chemotaxis of rhizobium phaseoli towards flavones and other related compounds

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CHEMOTAXIS OF *Rhizobium phaseoli* TOWARDS FLAVONES AND OTHER RELATED COMPOUNDS

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CONTENTS

ABSTRACT

The formation of nitrogen-fixing nodules on leguminous plants is the result of a highly specific interaction between the host plant and the soil bacterium Rhizobium. Two observations suggest that chemotaxis may play a role in the establishment of the legume-Rhizobium symbiosis: 1) The widespread occurence of Rhizobium species with flagella (Götz, 1982; Carlile, 1980)

2) The copious exudation into the soil surrounding legume roots of a wide variety of potentially chemotactic compounds which include amino acids, sugars and sugar-acids. (Beringer et.al., 1979). Recently the plant flavones luteolin (3,4,5,7 - Tetra - hydroxyflavone) and apigenin (4,5,7 - Trihydroxy-flavone) have been reported as inducers of Rhizobium nodulation genes (Peters, 1986, Rolfe 1986). We report here that R. phaseoli (RP8002) exhibits positive chemotaxis toward both flavones. Other diverse compounds tested elicited different responses.

An understanding of the influence of flavones in two events such as chemotaxis and nodulation efficiency can be valuable to clarify their action in the intricate legume-rhizobia interaction.

1 INTRODUCTION

1.1 Microbial movement. Microorganisms may be moved passively for long distances. They can be dispersed through the air by wind and eddy diffusion and in soil and aquatic environments by water movements. They may also be transported through air, water and soil on or in the bodies of insects and other animals. Many microorganisms are also motile, moving actively through the expenditure of energy. The rates of transport and distances travelled in this way are small compared with those resulting from passive movement, so motility tends to have been neglected by microbial ecologists. Active movement, however, can be guided by means of a sensory system, so that an organism can respond to their environment by swimming towards some chemicals and away from others (Adler, 1975). This movement, called chemotaxis, involves a number of steps. The bacteria must be able to sense information about the external environment, transmit this information across the cytoplasmic membrane to generate signals that converge on the flagellar motors (and to activate mechanisms that permit adaptation), and be able to swim.

To swim in the correct direction, bacteria must be capable of detecting differences in the concentration of attractants or repellents (Wolfe et al., 1987). Although the former bacterial behaviour is undoubtedly accepted, some soil microbiologists dispute whether bacteria actually swim in soil. Soil microorganisms which can detect and move toward a source of nutrients would have a selective advantage since nutrients are usually in short supply in the soil. Motile, chemotactic plant pathogens and symbiotic nitrogen fixers would have an advantage in reaching their host organisms by directed rather than random means.

1.2 Rhizobium morphology. According to Bergey's Manual of Determinative Bacteriology (Jordan, 1984) bacteria assigned to this genus are rod-shaped cells, 0.5 - 0.9µm. wide and 1.2 - 3.0µm. long without endospores and are gram-negative, aerobic and motile (due to the presence of up to six flagella).

The former genus Rhizbium has been recognised as consisting of two groups (fast growers and slow growers) based upon their growth rates on yeast extract. (Table 1).

Table 1 Classification of rhizobia (after Jordan, 1984)

Genus	Popular name and cultural characteristics	Species and varieties	Examples of hosts
Rhizobium	"Fast-growing rhizobia" Cultural characteristics: Colonies usually grow to 2-4 mm diameter within 3-5 days after inoculation on to standard yeast-mannitol-mineral salts medium. Produce acid on standard medium	R. leguminosarum var. viciae	Peas (Pisum)
		R. leguminosarum var. trifoli	Clovers (Trifolium)
		R. leguminosarum var. phaseoli	Beans (Phaseolus)
		R. meliloti	Lucerne, alfalfa (Medicago)
		R. loti	Trefoil (Lotus)
		R. fredii	Some soyabeans (Glycine)
Bradyrhizobium	"Slow-growing rhizobia" Cultural characteristics: Colonies do not exceed 1 mm diameter after 5-7 days on standard medium. Produce alkali	B. japonicum	Soyabeans (Glycine)

1.3 Importance of plant legumes. Plants of the legume family are second only to those of the grass family in their importance to humans. In developing countries, especially, the cultivation of legumes is the best and quickest way to augment the production of food proteins (Vietmeyer, 1986).

The ability of legumes to form association with bacteria that convert, or fix atmospheric nitrogen into a usable form leads to two features of importance to humans. Firstly, since nitrogen is not limiting to them, legumes incorporate more proteins into their tissues than other groups of angiosperms.Secondly the legume-bacteria association usually produces an excess of usable nitrogen in the soil. This means, in effect, that growing legumes can serve to provide a food crop and simultaneously to fertilize the soil.

1.4 Plant-host interaction. Rhizobium is responsible for maintaining this permanent protein supply in Leguminosae, through a very complex series of phenomena not completely understood, but that can be described as follows:

a) Exchange of molecular signals between host and microbe

b) Recognition of Rhizobium for a specific plant

c) Guided motility towards root hair

d) Attachment of Rhizobium to a root hair

e) Curling of the hair

f) Initiation of an infection thread

g) Growth of the infection thread

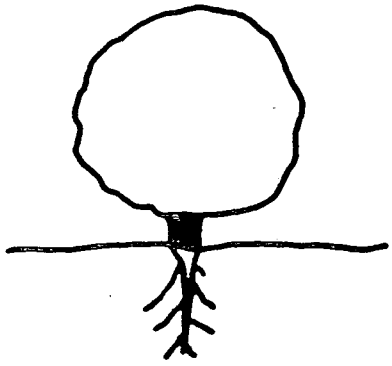
h) Deposition of membrane-enclosed bacteria into host cell

i) Transformation of bacteria into bacteroids

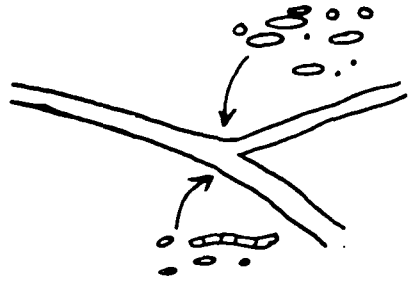
The exchange of molecular signals can have either a positive or a negative effect on the plant-microbe interaction that induces recognition from the bacterium to the plant and vice versa. Infective rhizobia attach to host root hairs or root cells genetically predisposed to become root hairs. Following attachment, infective rhizobia induce curling of the root hair by an unknown mechanism (Yao et al., 1969 and Callaham, 1981) The rhizobia become entraiped in the curl of the root hair, which appears important for initi-

tion of the infection process. The cell deposits new cell wall material internal to the point at which rhizobia are entrapped by the curled root hair (Calvert, 1984).

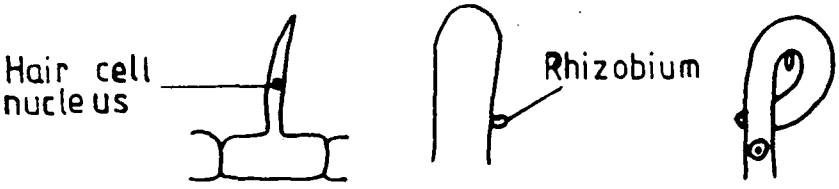
The new cell wall material is used to form a tubular structure called the infection thread. The infection thread grows as it follows the host cell nucleus to the base of the root hair cell. Root cortical cells are induced to divide and differentiate into nodule tissue. The infection thread passes through the cell wall of the root hair and begins to branch into the adjacent, newly divided cortical cells (Callaham, 1981). Rhizobia are released from the infection thread into the cytoplasm of cortical-cells and are surrounded by a host-derived peribacteroid membrane. The rhizobia differentiate into bacteroids. Mature bacteroids are capable of reducing atmospheric nitrogen into ammonia, which the plants assimilate for growth and development. Fig 1



Plant exudates



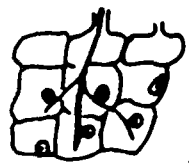
Initiation of motility



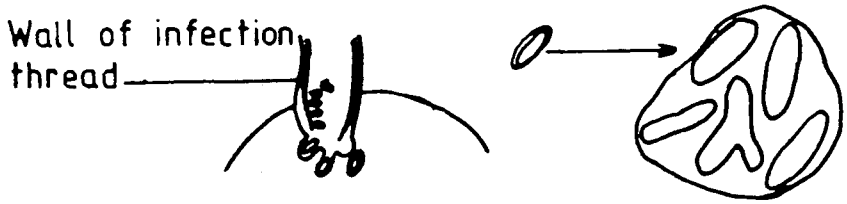
Rhizobium attaches to root hair

Root hair curls

Infection thread forms, appears to be directed by hair nucleus



Infection thread spreads, branching through cortex



Host membrane surronding infection thread

Rhizobium divide and differentiate into bacteroids

Fig. 1 Stages in the Rhizobium-legume interaction in clover (modified from Sprent, 1979).

1.5 Role of legume plant-exudates in chemotaxis and motility.

The micro-environment surrounding that portion of the soil under the direct influence of the plant root system is called the rhizosphere (Rovira , 1978). It may extend as much as 2cm. from the root surface. It is a zone rich in nutrients, particularly carbohydrates, aminoacids, and vitamins (Beringer et.al. 1979). This copious exudation from so diverse compounds, much of them nutrients, could explain the large and diverse populations of resident microbial colonists, most commonly, fungi and bacteria. Among them, Rhizobium is frequently present within and around the roots of certain legumes. That plant root exudates are chemoattractants was demonstrated using different species of Rhizobium and assessing its response towards various legumes and non legumes. Although both kind of root exudates attracted the different rhizobia, the bacteria showed a differential response in that they are attracted to the root exudate of some plants and showed no attraction toward others (Currier, 1976). Other authors have identified different chemicals produced by plant exudates which are chemotactically active.

For example, Burg et al., showed that R. meliloti was more attracted to amino acids than to sugars (Burg, 1982). In other case Rhizobium meliloti and R. lupini were optimally attracted to different aminoacids, and in general R. lupini exhibited greater chemotaxis towards carbohydrats (Goetz et.al., 1982). R. leguminosarum was attracted to a large range of sugars including D-glucose, cellobiose, and mannitol but not sucrose or trehalose (Bowra, 1981). R. leguminosarum was attracted toward the root exudates of P. sativum (Gaworzevska, 1982). Although Currier & Strobel, demonstrated that a glycoprotein, molecular weight 60,000, produced by birdsfoot trefoil roots attracted six different strains of Rhizobium (Currier, 1977), in most of the cases, only the fraction of the exudate containing substances with molecular weights less than 1000 showed a significant chemotactic activity (Gaworsevska, 1982).

1.6. Rhizobium nodulation (nod) genes and plant root exudates.

The nodulation process is a host-specific interaction in that each species of Rhizobium nodulates only one or a limited number of host plants. Bacterial gen s controlling nodulation have been located on large plasmids called Sym plasmids (Banfalvi, 1981 and Rosenberg, 1981). Based on complementation analysis of transposon insertion or deletion mutants and cloned fragments of the nod gene region, it was concluded that the genes nod A, nod B, nod C and nod B are common, i.e., are functionally interchangeable between different species of Rhizobium, whereas other genes code for host-specific nodulation functions (Innes, 1985 and Shearman, 1985). Recently, it was shown that the common nod A, nod B and nod C genes, which are required for root hair curling, as well as several host-specific nod genes, require a plant product for induction. The regulation of nod genes were studied by fusing fragments of the nod region with the lac Z structural gene of E. coli. It appeared that nod D is expressed constitutively (Innes, 1985 and Mulligan, 1985) and is subject to auto regulation Rossen, 1985). None of the other nod promoters studied was expressed in batch culture. In the presence of plant root exudates or seed exudates, however, promoter activity of common nod genes was observed in R. meliloti (Mulligan, 1985) and R. leguminosarum (Rossen, 1985), and promoter activity of common as well as host-specific nod genes was observed in R. trifolii (Innes, 1985). In all cases tested, the presence of a functional nod D gene was a prerequisite for induction (Mulligan, 1985 & Rossen, 1985). Another example of a bacterial response to plant compounds is the induction of the virulence (vir) genes of the opportunistic pathogen Agrobacterium tumefaciens. These genes are induced by acetosyringone (4-acetyl-2,6 - dimethoxyphenol) (Stachel, 1985) and other phenolic compounds (Bolton, 1986) whose presence in plant exudates is increased when the plant has been wounded (Stachel, 1985). Recently it has been demonstrated, that A. tumefaciens shows positive chemotaxis towards the same vir-inducer. Although chemotaxis can not be Ti-plasmid encoded, the findings indicated that at least

one Ti-plasmid function is involved in the specific chemotactic response to acetosyringone (Ashby, 1987). These plant factors are different from those identified as responsible for nod genes induction in Rhizobium.

Thus the plant signal compounds that have been identified as nod inducers can be classified as flavones. Although they are synthesised from the same precursors as the phytoalexin acetosyringone , they are structurally different. Among the plant released flavones to date identified as nod ABCD inducers are 7, 4'- dihydroxy flavone (geraldone) and 4'- hydroxy -7- methoxy flavone in white clover (Redman, 1986), luteolin in alfalfa (Peters, 1986) and apigenin –7-O-glycoside and several flavones in pea (Firmin, 1986).

The fact that plant exudates(small diffusible molecules) trigger the signal that allows Agrobacterium tumefaciens to recognise in nature a plant cell (Stachel, 1985) and that one of the Ti-plasmid functions has an important role in the specific chemotactic response to such compounds (Ashby, 1987), is clearly related with the findings in Rhizobium that the nod A, B, C genes are induced in the presence of plant exudates (Rosen, 1985). Moreover, those genes are expressed only in the presence of both the nod D gene and specific plant secreted compounds (flavones) (Djordjevic, 1987). In this work we studied the chemotactic response from Rhizobium phaseoli RP8002 towards apigenin and luteolin and some other compounds in order to increase our understanding of the role of these compounds in the specific plant-bacteria interactions in the soil and the role that such compounds may have in potentiating commercial microbial inoculants.

2 MATERIALS AND METHODS

2.1 Strains. Four different strains of Rhizobium were obtained from Dr. A. Johnstone (John Innes Institute) namely: Rhizobium phaseoli (AP8002), two Rhizobium leguminosarum (RL leg RCR 1040 and RL 16015) and Rhizobium trifoli (T83K3). They were maintained in L-Broth and Glycerol, frozen at -80 degrees C.

2.2. Growth of bacteria. To initiate growth, each culture was inoculated from the glycerol storage tubes in Rhizobium Initiation Medium (RI-medium) containing 0.5% (w/v) Tryptone, 0.3% Yeast Extract, 0.13% $CaCl_2 \cdot 2H_2O$ and 1.5% Agar. Single colonies were grown up in the former medium, incubated at 30 degree C during five days in petri dishes sealed with parafilm.

2.3 Isolation of motile bacteria. To achieve this, swarm plates were used, with the same medium, except that agar was added to a concentration of 0.25%, this medium was called Rhizobium Motility Medium (RM-medium). One single colony was spotted onto RM-medium plates, using sterile toothpicks. The inoculated plate was sealed with parafilm and incubated at 28 degrees C. After three days, bacteria grew outwards forming a ring and further plates were inoculated from the edge of the ring. This procedure was repeated twice and finally the edge scraped again and streaked onto RI-medium.

This procedure was repeated every 2 weeks.

2.4 Growth curves. Growth curves for each culture were monitored using a 250 ml. conical flask containing 100 ml. of RI-liquid-medium and inoculated with 200 µl of an overnight culture to an initial optical density $A_{590}=0.05$ The flask was incubated at 28 degrees C. in a New Brunswick G76 gyratory water tank set up at 125 rpm. Aliquots were removed at two hour intervals and the absorbance at 590 nm recorded. At the same time, one 100 µl sample was appropriatly diluted and plated on RI-medium for counting colonies.

2.5. Motility assessment. Two forms of motility assessment were employed. In the first one, petri dishes of RM-medium were centrally inoculated. Motile

and chemotactic cells migrated as a ring of increasing radius which was measured using a ruler. In the second one, microscopal observations were carried out from the culture previous to each chemotaxis experiment. One drop (about 50-µl) of the culture was enclosed in a convex slide and covered with a cover slip. Observations were made under 400x phase contrast optics using a Nikon Optiphot microscope.

2.6 Preparation of culture for chemotaxis assay. A single colony was inoculated into a McCartney bottle containing five mililitres of liquid RI-medium and grown as in Section 2.3. At A_{590} = 0.4 - 0.5 (about 3.7 x 10^{-10} cells ml^{-1}). Cells were concentrated by centrifugation (WIFUG Lab centrifuge 500E, Sweden) at 2500 rpm for 10 minutes, washed twice with chemotaxis buffer (0.1mm EDTA, 10mm KH_2PO_4, ph=7.0) andcarefully resuspended in this buffer to a final A_{590} of 0.2 - 0.3 (about $2x10^{-8}$ cells ml^{-1}).

2.7. Chemotaxis assay. The capillary assay for chemotaxis was performed by the principles of Adler (Adler, 1973). The system consisted of a glass plate (60 x 30 x 4mm) used as a base for a V-shaped capillary tube 40mm long, 1mm diameter. (B.D.H. Macfarlane Robson, London U.K.) with sealed ends (the bacterial well), the capillary tube (1-5µl capacity, A & J. Beveridge, Newcastle upon Tyne U.K.) containing the attractant and a cover slip. From one culture prepared as described before, 300 µl of bacterial suspension were enclosed into the V-shaped tubes. Capillary tubes were filled using a 3 µl ultra-micropipet (Monoject, Athy, Co. Kildare, U.K.) with the filter-sterilised solution of attractant at different concentrations dissolved in chemotaxis buffer. The capillaries were handled with forceps and after being loaded with the attractant, the upper end was sealed with grease. Each attractant concentration was performed with capillary tubes in duplicate. Negative control capillaries were filled with chemotaxis buffer alone and used to assess the background motility of bacteria. As positive control, liquid RI-medium was used in each experiment. After incubation for one hour at room temperature, the capillary was removed, its exterior was rinsed

with a thin stream of sterile water from a wash bottle, the sealed end was broken off and the content was expelled with the aid of the micropipet into Eppendorf tubes containing a solution of 1mM NaCl. Suitable dilutions were made and placed on petri dishes containing RI-medium to determine the number of bacteria per capillary tube, after 3 days incubation at 30 degrees C.
The inoculated plates were kept in plastic bags during the incubating time to avoid dessication. For thecolony count a Colony Counter (Gallen Kampf. CNW-325, Sussex, U.K.) was used.

2.8 Reagents and culture medium. Peptone was from BBL Microbiology Systems (Cocksville, USA) Yeast extract was from Oxoid (Hampshire, U.K.). Bacto-agar was from Difco Laboratories (Detroit, Michigan, USA). $CaCl_2$ H_2O, NaCl, KH_2PO_4 and EDTA were from Anala R. (BDH Chemicals Ltd. Poole, U.K.).
Luteolin and Apigenin were from Apin Chemicals Ltd.(Oxon, U.K.). Sucrose, Galactose, Glucose, Fructose, Maltose, Raffinose, Xylose, Umbelliferone and Naringenin were from Sigma Chemical Company (St. Louis Mo, USA). Acetosyringone was from Jansen Chemicals (Bersee Belgium). 3,4, - Dihydroxybenzoic acid, P-Hydroxybenzoic acid and Vanillyl alcohol were from Aldrich Chemicals (Milwaukee USA).

3 RESULTS

3.1 Motility assessment. Four strains of Rhizobium were subjected to motility and chemotaxis assessment using swarm plates of RM-medium. Strains Rhizobium phaseoli (PR8002) and Rhizobium trifoli (T86K3) were consistently more motile and chemotactic than the two strains of Rhizobium leguminosarum (RL1040 and RL16015). Moreover, R. phaseoli gave the greatest motility and chemotactic response in swarm plates as is depicted in Figs. 2 and 3. The experiment was repeated three times with consistent results.

The four strains were also assessed in their growth behaviour using the growth conditions and nutritional requirements stipulated in Material and Methods. The results are represented in Fig 4. From this curve, it can be seen that R. phaseoli (RP8002) showed the faster growth rate. Thus R. phaseoli was chosen as the test organism for the remaining experiments.

3.2 Chemotaxis toward sugars. To confirm the chemotactic ability of the chosen strain and set up the capillary tube conditions of future experiments, sucrose was used as attractant toward R. phaseoli in two different growth stages, i.e. : log and idio phase. The results are represented in Fig.5 (In this and subsequent figs. the dotted line represents control results obtained using liquid RI-medium as attractant).

Table 2 summarises the results obtained with various sugars tested as attractants for R. phaseoli. The response curves are represented in Figs. 6 and 7. Sucrose, raffinose and xylose act as good chemoattractants whereas glucose, galactose, fructose and maltose do not.

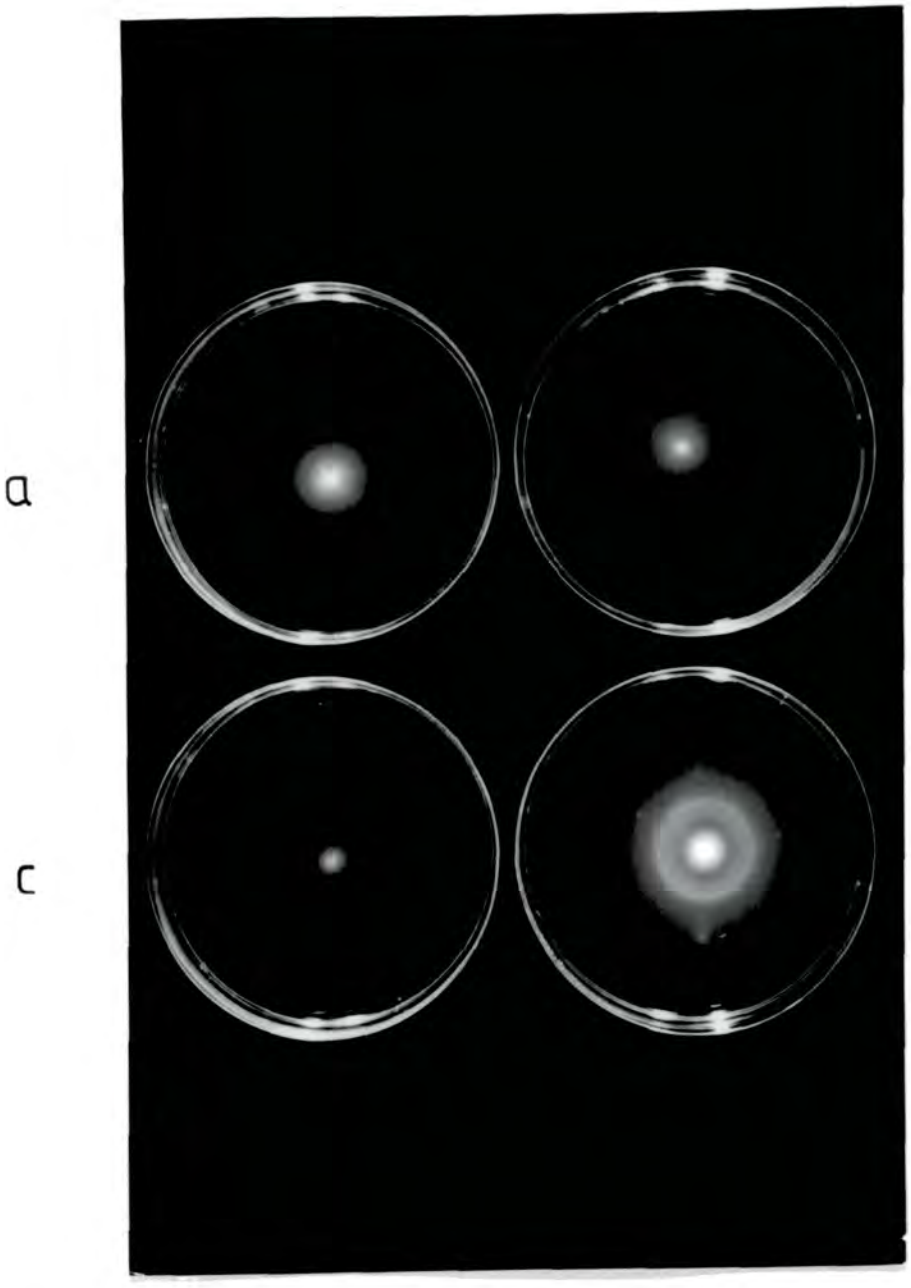


Fig.2 Motility and chemotaxis of Rhizobium in swarm plates: a, RL16015; b, T83K3; c, RL leg RCR1040; d, RP8002.

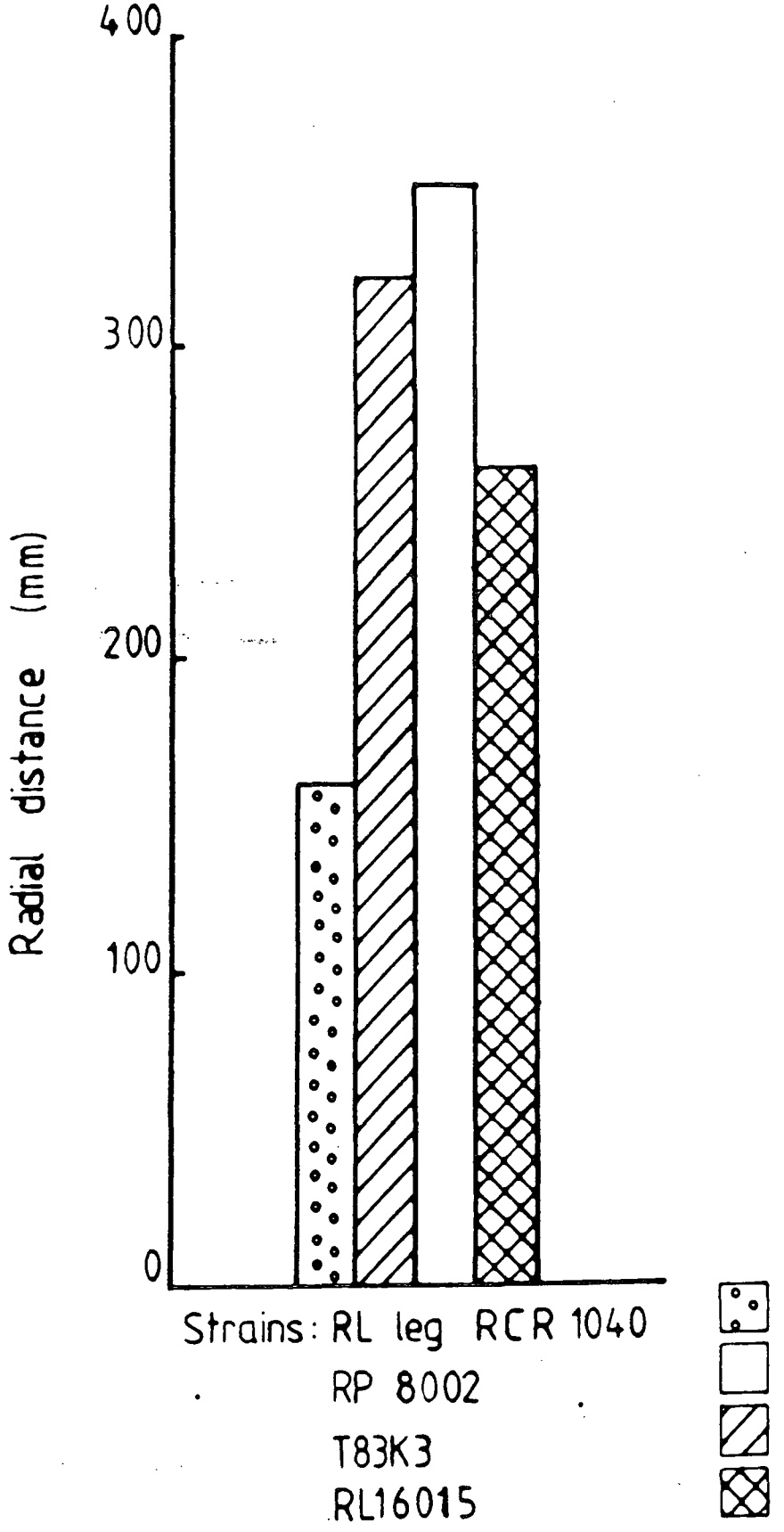
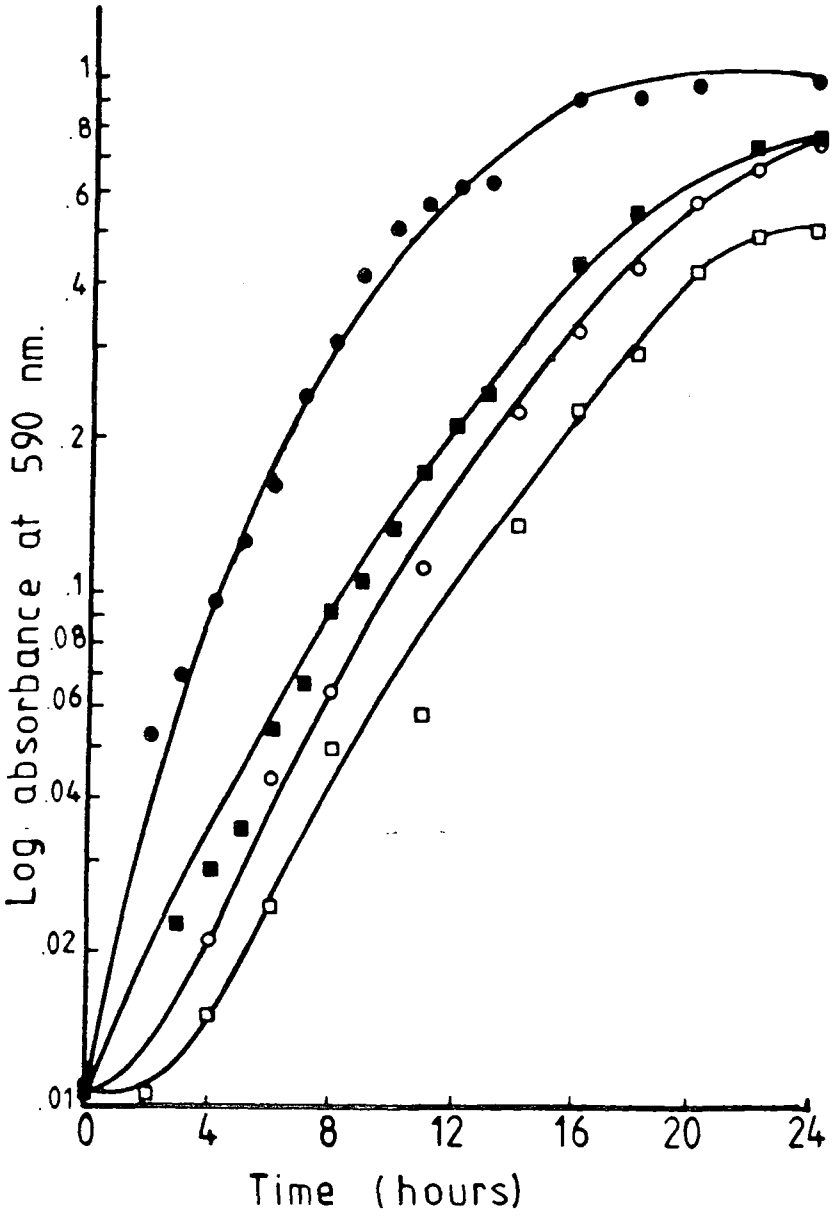


Fig. 3 Maximum ring radii of Rhizobium in swarm plates.



B. phaseoli RP8002 (●—●), R. trifoli T83K3 (■—■),
B. leguminosarum RL16015 (○—○), RL1040 (□—□).

Fig. 4 Growth curves of four different strains of Rhizobium.

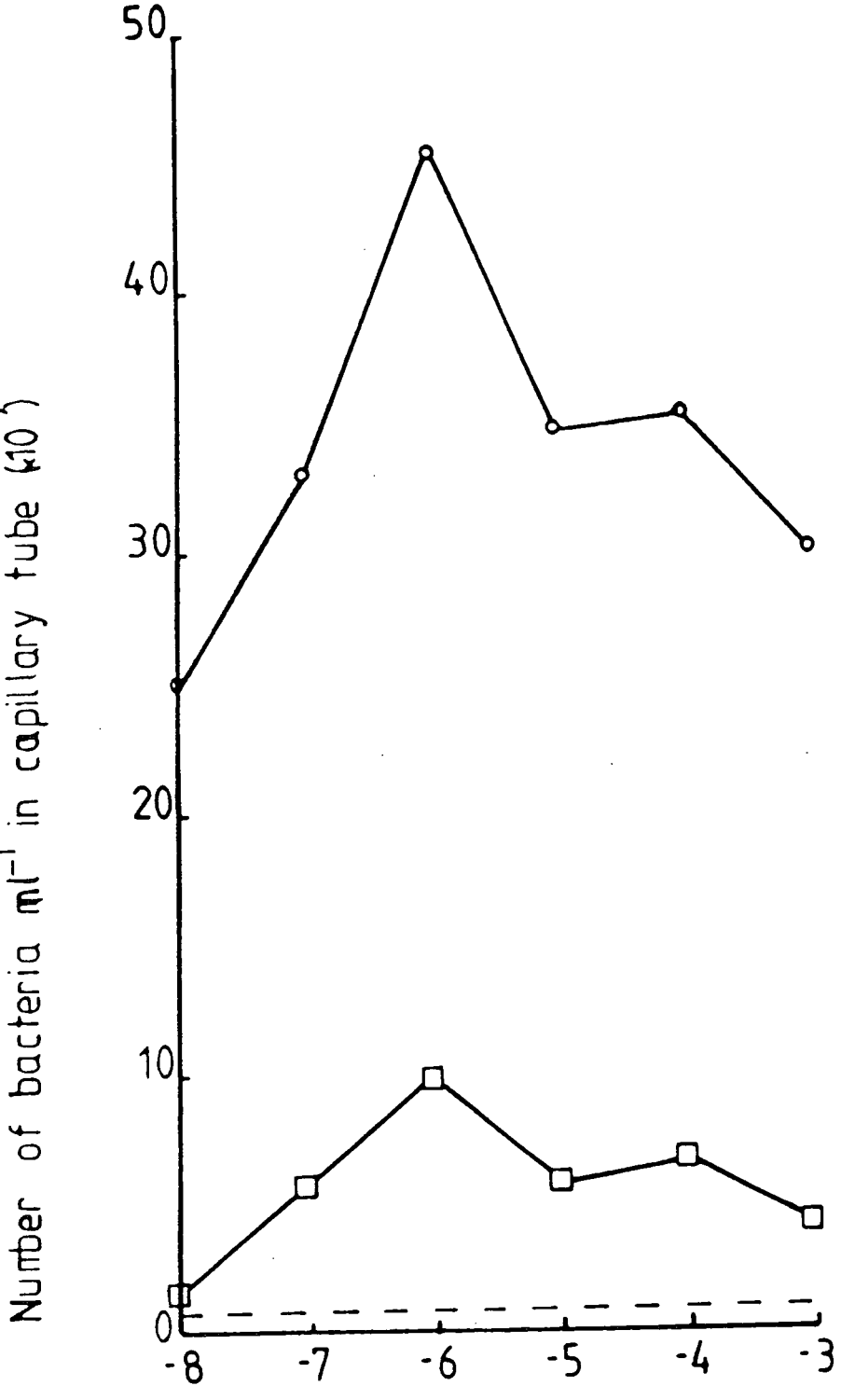


Fig. 5 Chemotaxis of R. phaseoli towards sucrose using log (□—□) and idiophase (○—○) cultures. The dotted line represents the negative control as described in Section 2.

Table 2 Maximum chemotaxis levels achieved with various sugars

Attractant	Maximum response		*Chemotaxis
	Concn. (M)	No. of bacteria in capillary tube ($\times 10^{-6}$)	
Sucrose	10^{-6}	8.6	+
Maltose	10^{-4}	2.4	-
Raffinose	10^{-5}	6.7	+
Xylose	10^{-4}	27.6	+
Galactose	10^{-6}	4.5	-
Glucose	10^{-5}	3.3	-
Fructose	10^{-4}	2.7	-

*Positive chemotaxis refers to a significant level of attraction greater than the negative control.

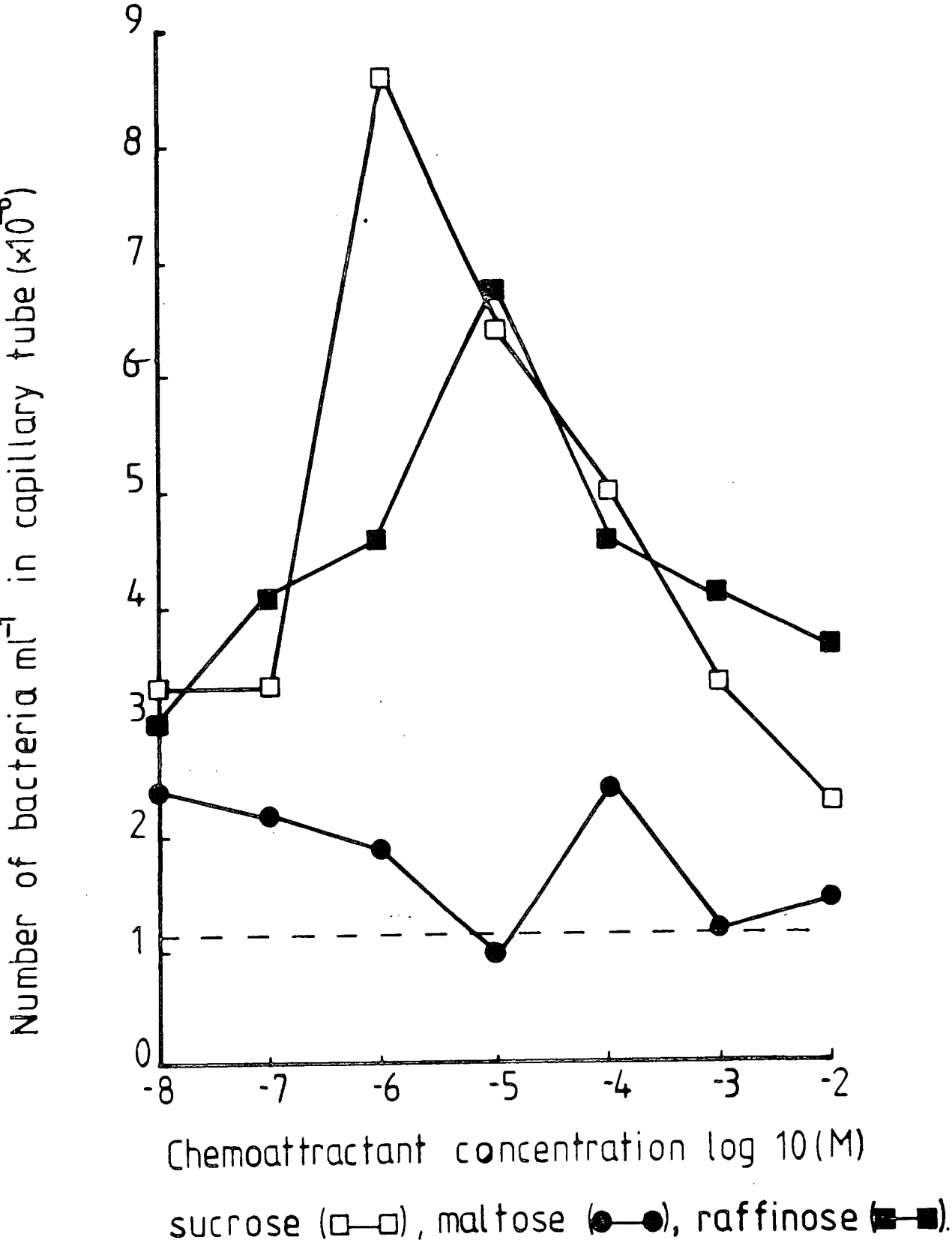


Fig. 6 Chemotaxis of R. phaseoli towards oligosaccharides.

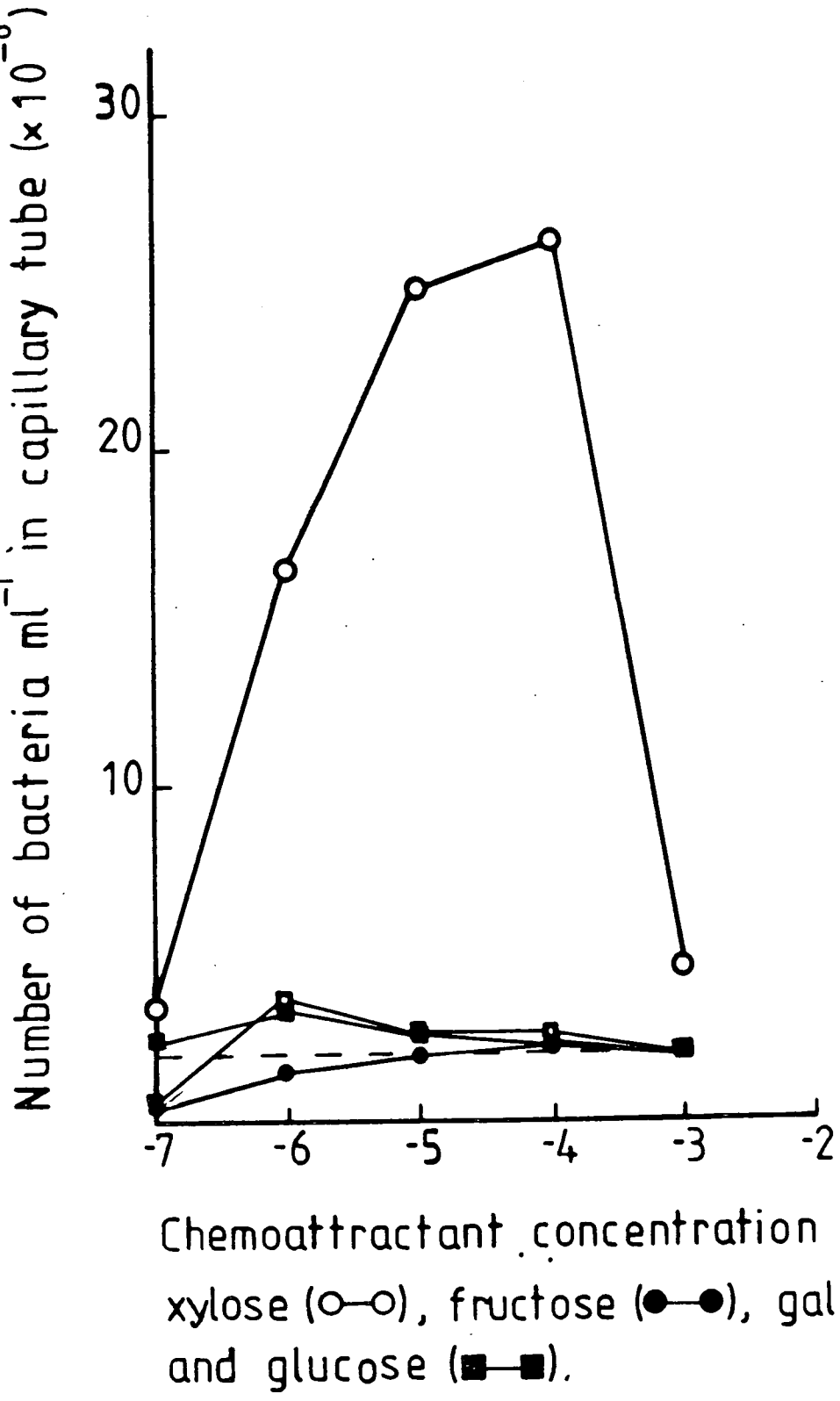


Fig. 7 Chemotaxis of R. phaseoli towards monosaccharides

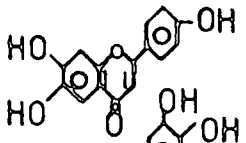
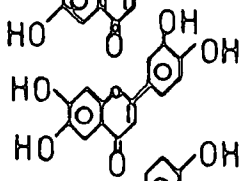
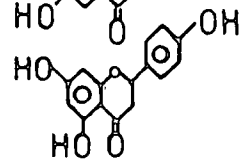
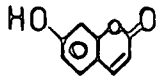
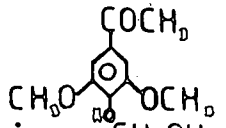
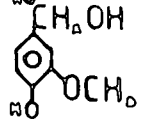
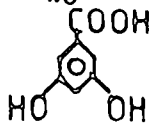
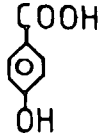
3.3 Chemotaxis toward flavones. Flavones are hydroxyderivatives of flavones itself.The most important members of the flavone group are: chysin (5,7-Dihydroxyflavone), apigenin (4',5,7-Trihydroxyflavone) and luteolin (3',4',5,7-Tetrahydroxyflavone). Apigenin and luteolin are among hundreds of identified flavonoid compounds found in plants and roots exudates, but only a few members of the family induce expression of nodulation genes in Rhizobium (Peters, 1986 and Firmin, 1986).

The chemotactic activity of Rhizobium phaseoli (RP8002) towards four flavones and other related compounds are summarised in Table 3.

In a preliminary experiment carried out in spite of the limited solubility of the compounds in water, both luteolin and apigenin gave a clear peak of response within 10^{-6} M concentration. (Fig.8). In this case, high bacteria concentration was maintained within the pool (A_{590}=0.77, approximately 7.2×10^{-10} cells ml^{-1}).

Solubility problems were overcome, using a mix of acetonitrile and dimethilsulphoxide (1:1). Neither solvent acts as a chemoattractant (data not shown). Apigenin gave a slightly higher response peak than luteolin both at a ten fold higher concentration of attractant (10^{-5} M), probably due to an improvement in solubility (Fig. 9).

Table 3 Maximum chemotaxis levels achieved with flavones and related compounds

Flavones	Structure	Maximum response: Conc. (M)	Maximum response: No. of bacteria ml^{-1} in cap. tube ($\times 10^{3}$)	Chemotaxis
Apigenin		10^{-5}	33.6	+
Luteolin		10^{-5}	23.5	+
Naringenin		10^{-3}	9.3	-
Related compounds				
Umbelliferone		10^{-3}	26.0	+
Acetosyringone		10^{-2}	121.0	+
Vanillylalcohol		10^{-6}	36.5	+
P-hydroxybenzoic ac.		10^{-6}	19.5	+
Di-hydroxybenzoic ac.		10^{-4}	11.6	+

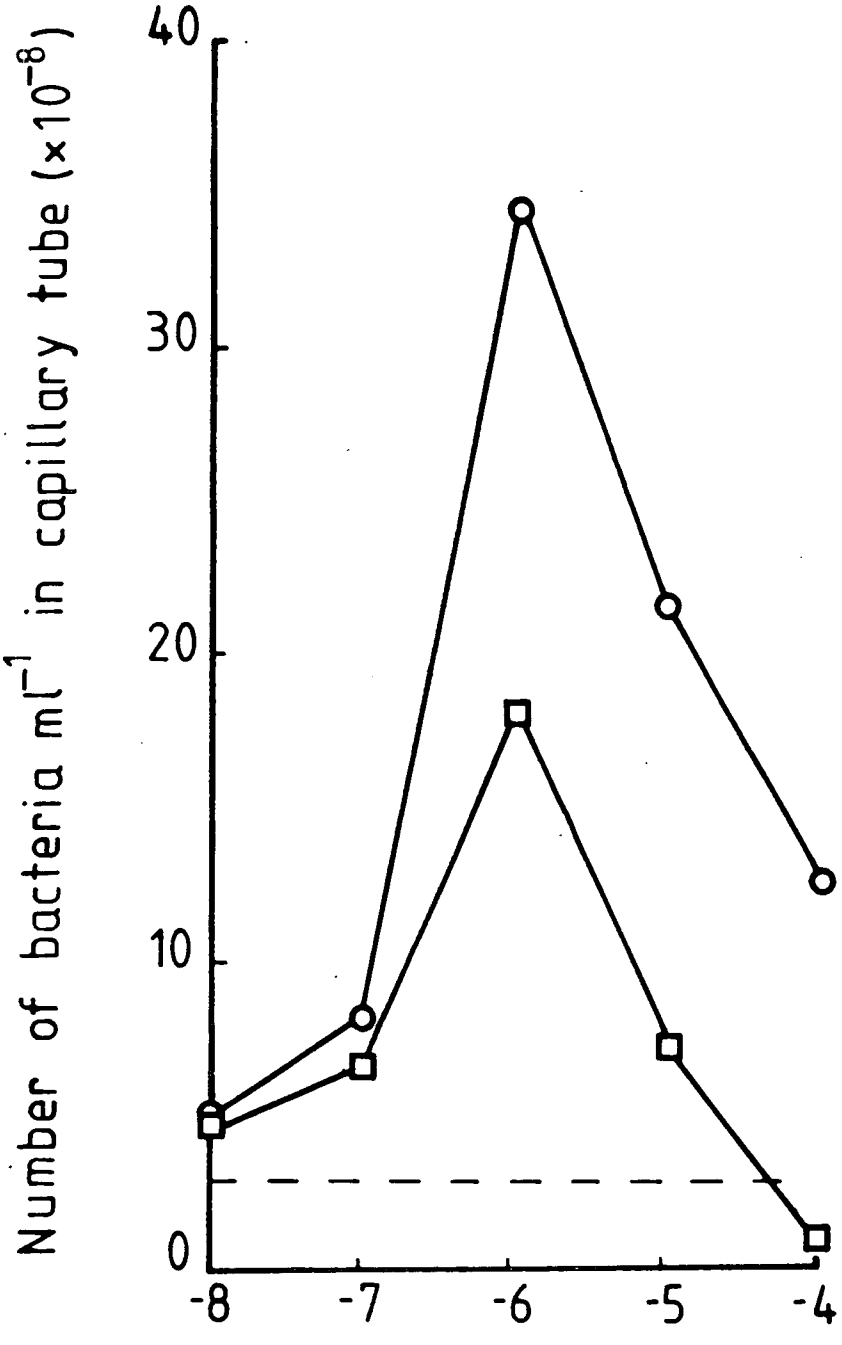


Fig. 8 Chemotaxis of R. phaseoli towards flavones.

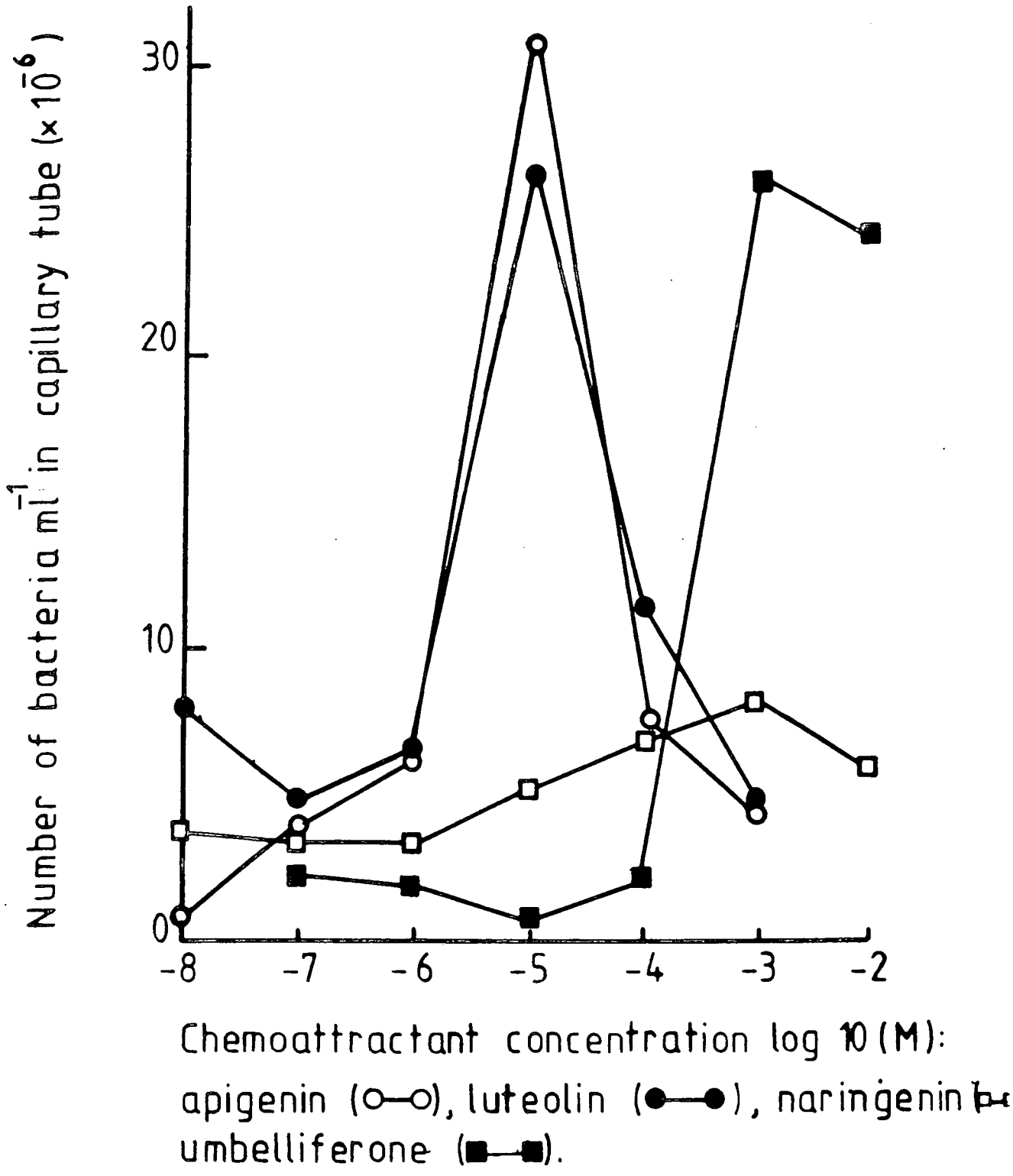


Fig. 9 Chemotaxis of R. phaseoli towards flavones and related compounds.

Finally, an assay using two different stages of growth (log- and idio-phase) was carried out employing the two flavones (Fig.10). Although the maximum peak concentration was maintained at 10^{-5} M, the high bacterial concentration gave poorer reproducibility. From these figures it was decided to use early stationary phase bacteria in further experiments.

3.4. Competition assays between apigenin and liteolin. It is unanimously accepted that attractants, that are closely related in structure compete with each other but not with structurally unrelated compounds (Adler, 1973).

. the experiments performed with luteolin and apigenin, one attractant at its optimum concentration was put into the capillary tube, and another attractant at a ten fold higher concentration was put into both the capillary and the bacterial suspension. The results of such experiments are described in Table 4.

From such data it can be seen that apigenin does not inhibit chemotaxis towards luteolin when the first of the compounds was put in the bacterial pool, but in the reciprocal experiment, luteolin does partially inhibit chemotaxis towards apigenin (approximately 70%) suggesting that the receptor which detects apigenin also is able to detect luteolin but that, in addition there is another receptor that detects luteolin but not apigenin (A "luteolin" receptor).

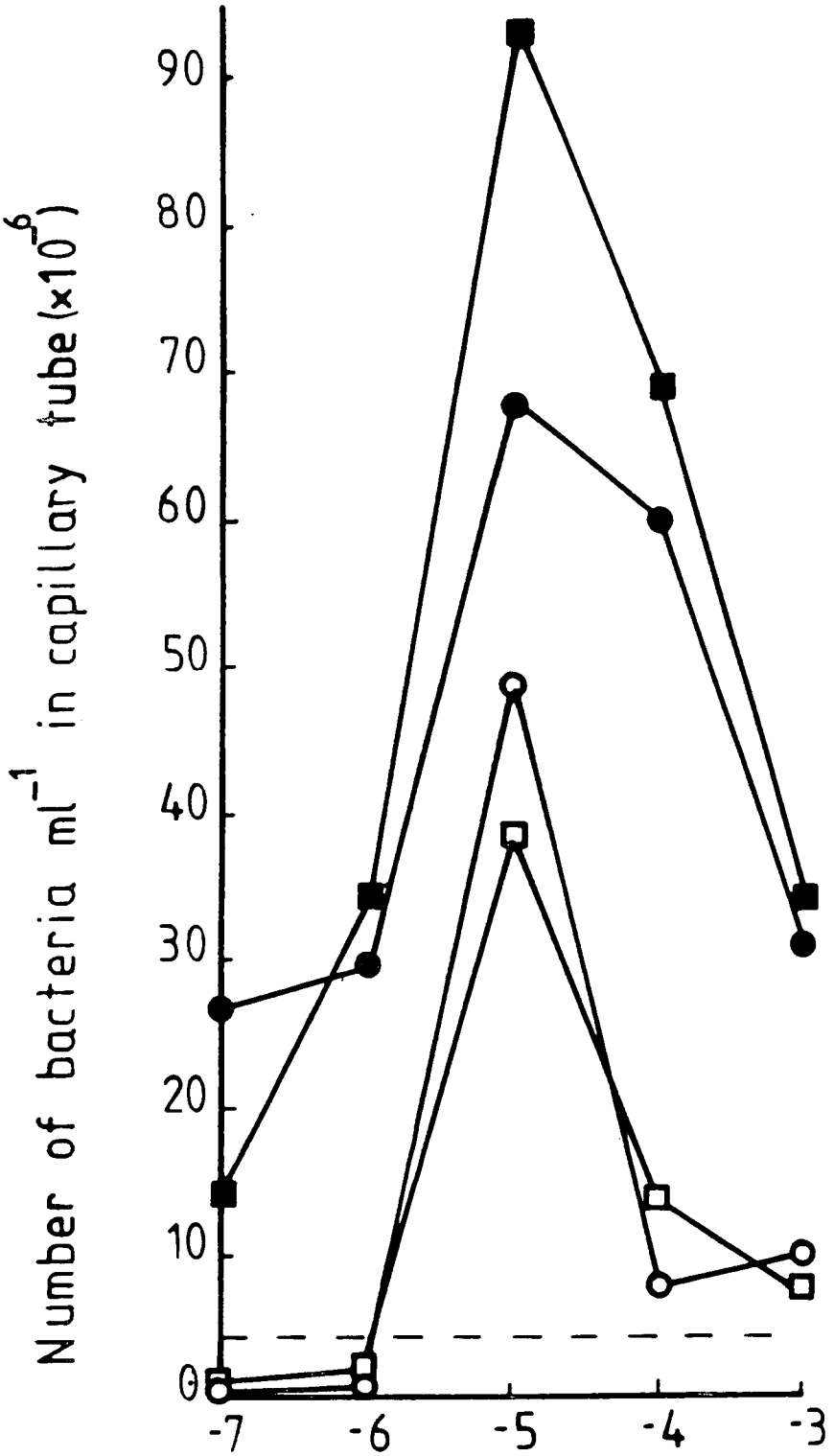


Fig. 10 Chemotaxis of R. phaseoli from two different growth phases towards flavones

Table 4 Competition between luteolin and apigenin for chemoreceptors

Attractant	No. of cells ml^{-1} ($\times 10^{-6}$)
Chemotaxis buffer	8.7
Apigenin (10^{-5} M)	28.3
Luteolin (10^{-5} M)	29.4
Apigenin-luteolin (10^{-5} M)	38.6
Apigenin-luteolin (10^{-5} M) apigenin saturation (10^{-4} M)	22.1
luteolin saturation (10^{-4} M)	6.9

3.5 Chemotaxis toward related compounds. Umbelliferone (7- Hydroxycumarin) and especially naringenin (4',5,7-Trihydroxyflavone) are structurally close to luteolin and apigenin, (see Table 3), but only umbelliferone showed appreciable chemoattraction at relatively high concentration (Fig 9).

3.6 Chemotaxis toward other compounds. Acetosyringone (3',5'-Dimethoxy-4' hydroxyacetophenone; AS) and vanillylalcohol (4-hydroxy -3- methoxybenzyl cohol; VA) are among the substances exuded by wounded plants. Recently both mpounds have been tested as attractants toward A. tumefaciens harbouring and without Ti-plasmid. The chemotactic response of a Ti-plasmid-harbouring strain, but the lack of it from the isogenic cured counterpart demonstrated that a Ti-plasmid determined function is involved in specific chemotaxis toward acetosyringone (Ashby, 1987). The experiments performed with Rhizobium toward AS and VA are represented in Fig11. AS exhibited a very potent response whereas VA is also chemotactic although in less degree and lower chemoattractant concentration.

Finally two structurally less related compounds ie. P-hydroxybenzoic acid (PHBA) and 3,4 - dihydroxybenzoic (DHBA) acid also present in root exudates were examined for a chemotactic response.

Only PHBA gave a typical chemotactic peak, with a maximum in 10^{-6} M, whereas the shape peak in DHBA was irregular but with an apparent maximum in 10^{-4} M. (Fig 12).

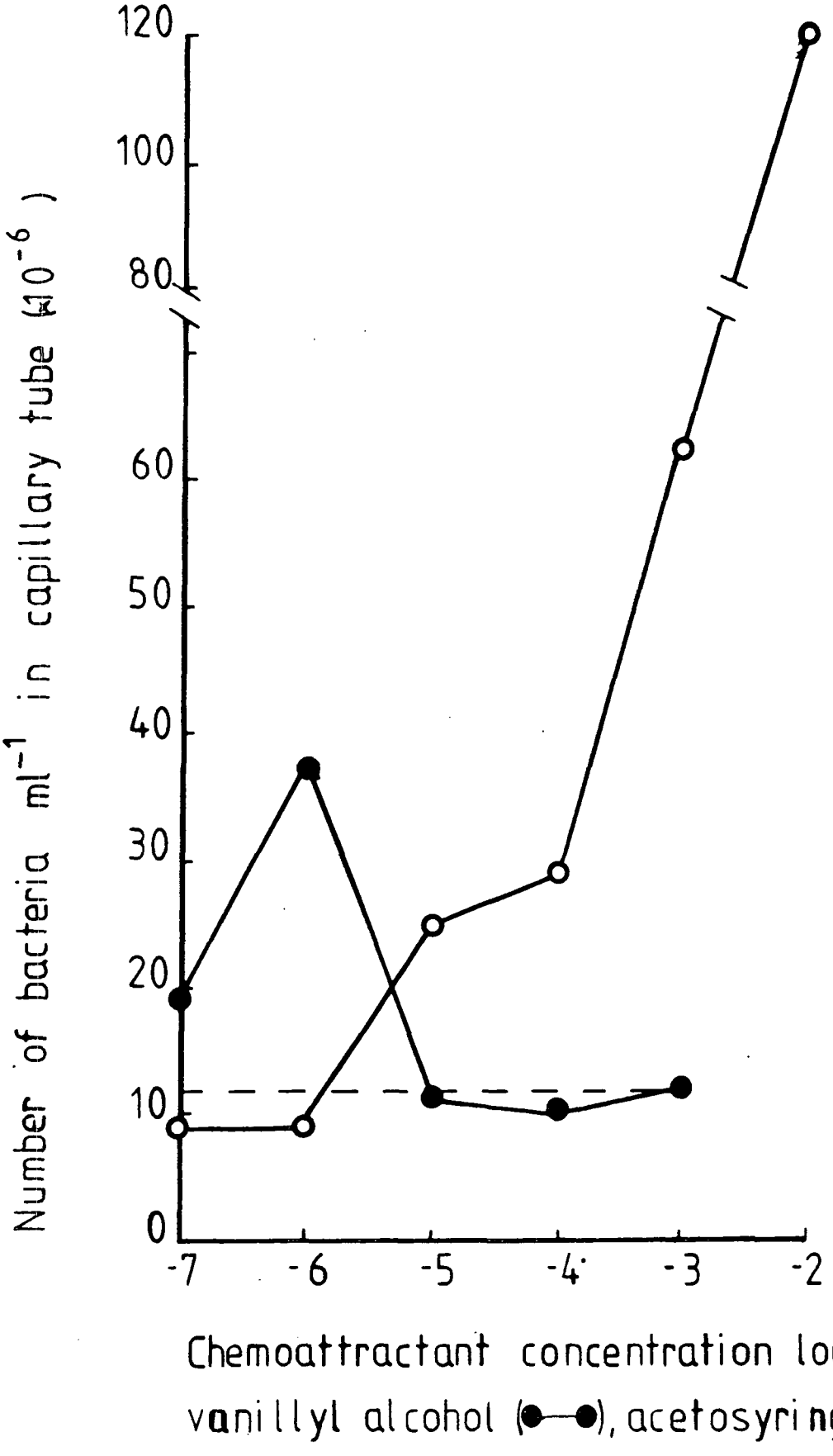


Fig. 11 Chemotaxis of R. phaseoli towards phenolic compounds

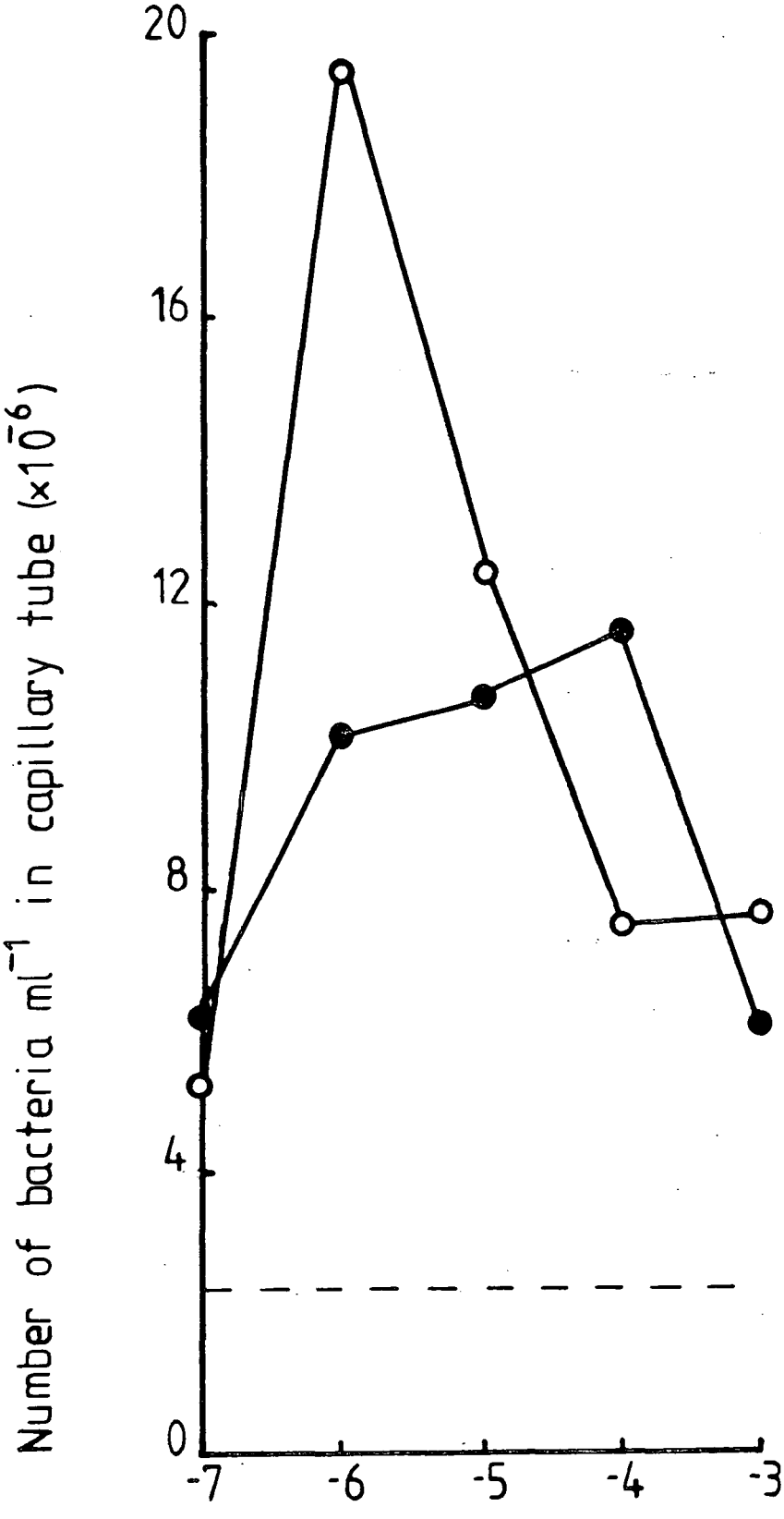


Fig. 12 Chemotaxis of R. phaseoli towards benzoic acids

4 Discussion

In order to choose the most efficient strain in terms of chemotaxis ability, swarm plates of RM-medium were used according to Adler (Adler, 1973). The method is simple and sufficiently sensitive to detect the most chemotactic and motile microorganism, and it can used to induce and maintain a motile population for long periods. R. phaseoli (RP8002) showed the greater capacity to move in swarm plates. The maximal distance achieved was 345 mm in 96 hours. Interestingly, the same strain showed the lowest duplication time in standard growth curves. Therefore, both criteria were used in selecting R, phaseoli (RP8002) for subsequent experiments.

Although, the four strains are fast growers, they showed marked differences in growth rate. This may be due to the lack of carbon source in the culture medium since certain taxes have been reported as inducible. This observation and the fact that growth conditions affect chemotaxis lead us to look for the optimal conditions. Although in general it is recommended that bacteria should be harvested in early log phase for the best chemotaxis (Adler, 1973). Our findings with Rhizobium gave the best response in the early part of the stationary phase (Figs. 5 and 10). The initial chemotaxis experiment using the capillary assay (Adler, 1973) was to confirm the chemotactic ability of the chosen strain using sucrose as attractant. This sugar is a major component in plant extracts and has been reported as a good attractant in a wide range of bacteria. In addition, in similar assays using A. tumefaciens toward different sugars, sucrose gave the most potent response (Loake, et.al. personal communication).

A range of different sugars (from monosacharides to trisacharides) were tested. Unlike E. coli, which in chemotactic tests gives very uniform responses, Rhizobium strains are often inconsistent with respect to motility and chemotactic sensitivity (Gaworzowska, 1982). This affirmation is particularly

valid when the attractants are sugars. Rhizobium phaseoli (RP8002) responded differently to different sugars. Thus, from the seven sugars tested, only sucrose, raffinose and xylose were chemotactically positive. Xylose gave the most potent response, and it gave the only response among all the monosaccharides tested, consistent with two previous reports in which a positive chemotaxis response was given by R. leguminosarum toward xylos. Moreover, the maximum response peak was similar (Gaworsewska, 1982 and Bowra 1981). (10^{-5} - 10^{-4} M). This data shows that the best sugar attractant tested when we consider the number of bacteria in capillary tube, is xylose. At the same time, R. phoseoli (RP8002) seems to have relatively weak responses to sugars.

With respect the flavones R. phaseoli (RP8002) showed positive chemotaxis toward apigenin, luteolin and umbelliferone but not toward naringenin. The effectiveness and consistency with which apigenin and luteolin induce nod genes in Rhizobium apparently depends on their structure. Thus, the compounds which produced a significant enhancement of nod A gene expression were all flavonoids with a hydroxyl group at the 7 position (Djordjevic, 1987). The attachment of the B-ring to C-2 is crucially important for induction of the nod A gene in R leguminosarum. Peters et.al., demonstrated the necessity for hydroxylation at the 3' or 4' positions (or both) of the B ring to induce nod ABC genes in R. meliloti. It seems that these characteristics are not essential for chemotaxis in R. phaseoli, since naringenin, which is more closely related, from the point of view of its structure, to luteolin and apigenin, was chemotactically inert.

In the competition assays carried out with apigenin and luteolin, the results clearly indicate that the presence of apigenin in saturating concentrations in both the capillary and the bacterial pool has no direct effect on the ability of Rhizobium phaseoli (RP8002) to chemotactically respond to luteolin. This suggests that the two flavones do not share the same chemoreceptors. However in the reciprocal experiment 75% of the chemotaxis ability is lost.

This suggests that the receptor which detects apigenin also is able to detect luteolin but that, in addition, there is another receptor that detects luteolin but not apigenin. An interesting enhanced effect in the chemotactic response was observed when both attractants were put into the capillary tube which strongly suggested a synergistic effect.

Other compounds tested which have been found in exudates of legumes(Redman, 1986) were umbelliferone and the benzoic acids PHBA and DHBA. Umbelliferone has been reported as a non-inducer of nod genes. (Redman, 1986 and Djordjevic, 1987). This compound was chemotactically active, although at higher concentration than the flavones. PHBA and DHBA gave a positive chemotactic response with maximumpeaks at concentrations of 10^{-6} and 10^{-4} M.

These results are relevant since umbelliferone is a key metabolite in the synthesis of flavones and benzoic acids are common breakdown products of flavones (Djordjevic, 1987).

Agrobacterium and Rhizobium spp. are closely related gram-negative heterotrophs, both are soil-dwelling bacteria which infect plants and produce alterations in the development of the host. The recent findings about the role of acetosyringone in vir-gene expression (Stachel, 1985) and from AS and VH in chemotaxis (Ashby, 1987) prompted chemotaxis assays toward both compounds. The recorded response in terms of number of bacteria in capillary tube was the highest from all the tested chemicals. In fact the peak achieved by AS was 100 times higher than for all other compounds. However, these responses should be taken with caution, because they were obtained in a high chemoattractant concentration (10^{-2} M).

AS has been reported to repress nod gene expression (L. Rossen personal communication). Thus the similarity in the response curves and chemotactic maxima of umbelliferone and AS could be significant. However, the reason why R. phaseoli should be so attracted to non nod-inducers remains a mystery.

5 CONCLUSIONS

Nodulation of legumes is an extremely complex phenomenon involving many different steps. Any of these steps may involve at once several distinct mechanisms showing a great specificity, in both the host plant and the bacterium. Furthermore, it has been shown that bacteria capable of exhibiting a chemotactic response usually find themselves at a selective advantage in unmixed environments (such as soil) with a mixed and large microbial population (such as the rhizosphere), where spatial or temporal chemical gradients persist, and where only micromolar quantities of the nutrients are present (Pilgram, 1976).

The initial communication between the bacterium and the plant appears to be the induction of Rhizobium nod gene expression by compounds secreted from the roots of legumes. This can occur in the absence of direct contact between the bacteria and the plant root. The fact that the molecules that can induce nod gene expression include substituted flavones such as luteolin (Yao, 1969), and apigenin (Zaat, 1987) and that the same molecules are chemotactically active, supports the assumption that specificity, if not absolutely necessary, at least plays an important role. However, additional experiments are suggested to assess the chemotactic activity and the nod gene expression relationship using a wider number of flavones. The former could help to understand the conditions necessary previous to the establishment of the nodulation process and the genetic basis for selection of certain Rhizobia by legumes. It may be possible to select rhizobia that are resistant to various unusual environmental factors, e.g. low soil ph,' toxicities, high or low temperatures. Genetic engineering techniques are contributing to the identification of which genes are involved in competition and chemotaxis, manipulating these genes appropriately, enhanced responses can be obtained towards attractants, adding better competitivity of microbial inoculants. These will contribute to facilitate the manipulation of both the host and

rhizobial genes in such a way that more efficient nodulation will be possible with specific legume-Rhizobium combinations, that will be of enormous value for agriculture.

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