SHORT COMMUNICATION

Motility and Chemotaxis towards Sugars in *Rhizobium leguminosarum*

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Motility in *Rhizobium leguminosarum* WU163 was dependent on the pH of the medium; addition of EDTA or treatment of the medium with a chelating resin was also required. Motility was improved by the addition of Ca²⁺ but not by Mg²⁺; both Zn²⁺ and Cu²⁺ abolished motility at low concentrations. Chemotaxis was demonstrated to L-arabinose, D-ribose, D-xylose, D-glucose, D-fructose, mannitol, sorbitol, dulcitol, lactose and cellobiose. The organisms were not chemotactic to either sucrose or trehalose under the conditions used.

INTRODUCTION

It is still not clear how an appropriate strain of *Rhizobium*, present in the soil as a saprophyte, reaches the rhizosphere of a legume whose roots it can infect. Two extreme situations can be considered: the first where contact between the developing legume root and a microcolony of rhizobia is purely by chance, and the second where chemoattractive substances produced by the plant guide rhizobia towards the root. Root exudation of sugars, amino acids and vitamins is well documented for legume roots (Rovira, 1965). *Rhizobium* as a genus is regularly motile, the flagellation being subpolar in the slow-growing strains and peritrichous in fast-growing strains (De Ley & Rassel, 1965), though the conditions required for motility have not been fully established (Currier, 1980). If rhizobia behave like other Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas* spp., chemotaxis would be expected to occur.

Previous studies have shown *Rhizobium* to be chemotactic to both root exudates and simple sugars. Currier & Strobel (1976a, b) showed that the birdsfoot trefoil strain of *Rhizobium* was chemotactic to a glucoprotein in root exudates but not to common sugars and amino acids. In soil such a protein seems likely to have a very restricted range of movement past charged particles and its significance remains to be assessed. Subsequent work has shown that the trefoil strain of *Rhizobium* is chemotactic to the simple sugars mannose, ribose, maltose, fructose, galactose and sucrose (Currier, 1980). Evidence presented by Gitte *et al.* (1978) suggests that a strain of *Rhizobium* from *Cicer* nodules was attracted to glucose, galactose, xylose, ribose and arabinose extracted from root exudates. Since concentrations of sugars are not given by these authors, it is hard to say whether the taxes they found are of physiological significance.

In this study, we report on factors which affect motility in *Rhizobium leguminosarum* WU163, and show that this strain is very similar to other Gram-negative bacteria in its chemotactic responses to sugars.

METHODS

Growth of bacteria. The organism used (*Rhizobium leguminosarum* WU163) was from the culture collection of the University of Western Australia. It nodulates peas effectively. It was maintained on yeast/mannitol agar (Vincent, 1970) and transferred every 2 weeks.
Chemostat cultures were grown in a minimal salts medium made by mixing 1 litre solution A with 1 ml solution B and 2 ml solution C after autoclaving the solutions separately at 109 °C for 45 min. Solution A contained (per litre) 1-5 g mannitol, 0-3 g sodium glutamate, 0-25 g MgSO₄·7H₂O, 0-36 g KH₂PO₄, 1-4 g K₂HPO₄ and 0-2 g NaCl; solution B contained (per litre) 15 g Na₂EDTA, 0-16 g ZnSO₄·7H₂O, 0-2 g Na₂MoO₄·2H₂O, 0-25 g H₂BO₃, 0-2 g MnSO₄·4H₂O, 0-02 g CuSO₄·5H₂O, 1 mg CoCl₂·6H₂O, 1 g thiamin·HCl. 2 g calcium pantothenate and 1 mg biotin; solution C contained (per litre) 10 g CaCl₂·2H₂O and 3-3 g FeSO₄·7H₂O. The chemostat had a 50 ml working volume and was maintained at 27 °C with constant aeration and stirring.

Motility measurements. Chemostat-grown cells were centrifuged at room temperature at 2500 g for 10 min, and resuspended by gentle rocking in 5 ml 10 mM-potassium phosphate buffer (pH 6-5) containing 0-1 mM-EDTA (motility medium). After a further centrifugation, the bacteria were resuspended in motility medium to give approximately 4·7 × 10⁷ cells ml⁻¹ and motility was checked microscopically. Motility assays were based on the method of Adler (1973) except that 2·5 cm × 4 mm i.d. glass test tubes were substituted for the cover slip–glass slide assembly. A 1 μl disposable capillary containing 0·75–1·75 cm trapped motility medium was placed in 0·1 ml bacterial suspension (4·7 × 10⁶ cells) in a test tube and incubated for 30 min at 25–26 °C. The capillary was then removed and the number of bacteria in the capillary was determined by plating its contents on yeast/mannitol agar. Three to five capillaries were used for each assay.

In some experiments EDTA was omitted from the medium and metal ions were removed by treatment with the chelating resin Chelex-100 so that the effects of adding low concentrations of heavy metal ions could be seen. The effect of medium pH was also tested. For pH values lower than 6·5 the buffer was 10 mM-citrate containing 0·1 mM-EDTA.

Chemotaxis measurements. Chemostat-grown cells were prepared as for motility measurements; the capillaries were filled with appropriate concentrations of attractant in motility medium. Assays were done with three to six capillaries per treatment and incubation was for 20 min at 25–26 °C.

Plate chemotaxis was observed in Petri dishes containing medium of the following composition (per litre): 2 g agar, 1·36 g KH₂PO₄, 0·4 g NH₄Cl, 0·24 g MgSO₄·7H₂O, 1 ml solution B, 2 ml solution C and metabolizable attractant at 1–10 mM; pH 6·5. Plates were incubated at 28 °C and the rate of formation and movement of rings of growth from a central inoculum was recorded.

Reagents. Sugars and derivatives for chemotaxis experiments were obtained from the following sources: L-arabinose, D-ribose, D-fructose, dulcitol, sorbitol, D-glucose and lactose from BDH; sucrose and mannitol from Ajax Chemicals, Sydney, Australia; D-xylene from G. T. Gurr Ltd, London; D-cellobiose and D-trehalose from Sigma. Thin layer chromatography on cellulose plates in propan-2-ol/ethyl acetate/water (3:1:1, by vol.) was used to check the purity of glucose, xylose, ribose, sucrose and lactose; it revealed no contamination. All sugars except cellobiose and trehalose were assayed for glucose content using the glucose oxidase reagents (Boehringer-Mannheim blood sugar analysis kit). D-Fructose and sucrose appeared to contain 0·09 and 0·91 % glucose, respectively. For chemotaxis experiments, sugars were filter-sterilized through 0·45 μm-pore-size Millipore membranes.

RESULTS

Motility. The washing regime used in preparation of the cells affected subsequent motility; membrane filtration was inferior to centrifugation as measured by accumulation of bacteria in capillaries. Accumulation was linear with density of the suspension up to an A₆₀₀ of 0·1 (1 cm cell). Accumulation increased with time of incubation up to 30 min at pH 6·5; the standard assay conditions selected were 30 min at pH 6·5 and 26 °C.

The pH of the motility medium had a significant effect on motility in R. leguminosarum WU163. Motility was optimal between pH 5·5 and 8·0, decreased sharply below pH 5·5 and was virtually absent at pH 4·5. Motility was also affected by the composition of the motility medium; omission of the chelating agent EDTA abolished accumulation. Addition of 1 mM-CaCl₂ doubled the accumulation (P < 0·05): 1 mM-MgSO₄ or 1 mM-glucose produced no significant effects.

Medium treated with Chelex-100 resin was used instead of medium with EDTA to study the effects of heavy metal ions; resin treatment was not significantly different from EDTA addition. CuCl₂ and ZnCl₂, at 10 μM, both inhibited accumulation almost completely, and CuCl₂ also lowered viability.

The minimum conditions for motility—0·1 mM-EDTA in 10 mM-phosphate buffer (pH 6·5)—were used in all subsequent chemotaxis assays.
Table 1. Chemotactic responses to sugars and sugar alcohols by R. leguminosarum WU163

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Threshold concn* (mM)</th>
<th>Concentration (mM)</th>
<th>Mean bacterial no. in capillary†</th>
<th>Plate chemotaxis</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Arabinose</td>
<td>0-001-0-01</td>
<td>0-1</td>
<td>79 200</td>
<td>+</td>
</tr>
<tr>
<td>D-Ribose</td>
<td>0-001</td>
<td>100</td>
<td>210 000</td>
<td>ND</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>0-001</td>
<td>0-1</td>
<td>68 400</td>
<td>ND</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>0-001</td>
<td>1-0</td>
<td>131 000</td>
<td>+</td>
</tr>
<tr>
<td>D-Fructose</td>
<td>0-001-0-01</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Mannitol</td>
<td>0-001</td>
<td>1-0</td>
<td>25 300</td>
<td>+</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>0-01-0-1</td>
<td>1-0</td>
<td>20 600</td>
<td>ND</td>
</tr>
<tr>
<td>Dulcitol</td>
<td>0-1-1-0</td>
<td>100</td>
<td>22 700</td>
<td>ND</td>
</tr>
<tr>
<td>Lactose</td>
<td>1-10</td>
<td>100</td>
<td>28 800</td>
<td>+</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>1-10</td>
<td>100</td>
<td>100 000</td>
<td>ND</td>
</tr>
<tr>
<td>Sucrose</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Trehalose</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, Not determined. —, No chemotaxis observed.
* Attractant concentration producing cell accumulation one standard deviation above the background accumulation.
† A background accumulation of 4200 cells has been subtracted.

Chemotaxis. In all experiments, background accumulation (Adler, 1973) was subtracted from the numbers in capillaries with attractant added. The threshold concentration was taken to be the attractant concentration producing one standard deviation above the background accumulation. With the chemostat providing a supply of cells of nearly constant physiological properties, the background accumulation was 4200 cells and the accumulation at the threshold concentration was 6500 cells.

A number of parameters of the chemotactic response were studied with 1 mM-mannitol as the attractant. Accumulation was time dependent with a plateau at 45 min. Conditions were standardized at 20 min incubation at 25-26 °C. Reproducibility was such that taxis to 1 mM-mannitol measured on three different days (four capillaries each day) showed a standard deviation for the number of bacteria accumulating of 20-30% of the mean for any day, and about 30% over the three days.

Table 1 summarizes the results obtained with various attractants. Definite positive chemotaxis was observed to L-arabinose, D-ribose, D-xylene, D-glucose, D-fructose, mannitol, sorbitol, dulcitol, lactose and cellobiose, while there was no demonstrable taxis to sucrose or trehalose. Plate chemotaxis confirmed the results from accumulation in capillaries (Table 1). A definite ring was formed if the sugar was an attractant while there was no movement of the inoculum if the sugar was not an attractant.

In this wild-type strain, no requirement could be demonstrated for exogenous methionine for chemotaxis towards glucose, although the bacterium showed significant chemotaxis towards 0-1 mM-L-methionine.

Discussion

The capillary assay for motility measures the approach to a situation where net movement of organisms in and out of the capillary is zero. Accordingly, the plateau reached between 30 and 45 min is to be expected.

Sensitivity of motility to pH agrees with results for other bacteria where the optimum pH for motility is close to the optimum pH for growth. Most strains of Rhizobium leguminosarum show optimal growth over the pH range 5 to 8 (Vincent, 1977); motility appears to be maximal at pH 5.5 and above for the strain we studied, compared with...
maximal chemotaxis between pH 7.0 and 7.5 for the rhizobia from birdsfoot trefoil (Currier, 1980).

The effects on motility of EDTA and chelating resin treatment of the medium seem likely to be via the same mechanism—the removal of heavy metal ions. Zinc and copper ions are known to have toxic effects on Rhizobium (Parker et al., 1977) and zinc and copper fertilizers sown with inoculated seed inhibit nodulation. The effects of these metal ions on nodulation may perhaps be as much due to inhibition of motility as to direct toxicity, especially at low concentrations.

The initiation of infection threads by rhizobia is apparently sensitive to both Ca$^{2+}$ deficiency and low pH (Munns, 1977). Two effects observed in our experiments could help to explain the field data—pH values lower than 5.5 are likely to inhibit rhizobial motility towards and along the root, and Ca$^{2+}$ may be important in improving motility. Whether Ca$^{2+}$ could overcome the inhibitory effects of low pH on motility was not studied, but this is obviously of relevance to the rhizosphere situation. For example, Rovira (1961) found that the rhizosphere to soil ratio of numbers of rhizobia around roots of Trifolium pratense increased from 1 to 44 when CaCO$_3$ was added to the soil, an event which might involve an effect on migration from the soil (motility) or multiplication in the rhizosphere.

A comparison of rhizobial motility with that of other bacteria can be made by calculating a motility coefficient ($\mu$) from the effective diffusivity equation of Segel et al. (1977). The value of $\mu$ for R. leguminosarum WU163 under our conditions was 0.19 cm$^2$ h$^{-1}$, compared with 0.20 cm$^2$ h$^{-1}$ for Pseudomonas fluorescens (Segel et al., 1977) and 0.25 cm$^2$ h$^{-1}$ for E. coli (Adler & Dahl, 1967), indicating that R. leguminosarum and P. fluorescens have similar motility, but are slightly less motile than E. coli.

The capillary method for determining chemotaxis is well documented (Adler, 1973). The substitution of a test-tube system for the slide method appears also to produce satisfactory results, with accumulation of bacteria a function of both incubation time and attractant concentration. Unlike the trefoil strain of Rhizobium (Currier, 1980), R. leguminosarum was chemotactic to glucose, xylose and arabinose but was not attracted to sucrose. Such differences in the chemotactic responses of different Rhizobium strains suggest that differential chemotaxis to sugars could play a role in the specific colonization of legume roots by rhizobia. Rhizobium leguminosarum appeared to respond to monosaccharides at lower concentrations than disaccharides. No chemotaxis was demonstrated to either sucrose or trehalose by R. leguminosarum under our conditions. However, since chemotaxis in E. coli (Adler et al., 1973) and Pseudomonas aeruginosa (Moulton & Montie, 1979) is inducible for some substrates, it remains possible that rhizobia grown on these substrates might show chemotaxis to them. Since the plate assay method did not reveal any moving rings after 30 h incubation on sucrose, such inducible chemotaxis appears unlikely for sucrose. Failure to respond chemotactically towards utilizable sugars is also common in other Gram negative bacteria (Adler, 1973; Moulton & Montie, 1979).

The significance of motility and chemotaxis in getting rhizobia into the rhizosphere of the appropriate legume remains to be assessed. Our work shows that they can respond chemotactically to simple sugars, some of them at quite low concentrations, while that of Gitte et al. (1978) indicates that the Cicer rhizobia respond to amino acids and sugars in root exudates, and that the magnitude of the response to amino acids is considerably greater than that to sugars. A similar difference in responsiveness to sugars and amino acids was found for chemotaxis in E. coli (Mesibov & Adler, 1972). Legume root exudates contain both these classes of compounds (Rovira, 1965) as well as other substances such as vitamins. Even though rhizobial mutants defective in motility or chemotaxis can nodulate after direct inoculation on to the root (Ames et al., 1980), the possibilities for chemotaxis being important for nodulation from the soil are clear; it is also possible that specific combinations of attractants may be able to selectively attract different rhizobia.
REFERENCES


