

MOTILITY, CHEMOTAXIS AND INFECTIVENESS
OF RHIZOBIUM

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To my father

ABSTRACT

The ability of the soil bacterium *Rhizobium*, in particular *R.leguminosarum*, to show oriented responses to chemical stimuli and the role of chemotaxis in the *Rhizobium*-legume symbiosis was investigated.

Rhizobium strains were found to show chemotaxis to a range of root exudates from both legumes and non-legumes. There was no evidence that chemotaxis to root exudates plays a role in host-symbiont specificity. Specific responses to low molecular weight components of the root exudates of the edible pea (*Pisum sativum* L.) were demonstrated with *R.leguminosarum*, its natural symbiont. No high molecular weight attractants were isolated.

Positive chemotaxis was shown to a large range of metabolites including all twenty common amino acids, nine sugars and four organic acids. Optimal responses were generally seen at 10^{-3} or 10^{-4} M. Negative chemotaxis was observed to a variety of repellants including metal cations, H^{+} and OH^{-} ions.

Competition experiments using motility and general chemotaxis mutants of *R.leguminosarum* clearly showed that non-chemotactic mutants nodulate pea seedlings less efficiently than the wild type and non-motile mutants still less efficiently. It was concluded that motility guided by chemotaxis facilitates infection and that motility and chemotaxis mutants will be less successful in nature than the wild type.

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CHAPTER I

INTRODUCTION

Mechanisms of Accumulation and Dispersal

The main advantage which motility confers on an organism is the ability to respond to external stimuli by movement towards or away from favourable and unfavourable environments. Accumulation at a site may be due to trapping of organisms that have arrived by random movement or by actual attraction where the path taken has been modified by a sensory response. Responses modified by sensory systems have been classified into two main types by Fraenkel & Gunn (1961); kinesis and taxis. In its loosest sense, a taxis refers to an active migration towards (positive taxis) or away from (negative taxis) a stimulus. However, Fraenkel & Gunn proposed that the term taxis should be used only to describe oriented movement towards or away from the source of the stimulus. Kinesis are responses to alterations in the intensity of the stimulus such that the rate of movement (orthokinesis) or the frequency of turning (klinokinesis) are modified, resulting in movement towards or away from the stimulus source. The distinction between these two types of behaviour may be useful when comparing behaviour in different organisms. But both true taxis (topotaxis) and kinesis may occur within a single organism. Discrimination between the two types of response is not always easy as it requires direct observation of cells or organisms. Thus difficulties may arise when using the restricted definition of taxis. One of the best examples is the so called 'chemotaxis' of bacteria, i.e. bacterial movement towards or away from chemical stimuli in the environment. This was studied for many years (see Adler 1975 for a review) before Berg & Brown (1972) first demonstrated that cells of the bacterium Escherichia coli turn less frequently when swimming up a gradient of a chemical attractant and more frequently as they swim down it. This was, therefore, not

true taxis but a klinokinetic response (Carlile 1975). Terminology has been covered in greater detail by Carlile (1980) and Mitch et al. (1977). In view of these factors, the term 'taxis' is used in this thesis in its broadest sense, to include taxes and kinesis but excluding accumulation due to trapping of organisms that have arrived at a site by random movement.

Rhizobium as a free-living soil organism

The Rhizobiaceae are a group of soil bacteria defined solely by their ability to nodulate leguminous plants. (The only non-legume which can be nodulated by Rhizobium is Trema cannabina; Trinick & Galbraith (1976).) Free-living rhizobia are aerobic, gram -ve rods 0.5-0.9 μm x 1.2-3.0 μm . They are normally motile and do not produce endospores (Vincent, 1977). The genus can be divided into two main types: fast and slow growers (Table I). Fast growing strains have a mean generation time of 2 to 4 h, produce abundant exopolysaccharide and are usually peritrichously flagellated (DeLey & Rassel, 1965). Fast growers include R.leguminosarum, R.trifolii, R.phaseoli and R.meliloti. Slow growing strains have a mean generation time of 6 to 8 h, produce little exopolysaccharide and generally have polar or sub-polar flagella (Graham, 1976). Slow growers include R.lupini, R.japonicum and the 'cowpea' rhizobia. Fast and slow growers can also be distinguished by internal antigen patterns (Vincent & Humphrey, 1970).

Many factors influence the survival of rhizobia in the soil (Nutman, 1975). These include: temperature, composition and moisture content of the soil (Tuzimjra et al., 1966); exudates from plant seeds and roots (Rovira, 1965a); antagonism by fungi, actinomycetes and bacteria; parasitism by Bdellovibrio sp., lysis by myxobacteria and

Table 1 Cross-inoculation groups of Rhizobia

FAST GROWING STRAINS

R.leguminosarumR.trifoliiR.phaseoliR.meliloti

REPRESENTATIVE HOSTS

Pisum sativum; Vicia fabaTrifolium repensPhaseolus vulgarisMedicago sativa

SLOW GROWING STRAINS

R.lupiniR.japonicum

'cowpea' Rhizobia

Lupinus luteus; Ornithopus sativusGlycine max

Slow growing forms which
cross-nodulate to variable
degrees among a great variety
of legume hosts.

predation by protozoans (Parker & Grove, 1970). In addition, *Rhizobium* bacteriophages occur widely in the soil; their influence on numbers is probably less important than genetic effects caused by transduction (Barnet, 1972). Free-living rhizobia are irregularly distributed throughout the soil and are generally more numerous in the rhizosphere of plants, especially legumes (Rovira, 1965b). Rovira (1961) found that the rhizosphere to soil ratio (R:S) for *R. trifolii* with red clover ranged from 8 to 200 whereas with the grass *Paspalum dilatatum* it was only 6 to 13. Similar observations were made by Nutman & Ross (1969).

In pure and mixed culture studies in agar planted with legumes, *Rhizobium* can multiply from 10^2 - 10^7 cells/ml in the outer rhizosphere to around 10^2 - 10^9 cells/ml in the inner rhizosphere (Vincent & Waters, 1953; Lim, 1963; Nutman, 1957). However, no consistent relationship between the host species and the ratios of the different strains of *Rhizobia* in the plant rhizosphere was observed. Non-nodulating or ineffective strains were stimulated to the same degree as effective strains.

There have been few studies on the proliferation of *Rhizobia* in competition with other soil microorganisms. Harris (1953) observed that certain fungi and bacteria markedly increased nodulation of clover by a poorly infective strain of *R. trifolii* but had little effect on the behaviour of a highly virulent strain.

Until recently, N_2 fixation by rhizobia was thought to occur only in symbiotic association with legumes. Conditions have been found however, in which some free living strains of *Rhizobium* will fix N_2 (Kurz and LaRue, 1975; McComb, Elliot & Dilworth, 1975; Pagan et al., 1975). The contribution of free-living N_2 -fixing rhizobia to the over-all total of fixed nitrogen remains to be assessed

but it is probably small, as with other free-living heterotrophs.

The Rhizobium-legume symbiosis

Rhizobium species form symbiotic relationships with a limited range of legume hosts (Beringer et al., 1979). Early work on the specificity of nodule formation led to their classification into cross-inoculation groups (Table I). Although specific mutual recognition is sufficiently precise in most temperate legumes to allow the definition of a species (e.g. Trifolium spp. are nodulated by Rhizobium trifolii), there have been cases of nodulation of "incorrect" hosts (Hepper, 1978). The great majority of legumes appear to be less specific with respect to infection by Rhizobium and therefore no comprehensive classification into species has been achieved (Graham, 1976).

The control of specificity is not entirely understood but is known to reside in both the rhizobium and the host legume. Adsorption of rhizobia to plant roots probably occurs by polar attachment (Dazzo et al., 1976) and may involve interaction between surface or polysaccharide capsular antigens of the bacterium and lectins produced by the root (Bohloul & Schmidt, 1974; Chen & Phillips, 1976; Sequeira, 1978). Immunochemical and genetic studies of cell surface components have demonstrated that the surfaces of infective R.trifolii and of clover epidermal cells, contain a unique antigen which is immunochemically cross-reactive (Dazzo & Hubbell, 1975). This cross reactive antigen (CRA) contains receptors which bind trifolin, a clover lectin which has been isolated from clover seeds and seedling roots and partially characterized (Dazzo et al., 1978). Dazzo & Hubbell (1975) proposed a lectin-binding model to explain specific recognition. According to this hypothesis, trifoliin recognises similar saccharide residues on R.trifolii and clover, and cross-bridges them to form a

molecular interfacial structure that initiates the specific adsorption of the bacterium to the root hair surface (Fig. I). Despite substantial evidence, the exact nature of the lectin-binding reaction and the origin of its components are still not fully understood (Planque & Kinje, 1977).

Specific lectin binding does not necessarily imply a 'successful' symbiotic relationship. For this to occur, legume and *Rhizobium* must interact at least at two levels: (i) Infection followed by nodulation must occur (an "infective" relationship); (ii) The nodule must become capable of fixing N_2 (an "effective" relationship). Both non-infective and ineffective strains of rhizobia are known. One of the main problems of legume agriculture is the relative ineffectiveness of wild rhizobia.

The processes of infection and nodulation are well documented (Dart, 1974). The adhesion of rhizobia to the root surface occurs in two phases (Dazzo, 1980a). In phase I attachment, a specific mechanism allows selective adherence. Substantial evidence supports the lectin-binding hypothesis of Dazzo & Hubbell (1975) which has already been discussed. In phase II attachment, other mechanisms firmly anchoring the bacterium to the root hair surface may operate during later pre-infective stages. Aggregated microfibrils associated with adherent bacteria are visible under the scanning electron microscope (Dart, 1971; Dazzo, 1980b). The composition of these fibrils is unknown but Napoli *et al.* (1975) suggested that they are the cellulose microfibrils produced by many rhizobial species. Alternatively, they may be bundles of low molecular weight, extracellular β -1, 2 glucans (Zevenhuizen, 1978).

There are two general mechanisms of penetration of legume roots

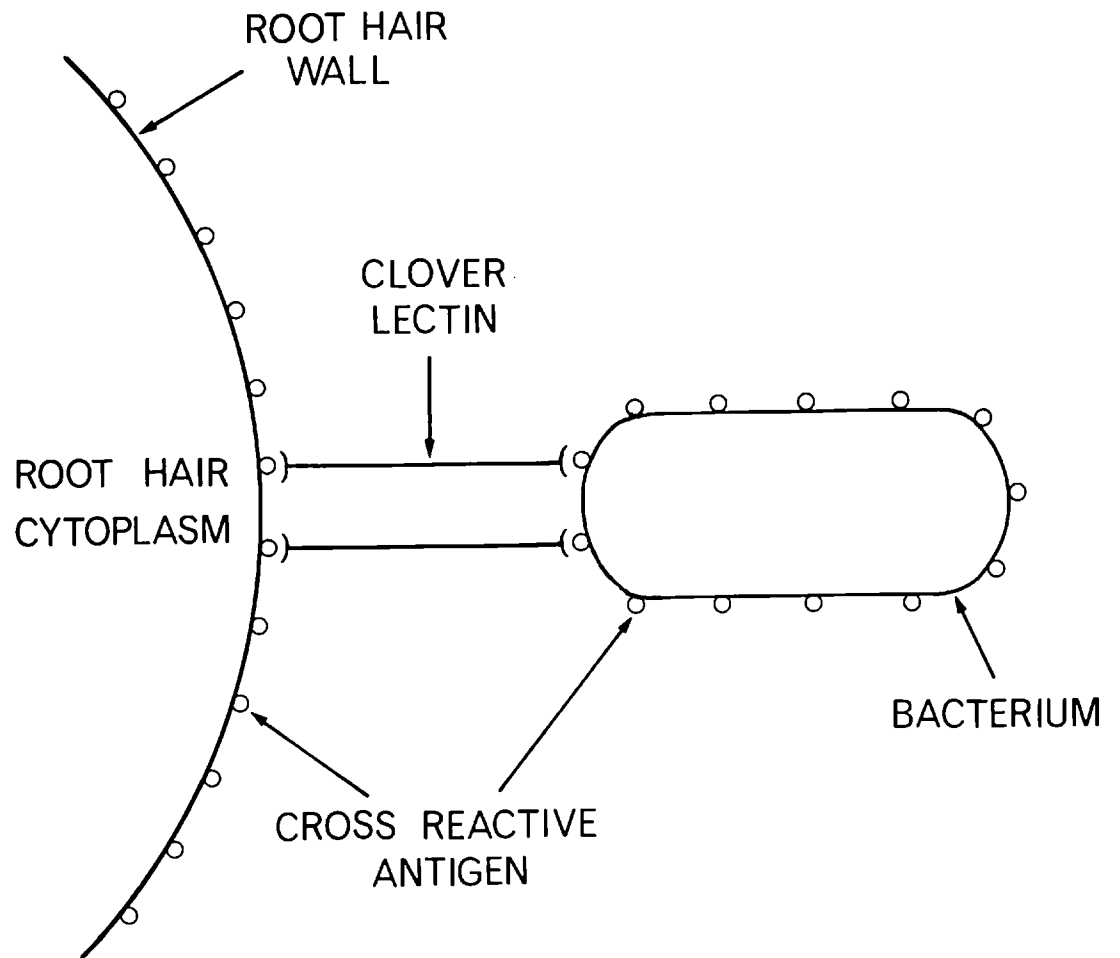


Fig.1. Lectin binding model to explain Phase 1 attachment.
Reproduced from Dazzo & Hubbell (1975)

by Rhizobia (Dart, 1975). For most fast-growing strains, adsorption to root hairs is normally followed by involution of the plant cell wall to form an infection thread (Kumarasinghe & Nutman, 1977) which grows through the cells of the root cortex by extension of its primary cell walls and the multiplication of bacteria within a slimy matrix. This slime is probably bacterial in origin (Sanders et al., 1978). The cells through which the branching infection thread passes are not themselves stimulated into cell division. Division of cortical root cells occurs at a point remote from the advancing infection thread (Newcomb, 1976). The alternative mode of infection is characterised by the absence of infection threads. Instead, access to the central root tissues is by intracellular penetration especially at the point of emergence of the lateral roots where the root epidermis is damaged. After penetration, the cortical cells start to divide and bacteria are dispersed in the developing nodule by continual division of infected root cells (Dart, 1975). After infection, bacteria are released from the infection thread directly into membrane envelopes where further division can occur. The rhizobia then differentiate into bacteroids which are often pleomorphic and larger than the free-living form, with an altered cell structure (Sutton et al., 1977).

Plant hormones probably also play an important role in the development of nodular tissue. Rhizobia have been shown to produce plant growth regulators which are implicated in infection and nodulation. Dullaart (1970) showed that the rhizobial symbiont of Lupinus luteus converts tryptophan into the plant auxin, indole-3-acetic acid. Pectolytic enzymes are also produced (Hubbell et al., 1978).

Oxygen, an essential requirement for ATP production to provide reducing power for N_2 fixation enters the nodules by diffusion.

Effective nodules are pink in colour due to leghaemoglobin, a myoglobin-like haemoprotein found only in legumes, probably situated between the surface of the bacteroids and the surrounding envelope. It seems to function by facilitating the diffusion of oxygen to the bacteroids to allow oxidative phosphorylation while maintaining a sufficiently low concentration of free O_2 to prevent oxygen inactivation of bacterial nitrogenase, the enzyme responsible for nitrogen reduction to ammonia (Appleby, 1974; Wittenberg et al., (1974).

Ultimately the success of this symbiosis depends on the expression of N_2 fixation through the activity of nitrogenase and the enzymes involved in the assimilation of the nitrogen fixed by it. The ability to synthesise nitrogenase and to regulate that synthesis is encoded in a cluster of genes termed the nif genes. Detailed discussion of nif is not appropriate here but the topic has recently been reviewed by Beringer et al. (1980). It is clear however, that the isolation and study of mutants will aid in the further understanding of the Rhizobium-legume symbiosis.

Molecular Biological Aspects of Chemotaxis

A great deal of work has been done, mainly with Escherichia coli and Salmonella typhimurium to elucidate the entire sensory transduction pathway from stimulus to response in molecular forms. Comprehensive reviews include Berg (1975); Hazelbauer & Parkinson (1977); Goy & Springer (1978); Macnab (1978, 1979) and Koshland (1980). Basically there are approximately 25 to 30 receptors on the surface of a bacterium which undergo conformational change on the binding of chemoeffectors. This conformational change transmits the signal to the flagellar apparatus. The signal is then processed through a signalling system which is composed of at least nine gene products and is common to all the many diverse stimuli. A signal processed

in this manner can be interpreted in terms of changing levels of a response regulator which alters the gear mechanism of the flagella motor. If the motor is reversed from its normal swimming mode the bacterium tumbles, thus converting the sensory stimulus into a behavioural response. Tumbling is suppressed when the bacterium travels up an attractant gradient and increases when it proceeds in an unfavourable direction thus generating a biased random walk.

General Ecological Aspects of Chemotaxis

The significance of taxes for motile organisms is emphasised by the diversity of known tactic stimuli. These have been reviewed by Carlile (1980) and include chemicals (chemotaxis), light (phototaxis), water (hydrotaxis), temperature (thermotaxis), gravity (geotaxis), magnetism (magnetotaxis), pressure (rheotaxis) and elasticotaxis which includes movement along lines of stress in the substratum (Stanier, 1942, 1947).

Chemotaxis is the response which has been most widely studied, reflecting the universal importance of the chemical composition of an environment to an organism. Chet & Mitchell (1976) review the ecological aspects of microbial chemotactic behaviour and Levandowsky & Hauser (1978) review the chemosensory responses of swimming algae and protozoa.

The most obvious role of chemotaxis is in nutrition and as an indicator of harmful conditions. Thus, ^{aerobic} bacteria generally show positive chemotaxis towards oxygen and to many amino acids and sugars (Baracchini & Sherris, 1959; Adler, 1966, 1975, 1976; La Marre et al., 1977; Mesibov & Adler, 1972; Moulton & Mountie, 1979). They are repelled by extremes of pH, certain ions and a range of organic substances some of which are products of anaerobic

metabolism and may serve as an indicator of overcrowded conditions (Tso & Adler, 1974; Ordal & Goldman, 1976; Young & Mitchell, 1973).

Many predatory microorganisms also show chemotaxis including the slime mould Badhamia utricularis which detects chemicals given off by the fungus Stereum hirsutum (Madelin et al., 1975). Chemotaxis may be involved in some host-parasite interactions, for example, the plant pathogenic bacterium Pseudomonas lachrymans shows chemotaxis to plant exudates (Chet et al., 1973). Similarly, Cameron & Carlile (1978) showed that zoospores of the fungus Phytophthora palmivora, a plant pathogen, are attracted by a range of fatty acids; alcohols and aldehydes, some of which are commonly produced by plants. Chemotaxis may also be involved in the infection of legume roots by symbiotic N₂-fixing bacteria of the genus Rhizobium (Currier & Strobel, 1976). This aspect will be considered in greater detail in the next section and the following chapters.

In none of the cases of predator-prey, symbiotic and host-parasitic relationships investigated to date has the chemotactic response been specific to host or prey alone. This is in contrast to the extreme specificity shown in mating interactions. Male gametes of the chytrid Allomyces for instance, show positive chemotaxis to sirenin, a sex attractant produced by the female gametes (Carlile & Machilis, 1965). Within a few minutes of fertilization taking place, however, this sensitivity is replaced by chemotaxis to amino acids which presumably aids the zygote in finding a suitable habitat (Machilis, 1969).

Although much more work is still needed to investigate and evaluate the role of such sensory responses under natural conditions, it is reasonable to assume that such complex systems would not have been evolved and retained if they were not of value to the organism concerned.

Taxes of the Rhizobiaceae

The widespread occurrence of chemotaxis by flagellate bacteria and the copious exudation of a wide variety of compounds into the soil by legume roots suggests that chemotaxis could have a role to play in the Rhizobium-legume symbiosis.

Currier & Strobel (1976) reported chemotaxis by a number of Rhizobium species to legume root exudates, but there was no one to one correlation between chemotaxis and nodulation. They failed to demonstrate chemotaxis of strains of R.trifolii to simple sugars and amino acids. Attraction of one strain of R.trifolii was attributed to a high molecular weight glycoprotein isolated from its host, birdsfoot trefoil, Lotus corniculatus, L. (Currier & Strobel, 1977a, b). In the soil such a protein seems likely to have a very restricted range of movement and its significance remains to be assessed. Gitte et al. (1978) showed attraction of a Rhizobium strain by exudates from its host Cicer arietinum L. (chickpea) and found chemotaxis to various sugars and amino acids detected in the exudate. However, since the concentrations of these simple molecules are not given it is difficult to comment on the physiological significance of this phenomenon.

Clearly considerably more work is needed to clarify the qualitative and quantitative aspects of chemotaxis in the Rhizobiaceae and its possible role in the establishment of the Rhizobium-legume symbiosis.

Aims and Approaches

The aim of the present work was to study two aspects of chemotaxis in the Rhizobiaceae: Firstly, to investigate the specificity of the response and the various components involved, and secondly to investigate the significance and the role of chemotaxis in the Rhizobium-legume symbiosis. The first aspect was investigated by

testing various species for chemotaxis to a range of root exudates from both host and non-host and then by selecting one species, R.leguminosarum, identifying the various constituents of its host root exudate and testing for chemotaxis to those metabolites.

The role of chemotaxis in the symbiotic relationship was studied by developing suitable motility and chemotaxis mutants and testing their ability to nodulate in competition.

CHAPTER 2

GENERAL METHODS

ORGANISMS AND ROUTINE CULTURE

Bacterial Strains

Rhizobium leguminosarum was isolated from a pea root nodule and designated N5. Rhizobium phaseoli and Rhizobium trifolii were strains 1231 and 6601 respectively from the collection of Rothamsted Experimental Station, Harpenden and were provided by Dr N. Brewin, John Innes Institute, Norwich as was Escherichia coli strain 1830. Escherichia coli strain C600 was provided by Dr D.G. Smith, Department of Botany and Microbiology, University College, London.

Maintenance of Bacteria

Rhizobium stock cultures were maintained on slopes of LA or TY medium. LA contained, per litre, 1.0g yeast extract (Oxoid), 10g mannitol, 0.5g K_2HPO_4 , 0.25g $MgSO_4 \cdot 7H_2O$, 0.1g NaCl and 3.0g $CaCO_3$. TY contained, per litre, 5g Oxoid Tryptone, 3g Oxoid Yeast extract, and 1.3g $CaCl_2 \cdot 6H_2O$. All media were made with distilled water using analytical grade reagents and solidified using 1.5% Oxoid no. 3 agar. The pH was adjusted to pH 7 if necessary. Sterilization was by autoclaving for 20 min at 121°C. Stock cultures were stored at 5°C for short-term storage or at -18°C under glycerol (20% v/v) for long-term storage.

E.coli strains were maintained on slopes of Oxoid nutrient agar and stored at 5°C.

Following frequent sub-culture Rhizobium may deteriorate in motility and ability to perform chemotaxis. Therefore, prior to experiments or preparation of stock cultures, migration on semi-solid media was used as a selection procedure to eliminate non-motile and non-chemotactic variants. Organisms were inoculated into the centre of semi-solid agar plates and allowed to swarm across the plate for 5-7 days (Plate I). Motile and chemotactic cells migrated in rings

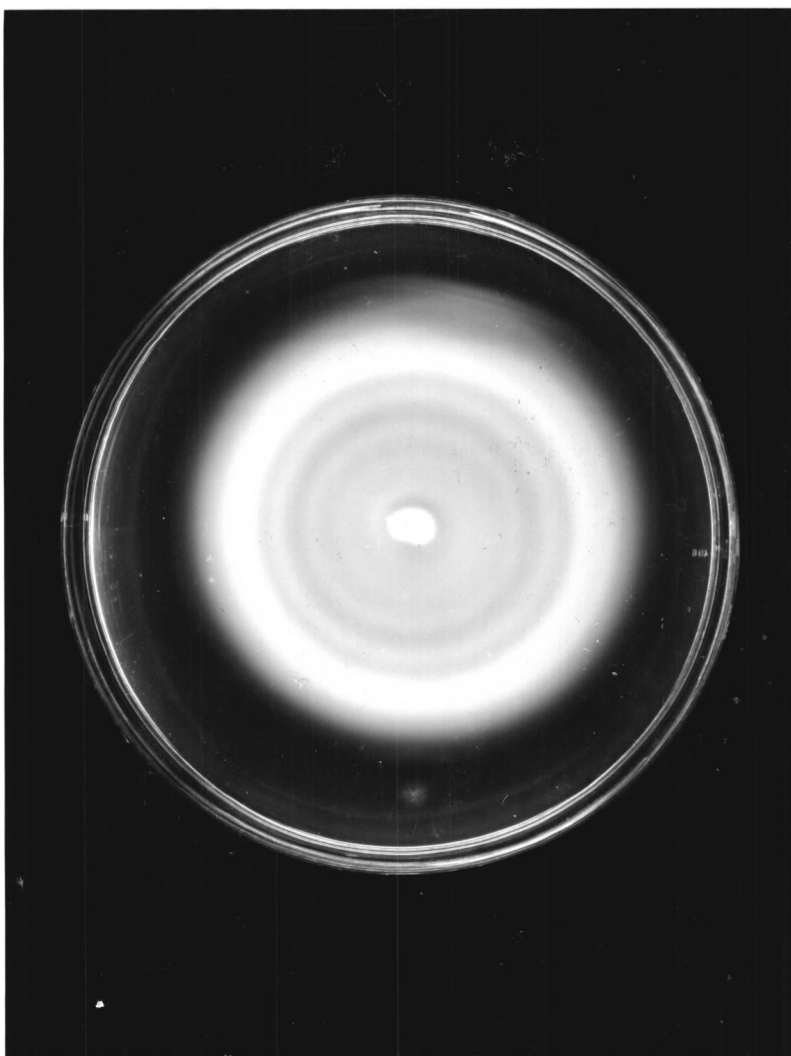


Plate 1. R.leguminosarum migrating on semi-solid agar.

of increasing radius across the plates and inoculum from these swarms was used for all experimental work (Adler, 1966^a). Semi-solid agar was minimal medium A gelled with 0.25% (w/v) Oxoid no. 3 agar (Table 2).

CHEMOTAXIS ASSAYS

Chemotaxis suspending media

Chemotaxis buffer for all *Rhizobium* strains was pH 7 potassium phosphate buffer, 10 mM; EGTA, 1 mM; CaCl_2 , 0.97 mM.

Chemotaxis buffer for *E.coli* was pH 7 potassium phosphate buffer, 10 mM; EDTA 0.1 mM.

Preparation of bacteria

Static liquid culture was used for *Rhizobium* work as this, unlike shaken culture, can favour motile and chemotactic cells (Smith & Doetsch, 1969; Pilgrim & Williams, 1976). For each experiment, 1 ml log phase inoculum was used to inoculate 100ml volumes of minimal medium A (Table 2) in 500 ml erlenmeyer flasks and grown at 24°C for 36-48 h to give an extinction coefficient of 0.5-0.8 at 450 nm in a 1 cm cuvette. Twenty ml volumes of this culture were centrifuged at 8000g, 10°C for 10 min and the pellet resuspended in chemotaxis buffer to a density of 5×10^8 cells ml⁻¹.

A similar procedure was adopted for *E.coli* except that since loss of motility and chemotaxis was not a problem, shaken liquid culture was used (1 ml log phase inoculum was used to inoculate 100 ml volumes of Oxoid nutrient broth in 500 ml erlenmeyer flasks and incubated overnight at 37°C on a shaker - radius of gyration, 4.5 cm; 200 rev. min⁻¹ to give an extinction of 0.5-0.8 at 450 nm). Cultures were centrifuged as for *Rhizobium*, the pellet suspended in chemotaxis buffer, recentrifuged and resuspended in fresh buffer.

Table 2. A defined minimal medium for the culture of Rhizobium:

Minimal Medium A

		Final Concentration
		g l^{-1}
Minimal Salts (MS)	Na Succinate	6.9
	Na Glutamate	1.1
	(+CaCl ₂ .2H ₂ O	0.11
	(K ₂ HPO ₄	0.11
	(MgSO ₄ .7H ₂ O	0.1
	(FeCl ₃ .6H ₂ O	3.3×10^{-3}
	(+Na ₂ EDTA	3.72×10^{-4}
	(Biotin	7.5×10^{-4}
	(Ca Pantothenate	7.5×10^{-4}
	(Thiamine HCl	7.5×10^{-4}

+ Minimal medium B contained 0.129 g l^{-1} CaCl₂.2H₂O and $3.80 \times 10^{-3} \text{ g l}^{-1}$ EGT

Minimal medium was adapted from the medium of Vogel & Bonner (1956).

All bacterial suspensions were examined microscopically to check motility prior to use in chemotaxis assays. E.coli were still 80-90% motile after three washes in chemotaxis buffer but Rhizobium cells tended to lose motility if centrifuged and resuspended more than once.

(b) Assay Procedure

Two main methods of chemotaxis assay were used, both based on the same principle, i.e. bacteria swimming from a chamber along a diffusion gradient of an attractant to a second chamber or a capillary which was the attractant source. The capillary method was used in early work but subsequently the chamber method was preferred.

(i) The Capillary method

This assay was based on the method of Adler (1973). 1 μ l capillary tubes, 3 cm long with an internal diameter of 0.2 mm (Microcaps from Drumand Scientific Co., USA) were sealed at one end in a bunsen flame, passed quickly through the flame several times and immediately plunged open end down into a bijou bottle containing 3 ml of the test substance dissolved in chemotaxis buffer. The capillaries drew up about 1 cm of liquid on cooling. Capillaries containing 0.75-1.5 cm of attractant were used in assays with no significant variation in results. After a few minutes, the capillaries could be inserted open end first into a chamber containing bacterial suspension. Assay chambers (20 x 6 x 1 mm deep) were filled with bacterial suspension and covered with coverslips taking care to avoid air bubbles. Both chambers and coverslips were sterilized using ether prior to use.

After incubation (60 min, 24°C for Rhizobium; 60 min, 30°C for E.coli) each capillary was removed and the exterior rinsed in a stream of sterile water. The sealed end was broken off and the contents squirted into a 1 ml aliquot of chemotaxis buffer using a rubber bulb. The number of organisms passing into the capillary during the incubation

period was then determined by one of three methods (see below)..

(ii) The blind well chamber assay

The blind well chamber method, in which migration of organisms occurs through membranes into test or control solutions, was first used for investigations of bacterial chemotaxis by Armitage et al. (1977). Chemotaxis chambers and unipore polycarbonate membranes (5 μ m pore size, 13 mm diameter) were obtained from Bio-Rad Laboratories. The bacterial suspension (0.15 ml) was pipetted into the lower well of the chamber. A polycarbonate membrane was fitted on top of the lower well and the upper well screwed onto it. The upper well was then fitted with 0.3 ml of the test material dissolved in chemotaxis buffer. The tops of the chambers were carefully sealed with parafilm (Gallenkamp) avoiding air bubbles and the chambers were inverted for incubation. This prevents gas production interfering with the results, allows the environment to remain aerobic and stops bacteria being forced through the membrane by pressure when the membrane is screwed down. Incubation was 90 min at 24°C for all *Rhizobium* strains; 60 min at 30°C for *E.coli*. After incubation the contents of the upper well were transferred to Eppendorf tubes for counting.

Bacterial counts

Three methods were used to determine the number of bacteria passing into the capillary or attractant chamber during chemotaxis assays.

(a) Plate count

0.1 ml samples of a series of dilutions were plated out onto VS agar. VS agar contained, per litre, 2.5 g sucrose, 0.5 g K_2HPO_4 , 0.16 g $CaSO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 0.02 g $FeCl_3 \cdot 6H_2O$ and 0.5 g yeast extract (Difco). After incubation (24°C, 48 h for *Rhizobium*

strains; 37°C, 12 h for E.coli) the number of colonies were determined with the aid of a colony counter.

(b) Coulter counter

For the blind well chamber method, 0.5 ml of the sample was diluted in 10 ml Isoton II (Coulter Electronics, Dunstable, Bedfordshire) which had been passed through a millipore filter (Millipore Ltd, Park Royal, London). Bacterial numbers were assessed using a Coulter counter (Coulter Electronics). In the case of the capillary assay, the entire contents of the capillary were squirted directly into the Isoton solution and carefully mixed.

(c) Haemocytometer count

A direct microscope count using a bacterial counting chamber also allowed observation of motility.

The viable count was normally used with either of the other two methods as a double check. A chemotaxis ratio was obtained by comparing the numbers passing into the test solution with the numbers passing into the control buffer. (Chemotaxis ratios are defined below). Each result was based on the averages for three test and three control chambers (or capillaries). A further control was the inclusion in each experiment of one chamber containing a known attractant to establish that the bacterial suspension was showing normal motility and chemotactic sensitivity.

Definition of Chemotaxis ratios

(i) Capillary Assay

$$\text{Chemotaxis ratio} = \frac{\text{Number of Bacteria in Attractant Capillary}}{\text{Number of Bacteria in Control Capillary}}$$

(ii) Blind well chamber Assay

$$\text{Chemotaxis ratio} = \frac{\text{No. of Bacteria passing through the membrane into the attractant well}}{\text{No. of Bacteria passing through the membrane into the control well}}$$

Significance of chemotaxis ratios

The numbers of R.leguminosarum passing through the membrane in the control chambers of 81 blind well chemotaxis experiments were analysed (Fig. 2). The mean value was 3.3×10^6 indicating that 4.4% of the bacteria passed through the membrane; the other values were normally distributed about this mean. Taking the mean value as unity, the standard deviation is 0.22. Thus any ratios above 1.66 will be significant at a 1% probability level. For the purposes of this study, all chemotaxis ratios obtained by the blind well chamber method were regarded as significant above 1.7. Insufficient data was available for comparable statistical analysis of control experiments using E.coli or other Rhizobium species, but because the variability in the numbers of bacteria passing through the membrane in the control chambers was similar, a chemotaxis ratio of 1.7 was also regarded as a positive response. Lack of data precluded analysis of the significance of capillary assay chemotaxis ratios but those obtained were generally much higher than blind well assay ratios.

(iii) Plate chemotaxis

Swarming on semi-solid media provides a simple method of demonstrating chemotaxis (Adler, 1966)^b. Rhizobium migrates through media containing a chemotactic agent as a ring or swarm of increasing radius. These rings were observed on semi-solid minimal medium (0.25% agar) but not on a modified minimal medium lacking succinate and glutamate. This modified medium contained minimal salts (Table 2) and 2.5 g l^{-1} sucrose. Ring formation on a modified medium to which a single substance had been added therefore provided evidence that the substance was chemotactic. This was used to confirm results obtained by the other assay methods.

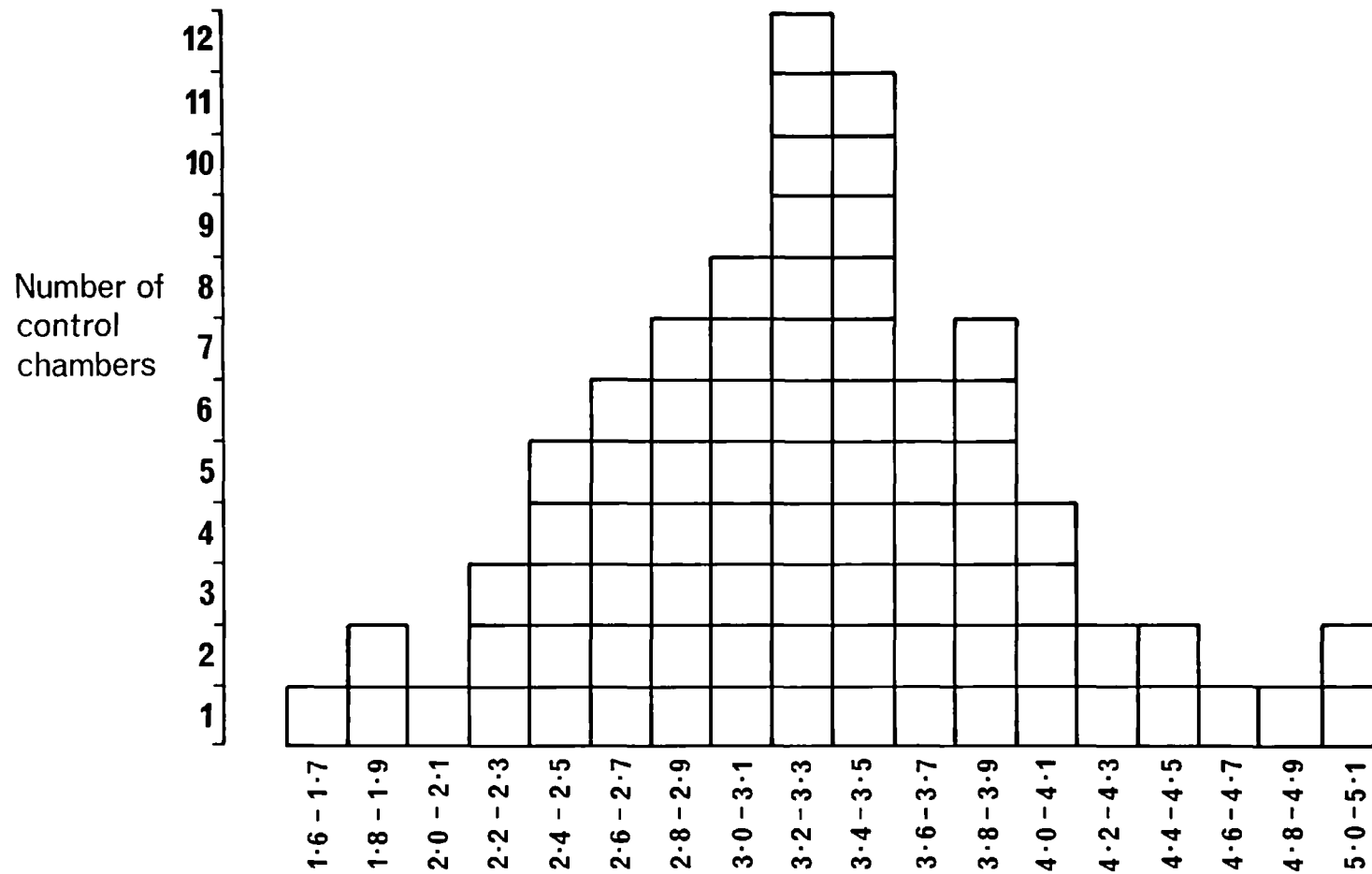


Fig.2. Summary of control data.

Legend to Fig. 2.

The histogram illustrates the numbers of bacteria (horizontal axis) passing through the polycarbonate membrane into the upper wells of the control chambers in 81 bind well chemotaxis assays.

The results are shown as the number of organisms $\times 10^6$.

The values are normally distributed about a mean of 3.28×10^6 with a standard deviation of 0.2.

Test plates were inoculated with motile and chemotactic cells from swarms on semi-solid medium. The rate of ring formation and the size of the rings gave some indication of the potency of the attractant.

ROOT EXUDATE PREPARATION

Plant Seeds

Root exudates were prepared from germinating seedlings of the following organisms:

Pea - Pisum sativum var Wisconsin Perfection

Clover - Trifolium repens, Aberystwyth strain S100

Broad bean - Vicia faba var Meteor

Mustard - Sinapsis alba, as supplied by Suttons Seeds Ltd.

Maize - Zea mays, "First of A" F' hybrid as supplied by Suttons Seeds Ltd.

(a) Pea Root Exudate

Peas were wetted for 5 min. with 75% (v/v) ethanol, washed in distilled water and surface sterilized for 30 min. in 10% (w/v) sodium hypochlorite solution (Beringer, 1974). Following six washes in distilled water, peas were incubated ^{on nutrient agar} for 48 h, 24°C in darkness to ensure sterility. Germination progressed only slightly during this period, with the radicle sometimes breaking through the testa. Seeds were then transferred to sterile distilled water (5 mm deep) in Erlenmeyer flasks and incubated at 24°C in dim light. At three days, when the radicle was about 10 mm long, the culture liquid was centrifuged (20 min., 8000g, 5 °C) to remove sloughed off cells, decanted and freeze dried to provide 0-3 day root exudate which was stored (5°C) and redissolved in distilled water as required.

Exudates from older seedlings were obtained by transferring 3 day seedlings, singly, to moist cotton wool over distilled water

in 25 ml "universal" bottles with their caps loosely screwed on. At 6 days, radicles were about 30 mm long with root hairs. The seedlings were then placed on muslin held taut between the lid and base of antibiotic assay plates (24 x 24 cm), with holes in the muslin to permit roots to reach a layer of water below. At 10 days, the roots had branched and the plumules emerged with the opening of the first leaf pair. Exudates (3-6 and 6-10 days) were obtained as for 0-3 day exudate.

(b) Other Root Exudates

Maize seeds were surface sterilized as above and transferred directly from Tryptone-yeast agar to moist cotton wool over distilled water in "universal" bottles. Mustard and clover seeds were surface sterilized for 20 min in 0.1% HgCl_2 , washed in sterile water, and transferred to moistened cotton wool over distilled water in 3 ml "bijoux" bottles. A few seeds were retained and incubated on TY agar to establish the absence of microbial contamination.

CHAPTER 3

ANALYSIS OF PEA ROOT EXUDATE

INTRODUCTION

Legumes exude a large variety of compounds into the rhizosphere. These include sugars, amino acids, organic acids, enzymes, vitamins, nucleotides, fungal stimulators, inhibitors and attractants (Rovira, 1956, 1965a; Elkan, 1961; Pearson & Parkinson, 1961; Chang-Ho & Hickman, 1970; Beringer, ^{et al} 1980). Many factors affect exudation such as plant species, plant age, temperature, light, nutrition, soil moisture, micro-organisms and the medium supporting the roots (Rovira, 1969; Ayers & Thornton, 1968; Boulter et al., 1966). As early as 1935, Virtanen & Laine demonstrated that nodulated legumes excreted nitrogenous substances including aspartic acid. Later, Nutman (1951) also reported that red clover, lucerne and vetch exuded amino compounds when nodulated or supplied with nitrate. More recent studies on legume root exudates include those of Gitte et al. (1978) on the cowpea Cicer arietinum and Chang-Ho & Hickman (1970) who studied 21 day pea seedlings.

Because of the discrepancies in published data on the composition of pea root exudates, a complete analysis was required at several stages of development in order to identify those components which could be of importance to *Rhizobium* in the soil.

MATERIALS AND METHODS

1. Fractionation of Crude pea root exudate

Ultrafiltration followed by ion exchange chromatography was used to fractionate exudate for further analysis. Crude pea root exudate was separated into high (>1000 daltons) and low (<1000 daltons) molecular weight fractions by ultrafiltration (10 ml standard UF ultrafiltration cell, 25 mm UM2 Diaflo ultrafiltration membrane, Amicon Corporation Ltd., Amicon House, Woking). The low molecular

weight fraction was adjusted to give a protein^{equiv.} concentration of $150 \mu\text{g ml}^{-1}$ using the Folin-Lowry Method (Herbert et al., 1971) and passed down a column of Dowex 50 (x-800) resin in the H^+ form. The effluent was passed down a column of Dowex 1 resin (acetate form) to give the neutral fraction. The cationic fraction was eluted from the Dowex 50 resin with $1 \text{ M NH}_4\text{OH}$ and the anionic fraction from the Dowex 1 resin with glacial acetic acid. The fractions were lyophilised for storage prior to further analysis.

2. Analysis of Fractions

(i) Cation fraction

Three different methods were used to separate and quantify the amino-compounds.

(a) Thin-layer chromatography

Two dimensional thin-layer chromatography on silica gel plates provided a useful method of identifying the components of the cation fraction. The solvent system for the first dimension was isopropanol: concentrated NH_4OH (67:33 v/v) followed by n-butanol-acetic acid-water (3:1:1 v/v) for the second dimension (Rai & Strobel, 1966). Amino acids were detected by spraying 0.1% ethanolic ninhydrin on the developed chromatograms and identified by their position relative to standards (Chaykin, 1966).

(b) High Voltage Paper Electrophoresis

High voltage paper electrophoresis (3000V, Whatman no. 1 paper) in pH 5 buffer (pyridine:acetic acid:water in the ratio 800:24:4) was carried out for 1 h on the cationic fraction. After air-drying, chromatograms were examined under UV light. The area containing the amino acids was removed and stitched to a second piece of Whatman no. 1 paper. This second chromatogram was run in pH 2.1 buffer

(8% acetic acid - 2% formic acid) at 3000V for 1 h. Amino-compounds were located using a ninhydrin-cadmium acetate stain (8.5 g ninhydrin in 85 ml acetone; 15 ml cadmium acetate solution, which had been made by dissolving 6 g cadmium acetate in 600 ml water and 300 ml acetic acid). Amino acids were identified by reference to the position and colour of standards.

3. Automatic analysis

The lyophilised cationic fraction from the exudate of a known number of pea seedlings was dissolved in 50 μ l 0.025 M HCl for qualitative and quantitative analysis in a Beckman automatic analyser.

(ii) Neutral fraction

Two methods were used to identify the sugars present in the neutral fraction.

(a) Thin-layer chromatography

Sugars were separated by 2-dimensional TLC using the following solvent systems (a) n-butanol:acetic acid:water (4:1:5 v/v) (b) Ethyl acetate:pyridine:water (8:2:1 v/v). Detection was by the method of Trevelyan et al. (1950) using a solution of silver nitrate in acetone followed by a saturated ethanolic 0.5 M NaOH spray. A second TLC plate prepared in the same way was sprayed with 0.3% ethanolic ninhydrin to detect any amino-sugars present.

(b) Paper chromatography

Paper chromatography was carried out on Whatman 3 MM paper using two solvent systems

(a) n-propanol:ethyl acetate:water (70:10:20 v/v),

(b) n-butanol:acetic acid:water (100:22:50 v/v)

Aniline hydrogen phthalate (1.7 g phthalic acid; 1 ml aniline; 90 ml ethanol; 5 ml glacial acetic acid; 5 ml 40% trichloroacetic acid) was

used to locate the sugars which were identified by reference to the colour and position of standards. Sugars were eluted in the n-propanol-ethyl acetate solvent for quantitative analysis. Reducing sugars with the exception of glucose, were estimated by the Somogyi-Nelson method (Nelson, 1944; Somogyi, 1952) and fructose and raffinose (after hydrolysis in 0.2 N oxalic acid) by the method of Bacon & Bell (1948).

Glucose was estimated with glucose oxidase and peroxidase using the method of Pollard (1982) and the chromogen ABTS (2,2'-Azino-di-3-ethylbenzthiazoline-6-sulphonate); (Werner et al., 1971).

(iii) Anion fraction

Both thin-layer and paper chromatography were used to identify the components of the organic acid fraction.

(a) Thin-layer chromatography

Two-dimensional TLC was carried out on the anion fraction using two solvent systems (a) n-butanol-acetic acid-water (4:1:1 v/v) (b) Ethyl acetate-pyridine-water (8:2:1 v/v). Components were detected by spraying the chromatogram with an ethanolic solution of bromo-phenol blue (Aronoff, 1961) and comparing the samples with prepared standards (Rai & Strobel, 1966). The Trevelyan et al. (1950) method was also used.

(b) Paper chromatography

Paper chromatography, on Whatman no. 1 paper, was carried out for 15 h in n-pentanol: 5 N formic acid (1:1 v/v). After air drying, chromatograms were baked at 90°C for 1 h and organic acids were detected on a reference strip by spraying with an ethanolic solution of bromophenol blue. After elution in water, organic acids were quantified by titrating the eluted compounds against 0.0144 N NaOH

using phenolphthalein as an indicator.

RESULTS AND DISCUSSION

Pea roots exude a wide range of low molecular weight compounds (Tables 3-5). The largest portion of this material is nitrogenous in nature and forms between 31-42% of the total dry weight (Table 6). Eighteen amino acids were identified in all three samples (Table 3). Production increased as expected with age of the root, from 9.8 $\mu\text{g}/\text{root}$ in the first three days of growth to 37.1 $\mu\text{g}/\text{root}$ in the 6-10 day sample. The extent of this increase varied considerably with individual amino acids; the production of some, like phenylalanine, did not greatly increase, while threonine increased from 0.3 $\mu\text{g}/\text{root}$ (0-3 days) to 5.5 $\mu\text{g}/\text{root}$. Threonine was also found in large amounts in 10 day pea root exudate by Rovira (1956). In each sample, the glutamine/glutamic acid/homoserine component made up the largest portion of the exudate. However, since glutamine is hydrolysed to glutamic acid in the amino-acid analyser and the homoserine peak is difficult to differentiate from that of glutamic acid, separate results were not obtained for those compounds. It is not clear, therefore, if any of the components was present in larger amounts or all three at equal concentrations.

There is no evidence to substantiate the claim of Van Egeraat (1975a) that homoserine accounted for 70% of all exuded material. In addition, R.leguminosarum failed to grow on homoserine as the sole nitrogen and carbon source as Van Egeraat (1975b) claimed. Serine was found in fairly large amounts in the 6-10 day sample as was shown by Ayers & Thornton (1968). Sherrod & Domsch (1970) also found serine together with homoserine and aspartic acid to be the predominant amino acids exuded by pea roots in liquid culture.

Analysis of the neutral fraction showed glucose, fructose and xylose to be the major components (Table 4). Rovira (1956) also found

Table 3 Amino acid analysis of pea root exudate ($\mu\text{g}/\text{root}$)

Amino Acid	Period (days) ⁽⁵⁾		
	0-3	3-6	6-10
Alanine ^{(1), (2)}	1.3	1.9	3.0
Arginine	0.6	1.3	3.0
(3) Aspartic acid ^{(1), (2)} /asparagine ^{(1), (2)}	1.1	1.4	1.7
Cysteine	0.1	0.1	0.8
(4) Glutamic acid ^{(1), (2)} /glutamine ⁽¹⁾ / homoserine ⁽¹⁾	1.6	2.0	5.5
Glycine ^{(1), (2)}	0.9	1.1	2.9
Histidine ⁽²⁾	1.3	1.8	2.6
Isoleucine	0.1	0.3	1.0
Leucine ⁽¹⁾	0.3	0.5	3.1
Lysine ⁽²⁾	1.2	1.6	2.7
Phenylalanine ⁽¹⁾	0.1	0.4	0.6
Serine ^{(1), (2)}	0.7	1.0	3.7
Threonine ⁽²⁾	0.3	0.6	5.2
Tryptophan ^{(1), (2)}	0.1	0.4	0.5
Valine	0.1	0.3	0.8
TOTAL	9.8	14.7	37.1

Legend to Table 3

This analysis was carried out in a Beckman automatic analyser using standard procedures.

- (1) Also detected by Thin Layer Chromatography (TLC); see text.
- (2) Also detected by high voltage paper electrophoresis; see text.
- (3) Asparagine is hydrolysed to aspartic acid during this analysis but both compounds were separated and detected by TLC.
- (4) Discrimination between glutamic acid, glutamine and homoserine is difficult. Glutamine is hydrolysed to glutamic acid which cannot be readily distinguished from homoserine by this method. All three amino acids were detected by TLC.
- (5) Samples of pea root exudate were collected over a period of days as described in Chapter 2.

Table 4 Sugar analysis of pea root exudate ($\mu\text{g}/\text{root}$)

Sugar ⁽¹⁾	Period (days) ⁽⁴⁾		
	0-3	3-6	6-10
(2) Fructose	1.7	2.3	5.8
(2) Glucose	1.5	3.0	6.3
(2) Maltose	0.8	1.4	3.0
Raffinose	0.4	1.2	2.3
(2) Ribose	1.1	1.6	2.3
Sucrose	0.1	1.2	1.8
(2) Xylose	1.3	2.8	5.4
(3) Unknown glucan	-	-	+
TOTAL	6.9	13.5	27.9

(1) Sugars were separated and identified by paper chromatography, eluted in n-propanol-ethyl acetate and analysed using suitable chemical methods (see text). Results are expressed as $\mu\text{g}/\text{plant root}$.

(2) Also detected by TLC (see text)

(3) Unidentified glucan, only found in 6-10 day sample

(4) Samples of pea root exudate were collected over a period of days as described in Chapter 2.

Table 5 Organic acid analysis of pea root exudate ($\mu\text{g}/\text{root}$)

Organic acid ⁽¹⁾	Period (days) ⁽³⁾		
	0-3	3-6	6-10
(2) Citric acid	1.0	1.7	2.6
(2) Fumaric acid	-	0.9	2.4
(2) Gluconic acid	0.7	1.6	3.8
Glycolic acid	-	-	0.6
(2) Malic acid	1.6	2.1	3.9
(2) Succinic acid	1.4	2.2	3.2
TOTAL	4.7	8.5	16.5

(1) Organic acids were separated and identified by paper chromatography and quantified, after elution in distilled water, by titration against 0.144 N NaOH (see text). Results are expressed as $\mu\text{g}/\text{plant root}$.

(2) Also detected by Thin layer chromatography (see text)

(3) Samples of pea root exudate were collected over a period of days as described in Chapter 2.

Table 6 The major components of pea root exudate ($\mu\text{g}/\text{root}$)

Component	Period (days)		
	0-3	3-6	6-10
Amino acids	9.8 (42)	14.7 (35)	37.1 (31)
Sugars	6.9 (30)	13.5 (32)	27.9 (23)
$\mu\text{g}/\text{root}$ Organic acids	4.7 (20)	8.5 (20)	16.5 (14)
Total low MW material	21.4 (93)	36.7 (87)	81.5 (67)
Dry weight crude exudate	23.1	42.2	121.3

1. Amounts are expressed as $\mu\text{g}/\text{plant root}$ and as a proportion (%) of total exudate solids.
2. The amounts of amino acids, sugars and organic acids are taken from Tables 5-7.
3. Some loss of material is to be expected during fractionation. Such losses were observed by Rai & Strobel (1966) during fractionation of sugar beet exudate by similar methods.

glucose and fructose to be produced in large amounts by 10 day old roots but he continued the experiments to show that by 21 days, roots were exuding only 20% as much sugar. No amino sugars were detected at any stage. A low molecular weight glucan was produced by 6-10 day old roots but this was not identified. Total sugar exudation increased from 6.9 $\mu\text{g}/\text{root}$ to 27.9 $\mu\text{g}/\text{root}$ for the 6-10 day sample (Table 6).

Six organic acids were identified and quantified in the three samples (Table 5). However, organic acids accounted for a small portion, only 14-20% of the low molecular weight material. With the exception of glycolic acid, which was only identified in the sample from the older roots, the remaining compounds were all found in similar amounts.

CHAPTER 4

POSITIVE CHEMOTAXIS TO ROOT EXUDATES

INTRODUCTION

It is generally accepted that the main factors for the stimulation of micro-organisms in the rhizosphere are the excretion of organic substances from seedling roots and the sloughing off of root hairs and epidermal cells. Munns (1968) demonstrated that when alfalfa grown in liquid culture was inoculated with homologous rhizobia, many more bacteria accumulated on the surface of the root than could be accounted for by multiplication of the inoculum. Chemotaxis to root excretions could explain this observation. Rhizobial growth is stimulated by the roots of most plants but generally more by legumes than non-legumes (Parker et al., 1977). Reports vary as to whether there is any specificity in stimulation by legumes for those bacteria capable of forming nodules in a species over non-nodulating bacteria. Peters & Alexander (1966) studied the effect of legume exudates on root nodule bacteria. They demonstrated that the effect of legumes in promoting bacterial growth in the root zone was not specific for the bacteria capable of inducing nodulation. Similar observations were made by Currier & Strobel (1976) who have recently attributed attraction of one strain of R.trifolii by its host, Birds foot trifoil (Lotus corniculatus L.), to a high molecular weight glycoprotein (1977a,b). Gitte et al. (1978) showed attraction of a Rhizobium strain by exudates of its host the chickpea (Acer arietinum L.) and found chemotaxis to the various amino acids, sugars and organic acids that they detected in the exudate.

In this investigation, the isolation of those components of pea root exudate responsible for chemotaxis has been attempted. In addition, the chemotaxis of a number of strains of Rhizobium to a range of legume and non-legume exudates has been tested in order to investigate the specificity of the response.

A. ATTRACTION OF R.LEGUMINOSARUM TO PEA ROOT EXUDATE

The chemotactic response of R.leguminosarum strain N5 to crude and fractionated pea root exudate was investigated.

MATERIALS AND METHODS

Chemotaxis assay

The chemotaxis assay was the blind well chamber method of Armitage et al. (1977) described in Chapter 2. Counting was done using the Coulter counter and checked with a viable count. A non-chemotactic control (chemotaxis buffer) and a known positive control (yeast extract or fresh pea root exudate) was included in each test.

Preparation of root exudate

Pea root exudates were prepared in sterile chemotaxis buffer as described in Chapter 2. For chemotaxis assays the concentration was adjusted to a protein content of $150 \mu\text{g ml}^{-1}$ using the Folin-Lowry method (Herbert et al., 1977).

Gel Filtration

Crude pea root exudate was separated into fractions of different molecular size by filtering on Sephadex G-10 and Sephadex G-15 gels (Pharmacia Ltd.). Filtration was carried out in distilled water to overcome problems of high salt concentrations when concentrating the eluate for chemotaxis assays. Fifty 1 ml fractions were collected. Selected fractions were tested for changes in sugar and protein concentration and for chemotactic activity.

The sugar content of selected fractions was analysed using the anthrone-sulphuric acid method (Plummer, 1978). 4 ml of anthrone reagent (0.2% anthrone in concentrated H_2SO_4) was added to the 1 ml sample and incubated for 10 min. in a boiling water bath. After cooling, samples were read at 260 nm against a reagent blank. This method measures hexoses and hexuloses only. The OD at 280 nm of every

fraction was measured to indicate protein content. Finally, fractions were pooled (Fig. 3) and tested for chemotaxis using the blind well chamber assay.

Ultrafiltration

Ultrafiltration of crude pea root exudate ($150 \mu\text{g protein ml}^{-1}$ in chemotaxis buffer) was carried out under pressure using a standard stirred UF ultrafiltration cell (Amicon Corporation Ltd.) with three types of ultrafiltration membrane: PM30, PM10 and UM2 (Amicon Corporation Ltd.). All fractions were restored to their original volume prior to testing for chemotaxis by the blind well chamber assay.

RESULTS AND DISCUSSION

R.leguminosarum strain N5 shows significant attraction (chemotaxis ratio 3.6) to fresh pea root exudate produced during the period 0-3 days by germinating pea seedlings (Table 7). A freeze-dried preparation redissolved and diluted to the same protein content in chemotaxis buffer gave a chemotaxis ratio of 3.8. Clearly 3 day pea exudate remains attractive after freeze-drying. All subsequent work on pea root exudates was carried out on freeze-dried material redissolved in chemotaxis buffer to give, unless otherwise indicated, a protein content of $150 \mu\text{g ml}^{-1}$.

Tests (5 replicates) were carried out on exudates produced by pea seedlings during the periods 0-3, 3-6 and 6-10 days. The chemotaxis ratios obtained (3.2, 3.0 and 3.4 respectively) did not differ significantly. Subsequent chemotaxis tests were confined to 0-3 day material.

Exudate from the same 3-day batch as was used to establish the effectiveness of freeze-drying was subjected to boiling (10 min.) and to autoclaving (Table 7). Chemotaxis ratios of 3.4 and 3.0 as compared with 3.8 were obtained. Since such treatments, which would denature

Table 7 The effect of various denaturing treatments on the potency
 of pea root exudate

Treatment	Chemotaxis ratio ⁽¹⁾
None	3.8
Freeze drying	3.8
Boiling (10 min.)	3.4
Autoclaving	3.0
Freezing	3.9
Pronase ⁽²⁾	4.6

(1) Chemotaxis ratios were obtained using the blind well chamber assay. Each result is the mean of 5 replicates.

(2) Pronase treatment: 5 ml of root exudate was incubated overnight at 40°C in 0.1 M potassium phosphate buffer at pH 7 with 1 ml of proteolytic enzyme from Streptomyces griseus (1 mg Pronase; Sigma Ltd., Poole, Dorset). Pronase itself gave a chemotaxis ratio of 1.8.

proteins, did not eliminate attractiveness, it is unlikely that a major part of the chemotactic effect is due to specific proteins. In order to check this result root exudate was treated with a proteolytic enzyme and tested for activity (Table 7). The product on testing gave a chemotaxis rate of 4.6 indicating that the treatment did not impair attraction. In fact, attraction may have been enhanced by the breakdown of proteins to release amino acids. The pronase preparation itself may have been slightly attractive (chemotaxis ratio 1.8).

Preliminary experiments with gel filtration indicated that only the fraction containing substances with molecular weights less than 700 daltons had chemotactic activity. Both amino acids and sugars were detected in this fraction (Fig. 3). No chemotactic activity for R.leguminosarum was detected in any of the high molecular weight fractions. Gel filtration was carried out with unbuffered exudate in order to avoid problems of removing excessive salt concentrations which would be obtained on concentrating the eluates for chemotaxis assays. In this unbuffered state it is possible that denaturation of high molecular weight compounds may have occurred, rendering potential attractants inactive. To overcome this difficulty, further study of the relationship between molecular size and chemotactic activity was carried out by ultrafiltration.

After fractionation by ultrafiltration, significant chemotaxis ratios were obtained only for the low molecular weight fraction (Table 8). This fraction ($150 \mu\text{g protein}_{\text{equiv.}} \text{ml}^{-1}$) was separated into its cation, anion and neutral components by ion exchange chromatography using the methods described in Chapter 3. The separate fractions were resuspended in chemotaxis buffer to the original concentration for

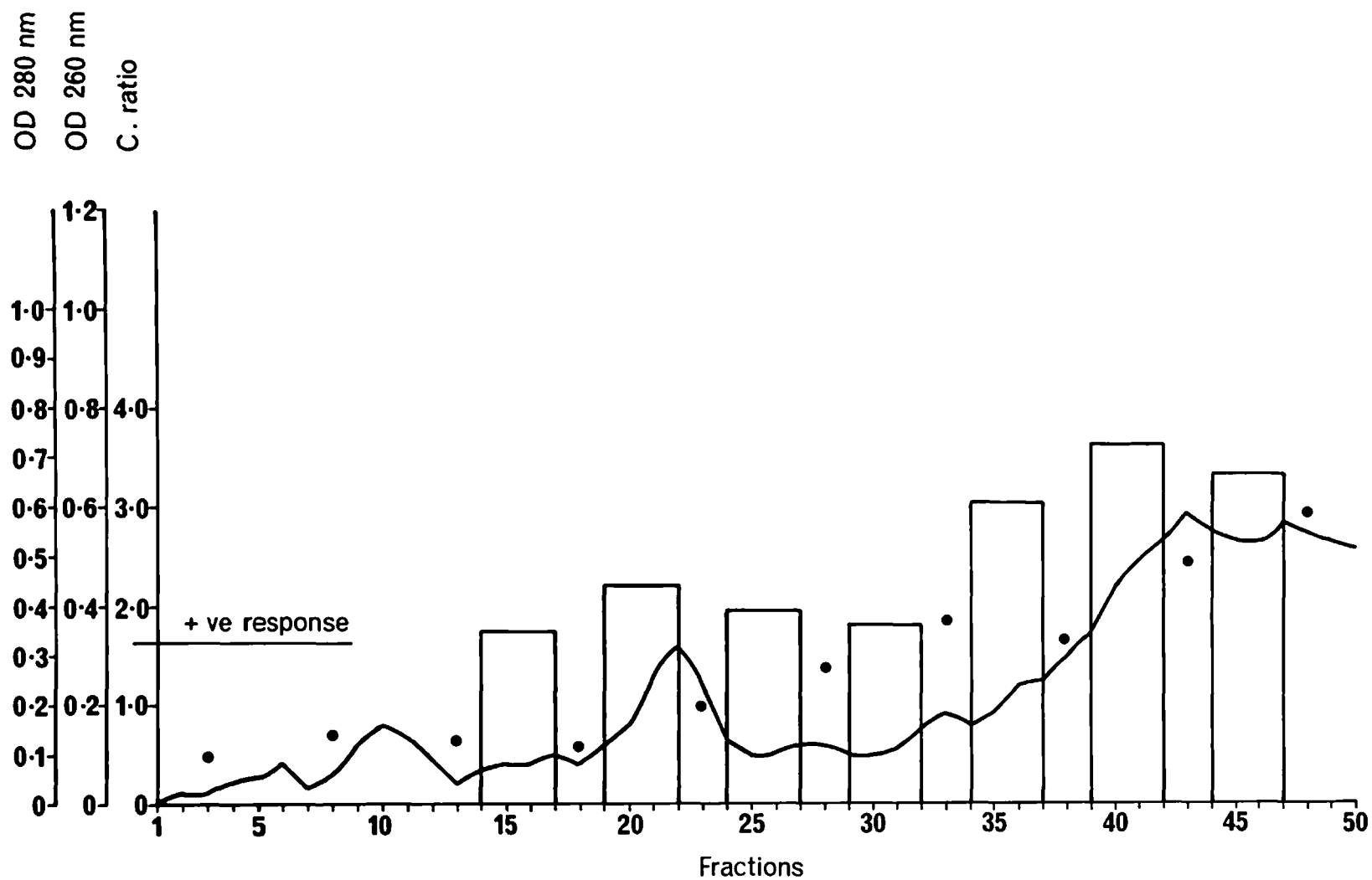


Fig.3. Fractionation of pea root exudate by gel filtration.

(a) Sephadex G-15

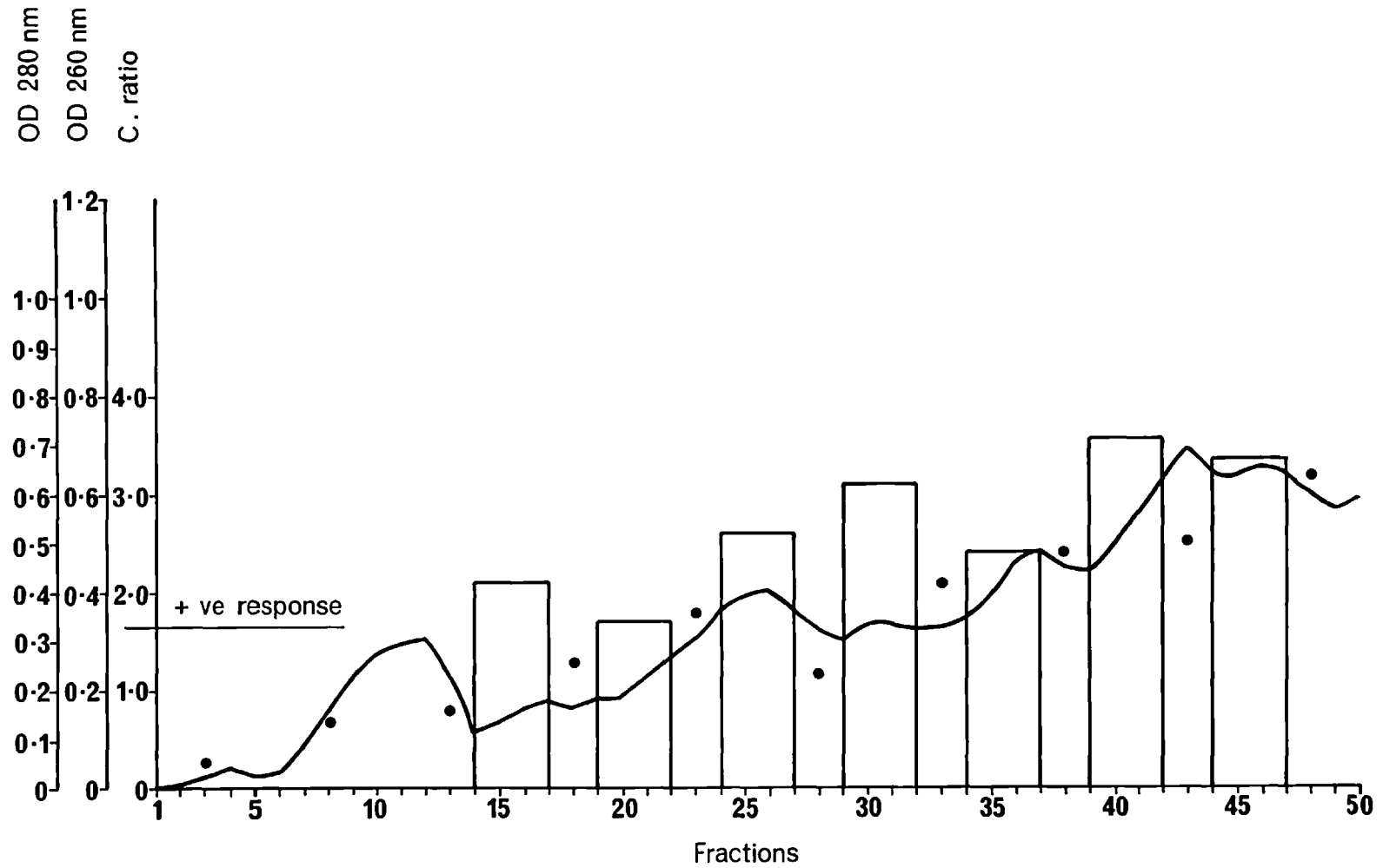


Fig.3. Fractionation of pea root exudate by gel filtration.

(b) Sephadex G-10

Legend to Fig. 3:

Crude pea root exudate was fractionated by gel filtration on Sephadex G-10 and G-15; see text.

————— OD of each fraction at 280 nm in a 1 cm cuvette

• • • OD of anthrone-sulphuric acid treated fractions at 260 nm
 [] in a 1 cm cuvette; see text

Chemotaxis ratios for the pooled fractions: 14-17, 19-22, 24-27, 29-32, 34-37, 39-42, 44-47. In each case, the void volume of the column (11 ml) i.e. the first 11 fractions containing molecules of a size excluded by the gel matrix, was also pooled and tested for chemotactic activity. For sephadex G10 this contained molecules with a molecular weight >700 daltons; for G15 it contained molecules with a molecular weight >1500 daltons. In both cases, this high molecular weight fraction showed no chemotactic activity.

Table 8 Molecular size and chemotactic activity

Molecular size ⁽²⁾ range (daltons)	Chemotaxis ⁽¹⁾ ratio
0-1000	2.7
1000-10000	1.7
10000-30000	1.6
>30000	1.6

(1) Chemotaxis ratios were obtained by the blind well chamber assay. Ratios >1.7 were regarded as significant (significance of chemotaxis ratios; see Chapter 2)

(2) Molecules were fractioned by size using ultrafiltration (see text).

chemotaxis assays. Assays were carried out on the unfractionated material, the separate and the recombined fractions by both blind well chamber and capillary tests (Table 9). All fractions showed significant chemotactic activity for R.leguminosarum, the highest ratios being obtained with the cationic and the lowest with the anionic fractions. The recombined fractions gave chemotaxis ratios similar to the unfractionated material.

B. CHEMOTAXIS OF RHIZOBIUM TO LEGUME AND NON-LEGUME ROOT EXUDATES

MATERIALS AND METHODS

Organisms

The strains of R.leguminosarum, R.phaseoli, R.trifolii and E.coli used in this investigation were those described in Chapter 2.

Root Exudates

Root exudates were prepared by growing surface sterilized seeds under aseptic conditions as described in Chapter 2. Exudates were obtained from pea roots and from two other legumes, clover (Trifolium repens, Aberystwyth strain S100) and broad bean (Vicia faba, variety meteor). Non-legume exudates were from another dicotyledenous species, mustard (Sinapis alba, Sutton Seeds Ltd.) and a monocotyledenous plant, maize (Zea mays, "First of All" F_1 Hybrid, Sutton Seeds Ltd.). Protein determinations on the exudates were carried out by the Folin-Lowry method (Herbert et al. 1977) and were followed by a series of dilutions in chemotaxis buffer to give a range of concentrations for testing.

Chemotaxis assays

Chemotaxis assays were carried out by the blind well chamber method described in Chapter 2. Two controls were included in each test; chemotaxis buffer (non-chemotactic) and $150 \mu\text{g protein ml}^{-1}$ pea root exudate (positive control).

Table 9 Chemotaxis of *R. leguminosarum* strain N5 to low molecular weight components of pea root exudate

Fraction	Chemotaxis ratio ⁽²⁾	
	Blind Well chamber	Capillary ⁽¹⁾ test
Unfractionated material	3.5	18.5
Cationic fraction	3.1	15.3
Neutral fraction	2.6	11.2
Anionic fraction	2.0	7.6
Recombined fractions	3.6	19.6

(1) The capillary test is based on the procedure of Adler (1973) and described in Chapter 2.

(2) Definition of chemotaxis ratios: see Chapter 2. Each result is the mean of 5 replicates.

RESULTS AND DISCUSSION

The ability of R.leguminosarum and other bacteria to show chemotaxis to a range of low molecular weight attractants suggests that exudates from plants other than pea would prove attractive to a range of microorganisms. It is clear from the results summarized in Table 10 (a-e) that this is the case. Escherichia coli was included as a test organism since it is not a plant symbiont. Thus, significant responses to root exudates of the same order as those obtained for Rhizobium strains serve to confirm the non-specificity of this phenomenon.

All exudates proved attractive to all the bacteria when tested at a dilution corresponding to a protein content of $75 \mu\text{g ml}^{-1}$ and most at $50 \mu\text{g protein ml}^{-1}$. With E.coli, significant attraction to maize root exudate was observed at a tenfold higher dilution. Each Rhizobium strain showed no greater response to its own host exudate compared to any other exudate. This is in agreement with the work of Currier & Strobel (1976) who reported chemotaxis by a number of Rhizobium strains to legume root exudate but no one to one correlation between chemotaxis and nodulation. Peters & Alexander (1966) also failed to find a specific effect.

The chemotaxis ratios obtained with the higher concentrations of broad bean exudate were less striking than those observed with other exudates for the entire range of organisms. E.coli, R.phaseoli and R.trifolii all failed to give a significant response at $75 \mu\text{g protein ml}^{-1}$ as did R.leguminosarum, the natural symbiont. There have been several previous reports of root exudates containing inhibitory materials including hydrocyanic acid, glycosides and saponins (Rovira, 1965) which could have a repellent effect on Rhizobia. Elkan (1961)

Table 10 a-e Response of Rhizobium strains and Escherichia coli to
a range of legume and non-legume root exudates

(a) Response to mustard exudate

Organism	Exudate concentration $\mu\text{g ml}^{-1}$ protein				
	300	150	75	50	5
<u>R. leguminosarum</u> strain N5	2.8	2.4	2.1	1.9	1.4
<u>R. phaseoli</u> strain 1231	2.5	2.9	2.3	1.8	1.6
<u>R. trifolii</u> strain 6601	2.9	2.4	2.7	2.0	1.4
<u>E. coli</u> strain C600	3.9	3.6	4.0	2.5	1.9

(b) Response to broad bean exudate

Organism	Exudate concentration $\mu\text{g ml}^{-1}$ protein				
	300	150	75	50	5
<u>R. leguminosarum</u> strain N5	2.1	1.8	1.9	1.5	1.4
<u>R. phaseoli</u> strain 1231	1.8	2.3	1.7	1.8	1.1
<u>R. trifolii</u> strain 6601	1.7	1.6	1.3	1.9	1.2
<u>E. coli</u> strain C600	2.9	2.2	1.6	1.8	1.2

(c) Response to pea exudate

Organism	Exudate concentration $\mu\text{g ml}^{-1}$ protein				
	300	150	75	50	5
<u>R. leguminosarum</u> strain N5	3.2	2.9	1.8	2.0	1.3
<u>R. phaseoli</u> strain 1231	3.3	2.7	2.8	1.5	1.2
<u>R. trifolii</u> strain 6601	3.7	2.7	3.0	1.4	1.3
<u>E. coli</u> strain C600	2.8	3.6	2.5	2.1	1.8

Table 10 (cont.)

(d) Response to clover exudate

Organism	Exudate concentration $\mu\text{g ml}^{-1}$ protein				
	300	150	75	50	5
<u>R. leguminosarum</u> strain N5	3.6	2.8	3.3	2.2	1.6
<u>R. phaseoli</u> strain 1231	3.1	2.6	1.8	1.3	0.9
<u>R. trifolii</u> strain 6601	3.8	2.9	2.4	1.9	1.2
<u>E. coli</u> strain C600	4.7	3.4	2.7	2.8	1.6

(e) Response to maize exudate

Organism	Exudate concentration $\mu\text{g ml}^{-1}$ protein				
	300	150	75	50	5
<u>R. leguminosarum</u> strain N5	3.9	3.5	2.7	2.7	1.7
<u>R. phaseoli</u> strain 1231	3.3	3.9	2.5	2.1	1.6
<u>R. trifolii</u> strain 6601	3.3	3.5	2.6	2.0	1.2
<u>E. coli</u> strain C600	4.1	3.6	3.7	2.4	1.8

Results are expressed as chemotaxis ratios obtained by the blind well chemotaxis assay method. Each result is the mean of 5 replicates. All ratios >1.7 are considered to be significant at a 1% level of probability (see Chapter 2 for an analysis of significance).

found that the exudate from a non-nodulating strain of soybean prevented nodulation of normally nodulating soybean strains. The occurrence of microbial inhibitors has also been reported by Kandasamy et al. (1974).

The results also confirm that exudates are active over a wide range of concentrations. This is to be expected if chemotaxis is to play a role in natural infections.

CHAPTER 5

POSITIVE CHEMOTAXIS TO SINGLE COMPOUNDS

INTRODUCTION

In view of the widespread occurrence of chemotaxis by flagellate bacteria (Carlile, 1980), motile Rhizobia could be expected to show chemotaxis to simple molecules. Currier & Strobel (1976), using a radioactive bacterial labelling technique failed to demonstrate chemotaxis of a strain of R.trifolii to common sugars and amino acids. However, subsequent work by Currier (1980) demonstrated chemotaxis of another trefoil strain to the simple sugars mannose, ribose, maltose, fructose, galactose and sucrose and Currier concluded that strains of Rhizobium vary in their responses; some are not affected by sugars and amino acids while others show positive chemotaxis towards them.

Gitte et al. (1978) used the Adler capillary assay method to show attraction of a Rhizobium strain by the various amino acids, sugars and organic acids found in the root exudate of its host, the chickpea (Cicer arietinum L.). Similar work by Dilworth & Bowra (1981) with a strain of R.leguminosarum and Gotz et al. (1982) using R.meliloti also demonstrated chemotaxis to small molecules.

The aims of the present study were to use a suitable method to quantify chemotaxis of R.leguminosarum to simple sugars and amino acids, especially those found in pea root exudate.

MATERIALS AND METHODS

Chemotaxis assay design

Two designs of chemotaxis assay were investigated. These are fully documented in Chapter 2. The first method, the capillary assay used by Adler (1973), involves placing capillaries of the test solution into a bacterial suspension and observing the accumulation of organisms in the capillary. Variations of this assay method were used to investigate Rhizobium chemotaxis by Currier & Strobel (1976, 1977a, b), Dilworth & Bowra (1981) and Gotz et al. (1982). The major disadvantage of this

method is that the diffusion gradients set up on introducing the attractant into the pool of bacteria are very easily disturbed by the slightest vibrations. In addition, the small capillaries can be difficult to manipulate.

The alternative method, the blind well chamber assay used by Armitage et al. (1977) was found to be less sensitive to external factors. In this method, a bacterial suspension is placed in a well, separated from solutions of the test chemical by a polycarbonate membrane. Chemotaxis is inferred when the number of organisms passing through the membrane in the test chambers is greater than the comparable number in the control chambers. This assay gave smaller chemotaxis ratios than the capillary assay but the results were much more reproducible.

Plate Chemotaxis

Swarming on a semi-solid modified minimal medium (Minimal salts + 2.5 gl^{-1} sucrose) containing the test chemical was used to confirm the assay results as described in Chapter 2. Using this method it was possible to observe if chemotaxis to a specific chemical could be induced.

Chemicals used in chemotaxis assays

Chemicals used in this study were of the highest purity commercially available and were tested without further purification.

RESULTS AND DISCUSSION

Chemoattractants

Preliminary chemotaxis experiments with R.leguminosarum strain N5, gave positive results with undefined mixtures such as yeast extract and brown sugar. Further work with low molecular weight compounds found in pea root exudate and related substances, showed attraction of Rhizobium to a wide range of amino acids, sugars and organic acids (Tables 11, 12, 13). Three amino acids, arginine, glycine and lysine gave significant positive chemotaxis ratios when the source concentration in the blind well chamber was 10^{-6} and all twenty were effective at 10^{-5} M. Optimal

Table 11 Chemotaxis of *R. leguminosarum* to amino acids

Amino acid	Concentration (M)					Conclusion
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	
Alanine	1.9	2.4	2.8	1.7	1.2	+
Arginine	1.7	2.9	2.6	2.5	1.8	+
Asparagine	2.2	2.7	2.4	2.1	1.5	+
*Aspartic acid	2.1	3.0	2.7	1.7	1.2	+
Cysteine	1.9	2.8	2.4	1.7	1.4	+
Glutamine	2.5	2.6	2.0	2.2	1.6	+
*Glutamic acid	3.1	3.6	3.2	1.7	1.6	+
Glycine	1.8	2.5	3.1	2.6	1.9	+
Histidine	1.9	2.7	3.0	2.6	1.6	+
Homoserine	2.0	2.5	2.6	1.7	1.5	+
Isoleucine	1.6	1.8	2.7	1.9	1.3	+
Leucine	1.8	2.1	2.7	2.1	1.4	+
Lysine	1.6	2.8	3.0	2.0	1.8	+
Methionine	1.9	2.9	2.2	2.0	1.3	+
Phenylalanine	1.7	2.0	2.8	2.2	0.8	+
Proline	1.8	3.3	2.8	2.4	1.6	+
Serine	1.8	2.0	2.7	2.5	1.3	+
Threonine	1.8	2.6	2.1	1.9	1.2	+
Tryptophan	1.5	2.5	2.2	2.0	1.4	+
Valine	1.9	2.1	2.5	2.3	0.9	+

*As Na⁺ salt

The results are expressed as chemotaxis ratios obtained by the blind well chamber chemotaxis assay method. Each result is the mean of 5 replicates. All amino acids were in the L-form. (+) indicates a significant response at a 1% level of probability. (See Chapter 2 for analysis of significance).

Table 12 Chemotaxis of *R. leguminosarum* to sugars

Sugar	Concentration					Conclusion
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	
L-arabinose	1.6	2.8	1.9	1.7	0.8	+
Fructose	1.7	2.5	2.2	2.4	1.2	+
Fucose	0.6	1.4	1.0	1.5	1.2	0
Galactose	1.9	2.5	2.9	1.9	1.5	+
Glucose	2.3	3.3	3.2	2.9	1.7	+
Lactose	1.7	2.0	2.6	2.3	1.5	+
Maltose	1.9	2.3	1.9	1.7	1.4	+
+D-mannitol	1.8	2.0	3.1	1.6	1.1	+
Raffinose	0.9	1.2	1.5	1.0	1.2	0
Ribose	1.6	2.8	1.9	1.7	0.8	+
Sucrose	1.4	1.3	1.5	0.9	1.2	0
Trehalose	1.1	1.3	1.4	0.9	1.2	0
Xylose	1.9	2.1	1.8	1.3	0.7	+

+sugar alcohol

The results are expressed as chemotaxis ratios obtained by the blind well chamber assay method. Each result is the mean of 5 replicates.

(+) indicates a significant response at a 1% level of probability.

(0) indicates no significant response. (See Chapter 2 for analysis of significance).

Table 13 Chemotaxis of *R. leguminosarum* to organic acids

Organic acid*	Concentration (M)					Conclusion
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	
Citric acid	1.9	2.3	1.8	1.7	1.6	+
Gluconic acid	1.8	1.9	1.7	1.7	0.9	+
Malic acid	1.7	2.0	2.4	1.8	1.3	+
Succinic acid	2.1	2.8	2.7	2.4	1.9	+
Glycolic acid	1.6	1.8	0.9	1.2	0.8	0

*as Na⁺ salt

Conditions: see legend to Table 12.

responses were seen at 10^{-3} - 10^{-4} M.

Positive chemotaxis was also observed with eight sugars and with mannitol, optimal concentrations again being in the range 10^{-3} M or 10^{-4} M. Sucrose, which was detected in pea root exudate, fucose and trehalose all failed to elicit a response. Since chemotaxis in E.coli (Adler et al., 1973) and in Pseudomonas aeruginosa is inducible for some substrates it was possible that Rhizobia grown on these substrates might show chemotaxis towards them. However, since plate chemotaxis assays did not reveal any moving rings after 48 h incubation on minimal salts medium containing sucrose, fucose or trehalose, indubitable responses seem unlikely. Failure to respond to some utilizable sugars is also common in other bacteria (Adler, 1973; Moulton & Mountie, 1979).

Positive chemotactic responses were observed to four of the five organic acids tested. Optimum results were seen at source concentrations of 10^{-3} to 10^{-4} M. Gluconic acid failed to elicit a significant response at all concentrations except 10^{-3} M where a small response was noted; the possibility of contamination by an attractant at this high concentration cannot however be excluded.

Optimum conditions for motility and chemotaxis

As in other bacteria, the optimum pH for motility in R.leguminosarum was in the same range as that for growth. Most strains of Rhizobium show optimal growth over the pH range 5 to 8 (Vincent, 1977). Good motility and chemotaxis in R.leguminosarum were observed between pH 6.5 and 7.5 and were optimal at pH 7. Similar results for the trefoil Rhizobium were demonstrated by Currier (1980) who showed that maximal chemotaxis in this strain occurred between pH 7.0-7.5.

Inclusion of a chelating agent (EGTA or EDTA) in both growth and chemotaxis suspending media was essential for good results. Very small traces of heavy metal cations seriously inhibited motility. The level

of free Ca^{2+} ions in Rhizobium chemotaxis buffer was regulated at 10^{-6}M using EGTA. Below 10^{-7}M free Ca^{2+} ions total cessation of motility was observed.

CHAPTER 6

NEGATIVE CHEMOTAXIS

INTRODUCTION

Negative chemotaxis, the movement of organisms away from chemicals was first described in bacteria by Engelmann (1881) and Pfeffer (1888) who reported taxis away from salts, bases, acids and alcohols. More recent work has shown negative chemotaxis in Rhodospirillum rubrum for certain amino acids and salts (Clayton, 1958) and in Pseudomonas fluorescens and other species for acids, bases, salts, alcohols and toxic chemicals (Smith & Doetsch, 1969; Doetsch & Seymour, 1970; Seymour & Doetsch, 1973; Young & Mitchell, 1973). Negative aerotaxis in three strains of Clostridia was demonstrated by Baracchini & Sherris (1959). The most comprehensive studies have been carried out with Escherichia coli (Tso & Adler, 1974) and Salmonella typhimurium (Tsang et al., 1973) which are repelled by extremes of pH, certain ions and a range of organic substances including hydrophobic amino acids at high concentrations, and various fatty acids. The latter, some of which are products of anaerobic metabolism, may serve as a signal for cells to escape from overcrowded conditions.

MATERIALS AND METHODS

(a) Blind well chamber assay

(i) Chemical-in-chamber method

The blind well chamber assay used to investigate positive chemotaxis (see chapter 2) may also be used to investigate negative chemotaxis. The procedure is exactly the same but, if the test chemical is a repellent, fewer than the background number of organisms will accumulate. In this situation, very small numbers of bacteria are involved and so the method becomes rather insensitive. Weak repellents could not be detected in this way. Known repellents were included in each assay to provide an additional control. Since insufficient data was available for statistical analysis,

chemotaxis ratios of less than 1.0 were taken to imply negative chemotaxis. The main advantage of this method over other negative assay techniques lies in the fact that it can be used for simultaneously screening for attractants and repellents.

(ii) Chemical-in-pond method

The blind well chamber assay can also be adopted to determine negative chemotaxis by mixing the suspected repellent into the suspension of bacteria in the lower well of the chamber. In this case the upper well contained only chemotaxis buffer. Bacteria 'escaped' into the upper chamber away from the repellent and were counted by the methods previously described. Chemotaxis ratios were calculated and those greater than 1.7 were regarded as significant for negative chemotaxis (significance of chemotaxis ratios: see Chapter 2). The greater the repellent effect the larger the chemotaxis ratio. Known repellents were included in each assay as additional controls. This method could be used to demonstrate fairly weak repellent effects.

(b) Chemical-in-plug method

In this method, the repellent is contained in plugs of hard agar (2%) which are placed into petri dishes of soft agar (0.25%) containing the test organisms at sufficient concentration to give visible turbidity. Negative chemotaxis occurs if the bacteria are repelled by the test chemical to give clear areas around the plugs. Repellent plugs were made by dissolving the test chemical in autoclaved and cooled melted agar (20 g l^{-1} agar in chemotaxis buffer) at a range of concentrations. The agar was poured into plastic petri dishes and allowed to solidify. Repellent plugs were cut out using a 6 mm diameter cork borer. The test organism was grown in minimal medium at 24°C for 36-48 h to give approximately $6 \times 10^8 \text{ cells ml}^{-1}$. Static liquid culture was used as this can favour motile, chemotactic cells (Smith & Doetsch, 1969).

Twenty-five ml volumes of this culture were washed once in chemotaxis buffer by centrifugation (8000 g, 10°C, 10 min.) and finally resuspended in the migration medium (chemotaxis buffer solidified with 0.25% agar) to give a final concentration of about 5×10^7 cells/ml. This cell-agar suspension was immediately poured into plastic petri dishes. After a few minutes to minimize convection due to cooling, agar plugs containing the repellent were planted into the cell-agar suspension using forceps. The plate was allowed to solidify and develop for 90 min. at 24°C. The distances from the edge of the plugs to the ring of bacteria were then measured using a magnifying glass to aid accuracy.

(c) Chemical-in-plate method

This method is basically the reverse of the chemical-in-plug method since in this case the repellent is contained in hard agar throughout the petri dish, while a turbid suspension of bacteria in soft agar is placed in wells. Approximately 20 ml volumes of 2% agar (2 g agar/100 ml chemotaxis buffer) containing the repellent at a range of concentrations, were poured into plastic petri dishes and allowed to solidify. Using a 9 mm diameter cork borer, holes were cut out of the agar. Bacteria were prepared as for the chemical-in-plug method and each hole was just filled with the test strain suspended in 0.2% agar containing chemotaxis buffer at 5×10^7 cells ml^{-1} . The plates were incubated at 24°C for 60 min. Bacteria escape from the repellent to form visible rings in the centre of wells.

Chemicals used in repellent assays

Chemicals used in this study were of the highest purify commercially available. Chemotaxis buffer was solidified with Oxoid no. 3 agar as required.

RESULTS AND DISCUSSION

Assay Techniques

In general, the chemical-in-pond method was the most useful since it was sufficiently sensitive to identify weak repellents and provided easily quantifiable results. Threshold values obtained by this method were often lower than those determined by other techniques. Chemotaxis ratios were generally lower than those for positive chemotaxis determinations by the same method. The reverse method, the chemical-in-chamber assay was more limited in its application since only strong repellents such as metal cations could be detected.

The chemical-in-plug method was successfully used to demonstrate the effect of H^+ and OH^- ions. By measuring the distance from the centre of the agar plug to the edge of the repellent zone, the method could be quantified. Concentration-response curves obtained in this way show the repellent effect of phenylacetate (Fig. 4), salicylate (Fig. 5) and both high and low pH (Fig. 6). The main advantage of this technique is that it allows a range of chemicals of different concentrations to be screened at the same time in identical conditions. The chemical-in-plate technique could not be quantified but was especially useful for testing a large number of colonies when screening for mutants (see Chapter 7).

Chemorepellents

A wide range of chemicals were screened for repellent activity (Table 14). Most of the test chemicals were effective repellents of E.coli (Tso & Adler, 1974) or B.subtilis (Ordal & Goldman, 1976). Similar patterns of repulsion emerged for R.leguminosarum with one major difference; the group of hydrophobic amino acids which are repellents for E.coli are attractants for R.leguminosarum. Most of the repellents tested were harmful to Rhizobium with some exceptions including

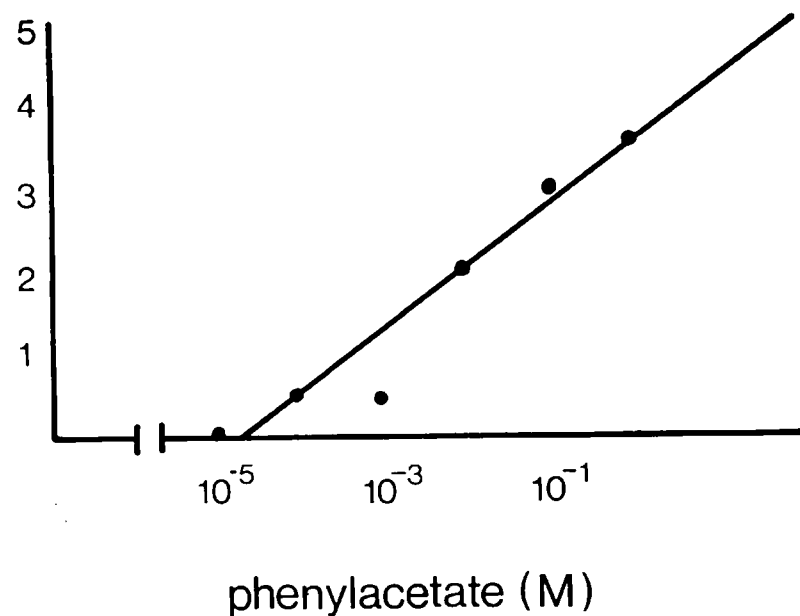


Fig.4. Concentration-response curve for phenylacetate (Na^+).

Concentration-response curve was produced by the chemical-in-plug method (see text). Results are shown as distances (mm.) migrated by the organisms from the edge of the repellent plug over a range of concentrations. Each result is the mean of five replicates.

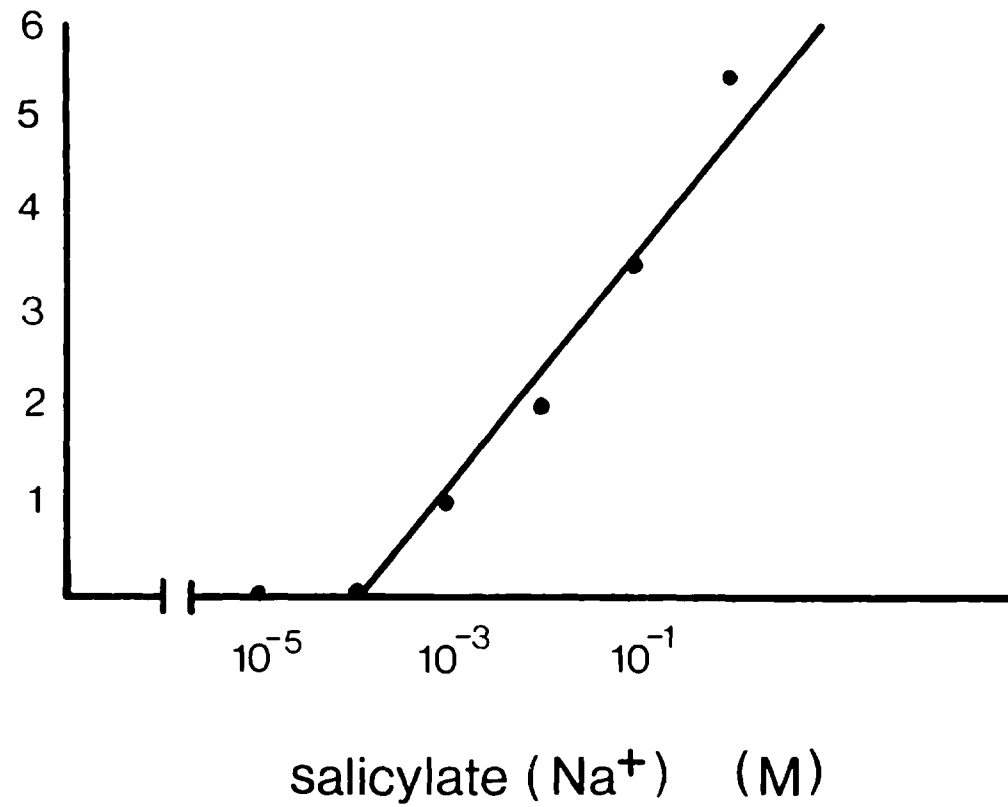


Fig. 5 Concentration-response curve for salicylate (Na⁺)
Conditions: see legend to Fig. 4.

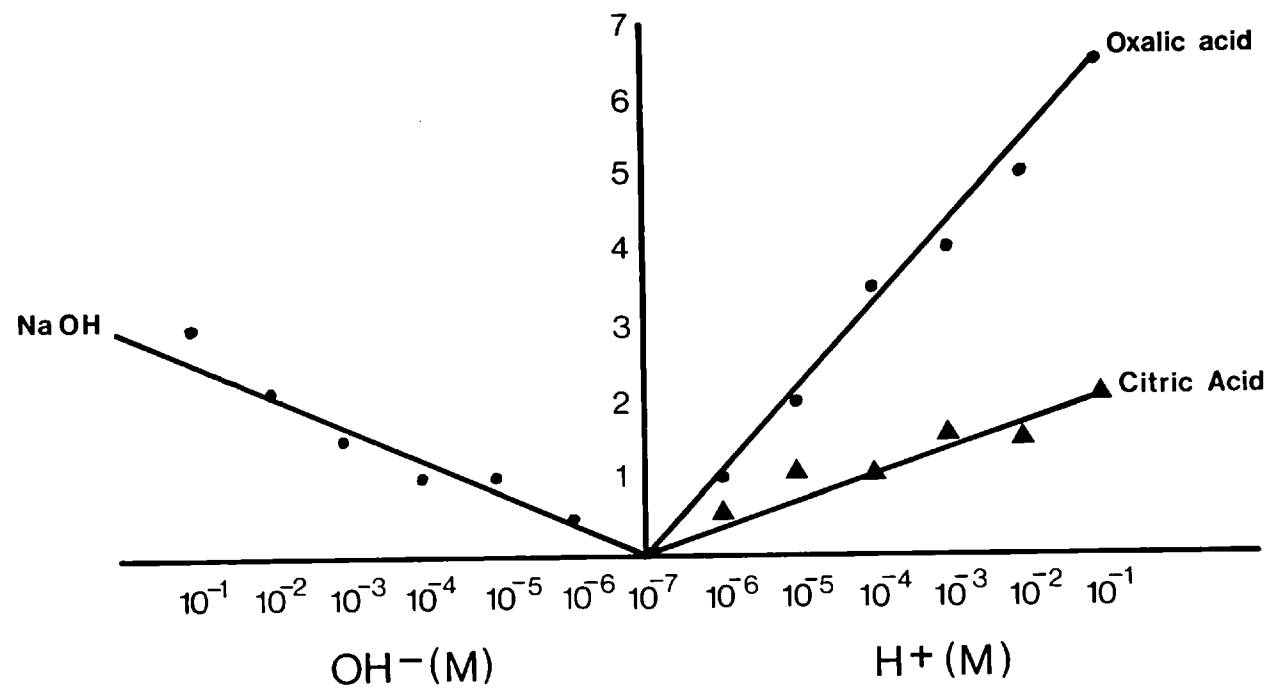


Fig.6 Concentration-response curve for H^+ and OH^- ions.
Conditions: see legend to Fig. 4.

Table 14 A summary of repellants for *R. leguminosarum*

Repellant	Chemotaxis ratios						Threshold
	(chemical-in-pond method)						M
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	M
Acetaldehyde	1.8	1.7	1.2	0.9	1.1	1.2	10^{-2}
Acetamide	1.7	1.8	1.7	1.3	0.5	0.7	10^{-3}
Acetate (Na^+)	1.9	2.0	1.7	1.8	0.2	1.1	10^{-4}
Acetyl salicylate	1.9	1.8	1.9	1.5	1.4	0.8	10^{-3}
Benzamide	2.0	1.8	1.9	1.9	1.7	1.2	10^{-5}
Benzyl alcohol	1.9	1.8	1.7	1.7	1.2	0.4	10^{-4}
n-butanol	1.8	1.8	1.6	1.5	1.6	1.1	10^{-2}
2,6 dibromophenol	1.3*	2.0	1.9	1.7	1.8	1.7	10^{-6}
2,4 dinitrophenol	1.5*	1.9	1.7	1.8	1.7	1.0	10^{-5}
Ethanol	1.8	1.7	1.3	1.5	1.4	1.1	10^{-2}
Ethylene glycol	2.0	1.8	1.7	1.1	1.3	0.8	10^{-3}
Folic acid (Na^+)	2.1	1.8	1.4	1.6	1.4	0.9	10^{-2}
Methanol	1.8	1.7	1.6	1.3	1.3	0.9	10^{-2}
Methyl benzoate	2.0	1.9	1.7	1.8	1.3	1.2	10^{-4}
NaCN	1.5*	1.8	1.9	1.9	1.7	1.4	10^{-5}
Phenol	2.1	1.8	1.5	1.6	1.4	0.8	10^{-2}
Phenyl benzoate	1.9	1.9	1.8	1.6	0.9	1.0	10^{-3}
INORGANIC IONS							
CuCl_2	2.2	2.1	1.9	2.0	1.8	1.5	10^{-6}
NiSO_4	2.1	2.0	1.9	1.8	1.5	1.4	10^{-4}
CoSO_4	2.3	2.0	2.0	1.8	1.9	1.6	10^{-5}
NON- REPELLANTS (	CrCl_3	1.7	1.5	1.5	1.1	1.4	10^{-1}
	CaSO_4	1.7	1.4	1.2	1.5	0.1	10^{-1}

Table 14 (cont.)

1. Results are expressed as chemotaxis ratios obtained by the chemical-in-pond assay method. Each result is the mean of 5 replicates. Ratios >1.7 are considered significant for repulsion at a 1% level of probability (significance of chemotaxis ratios: see Chapter 2).
2. Lack of response in some cases (*) was attributed to a $>90\%$ loss of motility.

acetate, a repellent with a threshold of 10^{-4} M. In contrast, phenol, which has been shown to completely inhibit growth of E.coli and Salmonella at 10^{-2} M (Tso & Adler, 1974), fails to repel E.coli for which it is an attractant, has only a weak repellent effect on R.leguminosarum (Table 14) but is a strong repellent for Salmonella (Lederberg, 1956). These results confirm that, as in other bacteria, there is no correlation between repulsion and harmfulness in R.leguminosarum.

Effect of pH

Work on proton gradients has demonstrated that bacteria can respond to temporal changes in pH (DeJong et al. 1976; Miller & Koshland, 1977). In R.leguminosarum as in other bacteria, e.g. E.coli (Adler, 1975) and Salmonella (Miller & Koshland, 1980) the organism swims towards optimal pH, in this case pH 7 (Fig. 6).

Cation repulsion

Metal ions are toxic and inhibit motility in many organisms including R.leguminosarum (Bowra & Dilworth, 1981). Of those cations tested, Co^{2+} , Ni^{2+} and Cu^{2+} were good repellents. Similar results have been found for E.coli but in this case Cu^{2+} ions are not repellents despite their toxicity (Tso & Adler, 1974). All three cations also inhibited motility of R.leguminosarum at low concentrations.

Throughout this study, the addition of a chelating agent (EDTA or EGTA) to all growth and chemotaxis media to chelate contaminating metal cations was essential for good motility and chemotaxis. Calcium ions which were found to be essential for motility in R.leguminosarum were not repellents. For optimum chemotaxis and motility in this organism, the level of free Ca^{2+} was regulated at 10^{-6} M using EGTA. When calcium levels dropped below this threshold, motility declined dramatically and ceased altogether below 10^{-7} M free Ca^{2+} .

CHAPTER 7

MUTAGENESIS

MATERIALS AND METHODS

Mutagenesis

Four methods of mutagenesis were used in order to produce a number of motility and chemotaxis mutants.

(i) Transposons mutagenesis

Transposons are discrete sequences of DNA, which can insert into DNA replicons such as chromosomes or plasmids (Kleckner et al., 1977). Insertion can be random and interrupted genes suffer complete loss of function. Some transposons carry drug resistance genes so that insertion can be mapped as drug resistant markers. PI group R plasmids such as RP4 can infect most Gram negative bacteria including Rhizobium species (Beringer et al., 1978a). Insertion of the lysogenic phage Mu into RP4 reduces the frequency of transfer between E.coli and Rhizobium 10^5 fold to about 10^{-7} (Boucher et al., 1977). This inability of RP4:: Mu plasmids to become established in R.leguminosarum can be used to transfer transposons into this species. TN5, a transposon coding for kanamycin resistance can be transferred by a plasmid carrying Mu into three species of Rhizobium (Beringer et al., 1978b). The plasmid used to transfer TN5 from E.coli to R.leguminosarum by this method was pJB4JI (pJB4JI::Mu::Tn5).

A streptomycin resistant mutant of R.leguminosarum strain N5 was obtained by plating out 0.1 ml of a Rhizobium culture onto a TY plate containing streptomycin ($200 \mu\text{g ml}^{-1}$). The mutant isolated from a colony on this plate was grown up in minimal medium B (18 h, 24°C) to give a log phase culture. A log phase culture of E.coli strain 1830 (E.coli pro met pJB4JI) was also prepared (6 h in nutrient broth, 37°C). 0.5 ml of each of the two cultures were mixed together and incubated on a sterile filter paper on a TY plate at 27°C for 18 h. After

incubation, the organisms were washed off the filter and resuspended in minimal medium B containing streptomycin ($200 \mu\text{g ml}^{-1}$) and kanamycin ($25 \mu\text{g ml}^{-1}$). Only R.leguminosarum cells which had acquired the transposon TN5 and hence resistance to both antibiotics were able to grow in this medium.

In mutations caused by the insertion of a drug-resistant element, the linear integrity of the affected gene has been disrupted. Insertion mutations will therefore normally result in complete loss of the function encoded by the damaged gene and can be recovered, at high frequency following low level 'mutagenesis' by exposure to a translocatable element. The Rhizobium cells growing in the antibiotic medium therefore all contained insertion mutations of some description. In order to isolate those insertions involving motility and chemotaxis genes, the culture was enriched for mot⁻ and che⁻ mutants by one of the selection methods described later in this section. Individual clones were then examined for swarm behaviour on soft agar plates.

(ii) Chemical mutagenesis

(a) Ethyl methane sulphonate (EMS)

This method was based on that of Parkinson (1976). R.leguminosarum strain N5 was grown in minimal medium B at 24°C to give a log phase culture. The cells were spun down (8000g, 10°C, 5 min.) and resuspended in chemotaxis buffer containing 3.5% EMS at 2×10^9 cells ml^{-1} . After incubation for 60 min. at room temperature the cells were washed three times in chemotaxis buffer, resuspended in minimal medium B and incubated overnight to allow segregation of mutations. A selection method was used to enrich the population for motility and chemotaxis mutants and then individual clones were tested for their swarm behaviour on soft agar plates (Minimal medium: distilled water, 3:1, 0.25% agar).

(b) N-methyl-N¹-nitro-nitrosoguanidine (NTG)

Motility and chemotaxis mutants of R.leguminosarum strain N5 were induced by treatment with NTG using a procedure based on that of Scherrer (1970) adopted by Beringer (1974). Log phase cultures of R.leguminosarum grown in VS medium (pH 7.2) at 28°C were treated with 200 mg NTG l⁻¹ for 1 h; the bacteria were washed three times in chemotaxis buffer, resuspended in minimal medium B and incubated at 24°C for 24 h to allow segregation and expression of mutations. The culture was enriched for non-motile, non-chemotactic mutants using one of the selection methods (see next section) and individual clones were picked into soft agar plates (Minimal medium: distilled water, 3:1; 0.25% agar) to screen for those with defective swarms.

(iii) Spontaneous mutations

Spontaneously derived non-motile mutants of R.leguminosarum strain N5 were isolated by enriching for bacteria which did not migrate in soft agar (see section method (i)).

Selection Methods

(i) Migration in soft agar

The centre of a semi-solid agar plate (Minimal medium or VS medium: distilled water, 3:1; 0.25% agar) was inoculated with bacteria. The swarm was allowed to grow for 5 days, after which time a sterile inoculating wire was inserted into the centre of the swarm and used to inoculate a second soft agar plate centrally. This procedure, which enriched for non-motile cells, was repeated several times. Finally, cells from the centre of the plate were streaked onto TY agar to isolate colonies. Individual clones were screened for motility using semi-solid agar.

(ii) Large Capillary method

& Dahl

This method, based on that used by Adler (1967) to compare random and non-random motility provided a means of isolating non-motile and non-chemotactic mutants. A large capillary tube (100 mm long, 1.2 mm internal diameter) was filled with diluted minimal medium (Minimal medium: distilled water 3:1) from end B to within 2 cm of the opposite end (Fig. 7). Agar was then added at end B by plunging the tube into hardened agar in a petri dish (15 g agar/1 distilled water) until the column of liquid had been moved to a few mm from end A. This space was filled with bacteria (about 10^6 cells) using a drawn out pasteur pipette. Next, 5 mm agar was added at end A, pushing out some of the agar at the other end. Finally, a 2-3 mm plug of plasticene was added at end A to prevent the column of liquid moving when the non-motile, non-chemotactic cells were removed. The tubes were incubated in a horizontal position at 24°C for 7-8 h. During incubation, a band of motile, chemotactic cells could be seen gradually moving across the capillary towards end B. After the incubation period, the tube was broken at the liquid/agar junction near end B and the contents of the capillary were removed in fractions, using a finely drawn out pasteur pipette. All the cells except for those contained in the 1 cm portion of the capillary nearest A were discarded. The remaining cells consisted of a population enriched for non-motile and non-chemotactic cells. These cells were plated out onto TY agar and individual clones were screened for motility and taxis on soft agar (Minimal medium or VS medium: distilled water, 3:1, 0.25% agar).

(iii) Preformed Liquid Gradient technique

The preformed liquid gradient technique of Aswad & Koshland (1975) allows the effective separation of generally non-chemotactic mutants from

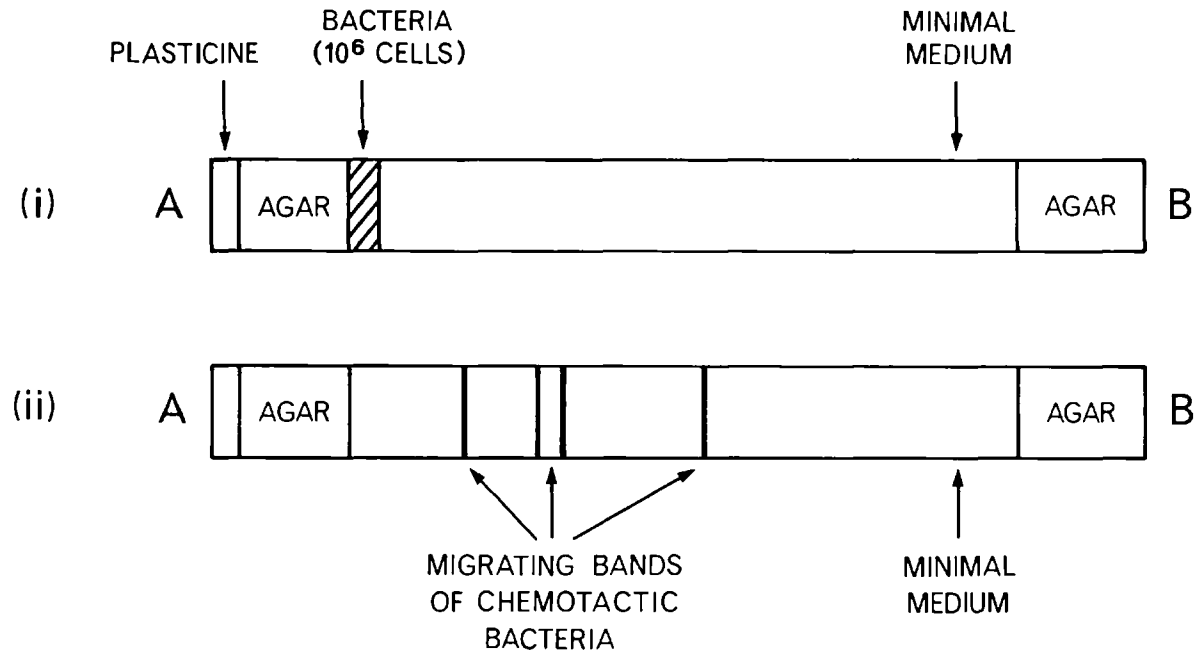


Fig.7. Isolation of mutants by the large capillary method.

The diagrams show the inoculated capillary tube (i) prior to incubation (ii) after 7 - 8h. incubation at 24°C.

both wild type and non-motile cells by floating a band of mutagenized wild type bacteria in a vertical gradient of liquid medium. A liquid gradient of yeast extract in chemotaxis buffer was constructed in a nitrocellulose tube (8 cm x 1.5 cm) which had been sterilized with ether. First 2.0 ml of yeast extract (150 mg yeast extract l^{-1} in chemotaxis buffer) was pumped in via a narrow plastic pipe extending to the bottom of the tube attached to a small pump and a gradient maker. This was followed by 2.5 ml of 150 mg l^{-1} yeast extract containing glycerol. The glycerol concentration was initially 0% (w/v) and was increased linearly to 1.0% at the end of the 2.5 ml pumping segment. Next, 0.2 ml bacterial suspension (5×10^7 cells/ml in 0.5 ml of a solution of 75 mg/l yeast extract, 1.5% glycerol) was pumped in, followed by 2.5 ml glycerol which increased linearly from 2.0% to 3.0% (Fig. 8). The glycerol gradient stabilized the attractant gradient against convection. The tube was incubated for 90 min. at room temperature (24-26°C) taking great care not to disturb the gradient. After incubation, 0.5 ml fractions were removed serially via a fine hole made in the bottom of the tube. Aswad & Koshland (1975) used this method to isolate mutants of S.typhimurium and found, as expected, that non-motile mutants did not show any significant change in distribution during incubation. Wild type cells responded to the gradient by moving up the tube slightly. The che⁻ cells, however, showed a preference for swimming down the tube instead of spreading symmetrically in both directions (Fig. 8). This was attributed to the influence of gravity. Using these results as a guide, individual clones grown on TY agar from fractions from the base of the tube were screened for che⁻ mutants using the soft agar technique and the blind well chamber assay. Similarly, the fraction isolated from the original point of inoculation was considerably enriched in

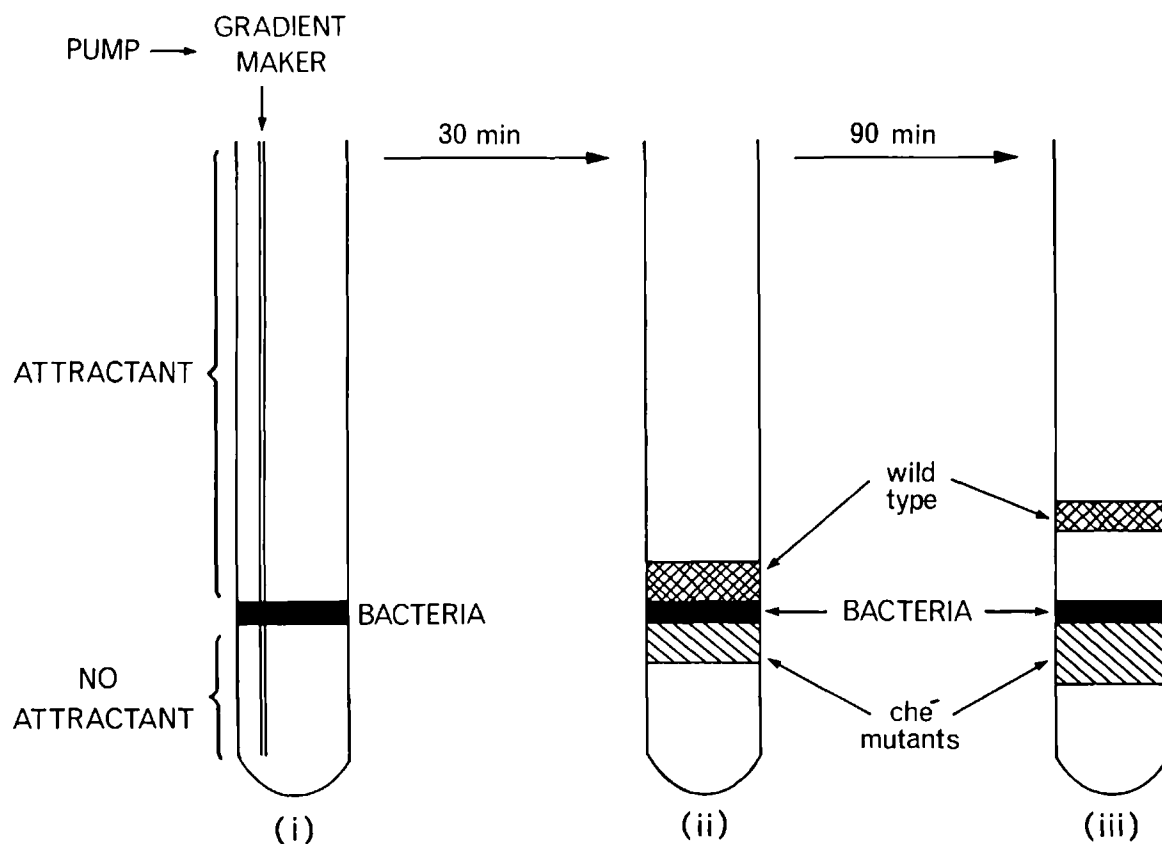


Fig. 8. The preformed liquid gradient technique.

The diagrams show the migration of wild type, non-motile (mot^-) and motile but not chemotactic (che^-) bacteria in a vertical step gradient of yeast extract: (i) prior to incubation (ii) after 30 min. incubation at room temperature (iii) after 90 min. incubation at room temperature. Wild type and mutant organisms were isolated in 0.5ml. fractions removed serially from the bottom of the tube after 90 min. incubation (see text).

mot⁻ cells and individual clones grown on TY agar, were screened by microscopic observation and migration in soft agar. Chemotactic, motile wild type cells isolated in the upper part of the tube were used in chemotaxis experiments. Yeast extract was chosen as the attractant since general, rather than specific, chemotaxis mutants were required.

(iv) Negative Chemotaxis

Several methods of measuring negative chemotaxis have already been described (Chapter 6). Of these methods, the chemical-in-plug technique (Adler, 1974) ^{Tso &} provided a useful way of isolating organisms with a general chemotaxis defect. Bacteria were mixed with 0.2% minimal agar and used to pour soft agar plates which were visibly turbid. Plugs of hard agar (1.5%) containing a repellent (10^{-4} M citric acid or 10^{-3} M Benzoate) were placed in the soft agar before it was allowed to set. Bacteria were repelled, creating a clear zone around the plug. To obtain chemotaxis mutants, a sterile 1 μ l pipette was used to pick up bacteria that remained in the clear zone. Organisms isolated in this way will include those with a general chemotaxis defect and mutants specifically defective in their ability to be repelled by the chemical repellent used. To isolate general chemotaxis mutants the organisms were streaked out onto TY plates and individual clones were screened for chemotaxis on soft agar.

The chemical-in-plate technique, described in Chapter 6, provided a useful way of screening large numbers of colonies for general chemotaxis mutants.

Isolation of Revertants

Motile revertants from non-motile mutants were isolated by streaking the cells through semi-solid medium (Vs or minimal medium: distilled water, 3:1; 0.25% agar). The petri-dishes were incubated

until a puff of motile bacteria appeared along the streak of non-motile organisms. Motile revertants were isolated from these areas.

Chemotactic revertants from non-chemotactic mutants were isolated by inoculating semi-solid agar plates centrally, and incubating until swarms began to form at the edge of the plate. Chemotactic revertants were re-isolated from these bands.

All mutants were thoroughly screened for motility (microscopic observation) and taxis (blind well chamber assays) prior to use in competition experiments. In addition, all strains were checked for nodulation (Chapter 8).

Streptomycin resistant derivatives

Streptomycin resistant derivatives of the chemotaxis (C_1, C_2, C_3) and motility mutants (M1, M2, M3) previously isolated, were obtained by plating out organisms onto minimal medium containing streptomycin ($200 \mu\text{g ml}^{-1}$). The mutants were designated SM1, SM2, SM3 (motility mutants) and SC1, SC2, SC3 (chemotaxis mutants). Individual clones were checked for nodulation, motility and taxis.

Electron microscopy

Electron microscope studies of the streptomycin resistant, non-motile strains were undertaken in order to determine whether the organisms were flagellated, and therefore general mot⁻ mutants or non-flagellated, fla⁻ strains. Preparation of carbon-coated, metal shadowed specimens was carried out using standard techniques (Short & Walker, 1976).

RESULTS AND DISCUSSION

Mutagenesis

A number of chemotaxis and motility mutants were isolated using the range of mutagenic and selection procedures which have been outlined. Six of these mutants were further screened and were selected

for use in competition experiments. Three motility mutants were designated M1, M2 and M3 and three general chemotaxis mutants C₁, C₂ and C₃ respectively. In addition, two revertants, RC1 and RM1 were isolated from their parent strains C1 and M1 (Table 15). The effectiveness of mutagenesis by the three available methods was investigated by comparing the increase in frequency of streptomycin resistant mutants to an untreated control. The relatively low rate of spontaneous mutations combined with the slow growth of R.leguminosarum across semi-solid media and the need for at least 7 or 8 sub-cultures made the soft agar technique on its own very time consuming.

Chemical mutagenesis substantially increased the rate of mutation; treatment with NTG giving the best results. EMS, which is widely used as a chemical mutagen for E.coli (Parkinson, 1976) and S.typhimurium (Aswad & Koshland, 1975) was not found to be a very good mutagen of Rhizobium. Only a 40-fold increase in the number of streptomycin resistant mutants was recorded compared to an untreated control. Similar results were found by Ames et al. (1980) who demonstrated that mutagenesis of R.meliloti using 3.5% EMS resulted in only a 75-fold increase in the number of rifampin-resistant mutants. Motility mutant M3 was isolated after EMS treatment (Table 15). Chemical mutagenesis with NTG gave better results resulting in a 1000-fold increase in streptomycin-resistant mutants. This technique has been widely used for Rhizobium mutagenesis by Beringer (1974). Mutants C1 and C2 were isolated after NTG treatment (Table 15).

The use of transposons as mutagenic agents has a number of distinct advantages over chemical methods. In addition to eliminating the need for using potentially harmful chemicals, transposon induced mutations can be mapped by mapping the position of the drug resistance carried on the transposon. This could prove

Table 15 Motility and chemotaxis mutants of R. leguminosarum strain N5

<u>Mutant</u>	<u>Mutagenesis</u>	<u>Selection</u>
M ₁	Transposon mutagenesis	Preformed liquid gradient
M ₂	Transposon mutagenesis	Large capillary
M ₃	EMS	Preformed liquid gradient
C ₁	NTG	Negative chemotaxis
C ₂	NTG	Preformed liquid gradient
C ₃	Spontaneous mutation	Migration in soft agar

very useful when mapping the genes concerned with chemotaxis.

^{et al}
Beringer (1978b) found that for R.leguminosarum, R.trifolii and R.phaseoli, the frequency of auxotrophy was about 0.3% when using TN5 as a generalized mutagen compared to a spontaneous rate of less than 10^{-8} . Using the same method it was found that transposon mutagenesis resulted in a 1500 fold increase in the frequency of streptomycin resistant mutations. Motility mutants M1 and M2 were isolated using this method.

Selection methods

After mutagenesis the proportion of motility and chemotaxis mutants in the population was increased by using a suitable selection method. Two of the methods involved migration on semi-solid media while the remaining techniques relied on liquid gradients. The main problem with swarm selection on semi-solid agar is that gradients necessary to draw the wild type away from the inoculum site do not exist prior to inoculation. They are only established once the bacteria at the inoculum site have had time to metabolize a significant amount of certain nutrients that serve as attractants. Wild type, non-motile and chemotactic mutants will all multiply at the same rate before these gradients are established. By this time, the inoculum is dense with wild type cells and separation becomes difficult. In addition, the use of semi-solid medium inhibits the movement of the bacteria and makes the separation rate slower than in liquid. Despite these drawbacks, swarm behaviour on semi-solid medium was a useful way of screening for both wild type and mutant organisms.

Failure to respond to repellents in hard agar plugs also depended on migration in soft agar but provided faster results. Since many of the cells found at the concentrated end of the repellent gradient were non-viable or showed very poor growth, isolations were made from the

centre of the repellent zone.

The large capillary method was based on the same principle as the swarm plate technique, but used a liquid gradient. The main problem encountered with this method was the difficulty in removing organisms from the horizontal tube without mixing. The preformed liquid gradient technique had none of the drawbacks of the other methods because (i) A preformed gradient was used (ii) Migration occurred in a liquid medium (iii) This technique provided conditions that would separate non-motile from non-chemotactic cells as well as non-motile from wild type cells. A further advantage was that separation was achieved in a relatively short time (90 min). Mutants M1, M3 and C2 were isolated after selection by this technique (Table 15).

All mutants showed growth rates comparable to the parent strain in liquid minimal medium (doubling time about 2.5 h). Chemotaxis mutants C1, C2 and C3 formed swarms on semi-solid media that were 4-5 times smaller than parental ones and lacked the typical bonding pattern. Revertants were identical to the parent strain in every aspect of swarm morphology.

Electron Microscopy

Electron microscope studies of mutants SM1, SM2 and SM3 showed the absence of flagella (Plates 2, 3, 4). These mutants were therefore classified as fla⁻ rather than mot⁻. Members of this class did not swim in liquid medium and failed to swarm in semi-solid agar.

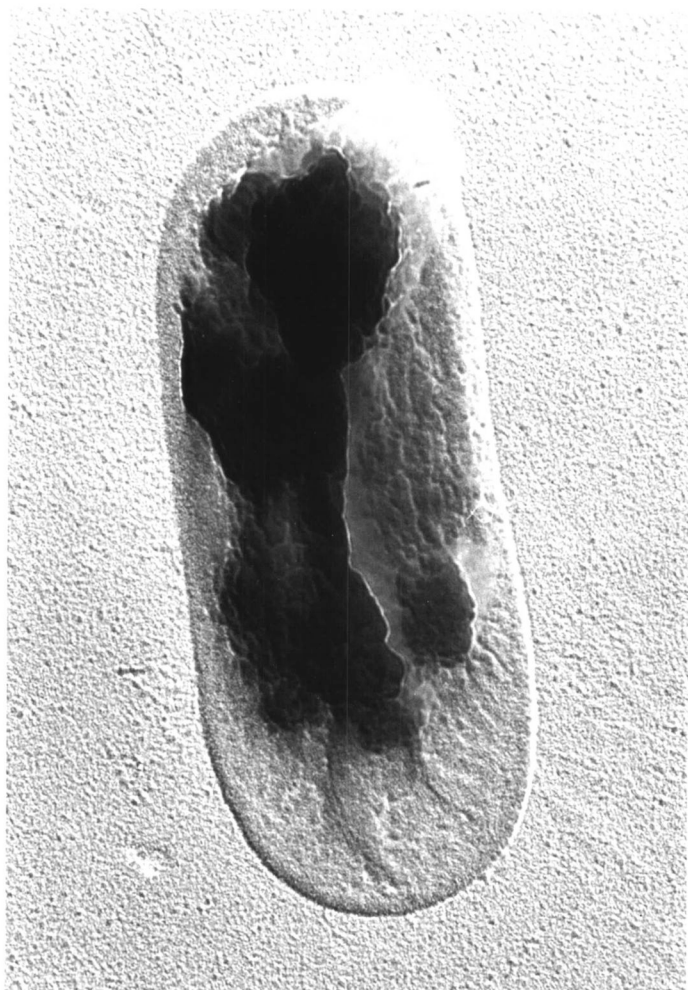


Plate 2. Electron micrograph of R. leguminosarum mutant SM1.

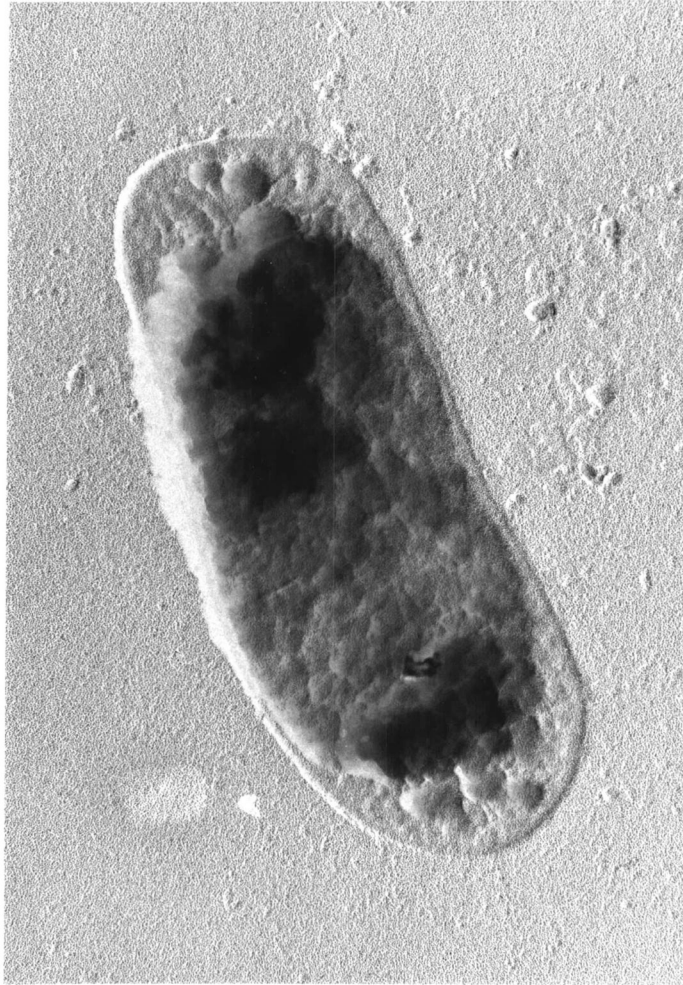


Plate 3. Electron micrograph of R.leguminosarum mutant SM2.



Plate 4. Electron micrograph of R.leguminosarum mutant SM3.

Chapter 8

COMPETITION EXPERIMENTS

INTRODUCTION

The significance of bacterial behaviour in ecological situations has not been well documented. Panopoulos & Schroth (1974) have shown that motility plays a role in the infection of plant tissue by motile pseudomonads. More recently, Freter et al. (1979) demonstrated the importance of motility and chemotaxis in the infection of intestinal tissues by cholera vibrios. The success of inoculant strains of *Rhizobium* will depend on their ability to compete with indigenous strains present in the soil with respect to infectivity.

In view of the evidence presented in this study for the non-specificity of chemotaxis of *Rhizobia* to legume exudates, it was necessary to investigate the role of motility and chemotaxis in natural infection. Napoli & Albersheim (1980) isolated non-motile mutants of *R. trifolii* and concluded that motility of *Rhizobium* was not required for nodulation of *Trifolium repens* by this strain. These results are consistent with those obtained by Ames et al. (1980). They have reported results from nodulation experiments performed with three clones of mutants of *R. meliloti* (mot⁻, fla⁻, che⁻) which indicate that all three types infect and produce nodules on alfalfa plants. This work establishes that motility and chemotaxis are not essential for infection but gives no indication as to whether motile, chemotactic strains are at an advantage compared to non-motile and non-chemotactic organisms. For this type of investigation, competition experiments using motility and chemotaxis mutants are needed.

MATERIALS AND METHODS

1. Growth media

Fahraeus (FP) medium for the growth of pea seedlings contained, per litre, 0.1 g, CaCl₂·2H₂O; 0.12 g, MgSO₄·7H₂O; 0.1 g, KH₂PO₄; 0.15 g NaHPO₄·12H₂O; 0.005 g Ferric citrate; 1 ml Gibson's trace elements.

Gibson's trace elements contained, per litre, 2.86 g H_3BO_3 ; 2.03 g $\text{MgSO}_4 \cdot 4\text{H}_2\text{O}$; 0.22 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$; 0.08 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$; 0.08 g $\text{H}_2\text{MOO}_4 \cdot 1\text{H}_2\text{O}$.

Nodulation Test

Inoculation and growth of peas was essentially as described by Beringer (1974). Peas (Pisum sativum var Wisconsin Perfection) were surface sterilized as indicated in Chapter 2, transferred to petri-dishes of TY agar and incubated for 7 days in the dark for pre-germination. Sterile, germinated peas were then transferred to 200 ml conical flasks containing 100 ml 0.5% FP agar. The flasks were inoculated with 10 ml sterile water containing about 10^8 cells of the test strain and incubated in the dark at 24°C until the etiolated plumules reached the cotton wool plugs (6-7 days). Using sterile tweezers, the plumules were pulled past the plugs so that the pea seed was above the agar surface. Flasks were then covered with black polythene to keep light from the plant roots and transferred to a greenhouse. Nodules were usually visible 3-4 weeks after inoculation.

Competition experiments

Competition experiments were carried out using the same method as for nodulation tests except that motility and chemotaxis mutants were mixed in various proportions with a known marked strain and used as the inoculant. This marked strain was R.leguminosarum strain 726, a rifampicin resistant organism. For each mixture, six plants were inoculated.

Additional controls included two uninoculated plants, 2 plants inoculated with strain 726 alone and two plants with the test strain alone. The performance of each mutant strain when mixed with 726 was compared to that of the parent wild strain.

Experiment A. Bacterial inoculum was grown in minimal medium (24°C , 18 h) to give a log phase culture and resuspended in chemotaxis buffer

at 10^7 cells ml^{-1} . One ml of this inoculum (or 0.5 ml of each strain in the case of a mixed inoculum) was added to 9 ml of sterile distilled water and used to inoculate each flask:

Experiment B: Bacterial inoculum was prepared as for experiment A except that cells were resuspended in chemotaxis buffer to give an inoculum containing 8×10^3 cells ml^{-1} .

Organisms

The mutant strains used in these competition experiments were streptomycin derivatives of the chemotaxis and motility mutants isolated as described in Chapter 7. These were SC1, SC2, SC3 (chemotaxis mutants) and SM1, SM2, SM3 (motility mutants). The wild type parent strain was a streptomycin resistant derivative of R.leguminosarum strain N5, designated SN5. Streptomycin resistant derivatives of the two revertant strains previously isolated (RC1 and RM1) were designated SRC1 and SRM1.

Nodule Analysis

Three to four weeks after inoculation, when the nodules were large enough for convenient handling, they were picked off the roots with tweezers, surface sterilized by immersion in sodium hypochlorite (10% w/v available chlorine) for 1 minute and washed thoroughly in distilled water. The sterilised nodules were crushed in 1 ml of 20% v/v glycerol and plated out onto TY plates. At this stage the samples could be frozen at -20°C for storage (Johnston & Beringer, 1975). Nodules containing strain 726 were identified by replica plating onto TY medium containing rifampicin (25 $\mu\text{g}/\text{ml}$) while those containing mutants were identified on TY medium containing streptomycin (200 $\mu\text{g}/\text{ml}$). The number of nodules of each type was counted and the results were compared to determine the relative ability of each mutant to compete with strain 726.

Statistical Analysis

The significance of the results was tested using a variance ratio analysis (Sprent, 1977).

RESULTS AND DISCUSSION

1. Growth rates of mutant strains

The growth rate of all the test strains and the wild type in liquid medium was shown to be approximately the same (doubling time about 2.5 h in minimal medium). This was important since the experimental design did not allow for differential growth which could have affected the results, especially at low inoculum levels.

2. Morphology of root nodules

All six mutant strains and both revertants successfully nodulated Pisum sativum. The average number of nodules produced per plant (usually between 12-20) did not differ significantly between mutant and wild type organisms (Table 16). The nodules produced by each strain could not be distinguished by size, shape or colour. Very small nodules from which no bacteria could be isolated were ignored. The data is therefore slightly biased in favour of isolates from larger nodules.

In order to show that nodulation by mutant strains was not due to reversion occurring during the infection process, the contents of a number of nodules produced by these strains were assayed for the swarm phenotype of the bacteria contained inside. The results showed that the bacteria recovered from these nodules exhibited the phenotype of the inoculant strain. Motility and chemotaxis are clearly not required for the infection of pea root hairs and the formation of nodules. This confirms the results obtained by Napoli & Albersheim (1980) for motility mutants of R. trifolii and those of Ames et al. (1980) who reported nodulation of alfalfa by non-motile and non-chemotactic R. meliloti.

Table 16 Nodulation of *Pisum sativum* by single strain inocula of *R. leguminosarum*

Test strain	Mean no. of nodules per plant ⁽¹⁾ ⁽²⁾
N5	14.2
726	14.9
SC1	15.5
SC2	16.2
SC3	12.8
SM1	17.1
SM2	15.5
SM3	13.4
SRC1	14.9
SRM1	15.3

(1) Each result is the mean of 12 plants

(2) Each single strain inoculum contained 10^7 cells per plant.

Competitiveness of mutant strains

The competitiveness of the test strains compared to strain 726 and to the parental type is summarized in Table 17 (see Appendix 1 for complete data; Appendix 2 for variance-ratio analysis). Strain N5 performed consistently worse than strain 726 in both experiments (85% as infective in experiment 1; 80% as infective in experiment 2). This was taken into account when comparing the infectiveness of the test strains to the parental type. Experimental design and conditions for experiments 1 and 2 were identical except for the size of the inoculum.

In both experiments, motility mutants failed to compete successfully with strain 726 and accounted for only 18-26% of the total infections. Therefore, the strains were only 25-42% as infective as the parental type. Strain SM3 performed slightly better than the other fla⁻ strains both times. With a fairly high initial inoculum (10^7 cells/plant), chemotaxis mutants accounted for 34-40% of the total infections and were 60-78% as infective as the parental strain. However, when the inoculum was decreased to 8×10^3 cells/plant, the competitive advantage of chemotaxis was much greater and che⁻ mutants were only 50-63% as infective as strain N5. With a smaller inoculum, the difference in performance of all six test strains compared to strain 726 was highly significant at a 1% level of probability (Variance-ratio analysis). The increasing competitive advantage of the wild type when present at decreasing levels in mixed inocula has also been demonstrated by Ames & Bergman (1981) using motility mutants of R.meliloti and by Hunter & Fairing (1980) with a slow migrating mutant of R.japonicum.

Both revertant strains SRC1 and SRM1 were able to compete successfully with strain 726 and were 104% and 100% respectively as infective as the parental type.

Table 17 Infectivity of non-motile and non-chemotactic
R. leguminosarum mutants

	Test strain	% Infections due to test strain	Infectiveness compared to	
			726	N5
	(1) SM1	23%	30%	35%
	(1) SM2	18%	21%	25%
	(1) SM3	26%	35%	42%
EXPERIMENT I				
	(5) SC1	40%	67%	78%
	(5) SC2	38%	62%	73%
	(1) SC3	34%	51%	60%
	(1) SM1	18%	23%	29%
	(1) SM2	18%	22%	27%
	(1) SM3	25%	33%	42%
EXPERIMENT 2				
	(1) SC1	31%	45%	57%
	(1) SC2	29%	40%	50%
	(1) SC3	33%	50%	63%
	(N5) RSM1	45%	83%	104%
	(N5) RSM2	44%	80%	100%

Control data

Experiment I: Inoculum size = 10^7 cells/plant

N5 was 85% as infective as 726 (see appendix 1A)

Experiment 2: Inoculum size = 8×10^3 cells/plant

N5 was 80% as infective as 726 (see appendix 1B)

Table 17 (cont.)

- (1) Significant difference between test strain and strain 726 at 1% level probability (F test; see appendix 2A and 2B).
- (5) Significant difference between test strain and strain 726 at 5% level probability (F test; see appendix 2A and 2B).
- (NS) No significant difference between test strain and strain 726.

Clearly, restoring chemotaxis and motility to an organism restores its competitive ability. These results indicate that actively motile, chemotactic cells in the soil could still be responsible for nodule formation on legume crops when greatly outnumbered by an applied inoculum of poorly motile or chemotactic cells. The advantage conferred by motility, especially when assisted by chemotaxis is considered.

CHAPTER 9

DISCUSSION

Composition of Pea Root Exudate

There have been several recent studies on the composition of legume root exudates, the most comprehensive including those of Chang-Ho & Hickman (1970) who analysed exudate from 21-day pea plants and Gitte et al. (1978) on the cowpea, Cicer arietinum. In the later analysis, which is most comparable to the work carried out in this study, cowpea seedlings were found to produce 106 μg of exudate per plant as compared to 121 μg found for 6-10 day pea plants in this work. Cation, neutral and anion fractions accounted for 63, 19 and 10 μg respectively compared to 37, 30 and 17 μg for the pea. Chang-Ho and Hickman (1970) found 90 μg of amino acids per 21 day pea plant. Their data on organic acids, expressed in terms of normality, is also comparable to that obtained in this study although results obtained for sugar analysis are about 100 times in excess of plausible values.

Studies on several types of seedling, both legume and non-legume, have demonstrated the presence of predominantly acidic amino acids in root exudates (Boulter et al., 1966; Miller & Schmidt, 1965; Richter et al., 1968; Vancura & Stanek, 1975). Analysis of the amino acid composition of pea exudate show that the absolute and relative amounts are dependent on plant growth conditions. Boulter et al. (1966) demonstrated that in liquid medium, glutamic acid formed the largest portion of pea root exudate while in exudate from pea seedlings grown in quartz sand, homoserine was the major constituent followed by aspartic acid and glutamic acid. Similar effects of the composition of the soil atmosphere on amino acid composition have been demonstrated by Ayers & Thornton (1968). Sugar and organic acid production is presumably similarly effected although it has been less studied.

Any analysis of root exudate can only therefore be applied to the exact conditions of the experimental model and may not be generalized. High molecular weight materials, which constitute an increasing proportion of pea root exudate as seedlings mature, may be partially degraded by soil micro-organisms and add to the low molecular weight components.

The zone of the root immediately behind the tip has been considered to be the major source of exudate (Pearson & Parkinson, 1961). Schroth & Snyder (1962) found that sugars and amino acids were exuded at the point of emergence of the adventitious roots. Van Egeraat (1975a) also demonstrated amino acid exudation at this time. The mechanisms responsible for the release of this wide range of organic compounds from the roots have not been thoroughly investigated. Exudation from intact roots which are functioning normally is of the order of 0.1-0.4% of the Carbon photosynthesised by the plants (McDougall & Rovira, 1965; Rovira & McDougall, 1967) but physical and chemical damage to the root increases this. It is not clear whether exudation is controlled by metabolic processes or is simply a leaking out of compounds over which the plant exercises no control.

Future studies on pea root exudate should simulate natural conditions at and preceeding the time of the infection as closely as possible. This could be done using both natural soil and soil sterilized by γ -radiation (Bowen & Rovira, 1976).

Chemotaxis of Rhizobium to single compounds

R.leguminosarum shows positive chemotaxis to a wide range of L-amino acids, sugars and organic acids thus bearing resemblance to other soil bacteria such as Bacillus subtilis, which responds to all 20 common amino acids (Ordal & Gibson, 1977). Chemotaxis

studies of other *Rhizobium* strains show similar patterns. Gotz et al (1982) found positive attraction of *R.meliloti* by 21 amino acids with thresholds similar to those recorded for *R.leguminosarum*. Similarly, Gitte et al. (1978) found attraction of a cowpea *Rhizobium* by 9 out of 10 amino acids tested. Only alanine failed to elicit a positive response.

In marked contrast to these results, Currier & Strobel (1977b) reported that one trefoil strain of *Rhizobium* was repelled by serine and aspartate, two were attracted by aspartate but most were indifferent to a range of small molecules. These results must be treated with some caution since *Rhizobium* strains are often inconsistent with respect to motility and chemotaxis. Known positive controls must therefore be included in all chemotaxis assays together with stringent checks of motility. These authors failed to take such precautions. Moreover, chemotaxis assays in that study were carried out using *Rhizobia* labelled with $U^{14}C$ glucose. It has been shown by Adler & Templeton (1967) that glucose in the growth medium of *E.coli* inhibits flagella synthesis. Any similar effects on *Rhizobium* would result in less than optimum motility.

Hunter & Fahring (1980) observed chemotactic migration of *R.japonicum* to the non-protein amino acid canavanine but not with the other fifteen amino acids tested. The assay technique in this case consisted of observations of migration in soft agar containing specific amino acids together with a general background of yeast extract. It was very difficult in plate chemotaxis experiments with *R.leguminosarum* to relate separate swarms of organisms to specific chemicals in a medium containing more than one chemoattractant. Changes in swarm behaviour and the observation of additional bands of adding single attractants to a medium containing yeast extract would not seem to be

a particularly reliable assay method. R.leguminosarum was not found to respond to any non-protein amino acids, including canavanine.

Sugar taxis in Rhizobium strains does not appear to be as comprehensive as amino acid taxis. In this study, positive chemotaxis of R.leguminosarum was observed to arabinose, fructose, glucose, lactose, maltose, mannitol (sugar alcohol), ribose and xylose but not to trehalose or sucrose. These results were confirmed by Bowra & Dilworth (1981). Gitte et al. (1978) found that galactose sugars, glucose and xylose were particularly good chemoattractants for the cowpea Rhizobium, while ribose and arabinose elicited a weaker positive response. In R.meliloti, Gotz et al. (1982) observed that carbohydrates such as glucose and mannitol produced only a weak response. This response was somewhat stronger in R.lupini, especially if the cells were grown in the presence of the carbohydrate prior to testing. Chemotaxis to sugars is also inducible in enteric bacteria such as E.coli (Lengeler, 1980) and in Pseudomonas aeruginosa (Moulton & Mountie, 1979) but was not observed for R.leguminosarum in this study. Currier (1980) with one trefoil strain of Rhizobium reported maltose, mannose and ribose to be strong attractants, fructose, galactose and sucrose to be weak attractants and five other sugars including glucose to be ineffective. Hunter & Fahring (1980) found arabinose to be an attractant for R.japonicum but no effect with other sugars. However, the reasons for regarding the results of these studies with suspicion have already been discussed.

Chemotaxis of Rhizobium to organic acids has been less studied. Gitte et al. (1978) reported attraction of the cowpea Rhizobium by gluconic, galacturonic and mannuronic acids. In this study, positive chemotaxis of R.leguminosarum has been observed to citric, gluconic, malic, succinic and glycolic acids when tested as the Na⁺ salt.

Another group of compounds which may be of importance to *Rhizobium* chemotaxis in nature are volatile substances. Hutchinson (1971) pointed out that volatile compounds are able to pass through gaseous as well as aqueous phases in the soil and may therefore be less exposed to absorption and microbial inactivation. In addition, they may be able to act at greater distances than non-volatile compounds. Fries (1973) reviews the effects of various volatiles on fungi, many of which show germination promotion or inhibition by such compounds emanating from the plant material. Cameron & Carlile (1981) observed that zoospores of Phytophthora palmivora were attracted by a wide range of volatile compounds many of which are known to be produced by plants. Since there is no published data on chemotaxis of *Rhizobium* to volatile attractants, this remains an area to be investigated especially in view of the rich spectrum of volatile compounds given off by pea roots (Fries, 1973).

The role of positive chemotaxis in infection will be discussed in a later section.

Negative Chemotaxis

A wide range of chemicals were found to act as repellents for R.leguminosarum including certain ions, extremes of pH and a range of organic substances. Many of the chemicals tested were also repellents of E.coli (Tso & Adler, 1974) or B.subtilis (Ordal, 1976). In *Rhizobium*, as in these other micro-organisms, harmfulness and the ability to repel an organism are not necessarily correlated. Not all of the repellents tested are found in the soil and so the advantage to the organisms of retaining such a response is not clear.

The survival value of avoiding high concentrations of H^+ and other cations is self evident, since at concentrations above their repellent thresholds these are lethal. Zinc and copper ions are known to have

toxic effects on *Rhizobium* (Parker, Trinick & Chatel, 1977) and zinc and copper fertilizers sown with inoculated seed inhibit nodulation. The effects of these ions on nodulation may be due as much to inhibition of motility as to direct toxicity, especially at low concentrations. Motility of *R.leguminosarum* was very poor outside the pH range 6.5-7.5. Munns (1977) has shown that the initiation of infection threads is sensitive to both Ca^{2+} deficiency and low pH. High local electrolyte concentrations are likely in the soil and a negative chemotactic response allows these conditions to be avoided.

Role of Chemotaxis and Motility in Infection

Movement of Rhizobia throughout the rhizosphere would appear to be essential for successful colonization of the rhizosphere and the infections of legumes. The evidence provided here suggests that this movement is not purely a random process but is directed by substances released by legume roots. The ability of *Rhizobium* species to respond to a wide range of low molecular weight components of root exudate may have important advantages. With a single compound, attraction up a concentration gradient occurs from a threshold concentration to a point at which indifference occurs. The ability to respond to gradients of many exudate components may provide continuous attraction from the greatest distance from the root at which an attractant is detected, all the way to the root surface. A mixture of attractants may produce a response at concentrations at which individual attractants are ineffective. The ability to respond to a wide range of readily diffusing low molecular weight attractants probably facilitates entry into the rhizosphere and approach to the root surface.

The control of specificity in the *Rhizobium*-legume symbiosis is not entirely understood. The lectin-binding model of Dazzo & Hubbell

(1975) provides a possible hypothesis (see Chapter 1). Clearly, chemotaxis to a wide range of common metabolites present in root exudates does not provide scope for specificity. A specific high molecular weight attractant with a very short range of action in the soil could, however, play a useful role in the final approach to the root surface. Currier & Strobel (1976, 1977a) reported the occurrence of a glycoprotein (molecular weight 60,000 daltons) isolated from the root exudates of birdsfoot trefoil, Lotus corriculatus which attracted six strains of R.trifolii. However, since no other studies of Rhizobial behaviour have detected similar large attractants and no significant chemotaxis of R.leguminosarum to the high molecular weight fraction of pea root exudate was observed in this study, the role of such a molecule remains to be assessed.

Motility is clearly a very important attribute to an organism when colonizing a rhizosphere since without it chemotaxis cannot occur. Gotz et al. (1982) showed that two strains of R.melitoli possessed an unusual type of flagella, termed "complex" flagella (Schmitt et al., 1974). These complex filaments differ from the common "plain" type in their fine structure, amino acid composition and consistency. In addition, the pronounced fragility of these flagella (Maruyama et al., 1978) and the conspicuous helical bands enforcing the filament structure suggest that these filaments are very rigid. When compared to motile cells of Salmonella typhimurium which possess similar numbers of plain flagella, Rhizobium cells were able to swim faster and longer in any one direction. In addition, complex flagella were superior in propelling bacterial cells through viscous environments. Hence, it seems probable that complex flagella are likely to confer a selective advantage to bacteria moving through the soil.

Ecological Advantage and Chemotaxis and Motility

The chemotaxis of *Rhizobium* to root exudates is a non specific interaction. *Rhizobia* showed similar results for chemotaxis to a wide range of both legume and non-legume exudates, a conclusion almost inevitable from the wide range of common metabolites present in all root exudates. The subsequent interaction which leads to the development of root nodules and the differentiation of *Rhizobia* into N_2 -fixing bacteroids is however very specific. The genetic structure of at least two *Rhizobium* species reflects this distinction between general and specific interactions. Whereas the genes for motility and chemotaxis are localized on the bacterial chromosome (Ames et al., 1980), genes required for specific infection, nodulation and nitrogen-fixation were shown to reside on large plasmids in strains of *R.leguminosarum* and *R.phaseoli* (Beynon et al., 1980).

The specific *Rhizobium*-legume interaction occurs independent of chemotaxis and motility. Recent studies with non-motile mutants of *R.meliloti* (Ames et al., 1980) and *R.trifolii* (Napoli & Albersheim, 1980) have shown that under laboratory conditions, both wild type and non-motile organisms were capable of nodulation and N_2 -fixation. Ames et al. (1980) also demonstrated infection of alfalfa plants by chemotaxis mutants (both *fla*⁻ and *mot*⁻) of *R.meliloti*.

Although chemotaxis and motility are not essential for nodulation, such complex systems would not have been evolved and retained if they did not have a role to play in natural infection. Ames & Bergman (1981) investigated the effect of motility on the competitive success of *R.meliloti* in nodule production and showed that motile strains formed more nodules than expected when mixed in various unfavourable ratios with either flagellated or non-flagellated, non-motile derivatives.

Similar work by Hunter & Fahring (1980) demonstrated that a slow-migrating mutant of R.japonicum nodulated less efficiently than the wild type. Competition experiments in this study with R.leguminosarum clearly show that non-chemotactic mutants nodulate less efficiently than the wild type and non-motile mutants still less efficiently. That motility is more important to an organism is to be expected since motility is essential for movement in the rhizosphere while chemotaxis is dependent on motility, and serves to provide a directional response.

It seems therefore that motility, guided by chemotaxis, facilitates infection and that motility and taxis mutants will be less successful in nature than the wild type.

APPENDIX 1

Appendix 1A (Data for Competition Experiment A)Control Data

INOCULUM STRAINS

		TYPE OF NODULE				
		N5	726	N5+726	T _{N5}	T ₇₂₆
	1.	8	9	0	8	9
N5 + 726	2.	12	6	1	13	7
1:1 ratio	3.	6	11	1	7	12
6 plants (1-6)	4.	9	8	1	10	9
T = Total	5.	6	12	0	6	12
	6.	7	11	0	7	11
TOTAL NOS. OF NODULES		48	57	3	51	60

% Infections due to N5 = 46%

$$\frac{\text{Infectiveness N5}}{\text{Infectiveness 726}} = 85\%$$

For the purposes of comparing results obtained with mutants SC1, SC2, SC3, SM1, SM2 and SM3 in direct competition with 726; N5 was considered to be 85% as infective as 726.

Inoculum size = 10^7 cells/plant

MOTILITY MUTANTSINOCULUM STRAINSTYPE OF NODULE

		SM1	726	SM1+726	T _{SM1}	T ₇₂₆
SM ₁ + 726	1.	3	12	0	3	12
1:1 Ratio	2.	2	15	0	2	15 % Infections due to SM1 = 23%
6 plants (1-6)	3.	5	11	0	5	11 Infectiveness compared to
T = Total	4.	1	17	1	2	18 strain 726 = 30%
	5.	4	12	0	4	12 strain N5 = 35%
	6.	6	9	1	7	10
TOTAL NOS. OF NODULES		21	76	2	23	78

		SM2	726	SM2+726	T _{SM2}	T ₇₂₆
	1.	5	10	0	5	10
SM ₂ + 726	2.	2	15	0	2	15 % Infections due to SM2 = 18%
1:1 Ratio	3.	3	15	0	3	15 Infectiveness compared to
6 plants (1-6)	4.	4	13	0	4	13 strain 726 = 21%
T = Total	5.	1	17	0	1	17 strain N5 = 25%
	6.	3	14	0	3	14
TOTAL NOS. OF NODULES		18	84	0	18	84

		SM3	726	SM3+726	T _{SM3}	T ₇₂₆
	1.	5	12	0	5	12
SM ₃ + 726	2.	3	17	0	3	17 % Infections due to SM3 = 26%
1:1 Ratio	3.	7	10	0	7	10 Infectiveness compared to
6 plants (1-6)	4.	2	18	0	2	18 strain 726 = 35%
T = Total	5.	4	14	0	4	14 strain N5 = 42%
	6.	8	11	0	8	11
TOTAL NOS. OF NODULES		29	82	0	29	82

CHEMOTAXIS MUTANTSINOCULUM STRAINSTYPE OF NODULE

		SC1	726	SC1+726	T _{SC1}	T ₇₂₆
SC ₁ + 726	1.	6	11	0	6	11
1:1 Ratio	2.	8	16	0	8	16 % Infections due to SC1 = 40%
6 plants (1-6)	3.	5	14	0	5	14 Infectiveness compared to
T = Total	4.	10	9	2	12	11 strain 726 = 67%
	5.	6	8	1	7	9 strain N5 = 78%
	6.	7	7	1	8	8
TOTAL NOS. OF NODULES		42	65	4	46	69

		SC2	726	SC2+726	T _{SC2}	T ₇₂₆
	1.	7	8	0	7	8
SC ₂ + 726	2.	4	10	5	9	15 % Infections due to SC2 = 38%
1:1 Ratio	3.	4	13	0	4	13 Infectiveness compared to
6 plants (1-6)	4.	8	7	2	10	9 strain 726 = 62%
T = Total	5.	4	12	0	4	12 strain N5 = 73%
	6.	5	8	3	8	11
TOTAL NOS. OF NODULES		32	58	10	42	68

		SC3	726	SC3+726	T _{SC3}	T ₇₂₆
	1.	7	12	0	7	12
SC ₃ + 726	2.	3	14	2	5	16 % Infections due to SC3 = 34%
1:1 Ratio	3.	7	9	0	7	9 Infectiveness compared to
6 plants (1-6)	4.	8	10	0	8	10 strain 726 = 51%
T = Total	5.	5	16	0	5	16 strain N5 = 60%
	6.	6	11	0	6	11
TOTAL NOS. OF NODULES		36	72	2	38	74

Appendix 1B (Data from Competition Experiment B)

<u>CONTROL DATA</u>		<u>TYPE OF NODULE</u>					NUMBER OF CELLS IN INOCULUM (Viable count)
		N5	726	N5+726	T _{N5}	T ₇₂₆	
A. N5 + 726							
1:1 Ratio	1.	6	7	0	6	7	
6 plants (1-6)	2.	7	12	0	7	12	
T = Total	3.	10	9	1	11	10	726 = 6.9x10 ³ cells/ml
	4.	8	11	0	8	11	N5 = 7.0x10 ³ cells/ml
	5.	8	9	1	9	10	
	6.	7	10	0	7	10	
TOTAL NOS. OF NODULES		46	58	2	48	60	

		N5	726	N5+726	T _{N5}	T ₇₂₆	
B. N5 + 726							
1:1 Ratio	1.	6	7	0	6	7	
6 plants (1-6)	2.	8	9	0	8	9	
T = Total	3.	5	8	1	6	9	726 = 8.2×10^3 cells/ml
	4.	9	13	0	9	13	N5 = 8.0×10^3 cells/ml
	5.	7	9	1	8	10	
	6.	10	9	1	11	10	
TOTAL NOS. OF NODULES		45	55	3	48	58	

		N5	726	N5+726	T _{N5}	T ₇₂₆	
c. N5 + 726							
1:1 ratio	1.	8	10	0	8	10	
6 plants (1-6)	2.	8	13	0	8	13	
T = Total	3.	7	8	0	7	8	726 = 8.7×10^3 cells/ml
	4.	7	9	0	7	9	N5 = 8.8×10^3 cells/ml
	5.	8	6	0	8	6	
	6.	5	10	0	5	10	
TOTAL NOS. OF NODULES		43	56	0	43	56	

Overall Total nos. of nodules: % N₅ nodules = 44%

N ₅	726	N5+726	T _{N5}	T ₇₂₆	$\frac{\text{Infectiveness of N5}}{\text{Infectiveness of 726}} = 80\%$
134	169	5	139	174	

In order to compare the results obtained for mutants SC1, SC2, SC3, SM1, SM2 and SM3 in direct competition with 726; N5 was considered to be 80% as infective as 726. Inoculum size = 8×10^3 cells/plant.

MOTILITY MUTANTSINOCULUM STRAINSTYPE OF NODULE

		SM1	726	SM1+726	T _{SM1}	T ₇₂₆	
SM1 + 726	1.	2	14	1	3	15	% Infections due to SM1 = 18%
1:1 Ratio	2.	3	13	0	3	13	Infectiveness compared to
6 plants (1-6)	3.	4	11	0	4	11	strain 726 = 23%
T = Total	4.	0	17	0	0	17	strain N5 = 29%
	5.	3	13	0	3	13	
	6.	5	10	0	5	10	
TOTAL NOS. OF NODULES		17	78	1	18	79	

		SM2	726	SM2+726	T _{SM2}	T ₇₂₆	
	1.	5	11	0	5	11	% Infections due to SM2 = 18%
SM2 + 726	2.	3	14	0	3	14	Infectiveness compared to
1:1 Ratio	3.	3	13	1	4	14	strain 726 = 22%
6 plants (1-6)	4.	4	17	0	4	17	strain N5 = 27%
T = Total	5.	1	15	0	1	15	
	6.	0	14	2	2	16	
TOTAL NOS. OF NODULES		16	84	3	19	87	

		SM3	726	SM3+726	T _{SM3}	T ₇₂₆	
	1.	3	17	0	3	17	
SM3 + 726	2.	4	11	1	5	12	% Infections due to SM3 = 25%
1:1 Ratio	3.	2	16	0	2	16	Infectiveness compared to
6 plants (1-6)	4.	8	11	0	8	11	strain 726 = 33%
T = Total	5.	4	15	0	4	15	strain N5 = 42%
	6.	6	13	0	6	13	
TOTAL NOS. OF NODULES		27	83	1	28	84	

CHEMOTAXIS MUTANTSINOCULUM STRAINSTYPE OF NODULE

		SC1	726	SC1+726	T _{SC1}	T ₇₂₆	
SC1 + 726	1.	1	12	2	3	14	
1:1 Ratio	2.	4	11	1	5	12	% Infections due to SC1 = 31%
6 plants (1-6)	3.	8	7	0	8	7	Infectiveness compared to
T = Total	4.	5	14	0	5	14	strain 726 = 45%
	5.	5	6	0	5	6	strain N5 = 57%
	6.	4	13	0	4	13	
TOTAL NOS. OF NODULES		27	63	3	30	66	

		SC2	726	SC2+726	T _{SC2}	T ₇₂₆	
SC2 + 726	1.	9	11	0	9	11	
1:1 Ratio	2.	5	14	0	5	14	% Infections due to SC2 = 29%
6 plants (1-6)	3.	3	16	0	3	16	Infectiveness compared to
T = Total	4.	6	10	0	6	10	strain 726 = 40%
	5.	5	12	0	5	12	strain N5 = 50%
	6.	4	17	0	4	17	
TOTAL NOS. OF NODULES		32	80	0	32	80	

		SC3	726	SC3+726	T _{SC3}	T ₇₂₆	
SC3 + 726	1.	3	14	0	3	14	
1:1 Ratio	2.	2	15	0	2	15	% Infections due to SC3 = 33%
6 plants (1-6)	3.	9	9	0	9	9	Infectiveness compared to
T = Total	4.	4	11	0	4	11	strain 726 = 50%
	5.	7	7	2	9	9	strain N5 = 63%
	6.	6	8	0	6	8	
TOTAL NOS. OF NODULES		31	64	2	33	66	

REVERTANTS

<u>INOCULUM STRAINS</u>		<u>TYPE OF NODULE</u>				
SRM ₁ + 726		SRM ₁	726	SRM ₁ +726	T _{SRM₁}	T ₇₂₆
1:1 Ratio	1.	7	9	0	7	9
6 plants (1-6)	2.	6	13	0	6	13
T = Total	3.	11	8	0	11	8
	4.	8	8	0	8	8
	5.	5	12	1	6	13
	6.	10	7	0	10	7
TOTAL NOS. OF NODULES		47	57	1	48	58

% Infections due to SRM₁ = 45%

Infectiveness compared to strain 726 = 83%; To strain N5 = 104%

<u>INOCULUM STRAINS</u>		<u>TYPE OF NODULE</u>				
SRC ₁ + 726		SRC ₁	726	SRC ₁ +726	T _{SRC1}	T ₇₂₆
1:1 Ratio	1.	8	9	0	8	9
6 plants (1-6)	2.	8	10	0	8	10
T = Total	3.	8	13	0	8	13
	4.	7	8	0	7	8
	5.	7	7	0	7	7
	6.	6	8	0	6	8
TOTAL NOS. OF NODULES		44	55	0	44	55

% Infections due to strain SRC₁ = 44%

Infectiveness compared to strain 726 = 80%; to strain N5 = 100%

APPENDIX 2

Appendix 2A Variance ratio analysis (F-Test) for competition Experiment 1

df = degrees of freedom

SS = Sums of Squares

MS = Mean Squares

F = Variance ratio

STRAINS SM1 + 726	df	SS	MS	F
Between samples	1	252	252	40.0
Within samples	10	63	6.3	
Total	11	315		

STRAINS SM2 + 726	df	SS	MS	F
Between samples	1	363	363	95.5
Within samples	10	38	3.8	
Total	11	401		

STRAINS SM3 + 726	df	SS	MS	F
Between samples	1	234	234	29.3
Within samples	10	80	8.0	
Total	11	314		

STRAINS SC1 + 726	df	SS	MS	F
Between samples	1	44	44	5.9
Within samples	10	75	7.5	
Total	11	119		

Appendix 2A (Cont.)

STRAINS SC2 + 726	df	SS	MS	F
Between samples	1	57	57	8.8
Within samples	10	.65	6.5	
Total	11	122		

STRAINS SC3 + 726	df	SS	MS	F
Between samples	1	108	108	20.4
Within samples	10	53	5.3	
Total	11	161		

Appendix 2B Variance ratio analysis (F-Test) for competition Experiment 2

df = degrees of freedom

SS = Sums of Squares

MS = Mean Squares

F = Variance ratio

STRAINS SM1 + 726	df	SS	MS	F
Between samples	1	310	310	66
Within samples	10	47	4.7	
Total	11	357		

STRAINS SM2 + 726	df	SS	MS	F
Between samples	1	385	385	116.6
Within samples	10	33	3.3	
Total	11	418		

STRAINS SM3 + 726	df	SS	MS	F
Between samples	1	270	270	51.9
Within samples	10	52	5.2	
Total	11	322		

STRAINS SC1 + 726	df	SS	MS	F
Between samples	1	108	108	13.9
Within samples	10	78	7.8	
Total	11	186		

STRAINS SC2 + 726	df	SS	MS	F
Between samples	1	209	209	42.6
Within samples	10	49	4.9	
Total	11	258		

Appendix 2B (Cont.)

STRAINS SC3 + 726	df	SS	MS	F
Between samples	1	91	91	10.5
Within samples	10	87	8.7	
Total	11	178		

STRAINS SRC1 + 726	df	SS	MS	F
Between samples	1	10	10	3.9
Within samples	10	26	2.6	
Total	11	36		

STRAINS SRM1 + 726	df	SS	MS	F
Between samples	1	9	9	1.6
Within samples	10	57	5.7	
Total	11	66		

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