Evidence for Bacterial Chemotaxis to Cyanobacteria from a Radioassay Technique

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Lyngbya birgei and Aphanizomenon flos-aquae elicited a significant chemotactic attraction of Aeromonas hydrophila compared with controls lacking cyanobacteria. There was a positive exponential relationship between biomass (chlorophyll a) of L. birgei and A. flos-aquae and chemotactic attraction of A. hydrophila. The assay equipment was simple and reliable and could be used to study bacterial chemotaxis in other species in situ.

The role of chemotaxis, the movement of bacteria in response to chemical gradients (2, 11), has been invoked to explain many consortia of cyanobacteria and bacteria (4, 9, 10). However, direct evidence for the role of chemotaxis in forming such consortia is lacking. In the current study, motile bacterial cells isolated from an eutrophic lake were used in laboratory experiments to show chemotactic attraction of bacteria to chambers containing intact filaments of N2-fixing and non-N2-fixing cyanobacterial species isolated from the same lake. These studies provide evidence for bacterial chemotaxis to cyanobacterial products, implying that chemotactic responses play a role in the formation of certain microbial consortia in nature.

The motile bacterium Aeromonas hydrophila (identity confirmed by Microbial ID, Inc., Newark, Del.) was used as the test bacterium. This species was the numerically dominant type (on the basis of Gram staining and cell and colony morphology during dilution plating) among those isolated from eutrophic Hebgen Lake, Montana. The non-N2-fixing cyanobacterium Lyngbya birgei and the N2-fixing cyanobacterium Aphanizomenon flos-aquae which were also isolated from Hebgen Lake were used in this study. These cyanobacteria dominated the water column of Hebgen Lake during September and October 1989, when our study was conducted (6).

Aeromonas hydrophila was grown in peptone agar (0.5% Bacto-Peptone [Difco] with 1.5 or 2.0% Bacto-Agar [Difco]) or lake water (pH 7.9 to 8.0) filtered through a polycarbonate membrane filter (pore size, 0.4 μm; Poretics Corporation, Livermore, Calif.) amended with either 0.5 or 0.25% Bacto-Peptone. When the bacteria were grown in liquid medium, 50 μM EDTA (J. T. Baker Chemical Co., Phillipsburg, N.J.) and 3 mM methionine (Calbiochem, Los Angeles, Calif.) were added to ensure motility of the bacteria (1, 4).

The cyanobacteria were used for experiments within 2 days of collection from water in the upper 20 cm of Hebgen Lake. Upon collection, the cyanobacteria were transferred to filtered (pore size, 0.2 μm) lake water and stored for up to 2 days at 12°C and a photosynthetic photon flux density of 200 μmol of quanta m⁻² s⁻¹ supplied by cool white fluorescent tubes. Bacteria were isolated from lake water samples and cyanobacterial washings (cyanobacteria were expressed three times through 20- and 22-gauge needles) by plating on nutrient agar (Difco) and amended lake water agar containing 0.1% Bacto-Yeast Extract, 0.25% Bacto-Dextrose, 0.2% Bacto-Peptone, and 2.0% Bacto-Agar in lake water filtered through a 0.4-μm-pore-size filter. Both media were autoclaved at 1.1 kg cm⁻² pressure for 15 min.

A. hydrophila was grown in 0.5% Bacto-Peptone in filtered (pore size, 0.4 μm) and autoclaved lake water in a 125-ml Erlenmeyer flask at 23 ± 1°C on a G10 Gyrotory shaker (New Brunswick Scientific Co., Inc., Edison, N.J.) at 60 rpm to a bacterial density of approximately 10¹⁰ cells ml⁻¹. One milliliter of this culture was transferred to 10 ml of 0.25% Bacto-Peptone in lake water with 3 mM methionine and 50 μM EDTA and grown with shaking as described above to obtain a cell count of approximately 10¹⁰ ml⁻¹. The bacterial culture was again transferred (1 ml in 10 ml of medium) into filter-sterilized (pore size, 0.2 μm) lake water (lacking Bacto-Peptone) containing 3 mM methionine, 50 μM EDTA, and [methyl-²H]thymidine (20 μCi; specific activity, 80.7 Ci mmol⁻¹; Dupont, Wilmington, Del.) and grown as described above for 4 h. This dilution series gradually lowered the amount of Bacto-Peptone which was necessary for efficient ³H labeling of the cells. The labeled bacterial suspension was then filtered onto a sterile 0.2-μm-pore-size membrane filter under gentle vacuum and washed three times with filter-sterilized lake water (pore size, 0.2 μm), and sufficient cells were resuspended in filter-sterilized lake water with 50 μM EDTA and 0.005% (wt/vol) Tween 80 (Sigma Chemical Co., St. Louis, Mo.) to bring the bacterial counts to 8.0 × 10⁸ cells ml⁻¹. The specific activity of a 0.5-ml aliquot of bacterial suspension was determined by standard liquid scintillation spectrometry following the addition of 7 ml of scintillation cocktail (Cytoscint; ICN Radiochemicals, Irvine, Calif.), using a Beckman LS-100C liquid scintillation counter. Because certain heavy metals are known to affect bacterial motility, the chelating agent EDTA was added; Tween 80 was used to prevent bacterial attachment to surfaces (4). Methionine was added to the bacterial suspension because it has been shown that chemotaxis of at least some strains or mutants of bacteria are stimulated in the presence of methionine (1).

Samples from essentially unialgal blooms of L. birgei and samples of A. flos-aquae (confirmed by microscopic observation) from Hebgen Lake were gently filtered onto polycarbonate membrane filters (pore size, 8 μm), rinsed from the filter with filter-sterilized (pore size, 0.2 μm) lake water, and gently passed through 18- and 20-gauge sterile hypodermic needles fitted on 20-ml syringes. The expressed sample was

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washed two more times on an 8-μm-pore-size membrane filter with filter-sterilized (pore size, 0.2 μm) lake water. This procedure removed most of the bacteria and debris associated with the cyanobacteria while dispersing the aggregates without causing visible damage to the filaments. The dispersed cyanobacteria were suspended in filter-sterilized lake water and preincubated at 25°C at 200 μmol of quanta m⁻² s⁻¹ supplied by cool white fluorescent lamps for 2 to 3 h before the chemotaxis assay.

The chlorophyll a (Chl a) assay was started by an initial extraction in 95% ethanol at 79°C for ca. 30 min; extraction continued overnight at 4°C (13). Either a spectrophotometer (Varian DMS 80) or fluorometer (Turner fluorometer model 112; Sequoia-Turner Corporation, Palo Alto, Calif.) was used to quantify the concentration of Chl a in the extract (8, 15). The spectrophotometric Chl a concentration was determined by using the extinction coefficient given by Wintermans and De Mots (15); the fluorometrically measured concentration was computed from comparison with pure Chl a from Anacystis nidulans (Sigma) in 95% ethanol.

Tygon tubing (formulation R-3603; VWR Scientific) with an inner diameter of 0.8 cm and a wall thickness of 0.16 cm was used as the reservoir for labeled bacterial cells. Holes of approximately 3.5 mm in diameter were made along the length of the tube by using heated steel wire. The distance between adjacent holes was 5 cm, leaving a length of 3 cm on one end and 5 cm on the other (Fig. 1). The tube was then autoclaved at a pressure of 1.1 kg cm⁻² for 10 min and positioned on a rigid styrofoam sheet using adhesive tape layers of absorbing paper so that the holes were parallel to the plane of the sheet. The end of the tube with the 3-cm extension from the last hole was closed with a metal clamp. The other end was raised slightly, using a piece of the rigid styrofoam.

After 2 to 3 h of preincubation, the cyanobacterial suspensions were degassed by using a vacuum of 120 mm of Hg (ca. 16 kPa) for about 5 min to remove existing bubbles and prevent their formation in the medium. After gentle mixing to ensure homogeneous suspensions, 0.5 ml (each) of the L. birgei and A. flos-aquae samples were drawn into sterile, disposable glass syringes (Glaspak B-D; Becton Dickinson, Rutherford, N.J.) of 2.5-ml capacity without gas bubble formation which otherwise caused erratic results. The tips of two replicate syringes per species were then randomly inserted into holes on the tube along with two control syringes that contained 0.5 ml (each) of degassed filter-sterilized (pore size, 0.2 μm) lake water. The six syringes were mounted horizontally on the styrofoam sheet following adjustment to ensure that the syringe contents completely filled the tips of the syringes. The freshly prepared radiolabeled bacterial cell suspension was then slowly introduced into the tube through the open end, using a sterile pipette, without introducing air bubbles so that the suspension filled all but the last 1 cm of the tube. Care was taken to ensure that no air bubbles which could trap bacteria were introduced into the tube. The open end of the tube was sealed, using a metal clamp. The whole apparatus (Fig. 1) was incubated horizontally at 23 ± 1°C for 3 h in the dark.

Two 0.5-ml portions of the initial radiolabeled bacterial sample were immediately fixed by using 0.05 ml of formalin to determine the initial radioactivity. Bacterial cell counts of the same initial sample were also determined with a hemacytometer to ascertain the initial specific activity of the bacterial suspension on a per cell basis.

Before initiation of the actual experiments, the equipment was tested for over 4 h with neutral red solution in the syringes and water in the Tygon tube and vice versa to verify that there was no significant bulk transport of the liquid from the syringes into the tube or from the tube into the syringes. We also verified that more than 95% of the bacterial cells in the suspension were motile when examined with a microscope at the beginning of the experiment.

After 3 h of incubation, the syringes were removed, the tips were carefully rinsed with distilled water, and the contents were transferred to separate scintillation vials. Formalin (final concentration, 3%) was added to each vial to kill cells immediately before the addition of 7.0 ml of Cytosol scintillation cocktail (ICN Radiochemicals). The radioactivity in each sample was determined by standard scintillation spectrometry, using a Beckman LS-100C liquid scintillation counter; the counting efficiency was determined by the external-standard-ratio method using [¹H]toluene as a reference. The data from two observations per treatment for three experiments (yielding a total of six observations per treatment) were combined in an analysis of variance to determine statistical differences among treatments.

Two additional controls were examined in a separate series of experiments to determine whether the chemical substances exuded from the cyanobacteria were present in the suspending medium after preincubation. These additional controls consisted of obtaining two replicate treatments of L. birgei (lake water that was filter sterilized [pore size, 0.2 μm] following 24-h incubation at 12°C and 300 μmol of photons m⁻² s⁻¹ with L. birgei and used in place of the lake water control described previously) and suspending medium exposed to A. flos-aquae under similar conditions. To further verify that bacterial motility led to the accumulation of labeled bacteria in the syringes, the chemotaxis assay was repeated by using radiolabeled bacteria immobilized with HgCl₂ (final concentration, 0.05% [wt/vol]). Microscopic observation confirmed that this concentration of HgCl₂ eliminated motility.

The chemotaxis assay was also carried out following the above procedure to determine the effect of cyanobacterial biomass on bacterial chemotaxis. After preincubation of the cyanobacteria, four different concentrations (i.e., Chl a levels) of cyanobacteria (two replicates at each concentration), together with a lake water control free of cyanobacteria, were used to determine the differential chemotactic response of A. hydrophila to cyanobacterial biomass.

The chemotactic response of radiolabeled A. hydrophila toward L. birgei and A. flos-aquae showed significant differences (P = 0.025) from lake water controls containing no
cyanobacteria (Table 1). Differences between cyanobacterial species were compared after normalization to the amount of Chl a. No statistically significant difference between L. birgei and A. flos-aquae occurred in the chemotactic attraction of A. hydrophila when the observations from the three experiments were pooled (Table 2). Within each individual experiment, bacterial attraction to A. flos-aquae was always greater than to L. birgei. In this experiment, the lack of significant differences between the cyanobacterial species, despite almost twofold-greater attraction to A. flos-aquae, results from relatively high variances associated with the L. birgei treatment. Greater attraction was expected to A. flos-aquae because Hebege Lake is N deficient (12), and extracellular N products from N2 fixation may cause greater attraction of microorganisms incapable of N2 fixation.

Microscopic observation at the end of the experiment showed that active motility of the bacterial cells was maintained in the suspension for at least 3 h. Degassing the cyanobacterial suspensions just before starting the experiment and avoiding gas bubbles in the syringe contents were essential because surface tension and physical blockage impeded bacterial movement. This experiment was conducted in the dark because preliminary experiments showed that gas bubbles were produced by cyanobacterial samples in the syringes when incubated under light (presumably through photosynthesis). The formulation of Tygon tube used in this study was tested for apparent toxic effects on the bacteria by growing the bacteria in cultures mixed with pieces of the tube material for 3 days. We found no significant difference in growth rate or motility of the treated bacteria compared with that of the control bacteria.

The suspending medium from either species showed no significant (P > 0.05) bacterial chemotactic attraction compared with the lake water control. The lake water control, therefore, appears to be an appropriate control for this assay. The chemotactic response of this same strain of A. hydrophila to amino acids and D-glucose has been demonstrated in a separate study (7). The lack of attraction by the suspending medium may result from rapid degradation of exuded material (presumably labile amino acids and carbohydrates [5, 10]) after the cyanobacteria were removed. Although our explanation for the lack of attraction by the suspending medium remains tentative, our experimental results indicate that the cyanobacteria must be physically present to elicit a response from bacteria.

The assay apparatus (Fig. 1) used in this study allowed the use of relatively large amounts of cyanobacterial samples compared with the amounts needed for capillary tube methods (1, 14). The inner diameter of the tip of the syringe used in our assay apparatus was 0.9 mm, which is comparable with that of capillary tubes used in previous studies (1, 3, 14). This diameter prevents bulk flow of liquid in and out of the syringe even though the syringe is larger, allowing more-homogeneous and larger samples to be used. When HgCl2-inactivated, labeled bacteria were used instead of the actively motile bacteria, the accumulation of radioactivity in the syringes was insignificant whether the syringes contained cyanobacteria or not. This strongly indicates that bacterial motility was responsible for the effects observed in our experiments.

There was a positive exponential relationship between biomass of both L. birgei and A. flos-aquae and the bacterial chemotaxis they elicited (Fig. 2). The responses, when modeled with an exponential model equation, yielded slopes of 0.014 and 0.039 dpm µg of Chl a−1 for L. birgei and A. flos-aquae, respectively; the regression coefficients (r2) for L. birgei and A. flos-aquae were 0.957 (P = 0.005) and 0.910 (P = 0.032), respectively. When the two slopes were compared by using a general linear model, there was a highly significant difference (P = 0.0006), confirming that A. flos-aquae shows a stronger response to A. hydrophila chemotaxis than L. birgei does. The stronger response of A. flos-aquae may be due to (i) additional products via N2 fixation (absent in L. birgei), (ii) less extracellular mucilaginous investments in A. flos-aquae compared with that of L. birgei, (6) or (ii) naturally higher rates of exudation of chemotactically active products by A. flos-aquae. The exponential increase in chemotactic response may reflect the requirement of a particular threshold biomass level required to produce the optimal concentration gradient or gradients of the chemotactically active substances exuded from the cya-

### Table 1. Chemotactic attraction of A. hydrophila to L. birgei and A. flos-aquae

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>dpm ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>74.04 ± 7.45</td>
</tr>
<tr>
<td>L. birgei</td>
<td>6</td>
<td>224.60 ± 58.76</td>
</tr>
<tr>
<td>A. flos-aquae</td>
<td>6</td>
<td>295.90 ± 77.51</td>
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</tbody>
</table>

a The data shown are pooled data from three experiments. Statistical significance was determined by using a one-way analysis of variance coupled with a least-significant-difference comparison.

b Number of observations.

c Mean disintegrations minute−1 ± standard error.

d Significantly different from value obtained with control (P = 0.025).

### Table 2. Comparison of chemotactic attraction of A. hydrophila to L. birgei and A. flos-aquae

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Net dpm µg of Chl a−1 ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. birgei</td>
<td>6</td>
<td>8.54 ± 3.22</td>
</tr>
<tr>
<td>A. flos-aquae</td>
<td>6</td>
<td>16.46 ± 3.70</td>
</tr>
</tbody>
</table>

a The data shown are pooled data from three experiments. Statistical significance was determined by using a one-way analysis of variance coupled with a least-significant-difference comparison.

b Number of observations.

c Mean disintegrations minute−1 (minus that of control) normalized to the amount of chlorophyll a ± standard error.

**Fig. 2.** Chemotactic response (disintegrations per minute) by A. hydrophila to various concentrations of L. birgei and A. flos-aquae biomass (Chl a). Error bars denote standard error (n = 2); when not shown, the error bars are smaller than the symbol.
nobacteria. These response curves imply that in nature, chemotactic attraction of bacteria to aggregates or planktonic masses of cyanobacteria could be higher than to individual filaments or dispersed cyanobacterial populations. Our study provides evidence for chemotactic attraction of a bacterium to two species of cyanobacteria isolated from a eutrophic reservoir and the dependence of bacterial chemotaxis on cyanobacterial biomass and presents a simple and reliable technique to test chemotaxis in other organisms. Importantly, this novel approach has the potential to be of value in studying bacterial chemotaxis under in situ field conditions.

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REFERENCES