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Pseudomonas aeruginosa Chemotaxis Associated with Blooms of N_2-Fixing Blue-Green Algae (Cyanobacteria)

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Pseudomonas aeruginosa (Schroeter) Migula, a numerically significant bacterium found during N_2-fixing blooms of the blue-green algae (cyanobacteria) *Anabaena* sp. in the Chowan River, North Carolina, was chemotactically attracted to amino acids when tested in a radioassay. The bacterium was labeled with $^{32}P_i$, and the disintegrations per minute determined by liquid scintillation counting were proportional to the number of cells accumulating in microcapillaries containing amino acids. Positive chemotaxis was observed toward all of the amino acids tested, although the degrees of response varied. Since many nitrogen-fixing blue-green algae secrete nitrogenous compounds, this attraction may be instrumental in establishing a symbiotic relationship between this bacterium and blue-green algae in freshwater.

Chemotaxis, the movement of a motile organism with reference to a chemical agent, is a widespread phenomenon in nature and may be important in initiating symbiotic relationships between blue-green algae (cyanobacteria) and heterotrophic bacteria in freshwater. The chemical attraction of the bacteria to the algae may result in the establishment of a specific microflora within the microenvironment bordering the algal cell (5). Bacterial growth is stimulated by the extracellular products of the algae, and, in turn, the bacteria modify the net excretion rate of algal products (12, 20). Algal-bacterial symbioses are mediated by the exchange of metabolic products (22). Bacteria provide the algae with CO_2, vitamins, growth factors, and buffering capacity (8). Algae supply bacteria with oxygen and extracellular products that serve as organic substrates for growth (16).

The chemotactic ability of *Pseudomonas aeruginosa* (Schroeter) Migula, a bacterium found to be associated with blue-green algal blooms in the Chowan River, North Carolina (K. K. Gallucci, M.S. thesis, University of North Carolina, Chapel Hill, 1981), to selected amino acids was tested by means of a radioassay, using ^{32}P-labeled cells. The technique is a modification of the Wellman and Paerl method (27) which estimates the numbers of ^{14}C-labeled bacterial cells which move into capillaries in response to attractant by means of liquid scintillation counting. The Wellman and Paerl method is less time-consuming and somewhat more sensitive than the dilution plating method of Adler (2) and is more amenable to field studies since it does not rely on sterile technique. However, partial loss of label does occur during the washing procedure and through bacterial respiration of the substrate (27). The method described here circumvents these problems by adding the label after the washing procedure and by using $^{32}P_i$, a label rapidly taken up but not readily released by the bacteria.

MATERIALS AND METHODS

Assay organism. *P. aeruginosa* (Schroeter) Migula, isolated from the Chowan River, North Carolina, was identified by the principal investigator, and this identification was verified by the Diagnostic Microbiology Laboratory of the University of North Carolina Memorial Hospital, Chapel Hill. It is a highly motile, metabolically diverse species commonly found in soil and water.

P. aeruginosa was chosen as a significant bacterial species found associated with the nitrogen-fixing blue-green algae of the Chowan River in July and August 1980. Up to 50% of the total isolates obtained from the bloom area during the sampling period were identified as *P. aeruginosa* (Gallucci, M.S. thesis).

Culture media. Pure cultures were routinely maintained on peptone agar, 1.5% (wt/vol) peptone in deionized water. Cells were transferred to 0.5% (wt/vol) peptone broth 12 to 15 h before the assay was begun. The wash medium contained 10^{-2} M sodium bicarbonate as a buffer and 10^{-4} M EDTA, which promotes bacterial motility by chelating heavy metals which inhibit motility (2). The final wash medium contained, in addition, 0.05% (wt/vol) Tween 80 (Difco Laboratories) to prevent bacterial attachment to the capillary tubes. The wash media were made up with filtered (GF/C), autoclaved Chowan River water, and final solutions were filter sterilized (0.45-μm porosity; Nuclepore Corp.). The pH of the solutions was adjusted to 7.5 with 0.1 N HCl. Amino acids (Sigma Chemi-

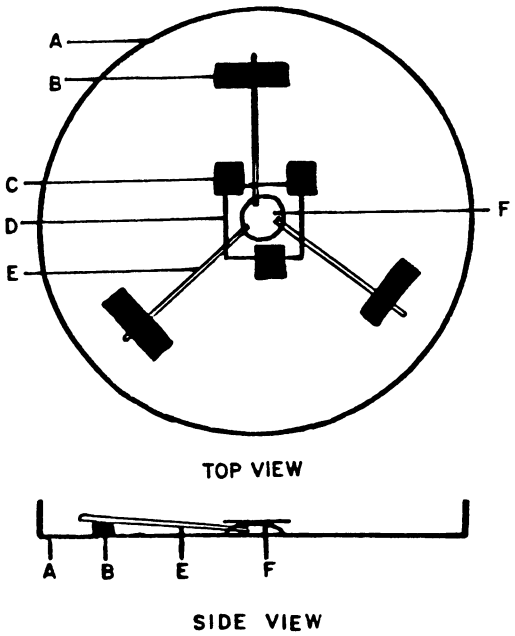

FIG. 1. Diagram of chemotaxis chamber used in experimental procedure. A, Petri dish; B, rubber support; C, rubber support; D, cover slip; E, capillary tube; F, labeled culture.

cal Co.) were all Sigma grade and of the L-configuration.

Preparation of labeled bacteria. For each experiment, *P. aeruginosa* was transferred from 1-day-old slants or broths to 125-ml Erlenmeyer flasks containing 10 ml of 0.5% (wt/vol) peptone broth for 12 to 15 h at 25°C on a rotary shaker until an optical density of 0.2 to 0.5 was reached in a 1-cm cuvette at 590 nm read in a Bausch & Lomb Spectronic 710 spectrophotometer. An optical density of 0.5 equaled 6×10^8 cells per ml. The relation between bacterial concentrations and spectrophotometric extinctions was determined by parallel microscopic cell counts, using the acridine orange direct count technique (7, 13).

The 10-ml culture was centrifuged at 2,000 × *g* for 10 min at 15°C. The supernatant was decanted, and the pellet was gently resuspended in 5 ml of wash medium. This was repeated twice more, followed by resuspension of the pellet in 5 ml of final wash medium. Cells in the pellet were periodically checked for motility, and normally more than 90% of the cells were motile. The final extinction at 590 nm was between 0.02 and 0.07, which corresponded to a bacterial concentration of 3.0×10^7 to 5.0×10^7 cells per ml. The suspension was transferred to a 125-ml Erlenmeyer flask to which was added 15 to 70 μCi of carrier-free ^{32}P as H_3PO_4 (ICN Corp.). The flask was incubated at 25°C on a rotary shaker for 30 to 60 min, after which a 100-μl sample was filtered through a 2.5-cm-diameter Sartorius filter (0.45-μm porosity), washed, placed in 10 ml of deionized water, and counted for Cerenkov radiation (10). The counts were compared with those obtained from an unfiltered sample prepared in the same manner to determine the radioactive uptake of the cells. Typically, the uptake in 60 min was 75 to 98% of the ^{32}P added and remained in that range for at least 2 h. The variation observed in the percent uptake was within the range of experimental error for this procedure. The cell suspension retained motility for up to 5 h at 25°C.

Chemotaxis assays. Modifications of the Adler (2) and Wellman and Paerl (27) methods were used. Samples of the labeled bacterial suspension (0.2 ml) were placed in the center of a series of disposable petri dishes (Fisher Scientific Co.) which served as testing chambers. Three rubber supports (2 mm in height) were placed around the culture sample (Fig. 1), and a glass cover slip was placed on top of the supports over the drop.

Both 1- and 2-μl capillaries (Microcaps, Drummond Scientific Co.) were used depending on availability. Adler reports that both sizes attract identical numbers of bacteria (2). The capillaries were washed in mild detergent (Sparkleen, Fisher Scientific Co.) and rinsed several times in acetone and deionized water. They were sterilized with dry heat at 100°C for at least 12 h and were always handled with forceps thereafter. One end of each capillary was sealed with a flame.

The attractant stock solutions were prepared from Sigma-grade amino acids (Sigma Chemical Co.) dissolved in final wash medium. These were diluted 10-fold in the final wash medium to yield concentrations of 10^{-1} to 10^{-6} M. Test and stock solutions either were

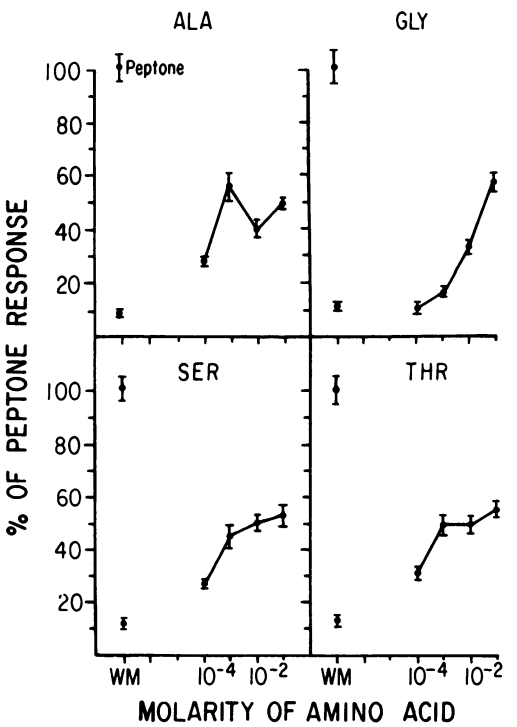

FIG. 2. Composite figure of all data obtained during chemotaxis experiments. Curves for each L-amino acid represent the average of all assays. The peptone response is shown in the upper left of each graph (Fig. 3 to 6). WM, wash medium.

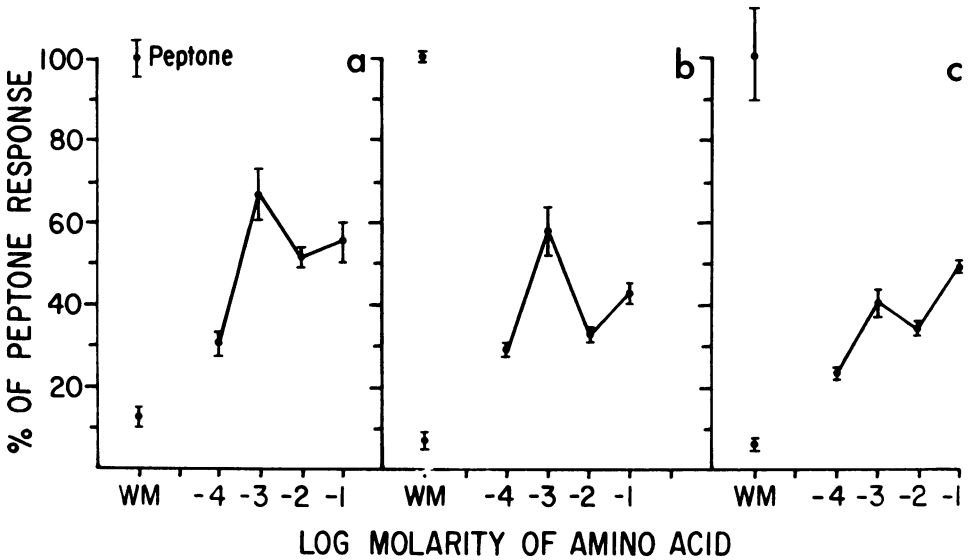

FIG. 3. Response curves for L-alanine assays run on (a) 11, (b) 12, and (c) 14 December 1980. WM, wash medium.

prepared fresh daily or had been frozen only once for less than 24 h. The solutions were drawn into the capillaries by passing one end quickly through a flame several times and then plunging it, open end down, into 1 ml of test solution. As the capillary cooled, the test solution was drawn up about 1 cm. Triplicate capillaries of each concentration of each amino acid test solution were inserted in the 0.2-ml bacterial suspension and were placed on the rubber supports to prevent the bacterial suspension from being drawn up along the capillary exterior. Triplicate capillaries containing 0.5% (wt/vol) peptone were used as controls.

The assays were incubated on a slide warmer maintained at 30 ± 1°C for 45 min. The capillaries were removed, rinsed, and placed in 10 ml of deionized water in a scintillation vial and counted for Cerenkov radiation. Each sample was counted for 10 min in a Beckman LS 7000 microprocessor-controlled scintillation spectrometer. The counting efficiency was 42%, and all results were converted to disintegrations per minute. Every assay was repeated at least twice on different days with different bacterial suspensions.

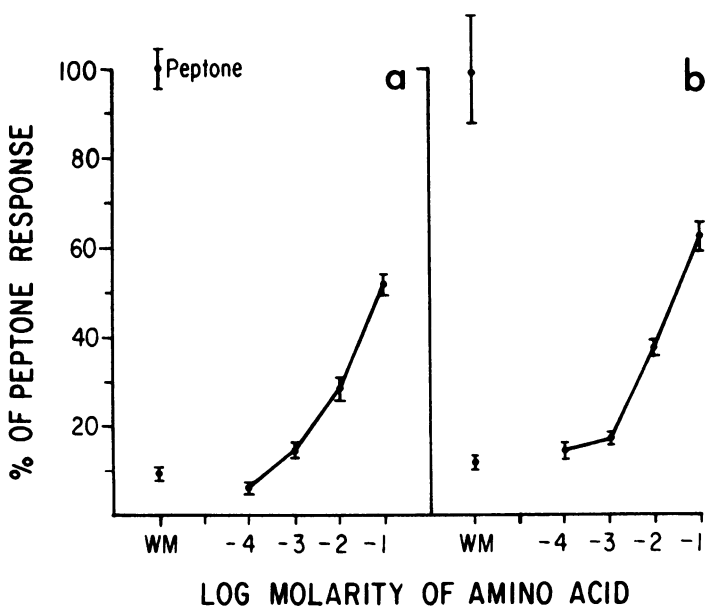

FIG. 4. Response curves for L-glycine assays run on (a) 15 and (b) 16 December 1980. wm, wash medium.

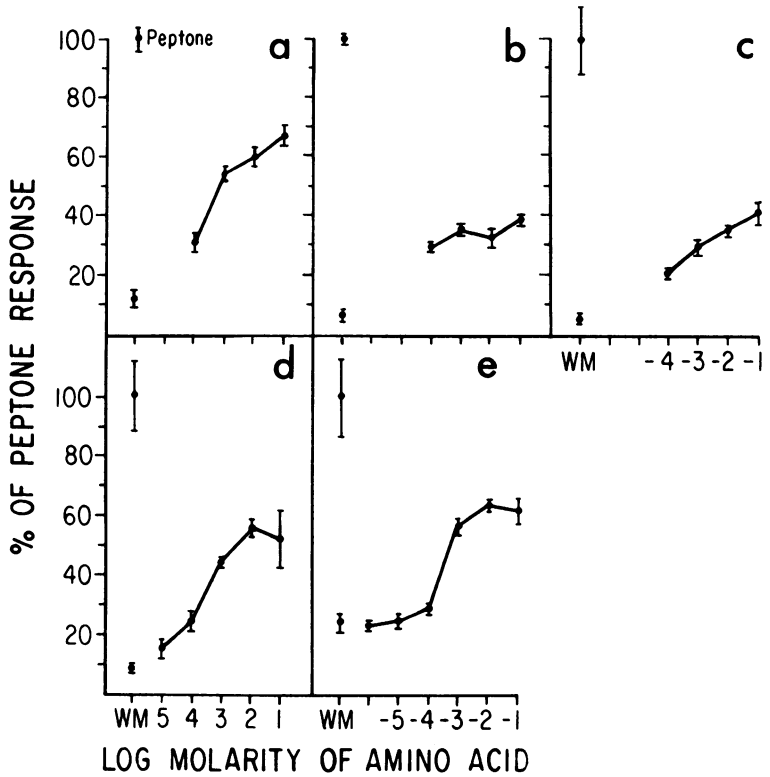

FIG. 5. Response curve for L-serine assays run on (a) 11, (b) 12, and (c) 14 December 1980 and on (d) 30 January and (e) 3 February 1981. WM, wash medium.

RESULTS

The effects of 0.5% (wt/vol) peptone and increasing molar concentrations of alanine, glycine, serine, and threonine on the attraction of *P. aeruginosa* are shown in Fig. 2. Each amino acid curve represents the average of all experiments. Concentrations higher than 10^{-1} M were not tested since the amino acids were only marginally soluble at these concentrations. The concentration-response curves were plotted according to amino acid molarity versus the percent response based on 0.5% peptone, a rich organic attractant used as a control in previous

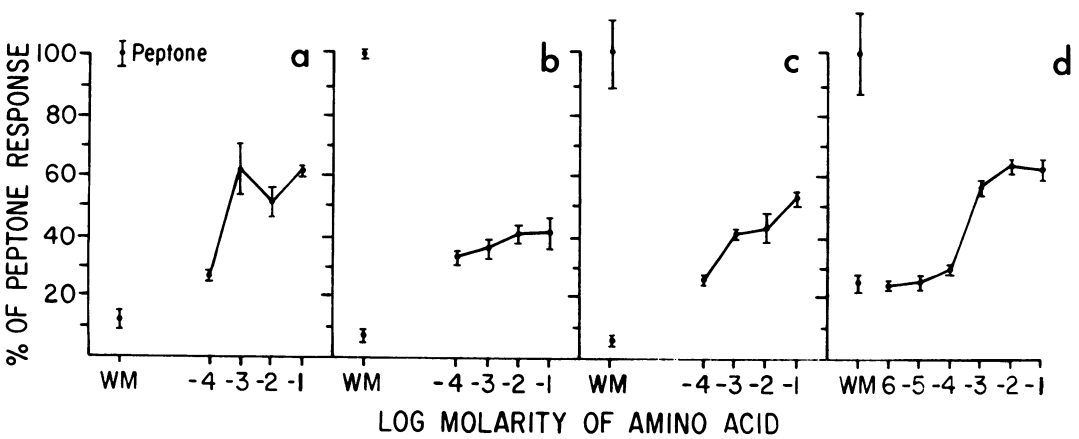

FIG. 6. Response curves for L-threonine assays run on (a) 11, (b) 12, and (c) 14 December 1980 and on (d) 3 February 1981. WM, wash medium.

studies (5). This concentration of peptone was used as the standard on which all responses were based since the quantity of radioactivity and the numbers of bacteria varied among the experiments. It is a more complete medium, was used to culture the bacteria, and consistently attracted the greatest number of bacteria. The response to peptone is shown in the upper left corner of each graph (Fig. 2 to 6).

The responses were measured in terms of disintegrations of ^{32}P per minute accumulating in the capillaries. These values were then compared with those obtained in the peptone capillaries and expressed as a percentage. The bars in all figures are standard error bars based on at least three replicates. Standard error ranged from ±1 to 6% and averaged 3% for all experiments shown here.

P. aeruginosa was chemotactic to all of the amino acids tested, although the degrees of response varied. In each assay, some of the values obtained exceeded those obtained for the final wash medium. The accumulation of bacteria in the wash medium capillaries was interpreted as random movement.

The peak concentration is where the maximum response to attractant occurs; the threshold concentration is the lowest concentration of attractant that results in an accumulation of bacteria greater than that obtained for the final wash medium, i.e., random movement (2). The peak concentrations varied among the amino acids. It was 10^{-1} M for glycine, serine, and threonine and between 10^{-3} and 10^{-2} M for alanine. The threshold concentration for glycine was determined to be between 10^{-4} and 10^{-3} M; for serine it was between 10^{-5} and 10^{-4} M in two assays; and for threonine, it was between 10^{-5} and 10^{-4} M in one assay. It was not determined for alanine.

DISCUSSION

P. aeruginosa is chemotactic to alanine, glycine, serine, and threonine. When the response system becomes saturated, curves tend to flatten out or plateau as in the responses to serine and threonine (Fig. 5 and 6). Curves that dip at higher concentrations may be the result of diffusion that occurs when the optimum concentration is actually outside of the capillary, with the corresponding accumulation of bacteria in that area. This probably occurred in the alanine assays since the peak concentration was between 10^{-3} and 10^{-2} M. Also, bacterial metabolism of the substrate interferes with the chemotactic response (2). Glycine, for example, is not metabolized by *P. aeruginosa* (Gallucci, M.S. thesis). It may have resulted in the most reproducible results (Fig. 4) since its metabolism did not interfere with the chemotactic response.

Chemoreceptors are the sensory devices that detect changes in the concentration of substrate and relay them to the flagella (1). Each may function for more than one amino acid, especially if they are structurally similar (3). Threonine (Fig. 6), which is not metabolized by *P. aeruginosa* (Gallucci, M.S. thesis), may be attractive because of its structural similarity to serine (Fig. 5), which has a similar response tendency and is metabolized by the bacterium.

This assay design provides advantages over the dilution plating technique of Adler (2) similar to those achieved by the Wellman and Paerl method (27). However, less label was lost and even more time was saved since the $^{32}P_i$ was added to the bacteria after the final wash step. The label was incorporated rapidly by the bacteria and was never washed out since all washing steps were done before the addition of the label. Bacterial adherence to the capillary exterior was minimized by the addition of Tween 80. By making use of the Cerenkov radiation of $^{32}P_i$, counts could be made in distilled water, thus eliminating any quenching problems of aqueous media in scintillation cocktails, as well as the costs of the cocktails themselves.

Alanine, glycine, serine, and threonine are all constituents in the extracellular products of blue-green algae (9, 15, 18, 24, 26, 28). *P. aeruginosa*, a numerically significant bacterial species in blue-green algal bloom areas of the Chowan River (Gallucci, M.S. thesis), was chemotactically attracted to these amino acids. This attraction may be instrumental in establishing a symbiotic relationship between the bacteria and blue-green algae in freshwater. The "phycosphere" of the algae controls the composition of the bacterial population by the release of extracellular products (5). The growth and location of this bacterium in the river are influenced by the presence of blue-green algae.

It is somewhat surprising that the most profound chemotactic responses were observed over a range of relatively high amino acid concentrations. These are substantially higher concentrations than those found in natural aquatic ecosystems (5, 11, 17, 19). Measurements of dissolved amino acids as well as other metabolically utilizable organic compounds, however, do not indicate the degrees of small-scale "patchiness" or microenvironments having relatively high concentrations of these compounds. Since it is known that phytoplankton actively or inactively (by lysis, for example) excrete amino acids, sugars, polypeptides, and polysaccharides (11), it is likely that elevated concentrations of these compounds (10^{-1} to 10^{-4} M) could exist in microenvironments bordering phytoplankton cells. These are regions where active

bacterial attraction and accumulation are often observed (6, 23).

The release of extracellular nitrogen which is ultimately derived from nitrogen fixation by blue-green algae is significant in freshwater (25, 26). Moreover, incorporation of labeled blue-green algal excretion products by bacteria attached to heterocysts of *Anabaena oscillarioides* has been observed by autoradiography (21).

P. aeruginosa is likely to be the dominant bacterium of the phycosphere of the heterocystous blue-green algae of the Chowan River. Furthermore, the nitrogen-fixing capability of *Anabaena flos-aquae*, a common inhabitant of the river, is greatly enhanced in the presence of *P. aeruginosa* when compared with axenic controls (Gallucci, M.S. thesis). The apparent enhancement may be due to the reduced O_2 concentrations created near the heterocysts by bacterial respiration of algal extracellular products (22), thereby promoting nitrogen fixation, an anaerobic process. The subsequent production of extracellular nitrogen in turn supports the bacterial population. Moreover, algal-bacterial mutual enhancements are widespread in nature (4, 14, 29).

Blue-green algal blooms present a very complicated ecological problem. One reason for their proliferation may be their intimate relations with bacteria. Algal-bacterial exchange within the phycosphere may serve to maintain the nuisance blooms that form on the surfaces of freshwater bodies, even in the absence of nutrient enhancement.

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