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Chemotaxis in the Marine Fungus *Rhizophydium littoreum*

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Zoospores of the marine chytrid *Rhizophydium littoreum* are attracted to a variety of substances common to their environment. In general, carbohydrates and polysaccharides elicited strong concentration-dependent positive responses. There was no direct correlation between all substances used as foods and those stimulating positive responses. The chemotactic activities of this organism should, however, tend to bring it toward concentrated food sources.

The sessile sporangium of the marine chytrid fungus *Rhizophydium littoreum* (2, 12, 13) releases zoospores when it matures. These zoospores may remain motile for 24 h or more, presumably until they come in contact with a substrate providing a suitable nutrient source. Light-directed movements for this organism have been described (13, 19), and it is assumed that positive phototaxis directs the zoospores vertically toward the photic zone, where food sources are abundant (10, 21, 24).

Once in the photic zone, zoospores may enter the microenvironment of potential food sources such as plants, animals, waste products, and decaying matter. Since distances to be travelled here in the microenvironment are small, it is likely that concentration gradients of chemicals could be maintained around the nutrient sources and that a positive chemotactic response could bring the zoospores closer to advantageous substrates. The littoral marine ecosystem might be compared with the soil-rhizosphere system, where phytopathogenic zoosporic fungi use such chemotactic responses to move up the gradient of exudate coming from host plants (8, 11).

While the marine coastal waters contain rather high concentrations of dissolved organic matter (17, 24, 25), the plants and animals that are sources of nutrients (exudates and decay) are potentially higher in nutrient content. It is advantageous for a marine fungus to be able to locate these high-nutrient microenvironments, where its growth potential is maximal. To determine whether *R. littoreum* has the ability to locate potential food sources chemotactically, the following study was made.

MATERIALS AND METHODS

R. littoreum (ATCC 36100) for chemotaxis experiments was maintained at 24°C in petri dishes (100 by 15 mm) containing approximately 25 ml of YPD (3) agar, 1 g of yeast extract, 1 g of peptone, 10 g of glucose, and 12 g of agar per liter of Instant Ocean Seawater (Aquarium Systems, Mentor, Ohio). At 48 ± 2 h after plates were inoculated with zoospores, the culture was flooded with sterile, pH 7.35 chemotaxis buffer (13.78 g of NaCl, 0.013 g of K_2HPO_4, 0.027 g of KH_2PO_4, 2.60 g of $MgSO_4 \cdot 7H_2O$, and 0.525 g of $CaCl_2 \cdot 2H_2O$ per liter of H_2O). Zoospores were washed three times by centrifugation in chemotaxis buffer to remove interfering chemicals. The zoospore suspension was adjusted to contain 1.2×10^7 to 4.0×10^7 zoospores per ml, and motility was checked microscopically. Suspensions showing less than 75% motile zoospores were not used.

The Adler chemotaxis assay, in which microbes migrate into a capillary micropipette (1), was modified slightly (18) to accommodate the larger size (cell diameter plus flagellum length of up to 31 μm) of the motile cells in this eucaryotic organism. A 2-μl micropipette (internal diameter, approximately 400 μm) was found to be suitable for the assay in preliminary experiments. The chemotaxis apparatus with active zoospore suspension was incubated for 45 min prior to sampling. All experiments were run in triplicate against triplicate controls containing only chemotaxis buffer. Experiments were carried out in a normally lighted lab (10 microeinsteins m^{-2} s^{-1}). Test chemicals were dissolved in chemotaxis buffer. All chemicals were of the highest purity available and were from Sigma Chemical Co. All amino acids were in the L configuration, and all sugars were D configuration. Algal tissue (*Bryopsis plumosa* and *Codium fragile*) was ground in chemotaxis buffer with a tissue homogenizer to obtain an extract containing approximately 0.1 g (dry weight) of plant per ml of liquid. The extract was diluted with chemotaxis buffer and sterilized with a washed membrane filter (0.2 μm pore size). The amino acid mixture used in chemotaxis experiments contained (in moles percent): serine, 21; glycine, 17; threonine, 16; valine, 15; alanine, phenylalanine, and glutamic acid, 5; aspartic acid, 4; arginine, histidine, and leucine, 3; lysine, proline, and tyrosine, 1; and isoleucine, 0.5 (9).

To categorize the relative strength of the chemotatic response, the mean response at each test concentration was compared with that of each control. The data were analyzed by Student's *t* test (Statistical Analysis System, SAS Institute). The responses were grouped according to strength of response, the strongest response (++++), $P > 0.0001$ to 0.0010; $P > 0.0011$ to 0.0100, good (+++); $P > 0.0101$ to 0.1000, moderate (++); $P > 0.1001$ to 0.1500, weak (+); and $P > 0.1500$, no response (0). Experiments were repeated three or more times in overlapping concentration ranges.

For growth measurements, a basal medium solidified with 12 g of Noble agar per liter and containing defined artificial seawater (3) was supplemented with 200 μg of thiamine, 2 μg of biotin, and 2 μg of vitamin B_{12} per liter. The positive controls contained 0.75 g of NH_4Cl per liter for nitrogen and 5.0 g of glucose per liter for carbon. In the experiments with nitrogen-containing compounds, the NH_4Cl was replaced by 2 g of the nitrogen-containing compound per liter. In the experiments providing carbon but no nitrogen, the glucose was replaced with 5 g of the test compound per liter. Both

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TABLE 1. Response of zoospores to chemoattractants, ranked by relative strength

Chemotaxis response score[a]	Substance	Concn (g/liter)	
		Best response	Threshold level
++++	Amylose	1.0×10^{1}	1.0×10^{-4}
	Bryopsis extract	1.0×10^{0}	1.0×10^{-4}
	Codium extract	1.0×10^{2}	$<1.0 \times 10^{-4}$
	Glycogen	1.0×10^{1}	1.0×10^{-4}
	Maltose	3.4×10^{1}	$<3.4 \times 10^{-5}$
	Starch	1.0×10^{1}	1.0×10^{-1}
+++	Bovine serum albumin (type V)	1.0×10^{-4}	—[b]
	Carboxymethyl cellulose	1.0×10^{-1}	1.0×10^{-3}
	Casein hydrolysate (acid)	1.0×10^{0}	1.0×10^{-4}
	Fructose	1.8×10^{0}	1.8×10^{-5}
	Glucose	1.8×10^{2}	1.8×10^{-3}
	Lactose	3.4×10^{1}	3.4×10^{-3}
	Mannuronic acid	1.9×10^{0}	1.9×10^{-4}
	Peptone	1.0×10^{0}	1.0×10^{-2}
	Yeast extract	1.0×10^{0}	1.0×10^{-5}
++	Amino acid mixture	1.0×10^{-2} (total)	1.0×10^{-2} (total)
	Cellobiose	3.4×10^{1}	3.4×10^{1}
	Fucose	1.6×10^{-1}	1.6×10^{-3}
	N-Acetyl-D-glucosamine	2.2×10^{1}	2.2×10^{1}
	Glycylglycine	1.0×10^{-2}	1.0×10^{-3}
	Glycylglyclglycine	1.0×10^{-2}	1.0×10^{-3}
	Mannose	1.8×10^{-1}	1.8×10^{-1}
	Mannitol	1.8×10^{-1}	1.8×10^{-3}
	myo-Inositol	1.8×10^{-2}	1.8×10^{-2}
	Sucrose	3.4×10^{0}	3.4×10^{-1}
	Trehalose	3.4×10^{1}	3.4×10^{-1}
+	Alanine	8.9×10^{-2}	8.9×10^{-2}
	Glycine	7.5×10^{-1}	7.5×10^{-6}
	Leucine	1.3×10^{-3}	1.3×10^{-3}
	Leucine/lysine/proline	1.0×10^{-2} each	1.0×10^{-2} each
	Nicotinic acid	1.2×10^{0}	1.2×10^{0}
	Pentaglycine	$1\ 0 \times 10^{1}$	1.0×10^{-3}
	Potassium acetate	1.0×10^{0}	1.0×10^{0}
	Serine	1.0×10^{-1}	1.0×10^{-1}
	Tetraglycine	1.0×10^{0}	1.0×10^{-3}

[a] Responses were scored as described in the text. The following substances gave a response score of 0: amylopectin, aspartic acid, carrageenan, chitin, glucosamine, glutamine, glycerol, histidine, leucine/lysine, leucine/proline, and phenylalanine.
[b] —, Not determined.

nitrogen and carbon sources were omitted from negative controls.

Growth was categorized as strong (++++) when the diameter of microscopically observed developing sporangia on plates was equal to or larger than that of the positive controls at 24 and 48 h. Moderate (++) growth was scored when growth was slower than in the control and the sporangia were smaller (down to 1/2 the diameter of controls) after 24 and 48 h of observation. Many in this category eventually achieved nearly the same size as the controls. Weak stimulation of growth (+) was scored when sporangia were less than 1/2 the diameter of the controls at 24 and 48 h. Typically, weak growth produced only a few zoospores per sporangium, whereas strong growth produced hundreds. In all cases growth stimulation included completion of the life cycle. No growth stimulation was scored whenever the life cycle was not completed in 1 week. In some cases zoospores in the no-growth category would encyst, germinate, and grow to a size equal to that of the weak-growth category. Second-generation zoospores were retrieved from all growth plates and replated on the same medium to ensure that initial observations were not simply lag-phase responses. No significant lag-phase results were observed.

RESULTS

Many of the compounds tested elicited positive chemotaxis, but only a few gave strong responses (Table 1). Glucose-based carbohydrates, such as potato starch, glycogen, amylose, maltose, and glucose, all produced strong responses at 0.01 g/liter. In addition, complex nitrogen-rich sources, such as bovine serum albumin, peptone, casein hydrolysate, yeast extract, and extracts from the algae *Codium fragile* and *Bryopsis plumosa*, stimulated good to strong responses. Strongly positive responses were concentration dependent, as demonstrated in Fig. 1. All of the above compounds readily supported the growth of *R. littoreum* (Table 2). Yeast extract, peptone, amylopectin, and algal extracts occasionally produced poor tactic responses because the zoospores rapidly encysted in the presence of the compound and thus could not enter the capillary to be counted.

Many individual amino acids supported the growth of this chytrid, but as a group they did not stimulate chemotaxis as well as did the carbohydrates or complex extracts from tissues (Table 2). Unlike carbohydrates (Fig. 1), amino acids did not produce a steeply sloped response to chemical

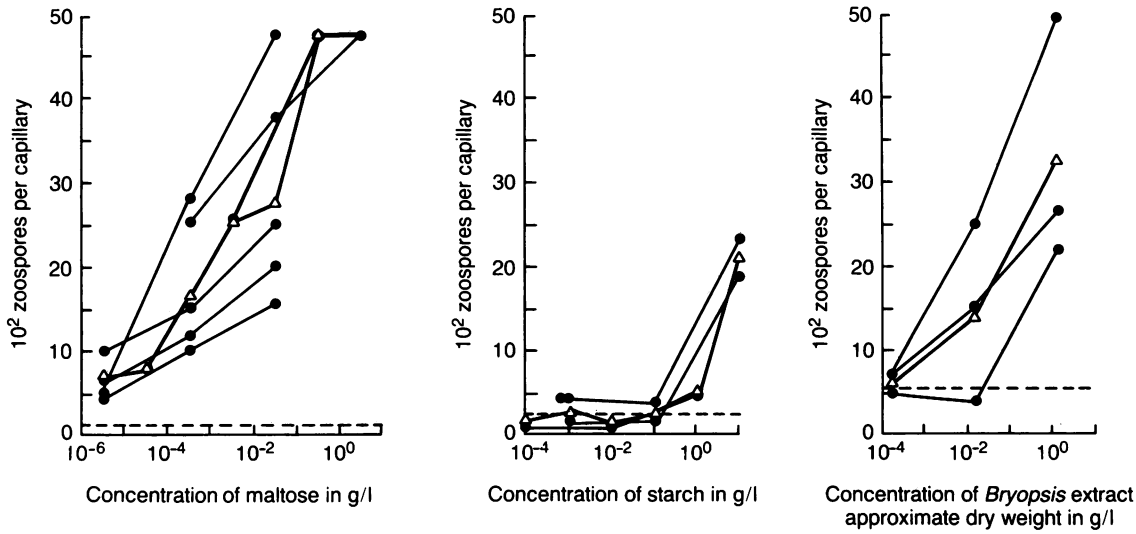

FIG. 1. Chemotactic migration of zoospores into capillary tubes with various concentrations of attractants. Symbols: – – –, control with chemotaxis buffer only; ●, individual experiments; △, average of all experiments.

concentration (data not shown). Threshold stimulation levels (20) for many carbohydrates were considerably lower than the concentrations producing the maximum response (Table 1). Amino acids had threshold levels of stimulation which were close or equal to those evoking the maximal response.

TABLE 2. Growth and chemotaxis

Growth response score[a]	Substance	Chemotaxis response score[a]
0	Histidine	0
	Leucine	+
	Glycine	+
	Serine	+
	Mannuronic acid	+++
	Potassium acetate	+
+	Lysine	+
	Phenylalanine	0
	Carboxymethyl cellulose	+++
	Lactose	+++
	Fucose	++
	Fructose	+++
	Trehalose	++
	Carrageenan	?
	myo-Inositol	++
	Mannitol	++
	Glycerol	0
	Tetraglycine	+
	N-Acetylglucosamine	++
++	Alanine	+
	Amylopectin	0
	Cellobiose	++
	Mannose	++
	Amylose	++++
	Maltose	++++
	Sucrose	++
+++	Proline	0
	Starch	++++
	Bovine serum albumin	+++
	Glucose	+++

[a] Responses were scored as described in the text.

The best stimulus by amino acids occurred with casein hydrolysate or when a mixture representing the amino acid composition of algal protein (9) was used. Carbohydrates such as lactose which only weakly supported growth gave better responses than individual amino acids or specific amino acid mixtures (Table 1).

Some compounds which are found in plant material (15) seemed to be strongly to moderately attractive to the zoospores. Mannuronic acid, fucose, cellobiose, mannose, mannitol, sucrose, trehalose, and *myo*-inositol all attracted zoospores. Carrageenan and glycerol, both associated with algae (15), did not promote chemotaxis. Although an extract of *B. plumosa* and *C. fragile* produce a strong response, the medium in which the algae were grown did not attract zoospores, indicating that substances from damaged plants may be more important than diffusing metabolites or exudates of healthy or undamaged plants.

Polysaccharides, glycogen, potato starch, and amylose stimulated stronger responses than the monosaccharides glucose, fructose, and fucose, and albumin was more stimulatory than individual amino acids. With the exception of cellobiose, the reducing sugars tended to be better attractants than the nonreducing sugars. Amylopectin did not appear to stimulate a positive response, but further experimentation showed that zoospores stopped swimming in the presence of amylopectin in the range of concentrations used. Glycylglycine and glycylglyclglycine were stronger attractants than glycine, but tetraglycine and pentaglycine did not continue the trend for more attractiveness in polymers of the amino acid.

Chemicals which supported growth were not necessarily positive chemoattractants, and chemoattractants did not necessarily act as sources of nutritional carbon or nitrogen (Table 2). Of the five compounds that did not stimulate growth, three were weakly attractive and one (mannuronic acid) was a good attractant. Conversely, proline, which stimulated strong growth as a sole nitrogen source, stimulated no chemotactic response.

Saccharides, including sugars, sugar acids, sugar amines, sugar alcohols, and the oligo- and polysaccharides, seemed

to be the strongest chemoattractors (Table 1). In general, amino acids served only as weak attractants.

Strong negative chemotaxis was not observed with any of the compounds tested, and the Adler assay would not necessarily recognize weakly negative chemotaxis. Compounds that would presumably be undesirable were not tested.

DISCUSSION

The results suggest that this organism can utilize chemotaxis for the purpose of moving toward potential food sources. Movement toward some compounds not used as nutrients and lack of movement toward some nutrients may indicate the greater importance of one type of sensory receptor over another. Thus, saccharides seem to be more important as a chemotatic cue than amino acids. Since *R. littoreum* has the ability to utilize ammonium in place of amino (3) nitrogen sources, carbon does not necessarily accompany the nitrogen source. Thus, finding carbon sources may be more important than finding amino acids. Since many carbohydrates support growth, it would be advantageous to respond to them.

Chemotaxis should confer a benefit or advantage to those microbes that possess it (6). For *R. littoreum*, we examined those compounds which might be associated with a beneficial (nutritious) environment (4). Most but not all of the compounds tested elicited positive chemotaxis. The benefit associated with these chemicals could indicate an inclination of *R. littoreum* toward either a parasitic or saprophytic nutritional mode. Kazama (12) showed that zoospores were attracted to killed *B. plumosa*. Observations in this lab (J.P.A.) have shown that the zoospores are not attracted to living *B. plumosa* in vitro unless it has been damaged. Our results further support that conclusion because *B. plumosa* exudates (in growth medium) elicit no response, but extracts of the cells do. Many of the compounds tested may be released when living materials are damaged by grazing or mechanical forces in the intertidal zone.

The observation that exposure to some compounds leads to rapid encystment, resulting in cessation of movement, is not unusual. The same response has been seen with other zoosporic fungi (7). In our experiment encystment was seen when very high concentrations of certain compounds were tested (Table 1). Such high concentrations would be likely to occur only in that part of the gradient very close to the exuding source. After encystment, the walls of the fungus become coated with a sticky substance (unpublished observation) which makes them adhere to virtually any surface. Neither zoospores nor developing thalli exhibit this characteristic in culture. This behavior would be likely to result in trapping of the encysted cells adjacent to their chemoattractant, and this could often be a food source.

Chemotaxis can be properly interpreted only if the phototactic response (19) is included. In phototaxis experiments it has been shown that at least some chemoattractants (and raw natural seawater) do not obscure the photoresponse in *R. littoreum* (19). The photoresponse thus could mask the chemoresponse during hours of daylight. Since all experiments on chemotaxis were done in average laboratory lighting, we conclude that that does not necessarily happen. If phototactic responses were dominant, perhaps at high light intensity, they would tend to bring zoospores from dark bottom waters toward the lighted photic zone with its abundant nutrients. Chemotaxis surely would be a strong influence to maintain zoospores near food sources, including those in rich sediments, during darkness. Once zoospores are in this zone, the importance of motility would be to direct the spore to the richest food source, where encystment and entrapment might occur. We suggest that both types of taxis are simultaneously functional and that the strength of the stimulus must control which response dominates.

Chemotaxis has been studied in only a few zoosporic fungi, and most of what has been described in the literature refers to the biflagellate plant pathogens (6, 11, 14) or the uniflagellate Blastocladiales (5, 7, 16, 22, 23). An extensive study of chemotaxis has been done on the ruminal phycomycete *Neocallimastix frontalis* (20). In those experiments it was shown that some carbohydrates, mostly mono- or disaccharides, produced good chemotaxis and that starches produced none. Amino acids also elicited no chemoresponse. We must conclude that while chemotaxis may be a general phenomenon in chytrids, the sensory system varies with each organism and is likely to be closely related to the selective pressures of the environment.

In *R. littoreum* both amino acids and carbohydrates stimulate chemotaxis, but amino acids are weak stimulators and not highly concentration dependent. This might suggest that amino acids work through a separate mechanism or that they are stimulating the carbohydrate receptor with only marginal reactivity. In *N. frontalis*, at least four carbohydrate receptors were found for different groups of carbohydrates (20).

Maltose was the most effective attractor tested. The threshold for stimulation was lower than that of any other compound tested. Since maltose is a primary breakdown product of many storage carbohydrates acted on by amylases, it may be common in the vicinity of those organisms. Maltose is apparently taken into the cell and metabolized quickly. In phototaxis experiments (19), maltose stimulated a faster photoresponse than controls, suggesting that the chemotactic response to maltose may be enhanced because the rate of swimming was increased.

If amylopectin, peptone, yeast extract, and albumin had not caused encystment, which interferes with scoring the test, they might have been rated among the strongest attractants. Since complex mixtures of amino acids and albumin gave stronger responses than one amino acid, it could be concluded that multiple-site stimulation brought about synergistic responses. It is also possible, as with the comparison of glucose with amylose, starch, glycogen, and maltose, that these molecules could simultaneously contact several receptors, causing the enhanced response. The experiments comparing glycine with several glycl peptides did not provide a clear answer to that question.

Overall, the responses of this marine fungus to compounds that one would expect to find in the coastal environment show that the organism is well adapted to moving in the direction of nutrient sources. Further work is needed to discover whether the organism has similar adaptations to avoid unfavorable environments.

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