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Chemotaxis of *Rhizobium spp.* to a Glycoprotein Produced by Birdsfoot Trefoil Roots

Abstract. Rhizobium spp. *show chemotaxis to plant root exudates. A glycoprotein has been isolated from the root exudates of birdsfoot trefoil,* Lotus corniculatus, *which, at micromolar concentrations, attracts six strains of rhizobia. This glycoprotein has been given the trivial name trefoil chemotactin and contains approximately twice as much protein as carbohydrate. Gel filtration of trefoil chemotactin on a Bio-Gel A-1.5m column gave a molecular weight of approximately 60,000. Trefoil chemotactin represents a new class of chemoattractants for bacteria.*

Nitrogen availability is critical for agriculture since available fixed nitrogen often limits field crop productivity. Commercial nitrogen fixation consumes large quantities of fossil fuels and energy (*1*). Biological nitrogen fixation uses the energy of sunlight and atmospheric nitrogen.

The genus *Rhizobium* is characterized by its ability to elicit nodules and fix nitrogen on legume roots. The bacterium-host interaction is specific in that a single strain of *Rhizobium* can infect only certain species, or even certain varieties within a species, of legumes (*2*). In fact, rhizobia are classified by their host range. The basis of this bacterium-host specificity is unknown.

It is known that the numbers of an infective strain of *Rhizobium* are selectively increased in the rhizosphere of a legume (*3*). Before nodulation the surface of the root is covered with a matrix of bacteria (*4*). Munns (*5*) showed that in liquid culture more bacteria accumulated on alfalfa roots in the first few hours after inoculation than could be accounted for by multiplication of the inoculum. Since rhizobia are generally motile (*6*), we set out to determine if chemotaxis of rhizobia to legume roots might play a part in nodulation. Earlier work in our laboratory showed that six strains of *Rhizobium* show differential chemotaxis to root exudates of legumes and nonlegumes (*7*). That is, a single strain of *Rhizobium* is attracted to some plant root exudates and is unaffected by others.

Crude birdsfoot trefoil root exudates attract trefoil *Rhizobium* strain 95C13 (*7*). Root exudates were collected from 10-day-old seedlings grown under sterile conditions in medium that contained no combined nitrogen. Surface-sterilized seeds were suspended above the liquid medium on an aluminum screen covered with cheesecloth. Clear root exudates were collected and filtered. Fractions of the root exudates were tested for fungal and bacterial contamination by plating on enriched media; no colonies were seen (*7*).

Chemotaxis was measured by counting the radioactivity in bacteria labeled by growth on [U-^{14}C]glucose that swam into a 5-μl micropipette dipped in a suspension of labeled bacteria (*7*). The micropipette contained nitrogen-free medium as control or a fraction of trefoil root exudate. Five replicates were used for each sample solution.

The work we now report was directed toward isolation and characterization of the compound in trefoil root exudate responsible for its attraction of trefoil *Rhizobium*. Treatment of trefoil root exudate with pronase or acid completely destroyed its ability to attract trefoil *Rhizobium* (*8*). Boiling the exudate for 10 minutes did not affect the activity. These results indicate that the active principle in the root exudate, which we have called trefoil chemotactin, is heat stable and contains peptide bonds.

The crude trefoil root exudate (1 liter) was concentrated tenfold, and two volumes of cold acetone were added. The precipitate was removed by centrifugation and the supernatant liquid, containing chemotactin, was reduced in volume 30-fold. Higher concentrations of acetone would precipitate chemotactin; however, it was difficult to resolubilize in water or buffer from the pellet. Hence, the acetone-soluble fraction (67 percent acetone) was extracted twice with double the aqueous volume of *n*-butanol, and then in the same manner with chloroform, and finally with ether. The

Table 1. Chemotaxis of the trefoil strain of *Rhizobium* to fractions of a birdsfoot trefoil root exudate. The chemotactic ratio, R, is calculated as the counting rate (disintegrations per minute) of the sample divided by that of the medium (control). The chemotactic ratio divided by the carbon content, R/CC is used as a measure of specific activity.

Test solution	Average disintegrations per 10 minutes	R	Carbon content (μg/ml)	R/CC
Nitrogen-free medium	258			
Concentrated trefoil root exudate*	689	2.67	3442	7.76×10^{-4}
Acetone supernatant	1080	4.19	2112	1.98×10^{-3}
Bio-Gel P-2 void volume	1052	4.08	141	2.89×10^{-2}
Ion exchange neutral	1950	7.56	266	2.84×10^{-2}
Bio-Gel A-1.5m, peak I	3148	12.20	26	4.69×10^{-1}

*The contents of ten 1-liter beakers—a total of approximately 800 ml of root exudate from 500 to 1000 10-day-old seedlings—were combined, filtered, and concentrated. A portion was lyophilized and assayed at each step of the purification (see text).

lipid-free aqueous extract, containing chemotactin, was reduced to 1 ml and fractionated on a Bio-Gel P-2 column. The void-volume peak was collected and passed through cation and anion exchange columns (*9*). Chemotactin was eluted from both of these columns with distilled water.

Chromatography of this fraction on a Bio-Gel A-1.5m column gave one biologically active peak (Fig. 1). Comparison of the elution volume of this peak with the elution volumes of proteins of known molecular weight gave an approximate molecular weight for trefoil chemotactin of 60,000 (*10*). Polyacrylamide gel electrophoresis and sodium dodecyl sulfate gel electrophoresis of this peak gave a single band on staining with Coomassie blue. High-voltage paper electrophoresis in phosphate buffer (*p*H 4.6) gave a single spot at the origin with ninhydrin and antimony trichloride sprays; hence the sample was not contaminated by a low-molecular-weight attraction. Throughout the purification procedure, the specific activity of the chemotactin preparation (on the basis of total carbon) was increased (Table 1), resulting in an apparent 600-fold purification.

Purified trefoil chemotactin was retained on a concanavalin A affinity column and eluted with 0.1*M* KCl or 0.1*M* α-methyl-D-mannoside (*11*). This indicated that chemotactin had the properties of a glycoprotein since concanavalin A binds compounds containing α-D-mannopyranosidic, α-D-glucopyranosidic, or α-*N*-acetyl-D-glucosaminidic linkages (*12*).

Purified trefoil chemotactin attracted all the six strains of rhizobia tested (Table 2). This was in contrast to the crude trefoil root exudate, which showed a differential response [table 2 in (*7*)]. In all cases the chemotactic ratio of the purified glycoprotein was greater than that of the crude root exudate. Initial results indicate that addition of crude trefoil root exudate to purified chemotactin reduces the latter's attraction of *Rhizobium meliloti* 102F51. Lectins from peanut and jack bean actively repel trefoil *Rhizobium* at $10^{-6}M$ (*13*). Negative chemotaxis has also been demonstrated for *Escherichia coli* (*14*).

The anthrone test for sugars (*15*) and the Lowry test for proteins (*16*) showed that purified trefoil chemotactin contains about twice as much protein as carbohydrate. Amino acid analysis of chemotactin after β-elimination in 0.1*N* NaOH and sulfite treatment showed that the amounts of serine and threonine were reduced and new sulfur-containing amino acids appeared (*17*). Hence, the carbohydrate and peptide portions of chemotactin are most likely linked through *o*-glycosidic bonds to serine and threonine. Carbohydrate analyses by gas-liquid chromatography of alditol acetates showed glucose, galactose, and mannose in the proportions 17 : 1 : 2.

Table 2. Chemotaxis of six strains of *Rhizobium* to trefoil root exudate (CC = 2500 μg/ml) or purified chemotactin (CC = 26 μg/ml).

Strain	Chemotactic ratio	
	Trefoil root exudate	Purified chemotactin
Trefoil	1.81	12.20
Sainfoin	1.60	13.06
Cowpea	3.87	5.64
Rhizobium japonicum	1.90	3.40
Rhizobium meliloti 102F51	NS*	23.62
Rhizobium meliloti 102F60	3.21	12.72

*No significant difference at the 95 percent confidence level between medium (control) and concentrated trefoil root exudate by Student's *t*-test.

In summary, we have shown that rhizobia are attracted by a glycoprotein produced and exuded by birdsfoot trefoil. This is of interest since other bacteria are not known to be attracted by macromolecules. *Escherichia coli* and *Salmonella typhimurium* are attracted by a variety of simple sugars and amino acids (*18*), while the trefoil *Rhizobium* strain 95C13 appears to be attracted only by the macromolecule trefoil chemotactin. A macromolecular attractant of rhizobia to roots might be advantageous to the plant through its low rate of diffusion. Since the diffusion rate is inversely proportional to the cube of molecular weight (assuming spherical molecules), glucose would diffuse approximately 3×10^7 times as quickly as trefoil chemotactin. The much lower rate of diffusion of the macromolecule results in a longer-lasting and steeper gradient of the attractant. The stability of the gradient might mean that less attractant has to be released by the plant.

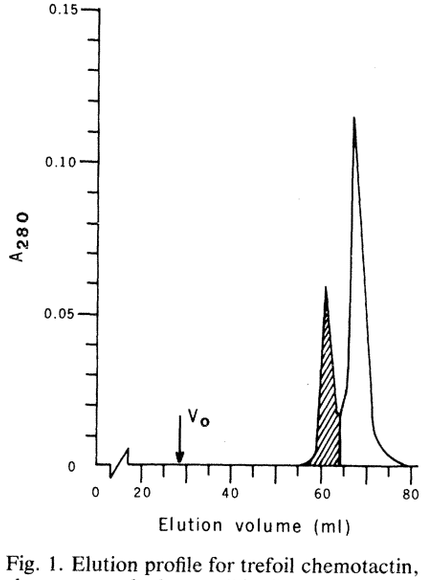

Fig. 1. Elution profile for trefoil chemotactin, chromatographed on a Bio-Gel A-1.5m column (0.9 by 94 cm) eluted with 10 m*M* phosphate buffer (*p*H 7.0) containing $10^{-4}M$ EDTA. One-milliliter fractions were collected. The peaks were pooled, dialyzed exhaustively against distilled water, and assayed for attraction of the trefoil strain of *Rhizobium*. The initial peak (fractions 59 to 64) showed chemoattraction (shading).

Chemotaxis of rhizobia toward the root surface may be the first step in the complex interaction that leads to nodulation and nitrogen fixation. Various strains of rhizobia show different specificities in attraction to plant root exudates (*7*). Therefore, chemotactin may be one of several similar glycoproteins produced by different legumes, with slightly altered and overlapping specificities. These different glycoproteins would attract the homologous strain of *Rhizobium* to its legume host. However, the specificity of chemotaxis is low [table 2 in (*7*)]; for example, a single strain of *Rhizobium* may be attracted to the roots of many plants that it does not nodulate. Hence chemotaxis of rhizobia to legume roots could be one in a series of fairly nonspecific steps, which, in total, result in the high degree of specificity seen in nodulation.

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9. The cation exchange column was Sephadex C-25 (1 cm by 5 cm, K^+ form); the anion exchange column was Sephadex A-25 (1 cm by 5 cm, phosphate form). The columns were prepared and washed with 100 ml of distilled water. The

Bio-Gel P-2 column void volume fraction (~15 ml) was passed through the C-25 column, and the column was then eluted with 20 ml of water. These fractions were combined and passed through the A-25 column, and it was eluted with 20 ml of water. Large amounts of yellow and brown contaminants were retained on the columns. These contaminants had no chemotactic activity.

10. The molecular weight standards used were: bovine immunoglobulin IgM (845,000), bovine IgG (169,000) (Sigma), human IgG (160,000), horse apoferritin (466,900), and bovine serum albumin (68,000) (Schwarz/Mann).
11. A column (1 cm by 5 cm) of concanavalin A–Sepharose (Sigma) was washed with 0.1*M* KCl or 0.1*M* α-methyl-D-mannoside and then rinsed with distilled water. Purified chemotactin was passed through the column, followed by a distilled water rinse. The chemotactin was removed by 0.1*M* KCl or 0.1*M* α-methyl-D-mannoside and dialyzed against water for 2 days.
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Incompatibility on *Brassica* Stigmas Is Overcome by Treating Pollen with Cycloheximide

Abstract. *Pollen of* Brassica *exhibited strong self-incompatibility. It did not germinate on, adhere to, or extend into the stigmatic tissues of the female parent plant. In contrast, pollen that had been treated with cycloheximide germinated on and penetrated into self-stigmas to the same degree as untreated pollen placed on cross-stigmas (compatible). Germ tubes from cycloheximide-treated pollen did not continue growth through stylar tissues and therefore were unable to effect fertilization.*

The translational inhibitor cycloheximide (*1*) only partially reduces or has no effect in vitro on the germination of pollen from *Lilium* (*2*), *Trigonella* (*3*), *Impatiens* (*4*), *Antirrhinum*, *Amaryllis*, and *Tradescantia* (*5*). In contrast, cycloheximide has blocked germination of *Tradescantia* (*5*) and *Lilium* and *Clivia* (*6*) pollens in vitro. This same contrast was obtained with *Brassica* by adding cycloheximide at different times to media containing germinating pollen (*7*). A high concentration ($2 \times 10^{-4}M$) of cycloheximide in the medium when the pollen was added did not inhibit germination while, paradoxically, strong inhibition resulted when the same high concentration was added to the medium 1 to 2 minutes after the pollen.

The germination in vitro in the presence of a high cycloheximide concentration suggested that new protein synthesis by pollen was not required for germination. The inhibition of germination caused by the delayed addition of the same high concentration of cycloheximide suggested that a protein or proteins synthesized by pollen between the time of placement of pollen in synthetic media and the addition of cycloheximide 1 to 2 minutes later might cause the inhibition of pollen germination.

These apparently protein-regulated variations of pollen germination in vitro might be involved with the regulation of incompatibility in vivo, since extracts of self-incompatible stigmas (that is, stigmas of the same plant that produced the pollen) had the same concentration- and time-dependent effects on pollen germination as cycloheximide (*7*). Extracts of cross-stigmas (compatible) had little or no effect (*7*). The extracts consisted of medium in which a given number (concentration) of stigmas had been incubated for about 10 minutes and then removed. Also, extracts from self- but not cross-stigmas rapidly (within 10 minutes) blocked the incorporation of ^{14}C-labeled leucine into pollen proteins (*7*). These results suggested that the endogenously synthesized pollen protein or proteins postulated to "turn off" pollen germination in vitro are involved with the regulation of self-incompatibility. Developmentally, the expression of incompatibility in *Brassica* in vivo is failure of the pollen to germinate on and adhere to the papillae of self-stigmas (*8*).

Additional evidence, obtained in situ, is now reported which supports the evidence obtained in vitro that germination of *Brassica* pollen does not require protein synthesis, whereas expression of self-incompatibility does. The prior treatment of *Brassica* pollen with cycloheximide overcame developmental expression of incompatibility: pollen so treated germinated and adhered to papillae of self-stigmas.

Pollen was treated in a "dry" state with cycloheximide as follows. Cycloheximide was dissolved in absolute methanol, and 0.1-ml portions were added to glass (Pyrex or Kimax) tubes (10 mm by 70 mm) to give the quantities indicated in Tables 1 and 2 and Figs. 1 to 3. The methanol was evaporated off by passing a gentle stream of air over the liquid surface while the tube was rotated. Heat applied to the base of the tube in a water bath at 60°C sped evaporation, which took approximately 30 seconds and coated 0.5 to 1.0 cm of the lower part of the tube with cycloheximide. Six fully dehisced anthers from one flower of greenhouse-grown *Brassica oleracea* var. *capitata* were added to each tube, and the pollen was released by tapping the tubes firmly against a wood bench. Emptied anthers were discarded by carefully inverting each tube. Most of the released pollen adhered to the cycloheximide-coated tube wall. From 30 to 60 minutes later, the pollen was

Table 1. Mean scores for relative number of pollen tubes detected in stigmatic and stylar tissues after treatment of either the pollen or the stigmas with cycloheximide (10 μg). The scores are averages of five or six pollinations. A score of 0.0 indicates no pollen tubes and 5.0 indicates 100+ tubes, as defined in more detail in Fig. 1. The untreated pollen represents a solvent control (methanol minus cycloheximide).

Experimental	Penetration of pollen tubes into stigmas and styles (relative score)			
	Self-pollinated		Cross-pollinated	
	Stigma	Style	Stigma	Style
Untreated pollen (control)	0	0	4.3	3.8
Cycloheximide-treated stigma	0.7	0.0	4.0	0.5
Cycloheximide-treated pollen	4.2	0.4	3.8	0.0