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Rapid Chemotaxis Assay Using Radioactively Labeled Bacterial Cells

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A rapid chemotaxis assay is described in which radioactively labeled cells of the assay organism are used to detect the number of cells trapped in capillaries containing attractant. The sensitivity and reproducibility of the radioactive technique is comparable to that of the dilution plating procedure of Adler (J. Adler, J. Gen. Microbiol. **14**:77-91, 1973), but is faster and also permits the results of the assay to be determined on the day that the assay is run. The method could be particularly useful for environmental studies and for field experiments, since it does not rely on sterile techniques for dilution plating.

Chemotaxis is a widespread biological phenomenon involved in a wide variety of behavioral responses such as feeding, sexual attraction, aggregation, and interactions between prey and predator, between host and parasite, and between symbionts. The chemotactic response can play an important role in community ecology (4). Often there is a need for a rapid method of assessing whether motile microorganisms are attracted (positive chemotaxis) or repelled (negative chemotaxis) by certain substances. The usual procedure, which is the basis of chemotaxis assays, was developed by Pfeffer (6, 7). Test substances in glass capillaries are placed into a suspension of motile microorganisms, and the movement of the microorganisms toward an attractant results in the accumulation of microorganisms around the mouth of the capillary (positive chemotaxis). The response can be observed microscopically or can be assessed in a quantitative manner (1, 2) by means of dilution plating of the capillary contents. The quantitative Adler method is very sensitive and reproducible, but the dilution plating technique is tedious and time consuming, and the results may not be available until 1 to 3 days after the assay, depending on the growth rate of the colony-forming units. More rapid assay methods have been developed by Armitage et al. (3) and by Hirschberg and Rogers (5). Armitage's group used a Coulter electronic particle counter to measure the proportion of motile bacteria passing through a polycarbonate membrane into a cell-free buffer. Hirschberg and Rogers used cells of *Chlamydomonas reinhardtii* labeled with $NaH^{14}CO_3$ for the chemotaxis assay and measured by liquid scintillation counting the number of cells which had moved into capillaries containing test chemicals. They report high variability in the assay (25%). This paper describes a similar modification of the Adler method in which the cells used for the assay were previously uniformly labeled with [^{14}C]glucose. This tracer has been used as a means of estimating the numbers of bacteria which moved into the capillary in response to attractant. The assay is sensitive, reproducible, and rapid.

MATERIALS AND METHODS

Assay organism. A strain of *Corynebacterium* sp. (UWO18 = (3B-5Y)), isolated from tar balls collected in the open Atlantic ocean (9) and identified at the National Culture Collection of Marine Bacteria, Aberdeen, United Kingdom, was used as the test organism. This strain is able to metabolize some component(s) of Kuwait crude oil for growth (D. Sidenberg, M. Sc. thesis, University of Western Ontario, London, 1980).

Stock cultures. The cultures were routinely maintained on seawater-yeast nitrogen base-oil agar containing 67 g of basal mineral salts medium designed for culturing yeasts (yeast nitrogen base; Difco Laboratories, Detroit, Mich.), 100 ml of distilled water, 20 g of Difco agar, 20 ml (2%, vol/vol) Kuwait crude oil, 1 g of Indulin C (to disperse the oil), and aged filtered seawater (FSW) to 1 liter and on Zobell's (10) marine medium 2216 (Difco).

FSW. Seawater obtained from the University of North Carolina Institute of Marine Sciences at Morehead City was filtered through a cellulose acetate membrane of 0.45-μm pore size (Millipore Corp., Bedford, Mass.) and then autoclaved and filtered through a Millipore membrane of 0.22-μm pore size.

Preparation of radioactively labeled bacteria for chemotaxis assay. Cells from seawater-yeast nitrogen base-oil agar slants were transferred to medium 2216 slants for 48 h. Cells from medium 2216 slants were inoculated into a 125-ml Erlenmeyer flask containing 50 ml of 2216 broth and grown for 20 h at

25°C on a gyratory shaker (Labline junior orbit shaker; Labline Instruments, Inc., Melrose Park, Ill.) at 150 rpm. Cells (100 μl) were taken from the 20-h culture and added to 5 ml of 2216 broth in a 10-ml screw-cap test tube. Three tubes were prepared. At the time of inoculation 100 μl (20 μCi) of uniformly labeled [^{14}C]glucose (specific activity, 333 mCi/mmol) (New England Nuclear Corp., Boston, Mass.) was added to each tube. The tubes were incubated in a horizontal position on a gyratory shaker as described above for 11 h, at which time a population of 6×10^7 cells per ml had developed, corresponding to an absorbance reading of 0.52 at 590 nm (Bausch and Lomb Spectronic 710 spectrophotometer). A 100-μl sample of cell suspension from each tube was filtered through a 2-cm-diameter Sartorius membrane filter, 0.2-μm pore size (Fisher Scientific Co., Raleigh, N.C.), washed, dried, and placed in 10 ml of a water-miscible scintillation fluid to determine the radioactivity in the cells at 11 h. Repeated experiments gave consistently high count rates in the order of 300,000 cpm in 100 μl of cells. The scintillation fluid contained 1,140 ml of dioxane, 1,140 ml of toluene, 750 ml of ethyl alcohol, 150 g of naphthalene, 15 g of POP and 0.3 g of dimethyl 1,4-bis-(5-phenyloxazolyl)benzene (H. W. Paerl, Ph.D. thesis, University of California, Davis, 1973). This scintillation cocktail was formulated to accept up to 1 ml of water without significant quenching. Typically, the addition of 1 ml of water decreased the counting efficiency for ^{14}C by 2 to 3%.

The remainder of the 11-h radioactively labeled cultures was centrifuged in the culture tubes at 3,400 × *g* for 10 min at 20°C. The supernatant was discarded, and the cells were resuspended in 5 ml of sterile FSW. The centrifugation and washing procedure was repeated twice. A drop of the final cell suspension in 5 ml of FSW was observed microscopically to confirm that the bacteria had retained their motility. After the final washing, a 100-ml sample of the washed cell suspension was taken at 0, 30, 60, and 90 min, filtered, dried, and placed in scintillation fluid as described above to determine whether any loss of label had occurred during the washing procedures or during the subsequent assay. The cell suspension could be stored at 4°C for up to 4 h and still retain motility.

Chemotaxis assays. The chemotactic response was evaluated by a modification of the quantitative method of Alder (2) for the accumulation of bacteria in microcapillaries. The bacterial suspension (0.2 ml) was placed in each test chamber by using a 1-ml disposable sterile syringe. Although Adler (2) had found that 1- and 2-μl capillaries gave the greatest response, 5-μl capillaries were used since one of the attractants, crude oil, is viscous at higher concentrations.

The capillaries (Microcaps; Drummond Scientific Co., Broomall, Pa.), were cleaned by washing in warm detergent and by rinsing several times in reagent-grade acetone (Fisher Scientific Co., Pittsburgh, Pa.) and then in distilled water. The microcapillaries were sterilized by dry heat at 150°C for at least 12 h and thereafter were always handled with forceps.

The test solutions were prepared from a stock solution of either Zobell's marine broth 2216 or Kuwait crude oil (20,000 μg/ml) in FSW. The stock solutions were diluted 10-fold in FSW to yield concentrations as low as 10^{-6} and 10^{-7}, respectively. The test solutions were prepared fresh for each assay and kept in tightly stoppered bottles. The test solution was drawn into the microcapillary by passing one end of the capillary through a flame and inserting the open end into the test solution. The solution was drawn up to about 1 cm within the capillary. Duplicate capillaries were placed in each 0.2 ml of bacterial test suspension.

The chemotaxis assays were run at 25 ± 1°C for 15, 30, and 60 min. At the appropriate time two capillaries were removed from the bacterial suspension, wiped carefully with a paper wipe, and each placed into 10 ml of scintillation fluid. The scintillation fluid used has the capacity to accept up to 1.0 ml of aqueous solution without decreasing the efficiency of counting. It was therefore more than adequate to compensate for any quenching problems from the contents of the 5-μl capillary. The efficiency was 92% with a [^{14}C]toluene internal standard for calibration purposes. Each sample was counted for 10 min in a Beckman LS 7000 Microprocessor scintillation spectrometer. The background count of the scintillation fluid was 32 to 34 cpm. Each chemotaxis assay was repeated at least twice with different suspensions on different days. Assays were terminated after 60 min, because at that time some of the bacteria were beginning to settle out of the suspension onto the bottom of the glass chemotaxis chamber.

Comparison with dilution plating method. When the number of bacteria trapped in the capillaries was estimated by the dilution plating method, the preparation of the cells for the assay and the assay conditions were similar to those described above, but the cells were unlabeled, and at the end of the assay each capillary was removed from the bacterial suspension and wiped carefully with a paper wipe, the sealed end was broken off, and the rubber bulb of the Drummond pipette was used to force the contents into 10 ml of 2216 broth. Serial dilutions of the capillary contents were made in 2216 broth, and 0.1-ml samples of each dilution were spread in triplicate on 2216 agar plates. The plates were kept at 25°C, and the colonies were counted after 48 h of incubation while they were still discrete. A capillary containing FSW was included in each test series as a control assay for random movement. Since the bacteria were suspended in FSW for the assay, no attraction to FSW in the capillary would be expected. Any accumulation in the capillary would therefore be due to random movement.

The relationship between the radioactive count and the number of colony-forming units was investigated by making dilutions of the washed labeled cell suspension. Two 100-μl samples of each dilution were filtered, washed, dried, placed in scintillation fluid, and counted; 100-μl samples of each dilution were plated in triplicate on 2216 agar plates, and the number of colony-forming units was counted after 48 h of incubation.

RESULTS

Corynebacterium sp. was chemotactically attached to Zobell's marine medium 2216, which contains salts in concentrations similar to those

found in seawater and peptone (5 mg/ml). The concentration response curve for a representative experiment is shown in Fig. 1. A similar reponse was observed with dilutions of a peptone solution (5 mg/ml) (unpublished data). Sterile FSW contains some component which attracts bacteria slightly, because the radioactive count is consistently and significantly ($P < 0.01$) higher (98 to 125 cpm per capillary) than the background count (32 to 34 cpm/10 ml of scintillation fluid). The chemotactic response observed at 10^{-6} and 10^{-5} dilutions of 2216 medium in seawater is of the same magnitude as seawater alone and can be explained by random movement of the population in seawater or in subthreshold levels of 2216 medium. The threshold for a detectable response to a component or components of 2216 medium occurred between 10^{-4} and 10^{-3} dilutions of the full-strength medium (0.5 to 5.0 μg of peptone per ml). The maximum response occurred with full-strength medium.

To relate the radioactive count to the numbers of cells, dilutions of radioactive cells in 2216 broth were made, and the radioactive count was determined for duplicate 100-μl samples from each dilution. Triplicate 100-μl samples of each dilution were also plated onto 2216 agar plates, and the number of colony-forming units was counted after 48 h. The relationship between the radioactive counts and the colony-forming units is shown in Fig. 2. The relationship is linear. No effect of radioactivity on viability was observed, since dilutions of unlabeled cells produced similar numbers of colony-forming units.

Utilization of radioactively labeled substrate during the course of the assay was not a significant factor, since the turnover time for glucose as determined by [^{14}C]glucose assimilation was 12.5 h at 25°C. This is the time for loss of half of the radioactivity in the cells. The kinetics for utilizaton of labeled glucose followed the expected pattern. Approximately 8.5% of the glucose assimilated was respired.

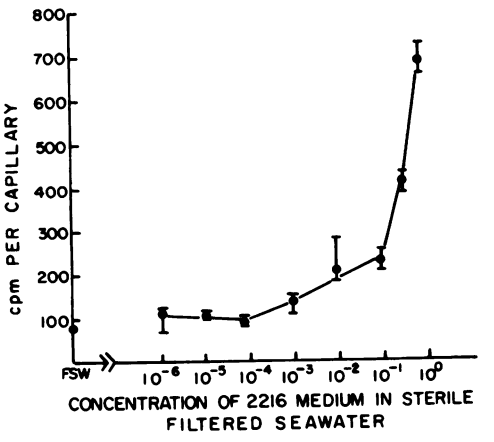

FIG. 1. *Chemotactic response of Corynebacterium sp. (UWO18) after 15 min of exposure to Zobell's marine medium 2216.*

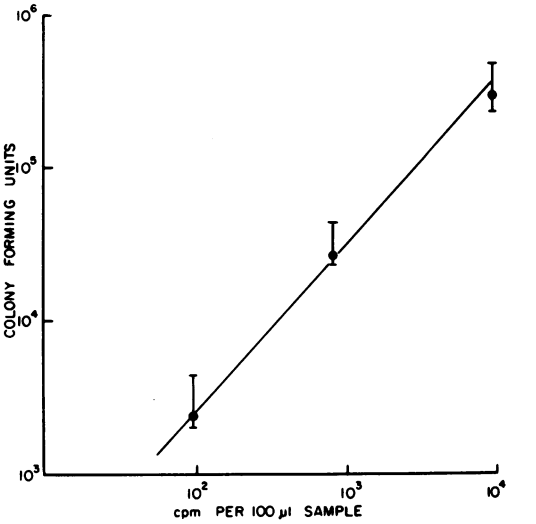

FIG. 2. *Relationship between radioactivity in cells and viable cell count.*

Corynebacterium sp. (UWO18) was also positively chemotactic to a component or components of Kuwait crude oil. A comparison of the number of bacteria which accumulated in the capillaries with the radioactive labeling technique and with the dilution plating method is shown in Fig. 3. The concentration response curves obtained by the two techniques are similar. After a 15-min assay the maximum accumulation of bacteria in the capillary occurred at the same concentration, i.e., at 2 μg of Kuwait crude oil per ml, with both techniques. Similarly, a decline in both concentration response curves occurred at concentrations greater than 2 μg/ml. The threshold responses were detected between 0.002 and 0.02 μg/ml with radioactively labeled cells and 0.02 to 0.2 μg/ml with the plate count technique. The radioactive technique is slightly more sensitive.

Figure 4 shows the accumulation of radioactively labeled cells with time. The concentration response curves at 15, 30, and 60 min show a phase shift with time. The maximum response occurs at 15 min with 2 μg/ml, at 30 min with 20 μg/ml, and at 60 min with 200 μg/ml. The greatest accumulation of radioactively labeled cells in the capillaries occurred at 60 min. However, by this time some cells from the suspension around

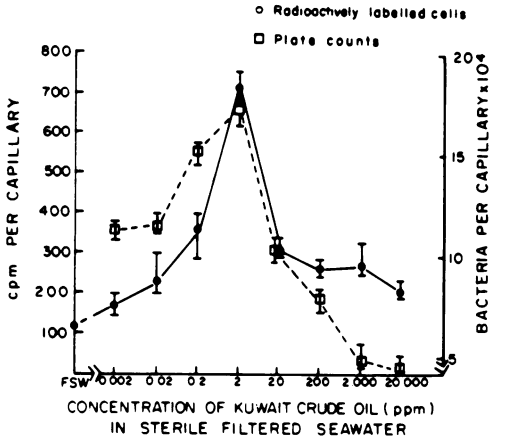

FIG. 3. *Chemotactic responses of Corynebacterium sp. (UWO18) after 15 min of exposure to Kuwait crude oil (micrograms per milliliter [ppm]).*

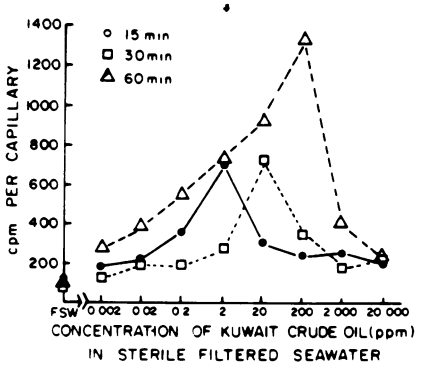

FIG. 4. *Accumulation of Corynebacterium sp. (UWO18) with increased time of exposure to Kuwait crude oil (micrograms per milliliter [ppm]).*

the capillaries were beginning to settle on the bottom of the assay chamber, so assays of longer duration were not undertaken. The rate of accumulation of radioactively labeled cells in the capillaries is shown in Fig. 5. At low concentrations, (0.02 to 2 μg/ml) more attraction was observed at 15 than at 30 min, possibly owing to dissipation of the gradient with time. The increased accumulation between 30 and 60 min at concentrations of 0.2 to 200 μg/ml may be due to increasing solubility of attractants in oil with time and development of gradients of attractants. At higher concentrations, 2,000 to 20,000 μg/ml, only a slight increase in attraction was observed with time. Inhibitory substances at higher concentrations may interfere with the chemotactic response.

DISCUSSION

The rapid chemotaxis assay, using radioactively labeled cells of the assay organism, gives

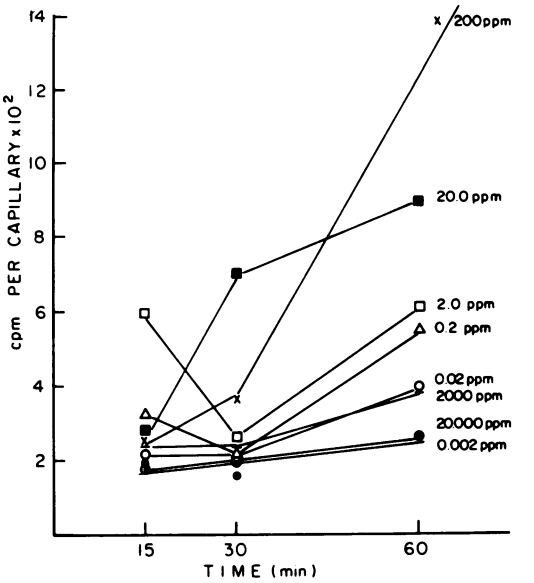

FIG. 5. *Rate of accumulation of radioactively labeled Corynebacterium sp. (UWO18) in capillaries containing various concentrations of Kuwait crude oil (micrograms per milliliter [ppm]).*

responses which are similar to those obtained with the quantitative Adler method (2) in which the estimates of cells trapped in capillaries containing attractant were obtained by dilution plating (Fig. 3). With radioactively labeled cells, duplicate experiments produced very consistent results (±8.5%). The advantage of our method is that the results of the assay can be obtained rapidly. The technique is simple and faster than the dilution plating method because the entire capillary can be inserted into the scintillation fluid in the vials, thus eliminating the breaking of the capillary and blowing out of contents into the dilution broth and dilution plating steps. The contents of the scintillation vials can be counted within 10 min, and the results are available on the same day that the assay is performed. The elimination of the time-consuming sterile technique of dilution plating permits more treatments to be handled during an assay. All of these features would make the technique particularly amenable for use under field conditions. Natural populations of aquatic motile organisms, collected in sealed bottles, could be injected with radioisotope; the sealed bottles could be reimmersed in the water to ensure uniform environmental conditions during uptake of the label. Labeled cells could be used for chemotaxis assays with various chemicals. The label in the organisms attracted into capillaries, once immersed in scintillation fluid, could be

stored until the vials could be sent to the laboratory for counting.

Although in general the radioactively labeled cell technique and the dilution plating technique give similar response patterns, there are some differences in the magnitude of the responses observed at the extremes of the range of concentrations. The radioactive technique is slightly more sensitive in detecting the threshold response. The explanation for the differences in the two curves in the higher concentration range (200 to 20,000 μg/ml) may be that the dilution plating method estimates viable cells and the radioactive labeling technique measures radioactivity in the total population. Possibly some loss in viability may occur at the higher concentrations of oil. When unlabeled cells were exposed to 20,000 μg of Kuwait crude oil per ml for 60 min and viability was estimated by dilution plating, the number of colony-forming units was 84% of that of untreated cells. Therefore, Kuwait crude oil at high concentrations is slightly toxic to the cells. Another factor which may contribute to the differences between the two estimates is that during dilution plating some cells may adhere to the walls of the capillary and would not be washed into the dilution medium. This would result in lower estimates of cell numbers in the dilution technique compared with the radioactive technique, in which all of the radioactivity in the cells in the capillary is counted. The differences between the two techniques, if this were the case, would be more marked at the extremes of the concentration range when fewer cells were trapped in the capillaries. The attraction of *Corynebacterium* sp. (UWO18) to Zobells' marine medium containing peptone was as expected. Nutrient broth is commonly used as an indicator of positive chemotaxis by other workers (4). The attraction of *Corynebacterium* sp. (UWO18) to Kuwait crude oil has been described elsewhere (Wellman, submitted for publication). One or more components of Kuwait crude oil can serve as a carbon substrate for the growth of this organism (Sidenberg, M.S. thesis). The concentration response curves (Fig. 3 and 4) for the attraction of *Corynebacterium* sp. (UWO18) to various concentration of Kuwait crude oil are similar to those reported by other workers for other organisms and other attractants (2, 4, 8). The phase shift with time (Fig. 4) is interesting since it suggests either that the bacteria rapidly become more tolerant of higher concentrations of attractant or that the organisms are metabolizing the attractant within 30 to 60 min and are thus effectively lowering the concentration of attractant diffusing from the capillary into the bacterial suspension as was reported earlier by Adler (2). Alternatively, any perceived phase shifts may be due to increasing solubility of attractants in oil with time, so that substances not perceived as attractants at 2 μg/ml are perceived at higher concentrations as gradients develop. In the experiment reported here the phase shift continued for up to 60 min, when the experiment was terminated due to settling out of some of the bacteria from suspension onto the bottom of the assay chamber. Consequently, for comparative purposes it is important that the time of the assays be the same.

The more radioactive label in the cells at the commencement of the assay, the more sensitive will be the detection of a chemotactic response. During the 30-min centrifugation and washing procedure to remove the 2216 medium in which the cells had been grown and to replace it with FSW, a considerable loss of label occurred with [^{14}C]glucose, from 324,000 ± 200 cpm in 100 μl of cells from 2216 medium to 98,500 ± 170 cpm in 100 μl of washed cells suspended in FSW. The loss may be due to removal of loosely bound labeled compounds from the cells since no lysis or loss of viability was detected. After the washing procedures, no further loss occurred during storage at either 4 or 25°C for 90 min, i.e., for the duration of the assay. Furthermore, the turnover time of the labeled pool, i.e., loss of half of the [^{14}C]glucose assimilated, was 12.5 h at 25°C, whereas approximately 8.5% of the glucose assimilated was respired. These results confirm the findings that loss of label due to metabolic activity (respiration) is insignificant during the assay time. Hence, the results of the assay can be relied on. The technique would be even more sensitive if the labeled compounds were not lost from the cells during the washing procedure. Experiments are currently underway to see whether labeling the cells with $^{32}PO_4$ and [^{3}H]- and [^{14}C]thymidine results in less loss during washing and thus greater sensitivity of the assay.

The technique of radioactive labeling of cells for chemotaxic assays can be used to rapidly screen a large number of test substances (naturally produced compounds or pure substances) for their ability to attract microorganisms. The technique can also be used to determine whether negative chemotaxis (repulsion) occurs by incorporating the repellant either into the capillary or into the bacterial suspension and measuring the decline in numbers of cells trapped in the capillaries compared with that observed with a positive attractant, such as nutrient medium. The technique should prove very useful for environment studies in which attraction of orga-

nisms to certain substrates is suspected to play a key role in the establishment of populations or where the application of repellants (e.g., antifouling agents) is to be used to prevent colonization and degradation by microorganisms.

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