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Rapid Method for Analyzing Bacterial Behavioral Responses to Chemical Stimuli

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A rapid method was developed to analyze bacterial behavioral responses to chemical stimuli. Digital image processing was used to detect the accumulation of bacteria at the mouth of a capillary containing an attractant. The accumulation of bacteria was determined from the total number of cells near the mouth of the capillary per videotape frame. This method was applied to measure the chemotactic response of *Pseudomonas aeruginosa* cells to serine, with results similar to those obtained by the classical capillary plating assay. The videotape method is much less time-consuming and makes it possible to assess the bacterial response to an attractant within a few minutes.

Motile bacteria sense and respond behaviorally to a wide variety of chemical stimuli, including amino acids, sugars, organic acids, and aromatic compounds (1, 2, 7, 8, 14, 15). Bacterial chemotaxis can be viewed as an important prelude to metabolism, prey-predator relationships, symbiosis, and other ecological interactions in microbial communities (5). An understanding of the mechanisms underlying responses to chemical stimuli also has practical implications, for example, for strategies aimed at using bacteria to detoxify polluted environments (7).

A widely used method for measuring chemotactic responses in bacteria is the capillary assay technique (3). In this procedure, a glass capillary tube containing medium plus attractant is immersed in a cell suspension in medium without attractant, and, after a suitable interval, the contents of the capillary are plated out and the colonies are counted. Although this procedure provides quantitative information on chemotaxis, it is time-consuming and the results tend to show great variability from trial to trial. Microscopic observation of bacterial motility can provide information required to fully understand how cells sense and respond behaviorally to the presence of chemical stimuli (4, 6, 7, 13). The present paper describes a computer-assisted method to rapidly analyze the accumulation of bacteria at the mouth of a capillary in response to an attractant. With this method, bacterial cells were videorecorded through a microscope, and the bacterial accumulation was analyzed from the video images to quantify the response to the attractant. This method made it possible to assess the bacterial response to an attractant within a few minutes.

MATERIALS AND METHODS

Media and chemicals. Nutrient broth was obtained from Difco Laboratories, Detroit, Mich. Nutrient agar and semisolid nutrient agar plates contained 1.5 and 0.3% agar (Difco), respectively. Chemotaxis medium contained 10 mM potassium phosphate buffer (pH 7.0) and 0.1 mM potassium EDTA. Chemotaxis medium, as well as all other solutions, was prepared with glass-distilled water. Attractant agar was prepared by adding 1.5% agar to chemotaxis medium containing an attractant. Organic acids were used as the sodium salts, adjusted to pH 7.0. Amino acids were of the L configuration, and sugars were of the D configuration.

Bacterial strain and growth conditions. The organism used in this work was *Pseudomonas aeruginosa* PAO1 (9). The strain was maintained on nutrient agar plates at 4°C. Motile strains were reisolated monthly by allowing them to swarm on a semisolid nutrient agar plate and then picking from the edge of the swarm. Bacteria, from a plate, were grown overnight in nutrient broth (7 ml in a 25-ml tube) at 37°C with shaking (100 to 250 rpm). Bacteria from the overnight-grown culture were inoculated to fresh medium (a 1% inoculum) and then incubated as described above to the exponential phase.

Chemotaxis assay. Cells were harvested by centrifugation in a 2-ml Eppendorf tube for 1 min at 8,000 × *g* at room

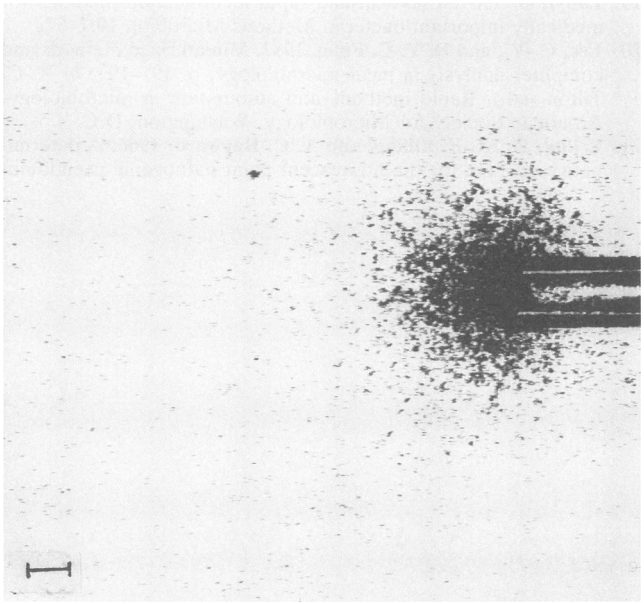

FIG. 1. Attraction of *P. aeruginosa* to serine. The capillary tube with a diameter of 10 μm contained serine at a concentration of 10^{-2} M. Bar = 10 μm.

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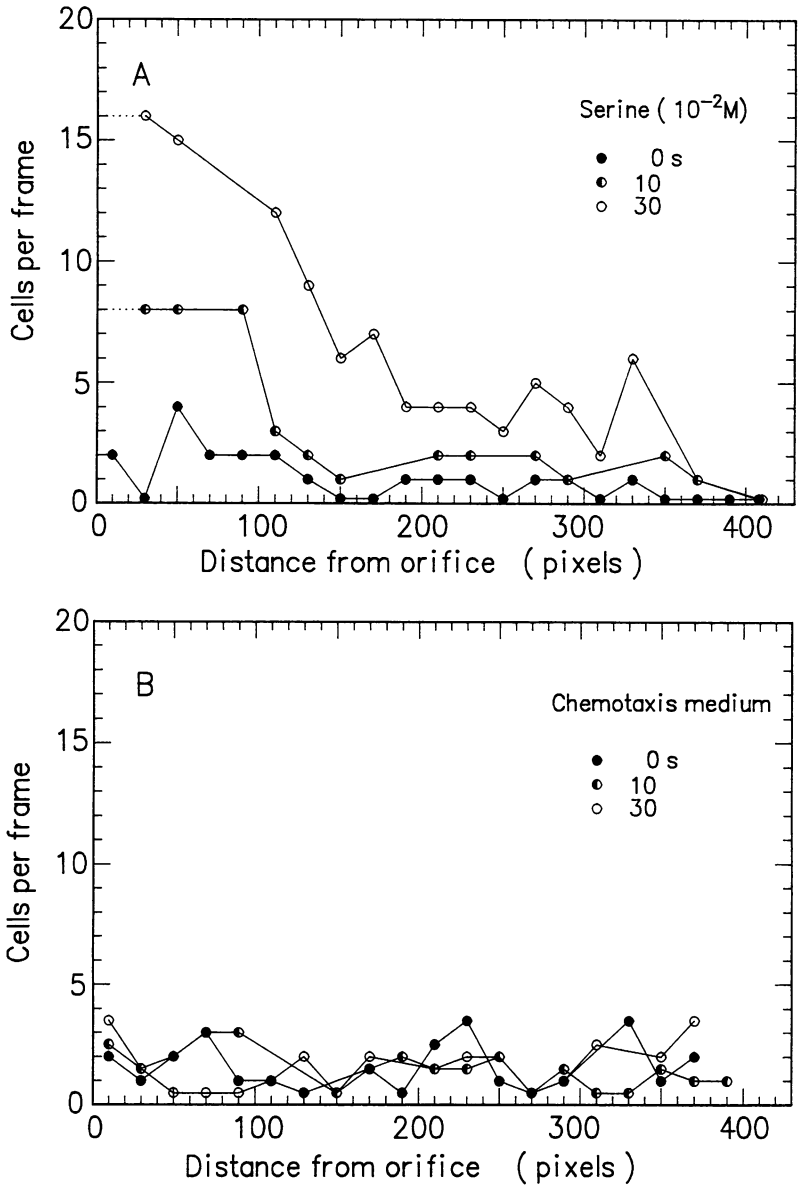

FIG. 2. Distributional patterns of bacteria around the mouth of the capillary containing 10^{-2} M serine (A) and only chemotaxis medium (B). Images were videotaped at 0, 10, and 30 s after the start of microscopic observation. A distance of 40 pixels is equivalent to 10 μm at this magnification (×300), and the scanned area per videotape frame is ca. 100 by 80 μm^2. The dotted lines in panel A represent an underestimation of the number of cells at the immediate vicinity of the orifice because of poor background discrimination.

temperature, and the pellet was gently resuspended in 1 ml of chemotaxis medium. The bacteria were again centrifuged and resuspended in chemotaxis medium to a concentration of ca. 6×10^8 cells per ml. When starved cells were required, this cell suspension was further incubated without shaking for at least 16 h before being assayed. A small chamber similar to that described by Adler (3) was used for microscopic observation of bacterial motility. The chamber was formed by laying a U-shaped glass tube (0.5 mm in diameter) as a spacer between two coverslips, and this setup was mounted on the stage of an IMT-2 inverted dark-field microscope (Olympus Co., Tokyo, Japan). The temperature in the chamber was controlled at 37°C with a microscope stage heating unit (IMT-2-HP; Olympus Co.). Glass capillary tubes with internal diameters of 10 to 40 μm were prepared from micro-injection glass tubings (1 mm in diameter; type G-1; Narishige Co., Tokyo, Japan) by using a microcapillary producing unit (PB-7; Narishige Co.). The capillaries having a constant diameter could be prepared by controlling the heating temperature and heating time according to the supplier's recommendations. The orifice sizes of the capillaries were measured microscopically with a stage micrometer. Neither end of the capillary was sealed. The capillary was plunged into a test tube containing attractant agar, which was kept molten at 60°C. The preferred length of the column of attractant agar in the capillary was about 10 ± 3 mm. Within this range of column length, no significant effect of length was observed on the attraction of bacteria. The exterior of the capillary was quickly rinsed with a thin stream of distilled water from a wash bottle and then inserted

into the chamber on the stage of a microscope. The capillary was carefully moved to a particular position in the field of a microscopic view by using a joystick micromanipulator (MN-151; Narishige Co.).

Microscopy. Cells in a 20-μl suspension were placed on a coverslip, and the coverslip was placed upside down on the U-shaped spacer to fill the chamber with the cell suspension immediately preceding the videotaping. Cells were videotaped through the phase-contrast microscope (magnification, ×300) with focus maintained at the mouth of the capillary. A heat reflection filter was used to reduce the effect of any temperature changes caused by the microscope lamp. The video image was recorded continuously by a video cassette recorder (BR9000; Hitachi Co., Tokyo, Japan).

Computer analysis. Videotapes were played back on a video cassette recorder and digitized with an image processor (LA525X; Pias Co., Osaka, Japan). The image processor detected areas of dark bacterial cells on a light background in a grid of 480 (vertical) by 512 (horizontal) pixels. Only those cells within the focus were recorded and processed further. The center of each cell area was calculated by the software program of LA525X and stored in a personal computer (PC9801; NEC Co., Tokyo, Japan). The cell-to-capillary distances were computed by a user program (DIST) designed for the present study. This user program was written in N_{88} BASIC and is available to investigators for their personal use. A distance of 40 pixels was equivalent to a length of 10 μm at a magnification of ×300, and the scanned area in the field of microscopic view was ca. 100 by 80 μm^2.

RESULTS AND DISCUSSION

P. aeruginosa cells accumulated toward serine (Fig. 1). Strong attraction was also observed for other amino acids, including alanine, arginine, glutamine, cysteine, and threonine. The bacterial movement was observed soon after the chamber was filled with the bacterial suspension, and a cloud of bacteria started to form at the mouth of the capillary by 10 s. Since the capillary was filled with an agar gel, no bacterial cells were detected inside the capillary.

The accumulation of bacteria was quantified by the changes in the distributional patterns of bacteria around the mouth of the capillary (Fig. 2A). Each point represents the number of cells which fall within a 10-pixel interval and is plotted at the midpoint of this distance interval. Immediately after the start of observation, the cells were randomly distributed and soon moved toward the capillary. The total number of cells per frame increased with time. The increase in the number of cells was most pronounced within the distance up to 100 pixels (25 μm) from the mouth of the capillary. After 10 s, a cloud of bacteria increased the brightness and decreased the contrast of the bacterial image near the orifice. Poor discrimination of the cells from the background resulted in the underestimation of the number of cells at the immediate vicinity of the orifice. Therefore, the curves at the immediate vicinity of the orifice are presented by dotted lines in Fig. 2A. In the absence of attractant, bacterial accumulation was not detected (Fig. 2B).

The degree to which bacteria accumulated was evaluated by the total number of cells per frame (Fig. 3). In the presence of serine, the total number of cells increased soon after the start of microscopic observation. The time lag

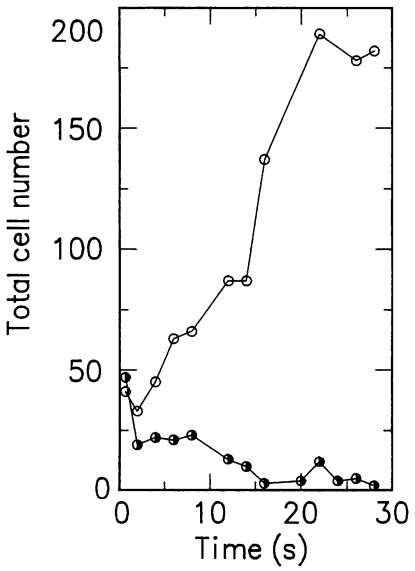

FIG. 3. Chemotactic responses of *P. aeruginosa* toward 10^{-2} M serine (○) and chemotaxis medium (◑). The diameter of capillaries was ca. 10 μm.

before bacterial response was less than 5 s. After 20 s, the total number of cells reached a plateau. However, the plateau did not necessarily mean that the bacterial accumulation saturated at this stage. We visually observed that the size of the cloud continued to increase even after 1 min. The small size of the scanned area (100 by 80 μm^2) may be a possible reason for the plateau. In the absence of serine, the total number of cells did not increase.

The response of bacteria was strongly dependent on the concentration of attractant in the capillary. Figure 4 shows the 60-s response to different concentrations of serine, which is similar to the classical *Escherichia coli*-type response to attractants (10). The response curve expressed in terms of

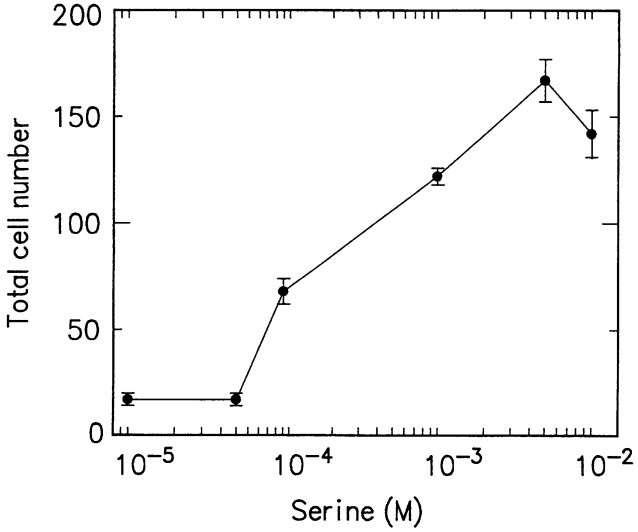

FIG. 4. Concentration-response curve for serine. Videotaped images were sampled at 60 s after the start of observation. Vertical bars represent the standard deviations of four different measurements. The diameter of capillaries was ca. 10 μm.

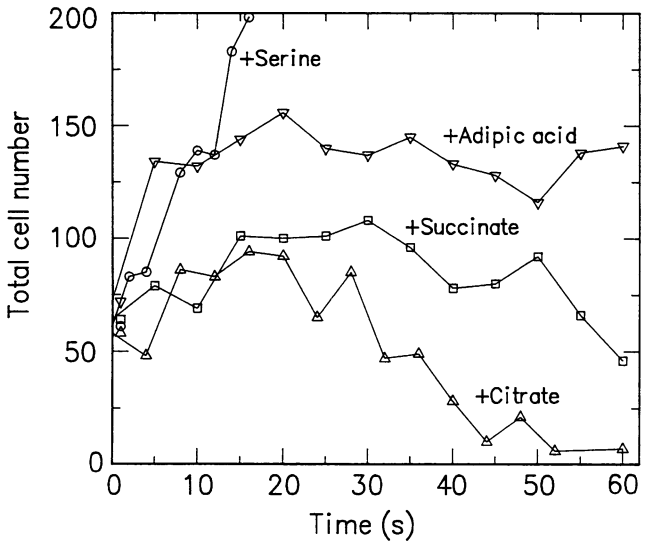

FIG. 5. Chemotactic responses of starved cells of *P. aeruginosa* toward adipic acid, citrate, serine, and succinate. The attractant concentration in the capillary was 10^{-2} M. The diameter of capillaries was ca. 40 μm.

TABLE 1. Comparison of chemotactic responses of starved cells of *P. aeruginosa* to various organic compounds[a]

Chemical stimulus[b]	Concn (mM) in capillary	Peak response[c] (cells/frame)	Time (s) for obtaining peak response
Organic acids[d]			
Adipic acid	10	140 ± 14	15[e]
Citrate	10	89 ± 4	15–20
Gluconate	10	116 ± 6	20–25
Glutamate	10	177 ± 15	20[e]
Succinate	10	105 ± 12	20–30
Amino acids			
Leucine	10	197 ± 6[f]	
Serine	10	219 ± 11[f]	
Threonine	10	207 ± 5[f]	
Valine	10	183 ± 7[f]	

[a] Cells were grown in nutrient broth and starved in chemotactic medium for 16 h.
[b] pH was adjusted to 7.0 with 1 N NaOH.
[c] Peak responses are the means ± standard deviations of four different runs.
[d] Sodium salts were used.
[e] Accumulation was saturated after the time presented.
[f] Since a bacterial cloud formed, response was measured at 20 s.

total number of cells per frame showed a peak at 5×10^{-3} M serine. The threshold concentration, the lowest concentration of attractant that gives a response greater than that obtained in the absence of attractant, was about 5×10^{-5} M. A similar value was also reported for the *P. aeruginosa* response to serine (12). As in the case of the classical assay technique, the peak and threshold values were dependent on the orifice size of the capillary and also the cell concentration of bacterial suspension used (data not shown).

The chemotactic responses of *P. aeruginosa* to a variety of chemical stimuli were analyzed by using the present method. In this series of experiments, the responses were found to be strong and more reproducible when cells were subjected to starvation (data not shown). Therefore, in this study, starved cells, which had been incubated in chemotaxis medium for at least 16 h at 37°C, were used for the assay. In order to detect weak responses, the capillaries having an orifice size (inner diameter) of ca. 40 μm were used throughout this series of measurements. The patterns of bacterial accumulation differed from attractant to attractant, and a cloud of bacteria was not always formed (Fig. 5). To compare the degrees of attractiveness, peak responses and times for obtaining the respective peaks are summarized in Table 1. Strong responses were observed with adipic acid, gluconate, succinate, and amino acids. As far as tested, significant responses were not detected with acetate, benzoate, formate, *p*-toluate, or sugars.

Our assay technique is different from Adler's classical technique in that the capillary was filled with attractant agar, and bacterial cells outside the capillary were counted. The use of a capillary containing attractant solution, which was employed in the classical technique (3, 11, 12), was not adequate for our rapid analysis, because an unexpected movement of water was often caused by physical factors such as capillary attraction and the density difference between solutions inside and outside the capillary.

The advantage of our method is that bacterial response to an attractant can be assessed within a few minutes, once all the appropriate instrumentation is set in place. Visual inspection of a monitor screen also can provide a rapid, qualitative assessment of whether a chemotactic response has occurred. With Adler's capillary assay technique, the incubation time for bacterial accumulation is typically 30 to 60 min (1–3). In the case of attractants that are transported and metabolized, the chemotactic response of bacteria would be affected by the metabolism of the attractant by the bacteria. The longer the incubation time is, of course, the greater are the influences on the response.

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