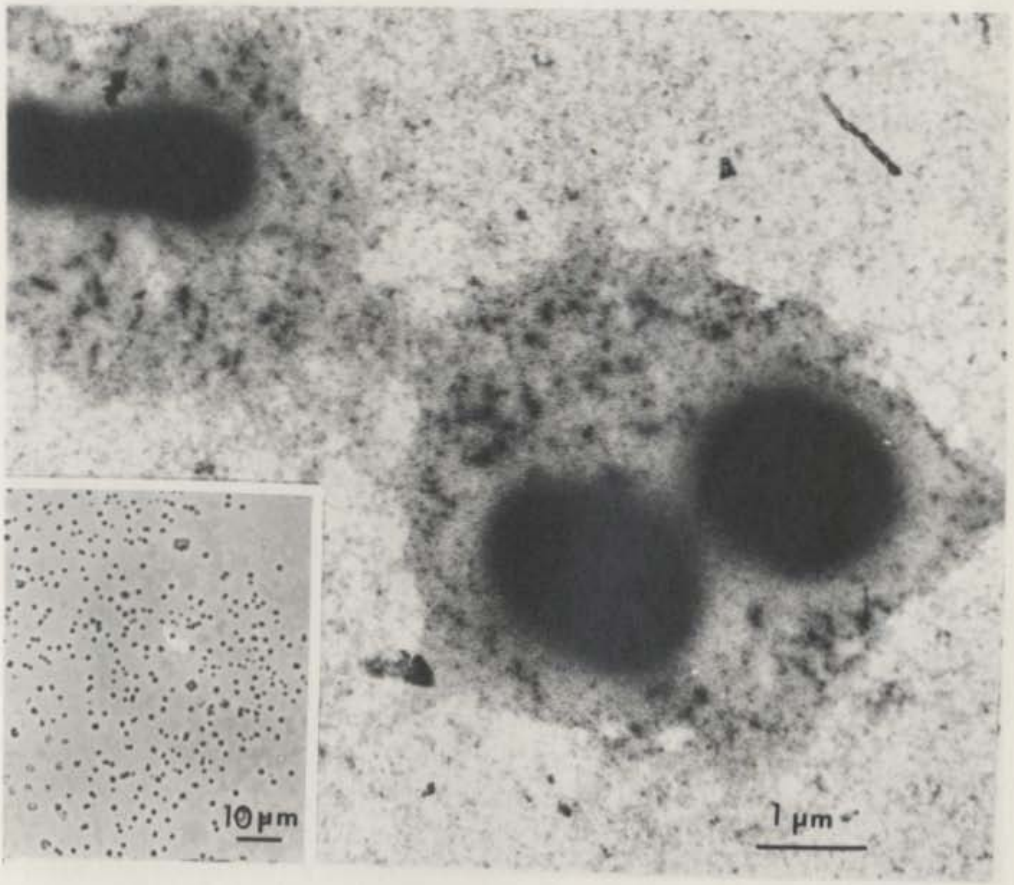


Fig. 11

whole mount preparations for electron microscopy (Fig. 7).

Repeated observations of bacteria with light and electron microscope revealed the presence of morphologically distinct types of cells in the broth culture of R. japonicum 61A76. The cells were either small rods, rounded to ovoidal in shape measuring 0.5 to 2.5 µm in size and they were either free or associated forming chains (Fig. 8). The other type of cells were rods measuring 3.5 to 5 µm in length, either free or often aggregated to form distinct 'star' shaped configurations (Fig. 9). Most often the rod form of cells which aggregated into 'stars' exhibited polarity by accumulating glycogen (Fig. 10) at one end. Such a polarity has been noted earlier (Tsien and Schmidt, 1977). Fig. 10 shows a whole mount of chemically unfixed and unstained 'star' which exemplifies heterogeneity in itself. There are two rods showing constriction due to impending cell division and only one of the cells in the 'star' is having a sub-polar flagellum and all five rod cells show variation in size.

The capsulated coccoids were very few in number (less than 1%) (Bal et al., 1978) in the broth culture of R. japonicum 61A76, which is a non-mucoid strain (Dazzo and Brill, 1979b). The capsulated cells had a distinct capsule surrounding the cells (Fig 8). Fig. 8 is a whole mount preparation of bacteria within the capsular sheath; some are in clusters (A) and others are in rows (B). In ultrathin sections the capsule shows loose fibrillar nature (Fig. 12).

Fig. 12. Ultrathin section of a capsulated coccoid cell of R. japonicum 61A76. Note the fibrillar connections between the capsule and the cell wall (arrows). Glycogen granules can be seen as storage material.

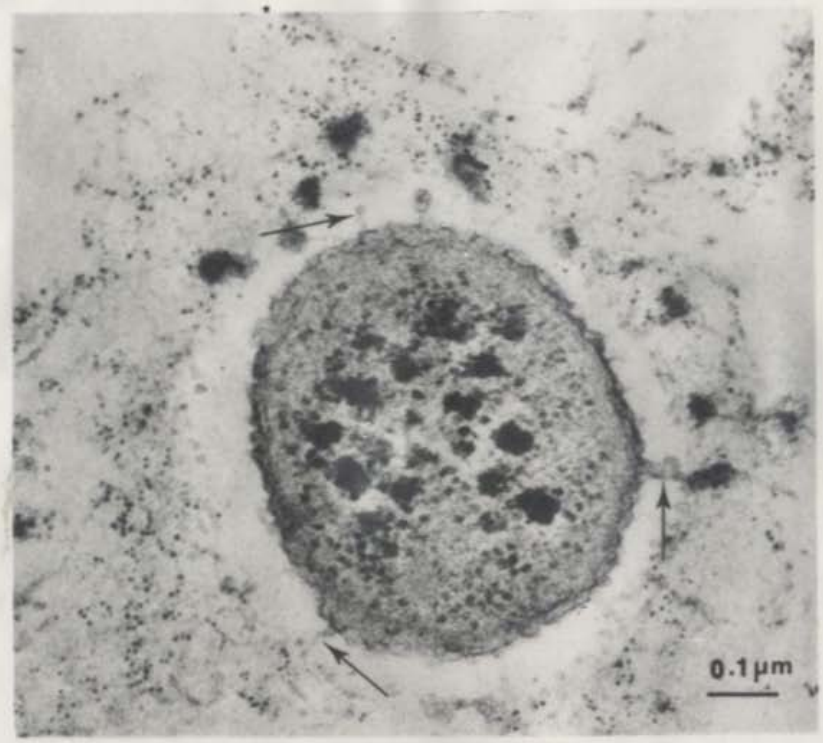


Fig. 12

Fig. 13. (Above) Electron micrograph of a rod cell of R. japonicum 61A76 showing the polar capsule (arrow). Glycogen (G) determines the polar end.

(Below) Ultrathin section of 'stars' showing the central common capsule (arrow) and the polarity exhibited by accumulation.

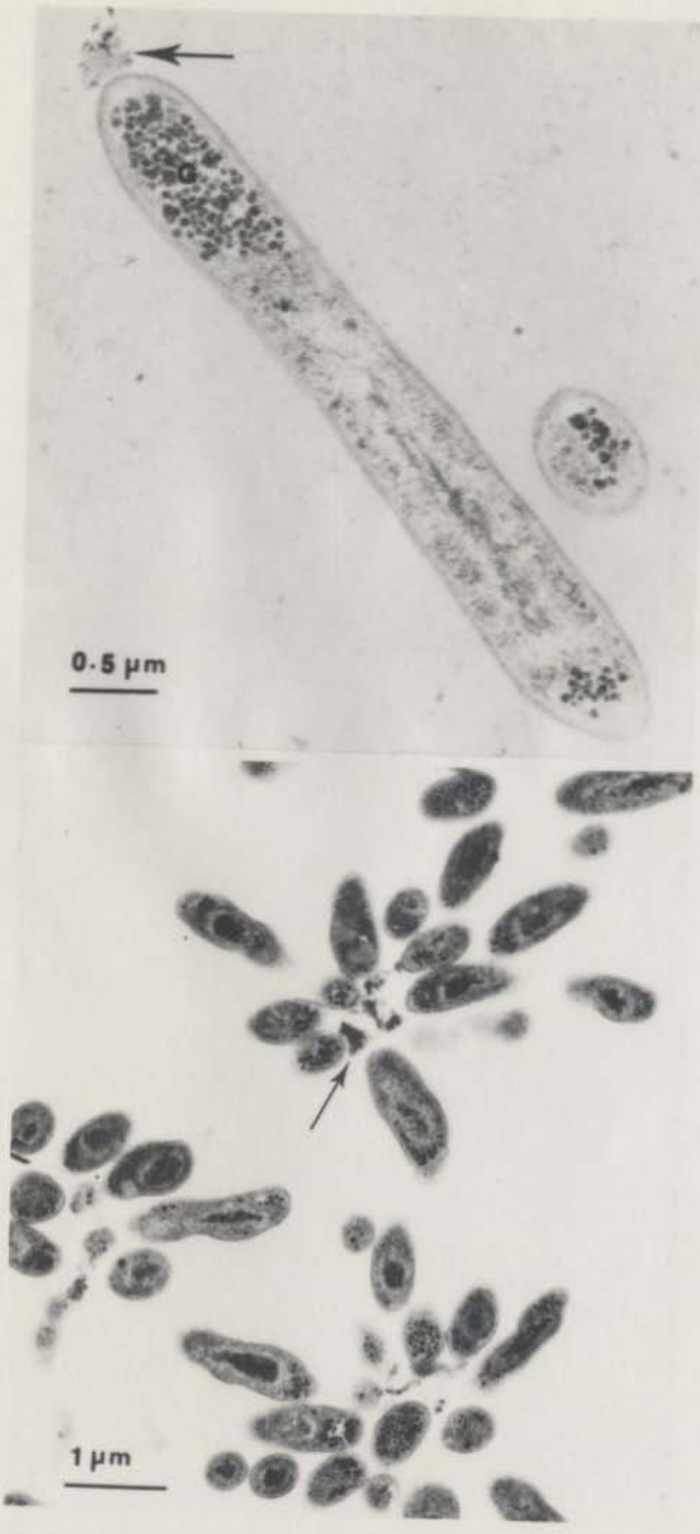


Fig. 13

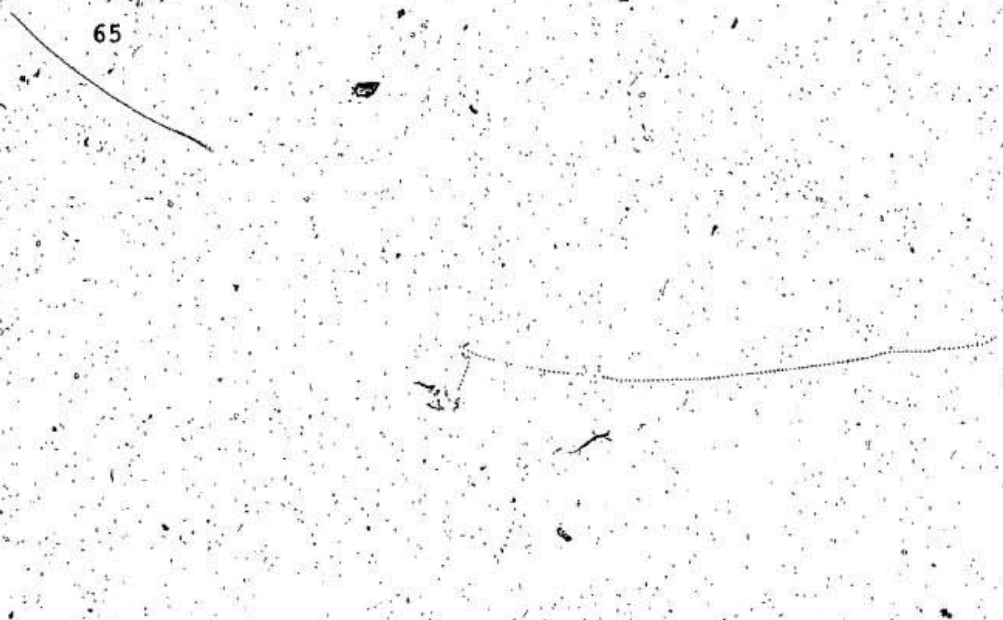


Fig. 14. Photomicrographs of differentially fractionated rods (stars) (above) and coccoid forms of cells (below) from the stationary phase of R. japonicum 61A76 broth culture.

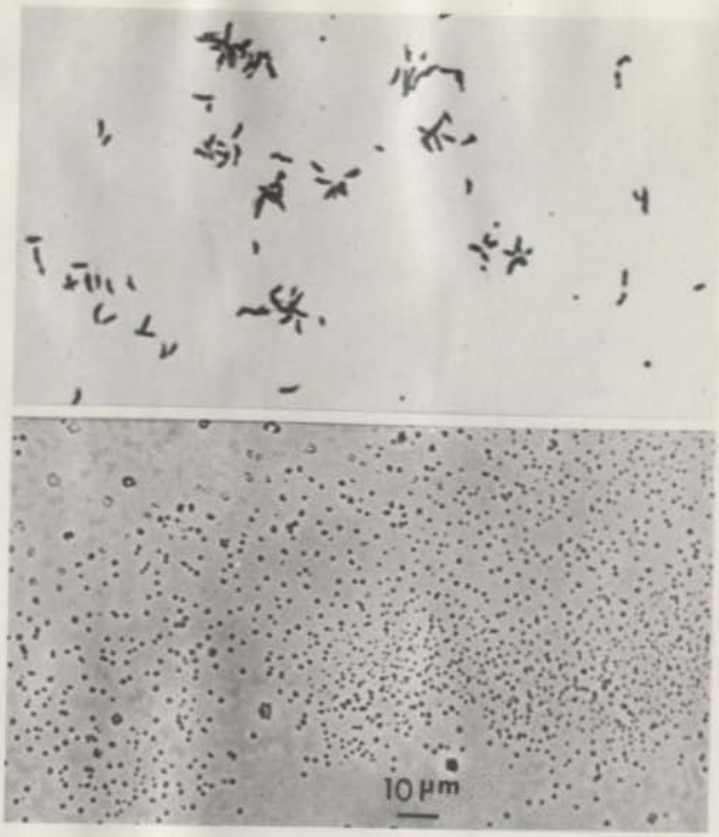


Fig. 14

Usually a gap appears between the cell wall and the capsular material and there is evidence for the secretion of capsular material as indicated by connecting capsular material between the cell wall and the capsule (arrows).

The capsulated rod forms exhibit a polar capsule which has been referred to as polar tips (Bohlool and Schmidt, 1976). The capsulated rods in ultrathin sections show polarity in the accumulation of glycogen (G) at the capsulated end (Fig. 13). The rods are often seen aggregated to form 'stars'. The rods are held by a common central polar capsular (arrows) material (Fig. 13). Ruthenium red did not stain the capsule in either rod or coccoid forms.

III. Fractionation and Characterization of Rods and Cocci

Isopycnic sucrose gradient (10 - 40%) centrifugation failed to produce clean reproducible fractions as they were seen overlapping. Therefore, the rod and coccoid forms of cells were fractionated by differential centrifugation at 12,000 x g for 10 mins and 115,000 x g for 60 mins, respectively, from a 24 hr stationary phase broth culture of R. japonicum 61A76 (Fig. 14), grown in yeast extract mannitol broth culture. R. meliloti, R. trifolii and R. leguminosarum, also grown in yeast extract mannitol medium, showed similar coccoid and rod forms on fractionation by differential centrifugation confirming the above observation and thereby establishing morphological

Fig. 15. Conversion of coccoid forms to rod forms, when allowed to grow in yeast extract mannitol medium for 72 hrs at 30°C at 220 rpm.

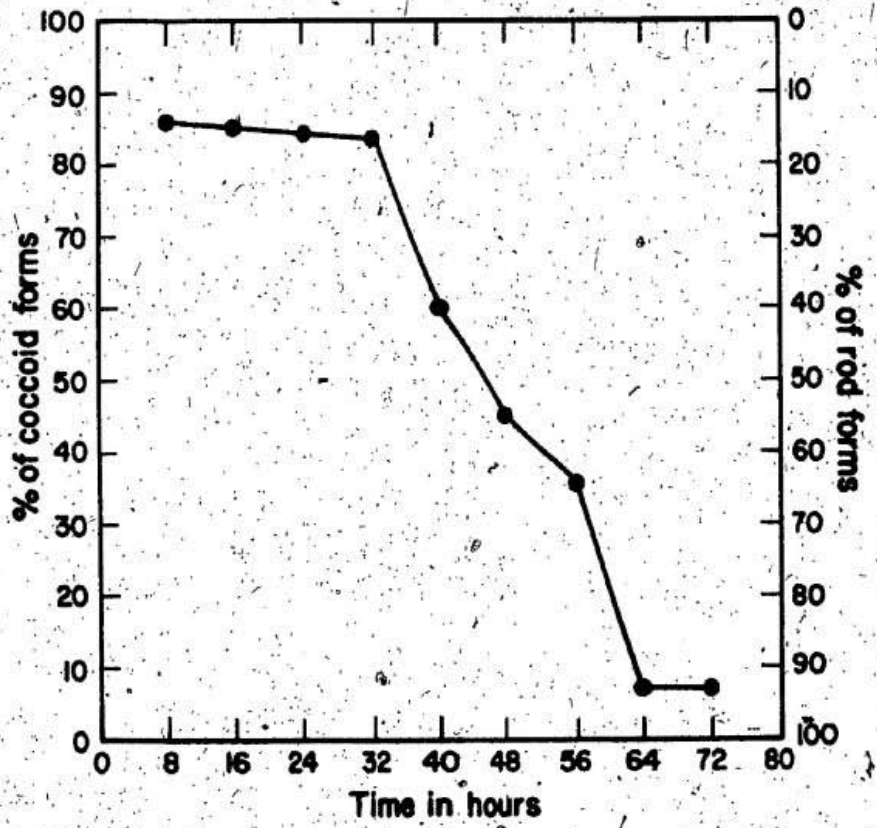

% of coccoid forms
% of rod forms
Time in hours
0
8
16
24
32
40
48
56
64
72
80
10
20
30
50
60
70
90
100

Fig. 15

Fig. 16. Conversion of rod forms into heterogeneous broth culture in yeast extract mannitol medium in 104 hrs at 30°C at 220 rpm.

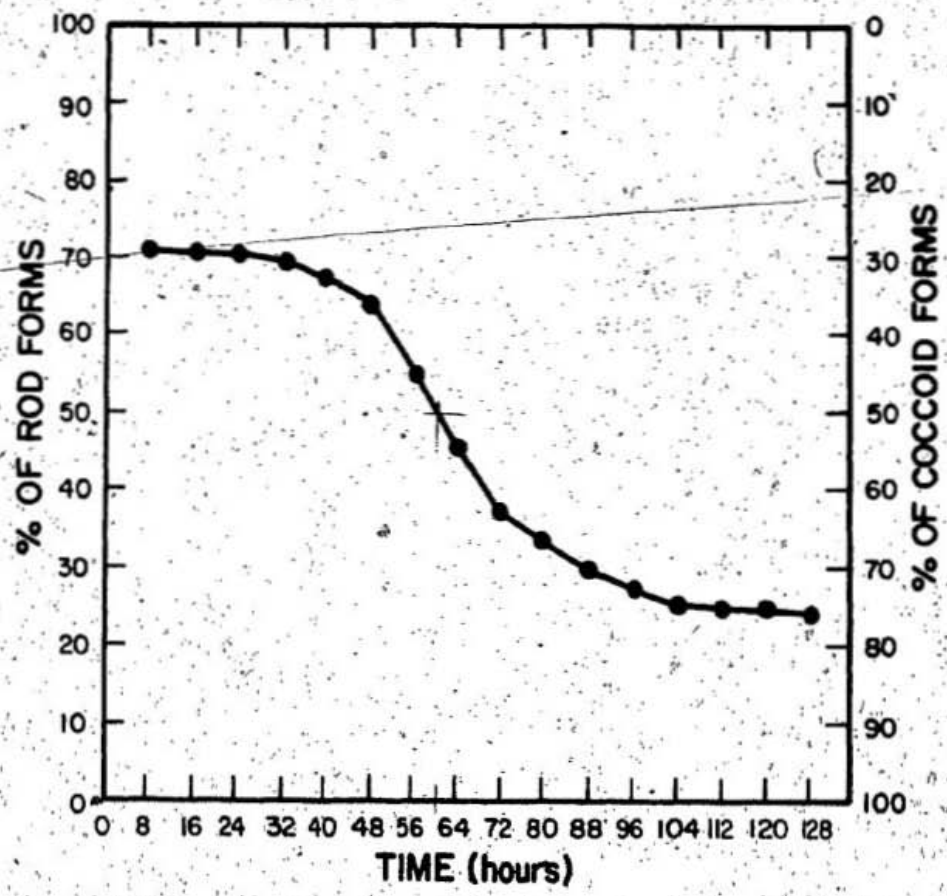

% OF ROD FORMS
% OF COCCOID FORMS
TIME (hours)
0 8 16 24 32 40 48 56 64 72 80 88 96 104 112 120 128
100 90 80 70 60 50 40 30 20 10 0
0 10 20 30 40 50 60 70 80 90 100

Fig. 16

Fig. 17. Incorporation of ^{3}H-Thymidine into rod and coccoid fraction of cells.

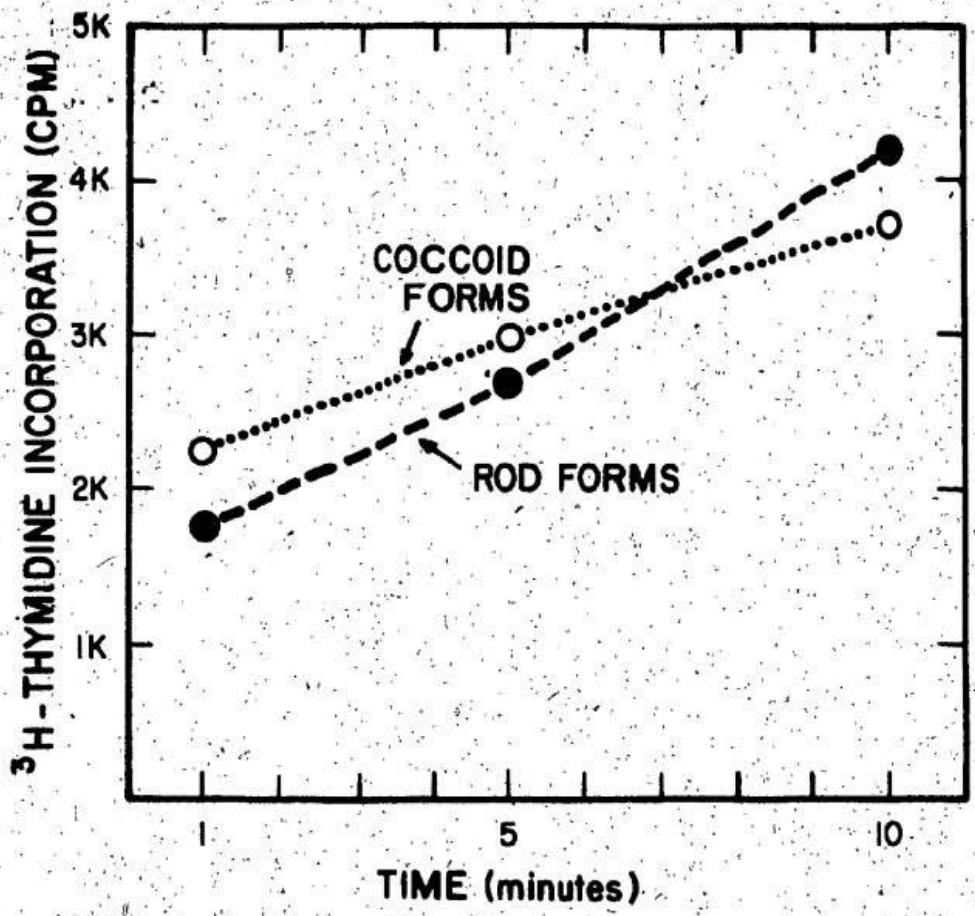

5K
4K
3K
2K
1K
^{3}H-THYMIDINE INCORPORATION (CPM)
COCCOID FORMS
ROD FORMS
1
5
10
TIME (minutes)

Fig. 17

heterogeneity in Rhizobium.

IV. Interconversion of Rods and Cocci in Broth Culture

The coccoid fraction of cells when allowed to grow in fresh medium was converted into a heterogeneous population of both rod and coccoid forms. Fig. 15 shows that over 90% of coccoid cells were converted into rods in the course of 72 hrs. Fig. 16 also shows that rod fraction cells (80% rods) also convert into a heterogeneous broth culture of rods and cocci over a period of time.

When the same fractionated coccoid cells were streaked on sterile yeast extract mannitol agar plates, they gave rise to single isolated colonies on incubation and these isolated colonies when observed by light microscopy revealed a large majority of rod forms and few coccoid forms.

V. ^{3}H-Thymidine Incorporation into Rods and Coccoid Forms

The results obtained from the above experiments are represented graphically in Fig. 17. It shows that both rods and cocci incorporate ^{3}H-Thymidine into their DNA and the increased labeling shows that both are undergoing the preparation (DNA synthesis) for cell division. This shows that both rods and coccoid forms are capable of undergoing cell division.

Fig. 18. Section of the root hair surface showing attachment of capsulated coccoid bacteria to the root hair surface. The root hair cytoplasm is plasmolyzed in this fixative and therefore not seen. Note the capsular material (arrows) and its attachment to the root hair surface; poly-β-hydroxybutyric acid (Pb) and glycogen granules can be seen.

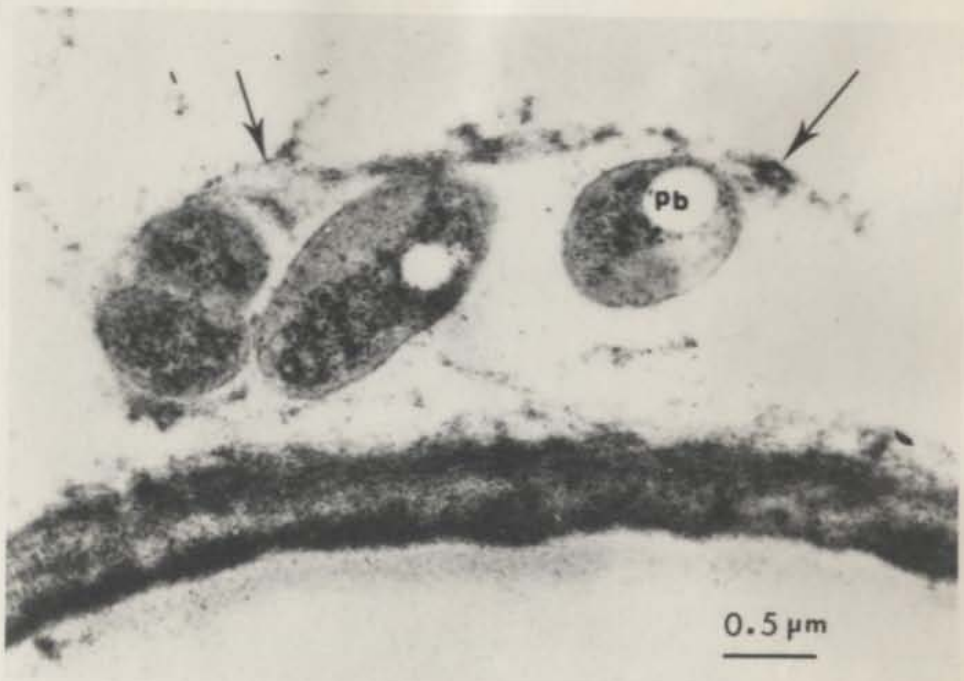


Fig. 18

Table 2: Nodulation of soybean infected with different fractions of *R. japonicum* 61A76 in modified Leonard's bottle jar assembly.

Fractions	Number of nodules
1. Coccoid	3.5 ± 5.2
2. Rod	5.5 ± 6.8
3. Heterogeneous	2.7 ± 3.6
4. Control without bacteria	ZERO

VI. Root Hair Attachment Studies

When the 5 day old soybean roots were dipped in a bacterial suspension and analyzed for bacterial attachment to the root hair surface, the number of root hairs showing the attached bacteria were low. Of the 1,798 root hairs counted, only about 5% revealed bacterial attachment and the number of bacteria attached per root was highly variable (1 to 16). Examination by light microscope showed that there was a random attachment of both coccoid and rod forms of cells. The rods showed distinct polar attachment perpendicular to the root hair surface. At the ultrastructural level, only the attachment of capsulated coccoid forms were visible in sections of root hairs (Fig. 18).

It was difficult to fix both the bacteria and the root hair using the same fixative. The root hairs were plasmolyzed as seen in Fig. 18 and therefore root hair cytoplasm was not visible in the particular area. The electron micrograph reveals the persistence of capsule (arrows) after attachment of the bacteria.

VII. Nodulation Experiments

The nodulation tests of 25 soybean plants in each case indicated that both the coccoid and rod fractions were capable of inducing nodule formation in soybeans (Table 2).

Fig. 19. Saturation curve of ^{125}I-soybean lectin binding to 100 µg dry weight of cells of *R. japonicum* 61A76. Note the drop in the curve after 400 µg/ml concentration of lectin.

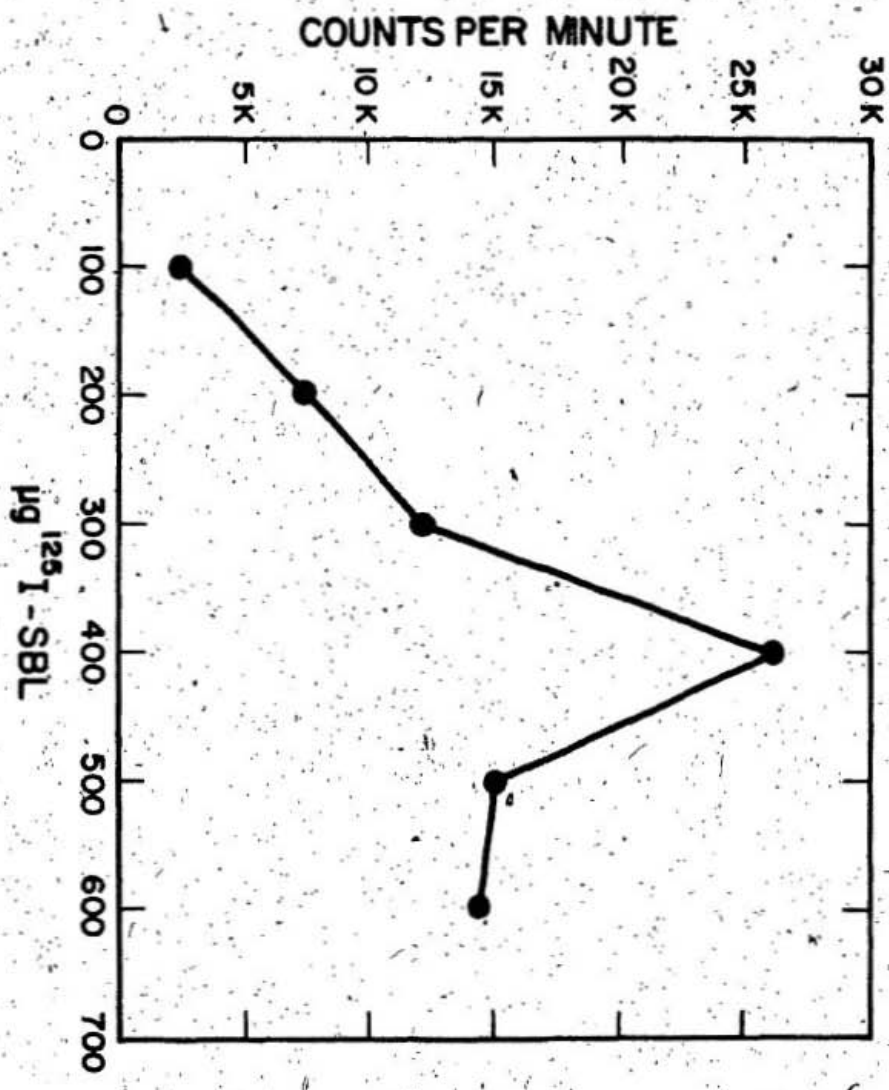

COUNTS PER MINUTE
0
5K
10K
15K
20K
25K
30K
0
100
200
300
400
500
600
700
µg 125I-SBL

Fig. 19

Table 3: The inhibitory effect of N-acetylgalactosamine and Galactose on ^{125}I-soybean lectin binding to Rhizobium japonicum 61A76.

	Experimental	Cpm
1.	350 µg of ^{125}I-soybean lectin (control)	29,439
2.	N-acetylgalactosamine (5mM)	224
3.	Galactose (50mM)	13,442

Table 4: ^{125}I-soybean lectin binding to rod and coccoid forms.

	Rod forms	Coccoid forms
Affinity constant	$1.0 \times 10^{5}\ M^{-1}$	$1.1 \times 10^{6}\ M^{-1}$
Number of soybean lectin molecules bound per microgram dry weight of bacteria	1×10^{15}	1×10^{14}

Fig. 20. Concentration dependence of ^{125}I-soybean lectin binding to the coccoid and rod forms of cells of R. japonicum 61A76, shown as a double reciprocal plot of the molar concentration of free and bound lectin.

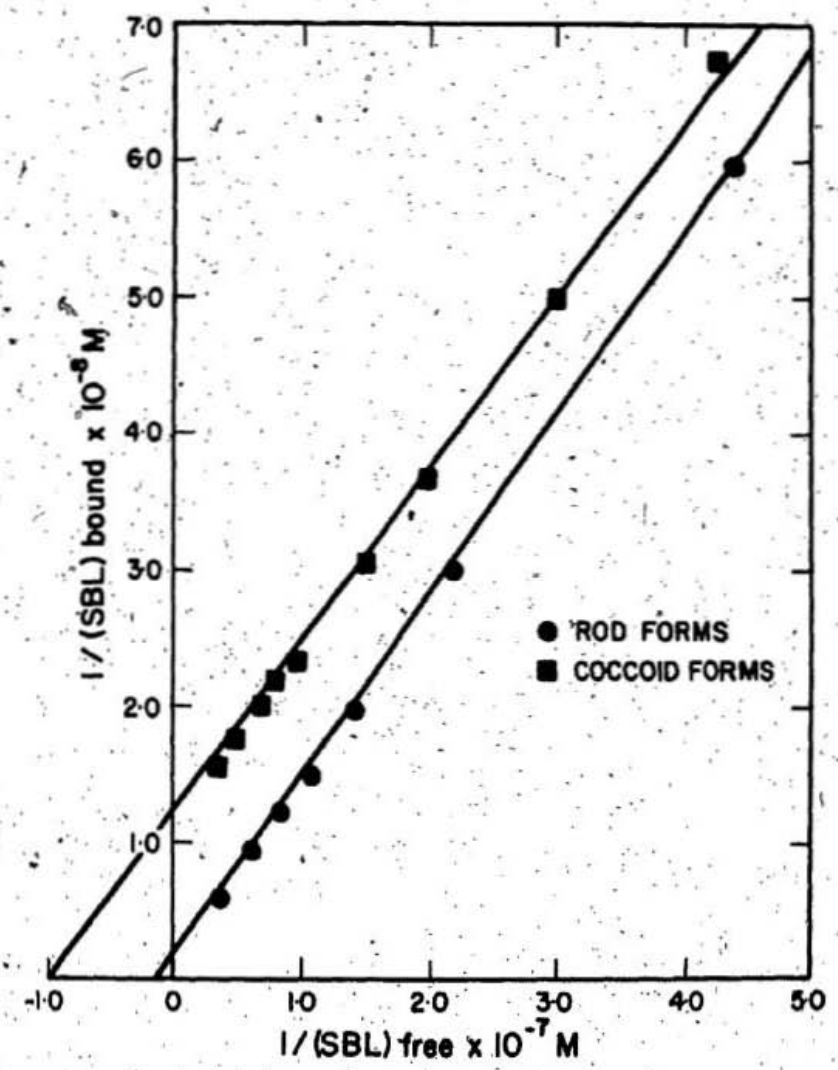

7·0
6·0
5·0
4·0
3·0
2·0
1·0
1/(SBL) bound x 10⁻⁹ M
ROD FORMS
COCCOID FORMS
-1·0
0
1·0
2·0
3·0
4·0
5·0
1/(SBL) free x 10⁻⁷ M

Fig. 20

Fig. 21. Growth curve of R. japonicum 61A76 in yeast extract mannitol broth culture (0.1% yeast extract) at 220 rpm with and without 350 µg per ml of soybean lectin.

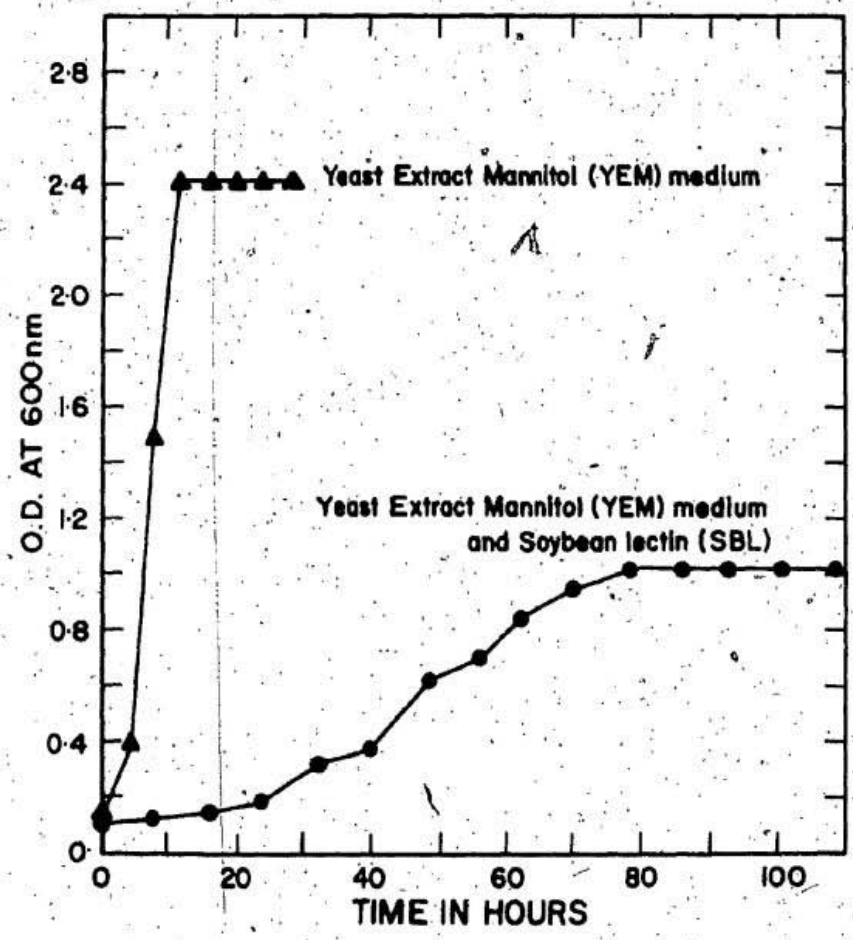

2·8
2·4
2·0
1·6
1·2
0·8
0·4
0
O.D. AT 600nm
Yeast Extract Mannitol (YEM) medium
Yeast Extract Mannitol (YEM) medium
and Soybean lectin (SBL)
0
20
40
60
80
100
TIME IN HOURS

Fig. 21

VIII. Labeled Soybean Lectin Binding Studies

(a) ^{125}I-Soybean Lectin Binding

The radioiodinated soybean lectin showed saturation at a concentration between 350 to 400 µg/ml with respect to its binding to the bacteria grown in yeast extract mannitol medium. There was a drop in lectin binding at higher concentrations (Fig. 19). The binding kinetics revealed an affinity constant of $4 \times 10^{7} M^{-1}$ and the average number of soybean lectin molecules bound per µg dry weight of cells was 9×10^{4}.

The control experiments set up with galactose and N-acetylgalactosamine showed that galactose at 50mM causes partial inhibition of soybean lectin binding and N-acetylgalactosamine at 5mM causes total inhibition of soybean lectin binding to R. japonicum cells in broth culture (Table 3).

The binding kinetics of ^{125}I-Soybean lectin in both coccoid and rod forms revealed minor differences (Table 4). Fig. 20 shows a monophasic linear relationship between $^{1}/_{(SBL)}$ bound and $^{1}/_{(SBL)}$ free over a range of molar concentrations employed.

(b) Effect of Soybean Lectin on the Growth of R. japonicum in Broth Culture

There were observable differences in the growth pattern of R. japonicum grown in yeast extract mannitol medium with 350 µg/ml of soybean lectin as shown in Fig. 21. The percentage of coccoid forms (70 - 80%) was higher

Fig. 22. Growth of R. japonicum 61A76 in soil extract medium broth culture with and without 350 µg per ml soybean lectin.

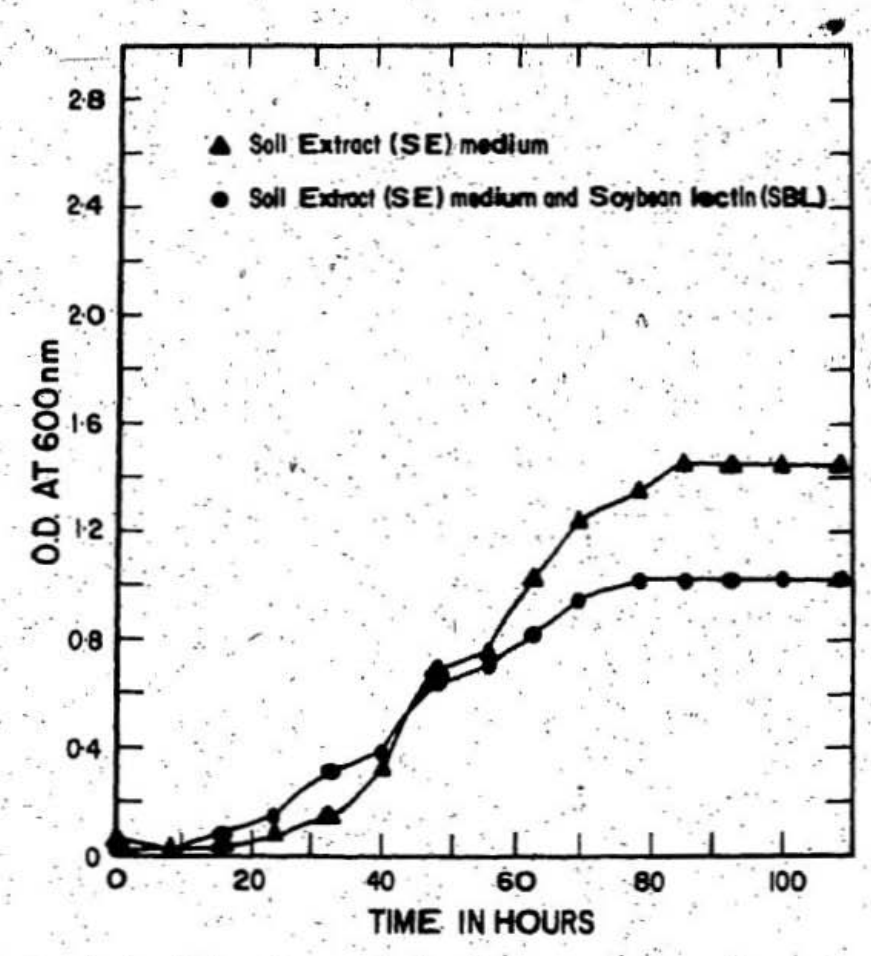

Soil Extract (SE) medium
Soil Extract (SE) medium and Soybean lectin (SBL)
O.D. AT 600nm
2·8
2·4
2·0
1·6
1·2
0·8
0·4
0
0
20
40
60
80
100
TIME IN HOURS

Fig. 22

Table 5: The characteristics of R. japonicum 61A76 grown in yeast extract mannitol (YEM) and soil extract (SE) medium.

	YEM Medium	SE Medium
Generation time	6.5 hr	14.5 hr
Percentage of coccoid forms	45	70
Size of coccoid forms	0.5-2.5 µm	1.5-3.0 µm
Size of rod forms	3.5-5 µm	4-5.6 µm

Fig. 23. Ultrathin section of the coccoid fraction of cells of R. japonicum 61A76 held together in a common capsular material.

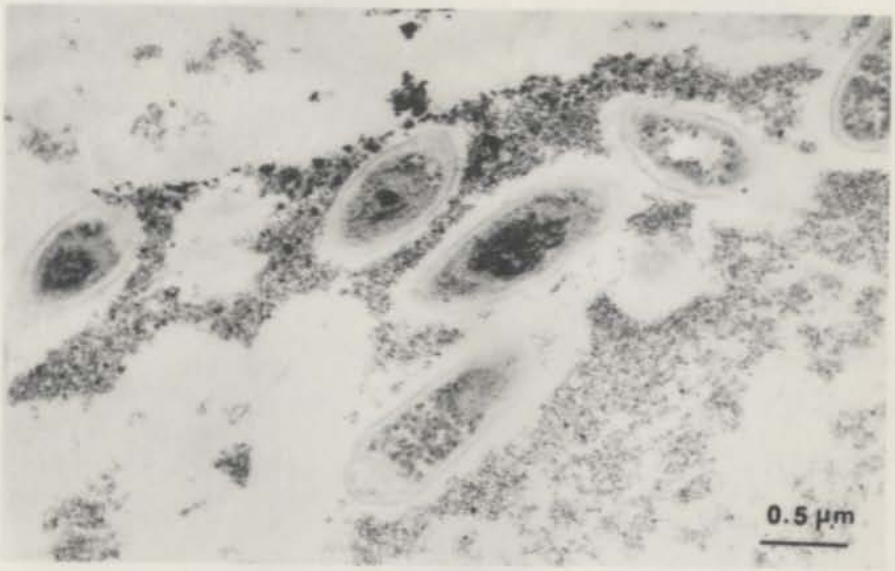


Fig. 23

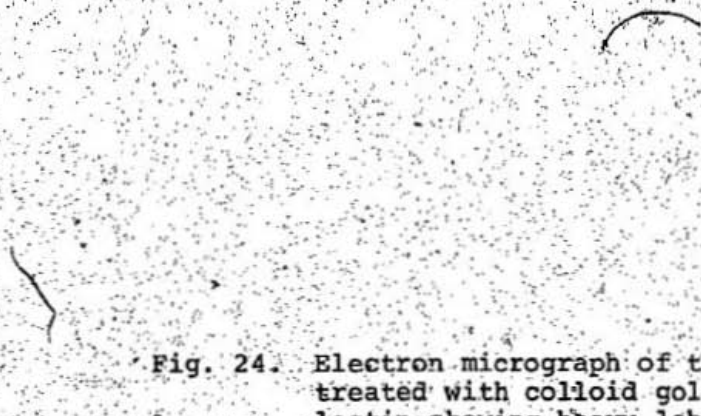


Fig. 24. Electron micrograph of the rod fraction treated with colloid gold labeled soybean lectin showing heavy labeling in the polar capsule (arrows).

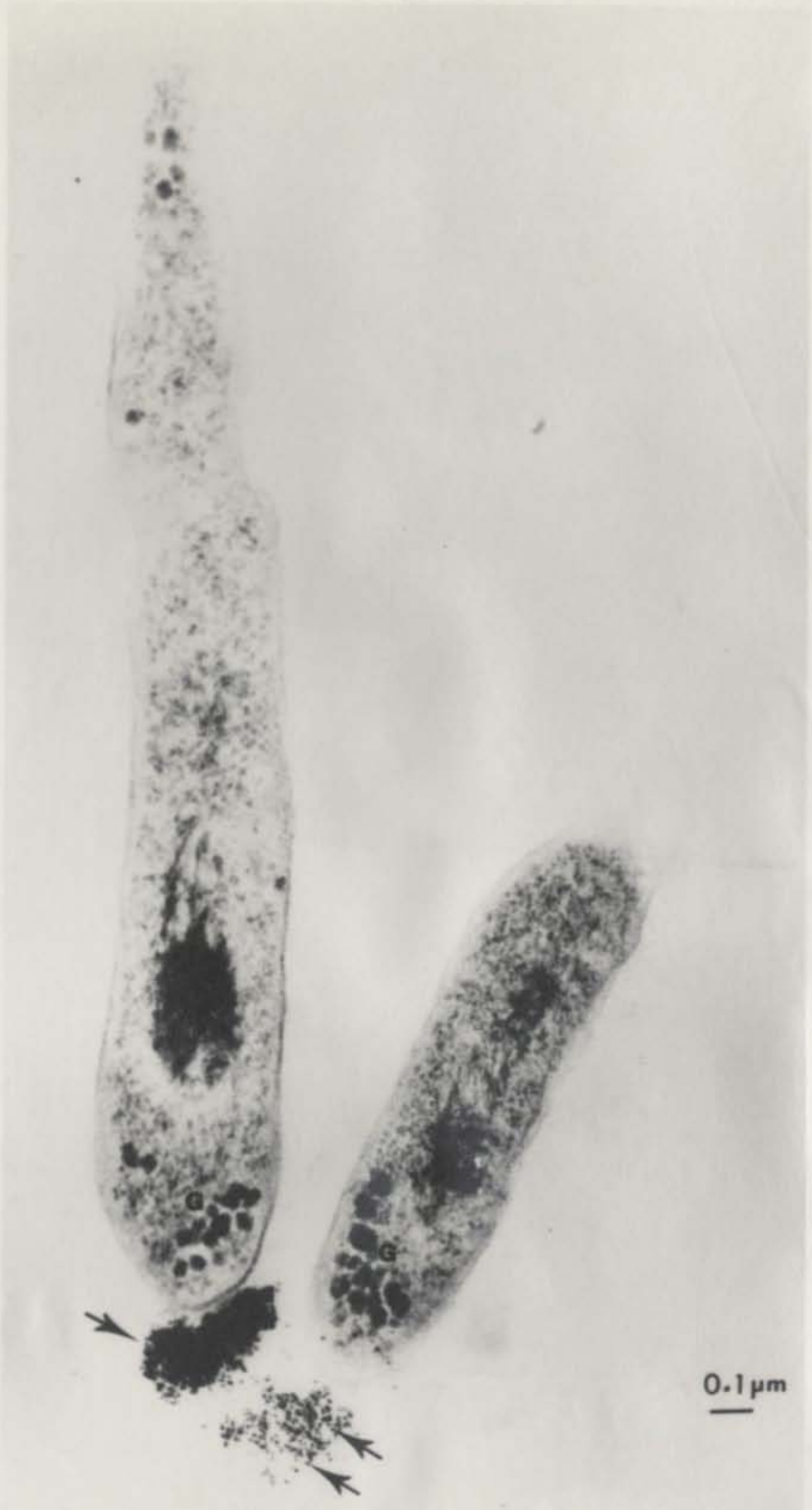


Fig. 24

Fig. 25. Electron micrograph of the rod fraction treated with colloidal gold labeled soybean lectin. Note the colloidal gold particles in the capsular material (arrows) at the polar tips of the rods and also the accumulation of glycogen (G) defining the polarity. The figure represents a section through the centre of 'star' showing the common capsular material.

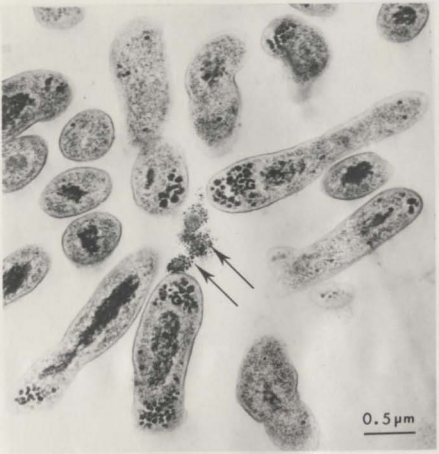


Fig. 25

Fig. 26. (Top) Capsulated coccoid cell showing ferritin-conjugated soybean lectin (arrow) in the amorphous capsule material. Lectin binding was carried out on fixed bacteria.

(Bottom) Capsulated coccoid cell showing ferritin-lectin binding (arrow). In this in vivo experiment, lectin binding was carried out in live bacteria, which were fixed after thorough washing. Note the fibrous nature of the capsule.

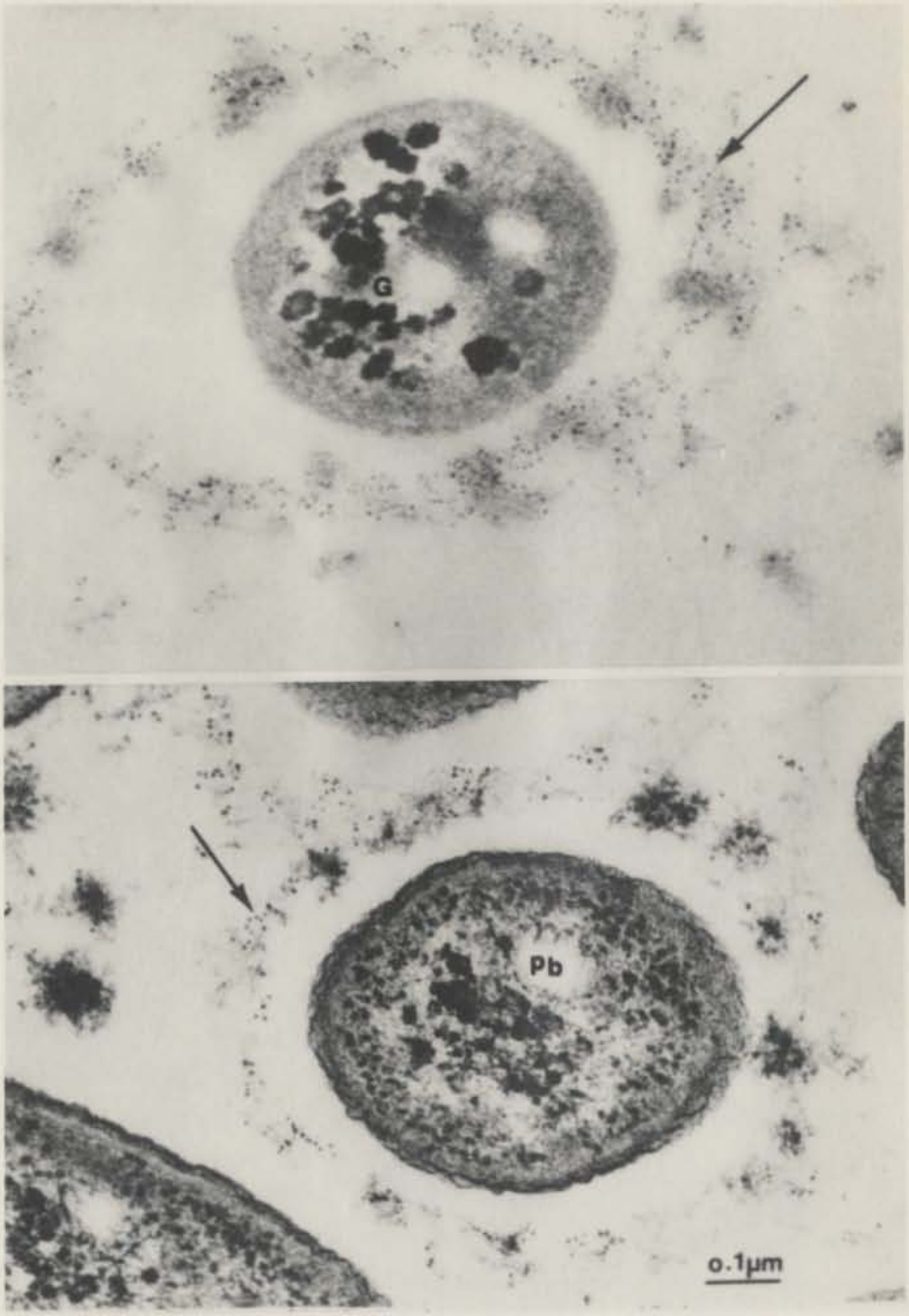


Fig. 26

than in the control medium seen in soil extract medium with and without 350 µg/ml of soybean lectin (Fig.22) Table 5).

IX. Labeled Soybean Lectin Binding Studies in Chemically Fixed Preparations

(a) Colloidal Gold Labeled Soybean Lectin

Ultrathin sections of aldehyde fixed bacteria treated with colloidal gold labeled soybean lectin revealed gold label in the capsular material surrounding the coccoid forms (Fig. 23). The rod forms showed gold labeling in the polar capsules (Fig. 24) and in the polar capsular material of the rods in the 'stars' (Fig. 25). In both the cases a gap appeared between the cell wall and capsular material.

(b) Ferritin Conjugated Soybean Lectin

When the cells were aldehyde fixed, washed and then treated with ferritin conjugated with soybean lectin for 1 hr, the label was seen distinctly in the capsular material of the coccoid forms (Fig. 26). The capsular material appeared loose and there was a gap between the cell wall and the capsule. When the treatment of ferritin conjugated soybean lectin was followed by fixation, the site of labeling remained the same (Fig. 26) but the capsular material appeared fibrillar. The gap described earlier between the cell wall and the capsule was also apparent in this preparation.

Fig. 27. Whole mount of a capsulated cell of R. japonicum 61A76 showing colloidal gold lectin label (arrows) around the capsule and in the loose capsular material (arrows).

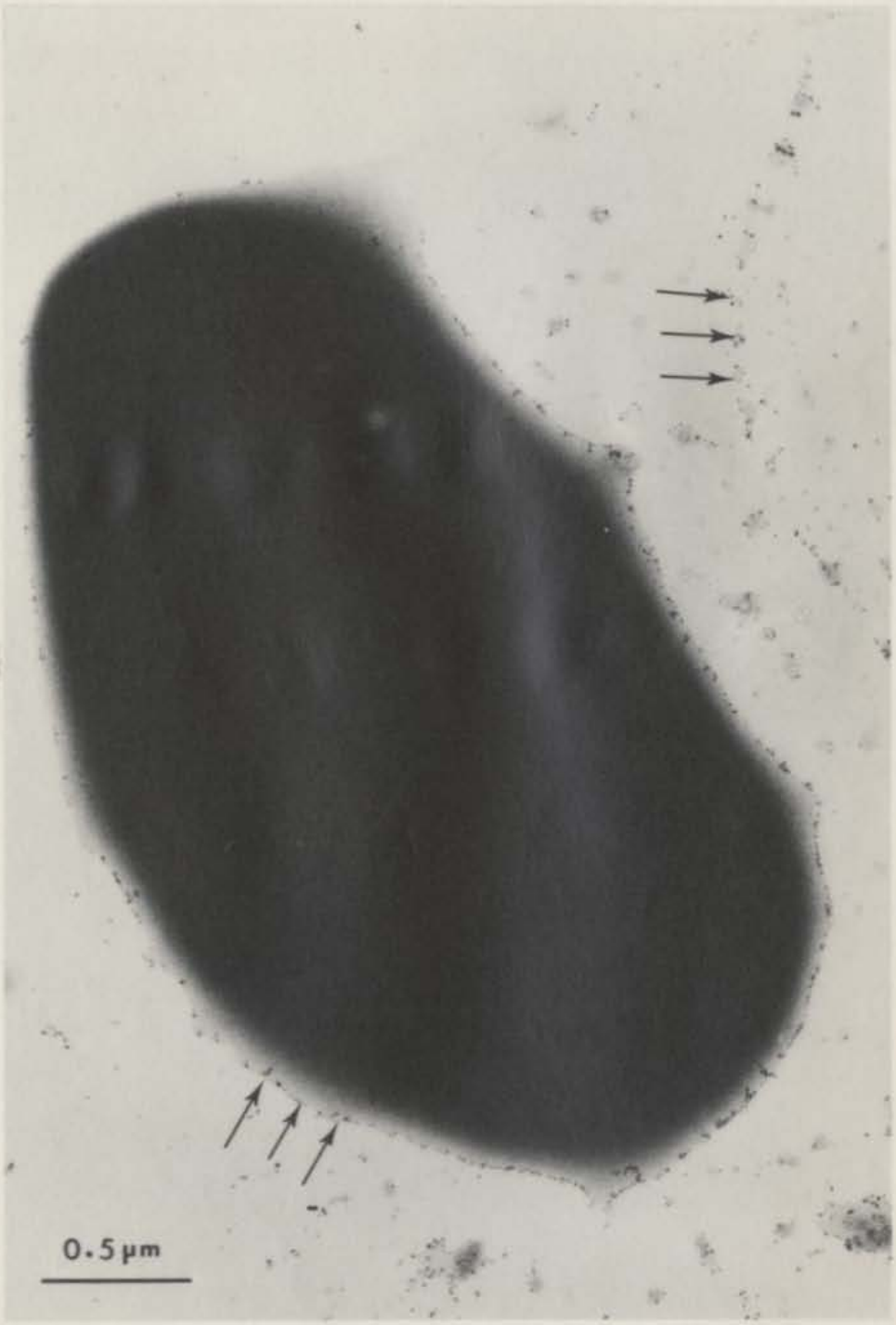


Fig. 27

Fig. 28. Whole mount of a non-capsulated R. japonicum 61A76 cell showing colloidal gold labeled soybean lectin label directly on the cell wall. The labeling can be seen in the form of clusters (arrows).

Fig. 28

Fig. 29. Whole mount preparation of R. japonicum 61A76 treated with colloidal gold labeled soybean lectin. The cells were neither chemically fixed nor stained. Note the two binding sites for soybean lectin (arrows) Viz., the primary binding site in the extracellular capsule and secondary binding site in the outer cell wall. Insert: a low power picture of the same.

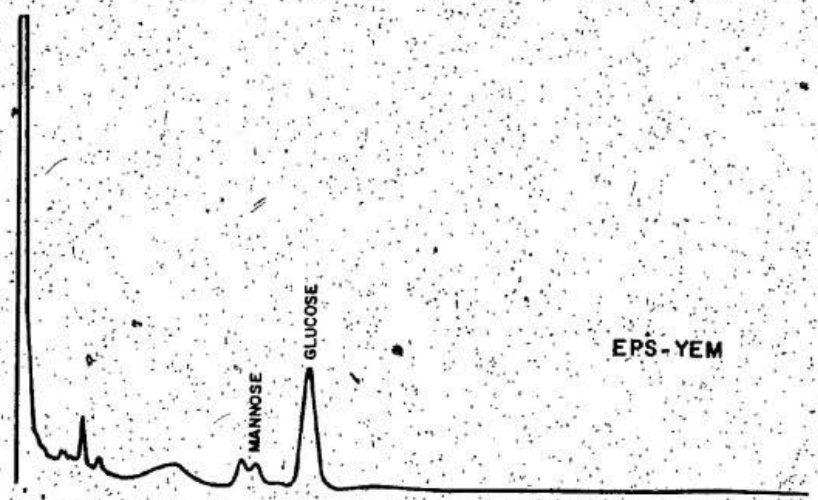
Fig. 29

X. Colloidal Gold Labeled Soybean Lectin Binding in Chemically Unfixed Whole Mounts of Bacteria

Whole mounts of bacteria made after treatment with colloidal gold labeled soybean lectin revealed similar labeling of the capsular material with soybean lectin. The capsule in Fig. 11 is massive showing gold label and surrounded by loose diffusing capsular material. The diffusion of capsular material labeled with gold lectin is clearly exhibited in Fig. 27 (arrows). The condensed capsular material which is bound to the cell is shown to be labeled. The gold labeling was also seen in close proximity to the cell wall (Fig. 28). Small coccoid forms without any capsule distinctly revealed coccoidal gold label on the cell wall. (Fig. 28 arrows). In most cases the label was clustered. Fig. 29 shows dual lectin binding to capsule and the cell wall.

In all the three labeled soybean lectin binding experiments N-acetylgalactosamine and galactose treated cells were used as controls. The results from ^{125}I-Soybean lectin binding experiments indicated a total inhibition of soybean lectin binding by N-acetylgalactosamine and partial inhibition by galactose. In electron microscopy, the bacterial cells treated with N-acetylgalactosamine were completely devoid of any gold or ferritin label.

Fig. 30. Gas-liquid chromatograms of alditol acetates of sugars from R. japonicum 61A76 extracellular polysaccharides. EPS-YEM=Extracellular polysaccharide extracted from the bacteria grown in yeast extract mannitol medium; EPS-S=Extracellular polysaccharide from the bacteria grown in soil extract medium.



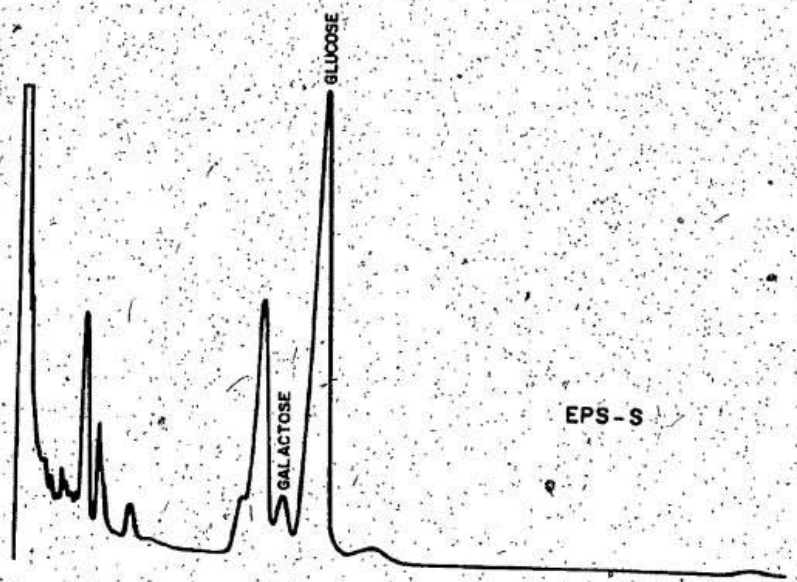


Fig. 30

Fig. 31. Gas-liquid chromatograms of alditol acetates of sugars from R. japonicum 61A76 lipopolysaccharides. LPS-YEM=Lipopolysaccharide from the bacteria grown in yeast extract mannitol medium; LPS-S=Lipopolysaccharide from soil extract medium.

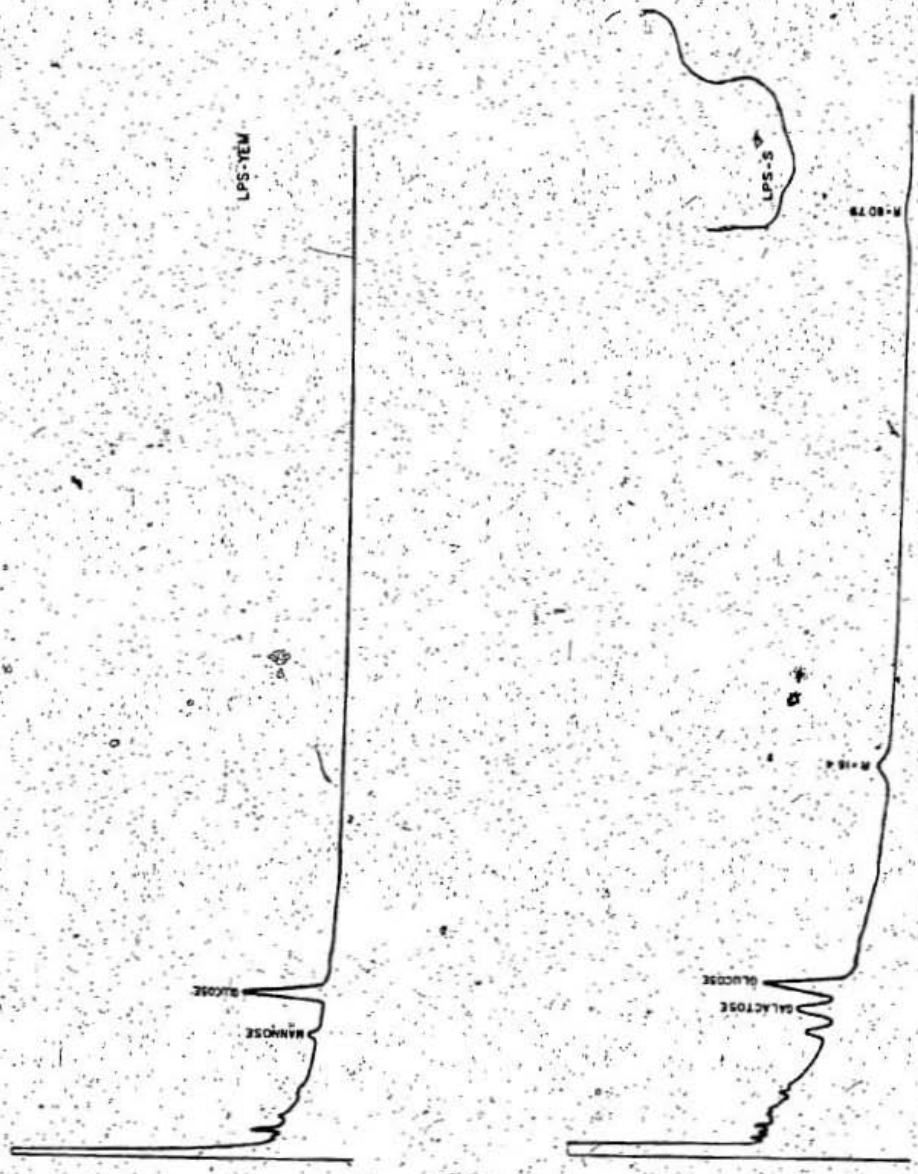
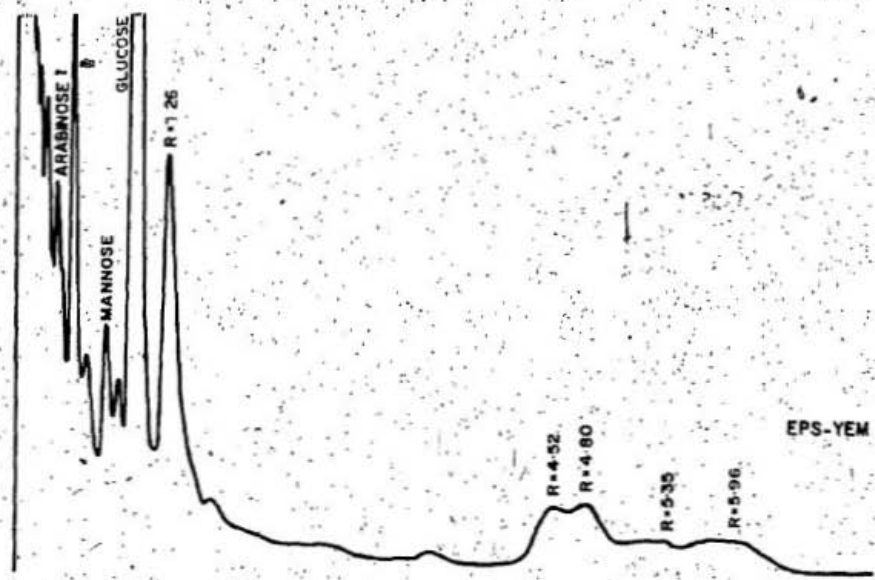

LPS-TEM
LPS-S
M-80 TB
M-16 K
GLUCOSE
MANNOSE
GLUCOSE
GALACTOSE
GLUCOSE

Fig. 31

Fig. 32. Gas-liquid chromatograms of amino sugar alditols from R. japonicum 61A76 extracellular polysaccharides. EPS-YEM= Extracellular polysaccharide from the bacteria grown in yeast extract mannitol medium; EPS-S=Extracellular polysaccharide from the bacteria grown in soil extract medium.


ARABINOSE I
MANNOSE
GLUCOSE
R•1·26
R•4·52
R•4·80
R•5·35
R•5·96
EPS-YEM

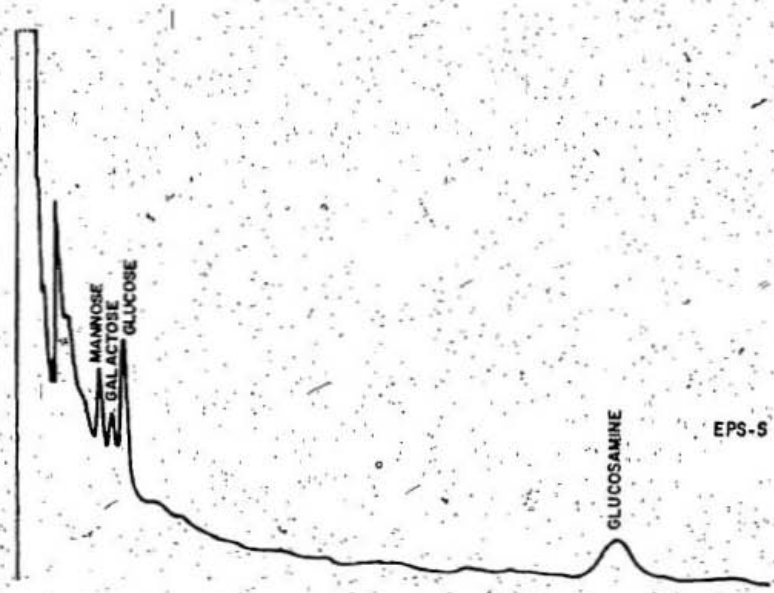

MANNOSE
GALACTOSE
GLUCOSE
GLUCOSAMINE
EPS-S

Fig. 32

Fig. 33. Gas-liquid chromatograms for amino sugar alditols from R. japonicum 61A76 lipopolysaccharides. LPS-YEM=Lipopolysaccharide from bacteria grown in yeast extract mannitol medium; LPS-S=Lipopolysaccharide from bacteria grown in soil extract medium.

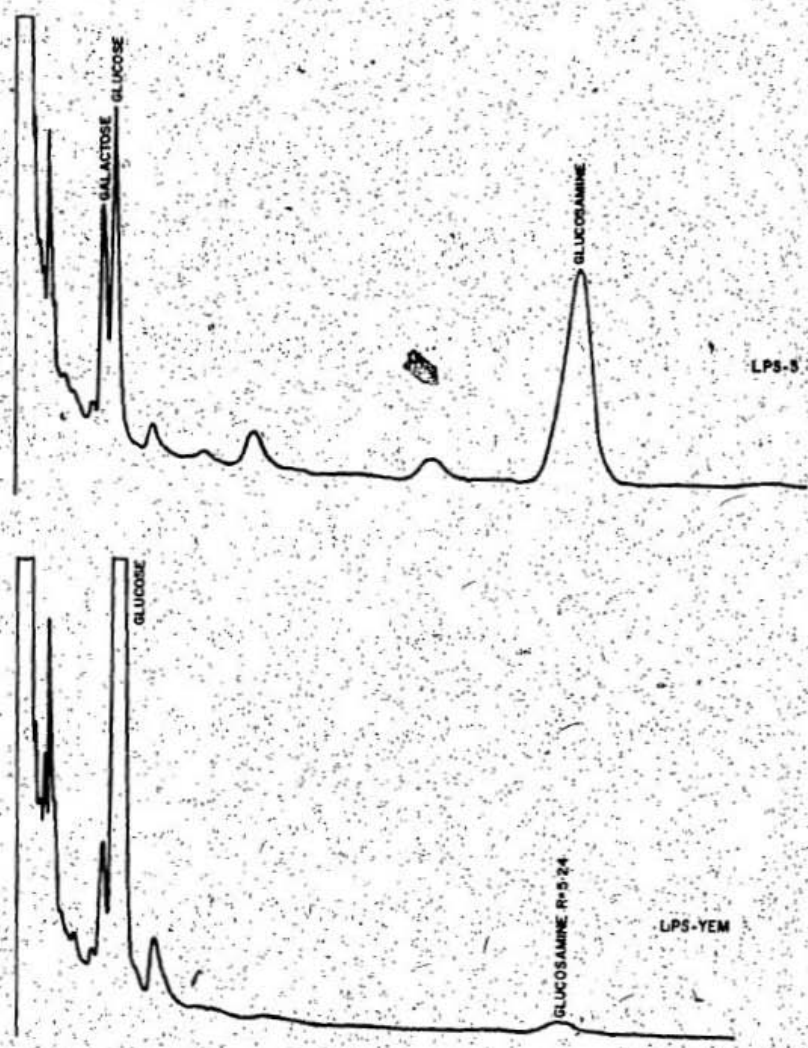

GALACTOSE
GLUCOSE
GLUCOSAMINE
LPS-5
GLUCOSE
GLUCOSAMINE R=5-24
LPS-YEM

Fig. 33

Table 6. Gas Liquid Chromatography (GLC) analysis of acetylated alditols (as moles %) of Lipopolysaccharides (LPS) and Extracellular Polysaccharides (EPS) of Rhizobium japonicum 61A76.

Culture media	Fractions analyzed	Relative retention time (glucose)						
		0.23	0.74	0.80	0.86	1.00	2.22	5.35
		rhamnose	unknown	mannose	galactose	glucose	heptose	glucosamine
Yeast extract mannitol (YEM)	Extracellular polysaccharide (from 1 litre of supernatant)	8.01	11.66	10.03	-	70.29	-	-
	Lipopolysaccharide yield = 0.6% dry wt. of cells	7.66	-	5.79	-	86.22	trace	trace
Soil extract (SE)	Extracellular polysaccharide (from 1 litre of supernatant)	9.82	-	29.3	5.59	52.77	-	-
	Lipopolysaccharide yield = 0.3% dry wt. of cells	-	-	10.04	23.55	47.03	11.08	8.03

Table 7. ^{125}I-soybean lectin binding to R. japonicum 61A76 grown in yeast extract mannitol and soil extract medium.

	YEM Medium	SE Medium
Affinity constant	4×10^7 M^{-1}	5×10^6 M^{-1}
Average number of soybean lectin molecules bound per microgram of dry weight of cells	9×10^4	9×10^7

XI. Carbohydrate Analyses of Extracellular and Lipopolysaccharides of R. japonicum 61A76 in Yeast Extract Mannitol and Soil Extract Medium

In order to detect the soybean lectin specific sugars (galactose and N-acetylgalactosamine), the sugar analyses of extracellular and lipopolysaccharides from R. japonicum 61A76 cells grown in yeast extract mannitol and soil extract media were carried out using gas chromatography (Figs. 30, 31, 32, 33). A comparison of sugars analyzed (Table 6) indicated that there was no detectable quantity of soybean lectin specific sugars in either of the fractions from the bacteria grown in yeast extract mannitol medium. But, the bacteria grown in soil extract medium showed detectable amount of soybean lectin specific galactose in both the fractions of polysaccharides. By paper or thin layer chromatography it was not possible to detect the presence of any acidic groups like uronic acid in the extracellular polysaccharides.

The radioiodinated soybean lectin studies (Table 7) indicated that there is an increased amount of lectin bound to the bacteria grown in soil extract medium when compared to those grown in yeast extract mannitol medium.

DISCUSSION

The intent of the present study was to examine how both the partners control specificity as mediated by legume seed lectins in soybean-*R. japonicum* interaction.

Although specificity operates at rhizosphere, rhizobial and legume root hair surface interphase, *Rhizobium* induced root hair curling, invagination of the root hair cell wall and infection thread formation, the present discussion will be strictly confined to *lectin mediated specific interaction*. Hamblin and Kent (1973) suggested that phytohemagglutinins from *Phaseolus vulgaris* might be involved in the specificity. They showed the presence of high lectin activity in the cotyledons by means of agglutination of human erythrocytes 'A' and the homologous rhizobia. The secretion of lectin on the root hairs was demonstrated by the attachment of erythrocytes in few localized regions of the root hair. When the nodulated roots below the nodule region were tested for lectin activity, it gave strong results. These results led them to suggest that lectins were capable of binding bacteria to the roots of *P. vulgaris* L. at sites suitable for the infection of the plant by the bacteria (root hairs). This was followed by Bohlool and Schmidt's (1974) extensive work on soybean lectin labeled with fluorescein isothiocyanate binding to 22 of 25

strains of Rhizobium japonicum which are capable of nodulating soybean. The lectin did not bind to any other Rhizobium species which do not nodulate soybeans. On the basis of their study, they suggested that legume seed lectins specifically interacts with the Rhizobium cell surface and may account for the specificity expressed between rhizobia and host plant in the initiation of the nitrogen fixing symbiosis.

Several subsequent studies identified other legumes which had lectins that bound specifically to the homologous Rhizobium Spp (Dazzo and Hubbell, 1975; Solheim, 1975; Wolpeft and Albersheim, 1976; Kamberger, 1979a, 1979b). These studies indicated that recognition may involve the binding of lectins to specific carbohydrates on the cell surface of Rhizobium. Appropriately, the work of Bowles and Kauss (1975) showed that legumes have multiple lectins with different carbohydrate specificities. In Rhizobium-clover symbiosis, the infective bacteria selectively adhere to the root hairs within hours after inoculation. Electron microscopic examination of the initial "docking" stages of attachment shows a common feature: the adherent rhizobial cell is surrounded by a fibrillar capsule in contact with electron-dense globular aggregates normally having a high affinity for the outer periphery of the cell wall of the legume root hair (Dazzo and Hubbell, 1975a; Bal et al., 1978). Immunochemical (Dazzo and Hubbell, 1975b; Dazzo and Brill, 1979) and genetic studies (Bishop et al., 1977; Dazzo and Brill, 1979)

suggest that this selective adherence may be initiated by a specific cross-bridging of antigenically related saccharide determinants on the surface of both the bacterium and the cell wall of the clover root hair by a multivalent, plant host-coded lectin called 'Trifoliin' (Dazzo et al., 1978). The receptor sites on clover roots which selectively bind both R. trifolii and its capsular polysaccharide accumulate at the growing tips of root hairs and diminish towards the base of the root hairs, thus matching the cell surface distribution of trifoliin on roots (Dazzo and Brill, 1977; Dazzo et al., 1978; Dazzo and Brill, 1979b).

Several experiments have shown that trifoliin and the antibody to clover root antigen bind to the same or similar overlapping determinants on R. trifolii and their exposure is necessary for the attachment of the bacteria to the root hairs. The sugar 2-deoxyglucose specifically inhibits both the agglutination of R. trifolii by either trifoliin or the anti-clover root antibody (Dazzo and Hubbell, 1975b; Dazzo and Brill, 1977). Also, this sugar specifically elutes trifoliin from intact clover root or from R. trifolii if precoated with trifoliin (Dazzo and Brill, 1977). Monovalent antigen-binding (Fab) fragments of immunoglobulin G antibody against the clover root antigens block the agglutination of R. trifolii by purified trifoliin, and these monovalent Fab fragments also block the binding of R. trifolii to clover root hairs (Dazzo and Brill, 1979). Intergeneric transformation studies indicate that genes

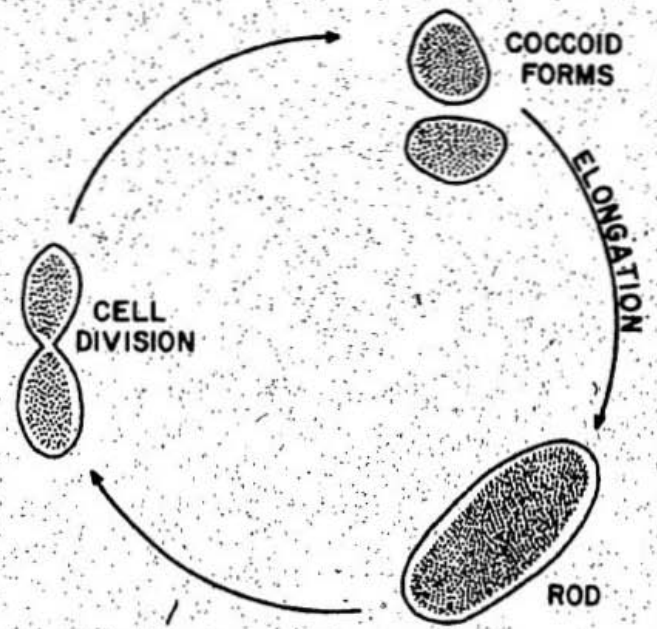
controlling the synthesis of the surface determinants on R. trifolii which bind trifoliin and anti-clover root antibody are co-transformed into Azotobacter vinelandii with a frequency of 100%, and only the hybrid transformants which carry this surface determinant bind in high numbers to clover root hair tips (Bishop et al., 1977; Dazzo and Brill, 1979). Because these hybrids selectively adhere to clover root hairs but do not infect them, it is clear that selective adherence via lectin binding to cell surface receptors, is only one of the requirements needed to trigger root hair infection. The selective ability of R. trifolii to adhere to clover robt hairs is influenced by conditions that affect the accumulation of trifoliin on the host root surface and the saccharide receptor on the bacterium (Dazzo and Brill, 1978).

The foregoing studies with Rhizobium-clover symbiosis strongly indicated the possibility of lectin mediation in the specificity and recognition between Rhizobium and legume interaction.

I. Culture Conditions and Growth Pattern in Rhizobium japonicum

The generation time of R. japonicum Nitragin 61A76 grown in yeast extract mannitol medium varies depending on the size of the inoculum. Rhizobium is a soil bacterium and it is quite reasonable to expect them to thrive very well in a soil based environment. There is experimental evidence that more number of Rhizobium occur in rhizosphere

Fig. 34. Schematic diagram of the life cycle of R. japonicum 61A76 in yeast extract mannitol medium broth culture. The top sketch shows the interconversion of rod and coccoid forms. The bottom sketch reveals the formation of 'stars'. The rod forms divide to give rise to coccoid forms accompanied by the synthesis of extracellular capsule and the cocci divide within the capsule resulting in the formation of a cluster of coccoid forms. These further elongate to form stars leaving behind a common central capsular material.


COCCOID
FORMS
ELONGATION
CELL
DIVISION
ROD

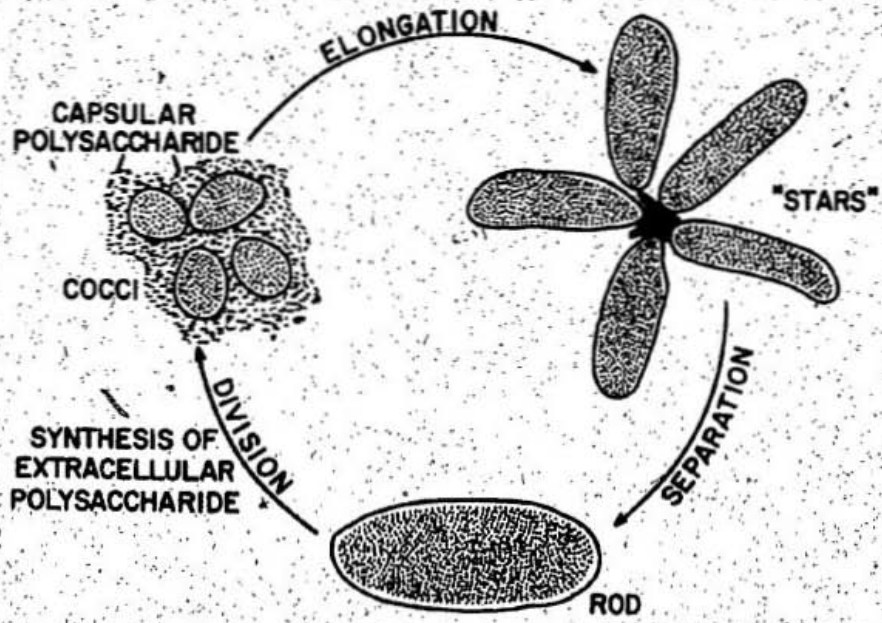

ELONGATION
CAPSULAR
POLYSACCHARIDE
"STARS"
COCCI
DIVISION
SEPARATION
SYNTHESIS OF
EXTRACELLULAR
POLYSACCHARIDE
ROD

Fig. 34

and rhizoplane environments (Dart and Mercer, 1964; Rovira, 1965, 1969; Bowen and Rovira, 1976; Bhuvaneswari and Bauer, 1978; Reyes and Schmidt, 1979; Schmidt, 1979). It seems that rhizosphere environment provides certain rhizobial specific growth factors (Rovira, 1969). Hence, the soil extract was chosen to monitor the growth pattern of Rhizobium japonicum. The bacterial growth is retarded as indicated by a mean generation time of 15 hrs. Since the soybean lectin binds to R. japonicum in broth culture (Bhuvaneswari et al., 1977) the growth pattern of R. japonicum 61A76 was studied in both yeast extract mannitol and soil extract medium with saturating concentrations of (350 µg/ml) lectin added.

The generation time increased from 6.5 hr to 20 hrs in yeast extract mannitol medium with lectin. There was an increase in number of coccoid forms of cells (70-80%) as opposed to that found in the control medium (30-40%). This is a clear indication that elongation of the coccoid forms to rod forms is inhibited. Under similar growth conditions, without the soybean lectin, the coccoid forms would have converted themselves into a majority of rod forms. It appears that coccoid and rod forms are two different phases of growth, as revealed by the interconversion studies of both rod and coccoid forms.

From the interconversion studies of coccoid and rod forms in liquid culture and on agar plates, it was possible to build a schematic diagram of the life cycle of R. japonicum cells (Fig. 34). A single coccoid cell undergoes elongation to form a rod which undergoes

further cell division to form cocci. Electron microscopic observations of the R. japonicum broth cultures have shown that a single capsulated coccoid form undergoes repeated cell divisions to give rise to a group of coccoid forms held together in a capsular matrix. Each one of them elongate to form 'stars' with a central capsular material (Fig. 34). These elongated rods separate and divide to give rise to cocci. Such a pattern of life cycle seems to be reasonable. A similar life cycle was also conceived by Bewley and Hutchinson (1929) and Hubbell (1962).

The increased number of coccoid forms in broth culture where soybean lectin was added, is indicative of the inhibitory effect of soybean lectin on elongation and formation of rods.

Similar retardation in growth of R. japonicum in soil extract medium with soybean lectin was observed as indicated by lengthening of the generation time to 20 hrs from 15 hrs. These results have important consequences from the point of view of 'infection' process. Apparently, capsulated coccoid cells are the "infective forms". It would be safe to assume that the chances of infection would increase in soil extract medium, which induces more capsulated cells to be formed. Usually, in natural soil, extracellular polysaccharide is subject to microbial degradation but in rhizosphere soil where Fe^{2+}, Ca^{2+} and Mg^{2+} are present, cationic complexes of polysaccharides are formed, which are not biodegradable (Lasik and

Gordiyenko, 1977). Therefore, such capsulated bacteria can thrive well. It appears that, at least in laboratory culture, soybean lectin facilitates formation of capsulated coccoid forms as revealed by the increased percentage of coccoid forms in soil extract grown R. japonicum.

II. ^{125}I-Soybean Lectin Binding Kinetics

Quantitative ^{125}I-soybean lectin binding studies carried out on R. japonicum 61A76 revealed that although the saturation point lies between 350-400 µg/ml of soybean lectin per 100 µg dry weight of cells, at higher lectin concentrations there is a drop in lectin binding. The drop beyond the saturation point is possibly due to scarcity of free lectin receptors on the cell surface (Bell, 1978). Such a phenomenon is exemplified in concanvalin A binding to erythrocytes (Linnemans et al., 1976) and hepatoma cells (Kaneko et al., 1975). One explanation that can be offered for the observed drop in lectin binding at higher concentrations is that the shearing forces created by surface molecular interactions would break up cell surface bonds and break down the cell surface receptors (Bell, 1978). It could, however, be due to negative cooperativity of lectin binding at high concentrations (Bhuvaneswari et al., 1977).

Quantification of soybean lectin binding kinetics showed an affinity constant of $4 \times 10^{7} M^{-1}$ which is in agreement with an earlier report (Bhuvaneswari et al., 1977). Rods and coccoid forms showed slight differences.

The amount of soybean lectin bound to R. japonicum cells grown in soil extract medium registered an increase over that of bacteria grown in yeast extract mannitol medium. This is an evidence for the appearance of increased lectin binding receptors in bacteria, cultivated in soil extract medium which is close to its natural environment. This led to the conclusion that a lectin mediated interaction between R. japonicum and soybean is better expressed in a soil based medium.

III. Heterogeneity in R. japonicum Broth Culture

Repeated observations of stationary phase broth culture cells under light and electron-microscopy revealed the presence of two morphologically distinct cells. Such an occurrence of different types of cells in pure cultures of bacteria are quite common (Wilson and Ashley Miles, 1966; Brinkenhoff, 1970; Clasener, 1972; Albersheim and Anderson Prouty, 1975). But their ultrastructural and functional characterization has been investigated only recently (Murray et al., 1965; Costerton et al., 1974; Cheng and Costerton, 1975; Irvin et al., 1975). It is only recently that such an attempt has been made in the bacteroid populations of soybean root nodules where four different functional and developmental types have been found (Ching et al., 1977). In free living cells of R. japonicum two forms were found, coccoid and rod forms. The coccoid forms varied in shape

from rounded to oval to small rods ranging in size from 0.5 - 2.5 µm in yeast extract mannitol medium and 1.5 - 3.0 µm in soil extract medium. The rod forms ranged in size from 3.5 - 5 µm in yeast extract medium and 4 - 5.6 µm in soil extract medium. Both the types of cells were distinguishable under both light and electron microscopes. Rod forms were often seen to aggregate to form stars. Heterogeneity within the 'star' is also evident in the whole mount preparation which has not been chemically fixed or stained. Out of five rod cells in the 'star' two of them are undergoing cell division which is evident from the constrictions. Only one of the rods had a sub-polar flagellum and all the five rod cells varied in size. It appears that for a 'star' to exhibit motility, it is sufficient if only one of them has a flagellum which can propel the entire constellation.

IV. Fractionation of Coccoid and Rod Forms

An attempt to fractionate these two types of cells with the help of sucrose density gradient centrifugation resulted in three overlapping fractions but the separations were not very clear. Therefore, differential centrifugation (12,000 x g and 115,000 x g) was employed to separate the two fractions. However, a slight contamination of rods and cocci in both the fractions were found. This fact has been borne in mind while discussing the functional characterization of these two fractions. Such

a heterogeneity is also characteristic of R. meliloti R. trifolii and R. leguminosarum and they could be separated by differential centrifugation. The heterogeneity was also evident in R. japonicum grown in soil extract medium.

V. Localization of Soybean Lectin Binding Sites

Soybean lectin binding studies were carried out using electron microscopy. Ferritin conjugated soybean lectin was used to localize extracellular capsule as the primary binding site for lectin. In Rhizobium studies the term capsule has been used to mean the loose extracellular polysaccharide (Dudman, 1968). Results of this study show that a clear distinction can be made between the loose and diffusing extracellular polysaccharide and a tightly bound massive capsular material. However, the labeled lectin binding studies strongly indicate that the materials are similar in nature from the point of view of lectin binding.

The capsular material is known to be a mucopolysaccharide (Dudman, 1964, 1968, 1972; Amarger et al., 1966; Zevenhuizen, 1971; Chaudhari et al., 1973; Hepper, 1975; Bjorndal et al., 1977; Dazzo and Brill, 1977). The absence of ruthenium red staining (Luft, 1968) suggests that the capsular polysaccharide is not acidic in nature. The specificity of soybean lectin lies in the presence of galactose and N-acetylgalactosamine residues of the capsular polysaccharide.

The thin layer and paper chromatography of extracellular polysaccharide from R. japonicum 61A76 grown in

both yeast extract and soil extract medium did not show any comparable acidic group when run with glucuronic acid as standard. However, there is a report of the presence of 4.0-methyl-D-glucuronic acid from R. japonicum strains 71A, CC708 and CB1795 (Dudman, 1978). In this study further chemical characterization of uronic acid was not carried out. The extracellular polysaccharides of different strains of Rhizobium japonicum and other slow growing rhizobia have shown extreme diversity in their composition (Dudman, 1976; Kennedy and Bailey, 1976; Kennedy, 1976; Jansson et al., 1977). Also, the degree of capsulation in various strains varies with the nature of the medium as well as conditions (Dudman, 1968).

It is noteworthy that in aldehyde fixed cells the ferritin conjugated soybean binds to the capsular material. The capsule, however, does not show direct contact with the cell surface in sections. The tenacity of lectin binding sites seems to become stronger because of cross-linkage by aldehyde fixation and can withstand extraction during processing for electron microscopy. In unfixed bacteria, although the primary lectin binding site remained unaltered, the capsule showed apparent fibrous nature. The gap between the capsule and bacteria seems to be an artifact of fixation.

These experiments were again repeated using colloidal gold labeled soybean lectin. The labeling of gold with soybean lectin was done by high speed centrifugation. Colloidal gold is electron dense and therefore easily

recognizable under the electron microscope. Particles of gold carry a net negative charge in water and their stability is maintained by electrostatic repulsion. The addition of strong electrolytes causes flocculation (Weiser, 1933). This can be prevented by adding a 'protective' protein colloid like soybean lectin. The proteins are adsorbed onto gold particles and are stabilized against subsequent flocculation by electrolytes (Horrisberger and Rosset, 1977). A detailed description of these methods concerning the versatility of gold method has been published by Horrisberger and Rosset (1977) and Horrisberger (1979).

In the gold method, the bacterial cells were treated with gold labeled lectin in suspension and were directly mounted on carbon stabilized formvar coated grids. The bacterial cells were neither chemically fixed nor stained thereby minimizing the formation of artifacts. The gold-lectin treated bacterial cells were also sectioned for transmission electron microscopy. It was evident that primary lectin binding sites were located in the extracellular capsule in both whole mounts and ultrathin sections. In certain non-capsulated bacteria, the gold lectin was intimately bound to the exposed cell wall. In most of these cells lectin binding is localized and clustered near a subpolar region of the bacteria. This binding has been designated as the secondary binding site.

In soybean lectin binding studies with ferritin-labeled lectin, the ultrathin sections did not reveal any

lectin binding to the cell wall. This may have been due to the solubilization of lipopolysaccharide of the outer cell wall during the alcohol dehydration process of the bacterial preparation for electron microscopy.

There is biochemical evidence for the presence of dual lectin binding sites (Bhagawat and Thomas, 1980) for peanut agglutinin; binding was reported to both EPS and LPS. The electron-microscopic study lends morphological evidence to the fact that soybean lectin also binds to both EPS and LPS. Therefore, there are two soybean lectin binding sites on R. japonicum cell surface; the primary lectin binding site in the capsular polysaccharide and secondary binding site in the cell wall lipopolysaccharide.

Mostly all the lectin binding cells had capsules. The capsulated cells were usually embedded in a common capsular matrix, presumably because the cells have divided within the capsular sheath. Chemically unfixed and unstained preparations of lectin treated cells showed that the capsular polysaccharide forms a compact sheath immediately surrounding the cells and are in turn surrounded by a loose material of polysaccharide. Both the compact sheath and loose material show soybean lectin binding indicating that both the materials may be similar in nature as far as their lectin binding property is concerned and that the capsule is in a dynamic steady state of secretion and diffusion.

VI. Root Hair Attachment Studies

The root hair attachment studies of bacteria supports the above electron microscopic observations that soybean lectin binds to the extracellular capsule and the outer cell wall of the bacteria. Only the capsular bacteria were found attached to the root hair surface after a thorough washing. The number of bacteria attached to root hairs was less than 5% of the 1,798 root hairs examined. This shows that R. japonicum 61A76 is a low lectin binding strain (Schmidt, E.L. Personal Communication). Less than 1% of the population possessed capsules. It took repeated scanning of a number of sections to locate a capsulated cell.

Attachment of capsulated bacteria to root hairs has been interpreted as the basis for specificity (Dazzo et al., 1976; Dazzo and Brill, 1977; Bal et al., 1978; Dazzo and Brill, 1979). Usually the coccoid forms held together in a common capsular matrix, seemed to attach firmly to the root hair surface (Bal et al., 1978).

It has been shown by Costerton et al. (1974, 1979) that in bovine rumen bacteria and bacteria firmly adhering to substrates under water in flowing streams or bacteria cultured on glass slides have an extracellular gummy polysaccharide by which these bacteria adhere or stick to the surface of substrates. That extracellular polysaccharide has been termed "glycocalyx". Bacterial pathogens often

have capsulated forms that are virulent and cause pathogenicity (Wilson and Ashley Miles, 1966; Clasener, 1972; Schuster and Coyne, 1974; Lippincot and Lippincot, 1975).

The rod forms of Rhizobium show polar attachment possibly due to the polar capsule. It appears that polar attachment, which can be observed by using the light microscope, does not withstand the preparations for transmission electron microscopy. Polar attachment is mechanically weak because the attachment is only by a polar tip. The polar attachment of rhizobia to root hairs seems to be a common phenomenon, but it is doubtful whether this reaction is typical for every infective bacteria (Chen and Phillips, 1976).

Although Chen and Phillips (1976) have repudiated attachment as the basis of specificity, Dazzo et al. (1976) have demonstrated a 3 - 10 fold selective adsorption to clover root hairs of infective bacteria over the non-infective rhizobia.

Dart and Mercer (1964), and Hubbell (1962) have suggested that the infective cells which are involved in invasion of the root hairs are coccoid swarmers. Therefore, from the root hair attachment studies it seems likely that

Fig. 35. A hypothetical sketch visualizing the attachment of capsulated R. japonicum 61A76 cell to the soybean root hair surface as mediated by soybean lectin.

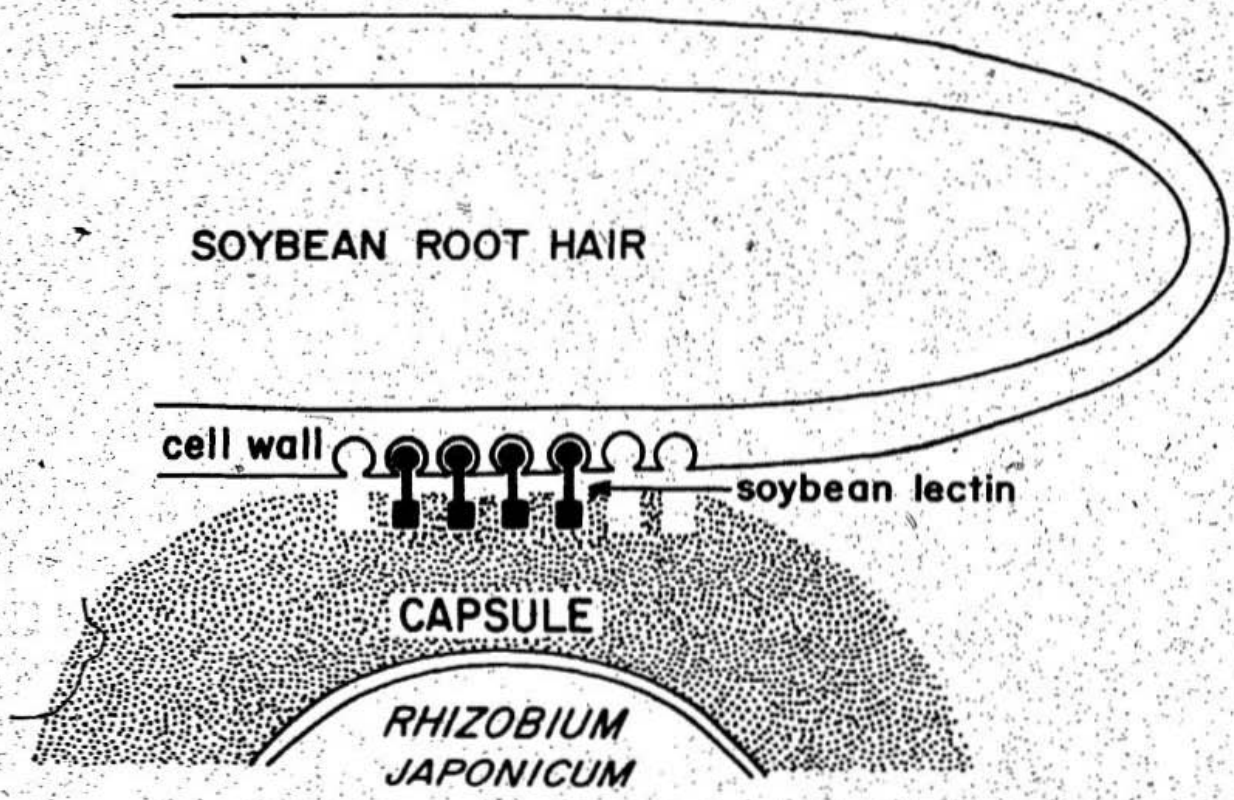

SOYBEAN ROOT HAIR
cell wall
soybean lectin
CAPSULE
RHIZOBIUM
JAPONICUM

Fig. 35

the capsulated coccoid forms firmly attach to the root hair surface and are the infective bacteria.

Involvement of lectin in the binding process implies either that the root hair surface secretes lectins or that there are specific sites in the root hair that have bound lectins. Recent reports of lectins as integral parts of the cell wall (Dazzo, 1977) lends support to the latter hypothesis. Evidence also comes from the reports of lectin or lectin-like activity from soybean roots (Pueppke et al., 1978; Pueppke, 1979; Kato et al., 1979; Bowles et al., 1979). Lectins have been shown to be structural components of the cell membrane system (Monsigny et al., 1979). Secretion of soybean lectin by root hairs has not yet been demonstrated, however glycoproteins responsible for Rhizobium chemotaxis in birdsfoot trefoil are known (Currier and Strobel, 1974). Soybean lectin has two reactive sites (Sharon, 1972), therefore it is possible that it may bind to the root hair surface on one side and to the bacteria on the other. Such a lectin mediated bacterial attachment to the root hair surface can be envisaged as shown in the schematic diagram (Fig. 35).

In animal systems, lectin receptor sites have been demonstrated both on surface and intracellularly for Con A and the processes of endocytosis or internalization of microbes have been demonstrated (Guillouzo et al., 1979). Pinocytosis and phagocytosis have been shown to be lectin mediated (Silverstein et al., 1977; Van Oss, 1978; Monsigny

et al., 1979; Ryter and Helio, 1979). Lectin mediated cell contacts in liver cells, erythrocytes and lymphocytes have been shown to have lectins between their contact points.

As the symbiotic association of Rhizobium and the legume only results in a nitrogen fixing beneficial partnership, it is proposed that the process by which Rhizobium enters into legume root nodule symbiosis be termed "internalization". Usually the term 'infection' is used in the context of pathogenicity or disease resulting from the invasion of microbes in both plants and animals. Therefore, the term 'internalization' of Rhizobium seems justified as the bacteria enter by a process of invagination of the root hair.

VII. Nodulation Studies

Nodulation studies showed that both coccoid and rod forms were capable of causing internalization and nodulation in soybeans. The variance which is greater than the mean indicates multiple infection of the same plant rather than randomly distributed internalization. The nodulation by rod forms could be explained on the basis that the differentially fractionated rods and cocci are not pure and moreover the rods can divide and give rise to cocci while establishing themselves in the rhizosphere of soybean. Such cocci derived from the rods due to their cell division could cause nodule formation in soybean.

VIII. Increasing of More Lectin Binding Receptors in R. japonicum

A comparative study of lectin binding properties of R. japonicum in two different laboratory culture media reveals that there is more lectin binding when the bacteria are grown in soil extract medium. This observation is important because of lectin mediated specific interaction and host recognition in soybean root nodule symbiosis. Specificity and recognition are inter-related and both are achieved in a multistep process. The first step is the establishment of Rhizobium in its host rhizosphere and multiplication therein (Fahraeus and Ljunggren, 1968; Rovira and Davey, 1974). It is well known that such a phenomenon indeed takes place (Reyes and Schmidt, 1979). Rhizosphere conditions provide a better environment for the expression of specificity and host recognition phenomenon (Hubbell, 1962; Dart and Mercer, 1964; Rovira, 1965, 1969; Bowan and Rovira, 1976; Schmidt, 1979). Rhizosphere/rhizoplane conditions have been shown to increase the soybean lectin binding to R. japonicum (Bhuvaneswari and Bauer, 1978). The increased soybean lectin binding in soil extract medium supports the conclusions of the above reports. Detectable amounts of soybean lectin specific hapten (galactose) found in both extracellular polysaccharide and lipopolysaccharide fractions of R. japonicum grown in soil extract medium indicates that soil extract medium increases the formation of soybean lectin specific binding receptor

sites in this mon-mucoid strain. In the yeast extract mannitol grown bacteria detectable amounts of galactose were not found, possibly because of the presence of a few capsulated cells. In earlier studies, less than 1% of the population of R. japonicum broth culture cells were found to bind soybean lectin as visualized by ferritin labeled soybean lectin at the electron microscope level (Bal et al., 1978). R. japonicum when grown in soil extract medium produced a higher percentage of coccoid forms; these forms have an increased number of total lectin binding receptors. From the carbohydrate analysis and ^{125}I-soybean lectin binding studies, it was possible to conclude that the soil extract medium was capable of inducing more lectin binding receptors in R. japonicum.

The R. japonicum in broth culture has two forms of cells, rods and coccoids. The capsulated coccoid forms are the "internalizing" cells. Soybean lectin has two binding sites on the cell surface of bacteria; the primary lectin binding site is localized in the capsular polysaccharide which comes in contact with lectin, before it can reach the secondary lectin binding site in the lipopolysaccharide in the cell wall. It is further hypothesized, that capsular receptors are responsible for attachment of the bacteria to the root hair surface and specificity and host recognition are mediated by the receptors on the cell wall lipopolysaccharide. It appears that lectin mediated specificity and host recognition are better expressed in cells grown

in the soil extract medium.

Since the bacteria and the plants interact to control specificity at a minimum of four different levels during the internalization process viz., rhizosphere, rhizobial attachment to the root hairs, response of root hair (curling) to bacterial internalization and "infection thread" formation, there is no need for absolute specificity at any given point throughout the members of the family leguminosae. Although the lectin hypothesis is an attractive concept it would be difficult to generalize its role throughout the nodulating legumes. Perhaps, it would be wiser to restrict the application of the lectin theory to a specific legume-Rhizobium symbiosis as every symbiotic system has not been thoroughly studied.

SUMMARY AND CONCLUSIONS

There is a high degree of specificity in legume-*Rhizobium* interaction which leads to the internalization of legumes by *Rhizobium* and eventual formation of a symbiotic root-nodule. Legume seed glycoproteins known as "lectins" or "phytoaemagglutinins" have been implicated in the host recognition phenomenon. Lectins are storage proteins which have the unique property of binding specifically to certain sugar haptens present on the cell surfaces. Little is known about the role of lectins in plants, however they are generally believed to be mobilized into the root system during seed germination and the establishment of the seedlings.

The present investigation has been carried out to elucidate the role of soybean lectin and the cell surface interaction between soybean and *Rhizobium japonicum* during the internalization process. Studies have been made on the rhizobial cell population to characterize the internalizing cells and to localize lectin binding sites.

There is heterogeneity of cells in broth culture cells of *R. japonicum* 61A76. Small coccoid forms and large rod forms could be distinguished by both light and electron microscopy. The coccoid forms were observed to be either single and isolated or were found associated in chains. They were either capsulated or non-capsulated and it was not uncommon to see a group of coccoid cells held together in a common capsular matrix. On the other hand, rod forms were

free and non-capsulated or were in aggregates forming 'stars'. The rods had polar capsules. It was possible to separate these two morphologically distinct fractions in the broth culture of R. japonicum by differential centrifugation.

The rod and coccoid fractions when cultured, separately in a fresh nutrient medium, developed into a heterogenous population. I propose that rod forms arose by the growth and elongation of coccoid forms; the rod forms then divided to produce coccoid-shaped cells. The two forms of R. japonicum are no more than two different phases in the life-cycle. Extensive electron microscopic observations indicated that capsulated coccoid forms undergo repeated cell divisions, which resulted in the formation of several coccoid cells held together in a capsular matrix. Elongation of such coccoid cells from within the matrix resulted in the 'star' formation. It was observed in experimental studies that both coccoid and rod forms showed attachment to the soybean root hair. The rod forms showed distinct polar attachment possibly due to the polar capsule. In the ultrastructural study of the attachment process the coccoid forms were clearly seen attached to the root hair whereas rod forms were not seen, which indicated that the rods were unable to withstand the rigours of preparative procedures for electron microscopy. This suggested that polar attachment was weak and rod forms might not be involved in the internalization process. The firmly attached

capsulated coccoid forms appeared to be the internalizing cells.

Radioiodinated soybean lectin binds to both forms of cells and the affinity constant was not significantly different in the two forms. When the bacteria are cultivated in both yeast extract mannitol and soil extract media, there was a significant difference in the lectin binding kinetics with the soil extract medium grown bacteria showing increased lectin binding.

Using ferritin conjugated and colloidal-gold labeled soybean lectin, it was possible to localize the primary binding sites of lectin in the capsule of the bacteria. In the capsulated coccoid forms the ferritin and gold labeling was seen distinctly all round the cells whereas in the rod forms, the labeling was strictly confined to the polar capsule. In addition, gold labeled soybean lectin was also seen bound to the outer cell wall of the bacteria indicating a secondary binding site.

The gas chromatographic analysis of extracellular and lipopolysaccharides of R. japonicum 61A76 grown in yeast extract-mannitol medium and soil extract medium, were carried out separately. The polysaccharides from the yeast-extract mannitol medium grown bacteria did not reveal any detectable amount of soybean lectin specific sugar (galactose or N-acetylgalactosamine) whereas the bacteria grown in soil extract medium showed the presence of galactose in both extracellular and lipopolysaccharides. Concomitantly, the bacteria grown in soil extract medium showed more lectin

binding as revealed by ^{125}I-Soybean lectin binding studies.

More lectin binding receptor sites (galactose) are increased in the LPS and EPS of R. japonicum, when grown in soil extract medium.

In conclusion, (1) there are two distinct forms of cells, i.e., rods and cocci in the R. japonicum broth culture; (2) there are two soybean lectin binding sites in R. japonicum, (a) primary binding sites localized in the capsule, (b) secondary binding sites localized in the cell wall lipopolysaccharide; and (3) more lectin binding receptor sites are increased in bacteria grown in soil extract medium.

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