A Diffusion Gradient Chamber for Studying Microbial Behavior and Separating Microorganisms

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The natural habitats of most microbes are dynamic and include spatial gradients of growth substrates, electron acceptors, pH, salts, and inhibitory compounds. To mimic this diffusive aspect of nature, we developed an analytical diffusion gradient chamber (DGC) that can be used to separate, enrich for, isolate, and study the behavior of microorganisms. The chamber is a polycarbonate box containing an arena (5 by 5 by 2 cm) into which is cast a semisolid growth medium. Continuously replenished solute reservoirs positioned on each side of the arena but separated from it by a porous membrane enable the formation throughout the gel of multiple, intersecting gradients of solutes in two dimensions. With glucose as the solute, a gradient which spanned a 100-fold range in concentration was established across the arena in about 4 days. The shape of the glucose gradient was accurately predicted by a mathematical model based on Fickian diffusion. The growth and migratory behavior of *Escherichia coli* in response to imposed gradients of attractants (aspartate, α-methyl aspartate, and serine) and a repellent (valine) were examined. Cells responded in predictable ways to such gradients by forming distinctive growth and migration patterns in the DGC. This was true for wild-type *E. coli* as well as specific chemotaxis and motility mutants. The patterns yielded information about the threshold concentration of chemoeffectors needed to elicit a response as well as their saturating concentration. It was also evident that the metabolism of attractants significantly affected the gradients and, hence, the movement of cells. Finally, it was possible to separate *E. coli* and *Pseudomonas fluorescens* in the DGC on the basis of their differential responses to gradients of various chemoeffectors.

Microbial life on the earth exists in spatial gradients of solutes, light intensity, temperature, pH, oxygen, and viscosity. Not surprisingly, many microbes have evolved mechanisms to position themselves in regions of gradients that favor their growth and/or survival. For example, the chemoheterotrophic sulfide-oxidizing bacterium *Beggiaota* sp. responds readily to gradients of oxygen and sulfide and, in aquatic environments, positions itself in a microoxic niche where it can oxidize sulfide diffusing up from anoxic zones (17, 19). Another example is seen with the purple and green sulfur phototrophs, which form well-defined zones of growth (blooms) in the water column of stratified lakes in response to gradients of light (quality and quantity), sulfide, oxygen, and pH (20).

Such positioning mechanisms usually involve some sort of taxis, which includes a mobility component (e.g., flagellar or gliding motility and buoyancy regulation, etc.) and a chemo- and/or photosensory component. Undoubtedly, the best-understood positioning mechanism is chemotaxis, which has been studied extensively in *Escherichia coli* (for reviews, see references 4, 5, 15, and 16). It is now well understood that *E. coli* cells move in a random walk consisting of runs and tumbles. *E. coli* is capable of biasing its random walk by increasing its run time when swimming up a gradient of a chemotactant or down a gradient of a repellent. The end result is more-rapid net migration toward an attractant or away from a repellent.

Despite our recognition of positioning phenomena and their probable importance to microbial growth and survival in nature, most systems employed for the isolation, cultivation, and study of microbes in the laboratory are homogeneous and make no attempt to imitate the dynamic gradients found in natural habitats. Consequently, we have only a limited understanding of the behavior of microbes confronted with multiple gradients of environmental substances (13, 26). Indeed, it may well be that natural habitats hold a variety of organisms capable of growth only within a very narrow range of environmental conditions. Such stenobiotic organisms might constitute much of the untapped diversity believed to exist in nature and represent organisms that have eluded isolation with conventional, homogeneous culture systems. In addition, it is possible that the physiological responses of organisms growing in nutrient gradients may differ from those growing in homogeneous culture (13, 21).

One approach to mimicking the spatial and temporal heterogeneity of natural habitats is to use chambers containing gel-stabilized media within which chemical gradients may be imposed. Pioneers of this approach include Caldwell, Wimpenny, and others (whose work is reviewed in reference 25), who created one- and two-dimensional gradients (7, 8, 23, 27, 28) and used these to study the growth and behavior of pure or defined mixed cultures of bacteria as well as of microbial communities in situ in sediments (6, 18). Another approach is to entrap chemoeffectives solutes in slow-release polymers that allow the establishment of long-term gradients in soft agar plates (14). Recently, a gradient plate that allows the rapid establishment of two-dimensional diffusion gradients in a thin agar slab was developed (29). This system is well suited for the study of diffusional dynamics and microbial responses within biofilms but is less well suited for studies of motility and chemotaxis.

In an effort to extend the development of gradient systems for microbiological studies, we have developed a simple diffusion gradient chamber (DGC) capable of establishing long-term continuous gradients of small soluble molecules or gases. This article describes the construction and use of such cham-

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bers along with a theoretical and empirical evaluation of the
diffusive behavior of solutes within them. In addition, the
migratory response of E. coli to gradients of known attractants
in the chamber was characterized, and we demonstrated the
separation of two known species of bacteria in the chamber on
the basis of their motility and chemotactic properties.

MATERIALS AND METHODS

DGC. The DGC illustrated in Fig. 1 is part of a gradient
system (described below) now produced commercially by Koh
Development, Ann Arbor, Mich. The main chamber, which
consists of an arena and the recesses that hold the reservoirs,
is machined from a block of polycarbonate (PC). There is an
opening between each recess and the arena. The reservoirs are
also machined from PC and contain stainless-steel inlet and
outlet ports (1.0- and 2.5-mm inside diameters, respectively). A
0.05-μm-pore-size PC filter membrane (Poretics Corp., Liver-
more, Calif.) and a Viton gasket are placed between each
opening into the arena and its corresponding reservoir. The
reservoirs are secured to the DGC with thumbscrews. The
membrane allows diffusion of small molecules from the reser-
voirs into the gel, which is contained in the arena, and it
prevents organisms from moving from the gel into the reser-
voirs. The volume of each reservoir is 3 ml. The total volume
of the arena, excluding the reservoirs, is 70 ml. The arena is
filled with 40 ml of 0.15% agarose gel-stabilized medium via a
fill port. This creates a layer (1.5 cm thick) that covers the
openings between the arena and each reservoir. The lid and
bottom plates are clear PC and are fastened by screws. Some
useful features of this design are as follows. (i) The assembled
DGC can be autoclaved and kept free from contamination
during filling of the main chamber and operation. (ii) By
continuous purging of the headspace with N₂ or other gas
mixtures through gas ports, the atmosphere in the chamber can
be controlled. (iii) The transparent lid and bottom plate allow
microbial growth and migration to be observed and recorded
photographically and also permit illumination for growth of
light-dependent microbes.

Gradient system. The DGC was placed on a transilluminator
box (TB) which can accommodate up to three gradient
chambers. The dimensions of the TB were 41 by 27.5 by 10.5 cm; the
two long sides each held a 12-in. (ca. 30-cm) fluorescent light
fixture (single 8-W, cool white bulb). The insides of the TB
were white, and a piece of black felt was placed on the bottom
of the TB to provide contrast. This design provided cool,
diffuse, even illumination from beneath the DGCs, which was
essential for visualization of microbial growth patterns in the
chamber(s). The TB also had a bracket for mounting a camera
above the DGCs to record growth patterns photographically.

Solutes contained in Erlemeyer flasks were continuously fed
through the reservoirs of the DGC via 0.8-mm (inside diam-
ter) tubing. The flow rate was controlled with a multichannel
peristaltic pump (Watson-Marlow, Inc., Concord, Mass.). An
effluent chamber was mounted on a stand next to the TB and
served three functions. (i) It consolidated all of the reservoir
outflows into one large waste container. (ii) It served as a
sterile break in the liquid flow. (iii) It permitted control of back
pressure in the chamber reservoirs by varying the height of the
inlet of the effluent chamber relative to the height of reservoir
outlets. This was critical since excessive back pressure may
cause bulk flow of liquid through the membrane, thereby
flooding the gel; however, insufficient back pressure may cause
siphoning of liquid from, and shrinkage of, the gel.

The DGC, effluent chamber, and feed sources were each
autoclaved separately with open ends of tubing capped to
maintain sterility. After sterilization, the DGCs were set on the
TB, the feed lines were connected to the reservoir inlets, and
the effluent tubes were connected from the reservoir outlets to
the effluent chamber. Sterile molten agarose medium was
cooled to 40°C and then injected into the arena of each DGC
via the fill port with a sterile 50-ml syringe equipped with a
14-gauge cannula.

Evaluation of gradient formation in DGCs. A glucose
gradient was established by using a source feed that contained
approximately 10 mM glucose in deionized H₂O. Diametri-
cally opposed to the source reservoir was the sink reservoir,
which was fed with deionized H₂O only. The two side reservoirs
were sealed off by substituting a piece of silicone sheeting (Silastic;
Neither the feed solutions nor the chamber was autoclaved for
these experiments; instead, 0.01% KCN was added to the gel
(0.15% agarose in deionized H₂O) and to the source feed to
inhibit microbial growth. After the gradient was initiated, the
chamber lid was removed at prescribed times, and samples of
the agarose gel were taken from points along a transect (measured with a ruler) down the center line of the arena.
Sampling was done by using a 1-ml syringe equipped with a
25-gauge needle. The maximum sample size was 200 μl. Just
prior to assay, the samples were melted in a microwave oven and
subsampled with a pipettor while still warm to ensure
accurate volume measurement of the agarose. Glucose was
quantified by reaction with glucose oxidase and horseradish
peroxidase by using a commercial assay kit (Sigma Chemical
Co.).

Bacterial strains. The following E. coli strains (all strepto-
mycin resistant) with relevant phenotype and genotype were
used: HCB 33 (= RP437), wild type for motility and che-
taxis (30); HCB 137 (= RP3098), nonmotile, lacks flagella
because of a deletion in flagellin genes (ΔflaI-flaH) (30); HCB
437, nonchemotactic, contains a deletion of its signal transduc-
ing chemotaxis genes (ΔcheAΔcheZ) (30), rotates its flagella
exclusively counterclockwise and runs only (30); HCB 483,
nonchemotactic (ΔcheAΔcheZ) but is capable of runs and
tumbles (30); RP 2359 (tur), deleted for the aspartate che-
taxis receptor protein. These strains were maintained either on

FIG. 1. Diagram of the DGC. An exploded view of one of the
reservoir units is shown on the right side. See Materials and Methods
details.
tryptone medium or M63 minimal salts (30) with 10 mM glycerol. For E. coli strains, the M63 medium was supplemented with the amino acids required for growth (histidine, leucine, methionine, and threonine) and streptomycin as described previously (30).

*Pseudomonas fluorescens*, ATCC 13525, was obtained from the American Type Culture Collection, Rockville, Md.

**Response of E. coli to gradients.** For chemotaxis experiments with *E. coli*, the M63 medium supplemented with the required amino acids (2 μg/ml) and glycerol (2 mM) was used as the basal medium for source and sink feeds as well as for the gel-stabilized medium contained in the arena. In addition, the gel-stabilized medium only was supplemented with streptomycin (125 μg/ml). The source feed contained the chemotacticant. The chambers were sterilized and set up as described above. The source and sink feeds were pumped through the respective reservoirs at a flow rate of 2.5 ml/h. For all experiments, the DGCs were kept in a 30°C constant-temperature room. After a gradient was established for 12 to 20 h, the center of the arena was inoculated with a mid- to late-log-phase culture of *E. coli* grown at 30°C in M63 medium containing 10 mM glycerol. Inoculation was accomplished by lifting the lid of the chamber and inserting a pipette tip containing 7.5 μl of culture into the bottom of the agarose and then withdrawing it while simultaneously expelling the inoculum. The lid was replaced immediately. After inoculation, photographs were taken at regular intervals (see below) to record the growth patterns of the cells.

**Separation of E. coli and P. fluorescens.** Three DGCs with M63 medium plus 2 mM glycerol and amino acids were set up in parallel, as described above; streptomycin was omitted. Gradients of aspartate (0.1 mM at the source) and valine (0.25 mM at the source) were initiated from reservoirs diametrically opposed to one another. The other two reservoirs were sealed off. Gradients were allowed to establish for 18 h prior to inoculation with *P. fluorescens* and/or *E. coli* HCB 33, each grown to mid- to late log phase (optical densities at 600 nm of 0.48 and 0.40, respectively) on M63 medium containing 10 mM glycerol. One DGC was inoculated with 2.5 μl of *P. fluorescens* only, another was inoculated with 2.5 μl of *E. coli* only, and a third was inoculated with 5 μl of a 1:1 mixture of the two bacteria. Growth and migration patterns were recorded photographically (see below). When it appeared visually that good separation of the two species had occurred, 10-μl samples were removed aseptically with a pipettor from selected points in the gel, diluted, and spread in duplicate onto plates of tryptone medium. Two sets of spread plates were made for each sampling site, one that contained streptomycin (125 μg/ml) and one that did not. The plates were incubated at 30°C for 48 h, after which time CFUs were enumerated. To differentiate between *E. coli* and *P. fluorescens*, the numbers of CFUs in the medium with streptomycin, which permitted growth of *E. coli* only, and the medium without streptomycin, which permitted growth of both species, were compared. In addition, 20 random colonies were picked from each set of plates and tested for their oxidase reaction by using a commercially available oxidase test reagent (Difco Laboratories, Detroit, Mich.).

**Photography and image analysis.** A Minolta Maxxum 7000i 35-mm camera equipped with an interval timer and autowinder that permitted automatic exposure at a preset interval was used for photography. The TB light was also set on an interval timer synchronized with the camera intervalometer. Kodak T-MAX 400 black-and-white print film was used at ASA 800; the exposure was f 9.5 at 1/60 s. For image analysis, the black-and-white photographs were scanned as 4-bit grayscale images into a Macintosh LC III computer. Density profiles of microbial growth and measurements of the movement of the growth front were made by using NIH Image v. 1.52 (available as public-domain software via anonymous ftp from zippy.nlm.nih.gov). Alternatively, measurements of the migration front were made directly from the black-and-white prints with calipers to the nearest 0.1 mm. In either case, the corresponding migration rates were corrected for magnification due to the printing enlargement process to give actual values. For all reported time series, time zero was considered the time of inoculation and not the time the gradient was initiated.

**Chemicals.** For chemotaxis experiments, chemically synthesized L-aspartic acid, L-valine, and L-serine were purchased (K&K Fine Chemicals, Costa Mesa, Calif.). All other chemicals were of reagent grade.

**Mathematical modeling.** Each reservoir on the chamber constitutes a potential source or sink for a solute of interest. A continuous solute gradient is established across the arena from a source (high concentration) to a sink (low concentration) by simulation of molecular diffusion. Within a four-sided chamber, at least four intersecting gradients can be established in two dimensions (the x and y axes).

Consumption (or production) of chemoactive solutes by cells can affect the local gradients and, hence, the migration rate of the cell. Thus, even though the DGC is relatively simple to use, the interrelated physical and biological events that occur simultaneously within the gradient chamber make it an inherently complex system. For this reason, a mathematical model, aimed at predicting the simultaneous biological and physical events taking place in the chamber, has been developed to complement the empirical studies. The model consists of non-steady-state conservation equations for the chemoactive compounds coupled with constitutive equations describing the rates of migration and chemical reaction of these species.

The non-steady-state conservation equation describing the simultaneous accumulation, transport, and reaction of the substrate (S) in the semisolid gel is:

\[ \frac{\partial S}{\partial t} = -\nabla \cdot N_i + r_i \]  

where \( N_i \) is the substrate flux vector, \( r_i \) is the volumetric production rate of \( S \), and \( t \) is time. Because there is no bulk convection of fluid through the semisolid gel and the substrate concentrations are presumed to be relatively dilute, the substrate flux can be expressed by using the following form of Fick’s law of diffusion:

\[ N_i = -D \nabla S \]  

where \( D \) is the molecular diffusion coefficient. Combining equations 1 and 2 gives:

\[ \frac{\partial S}{\partial t} = D \nabla^2 S + r_i \]  

Boundary conditions need to be specified to calculate the concentration profile across the gel. The membrane between the liquid reservoir and the gel may provide a significant resistance to substrate mass transfer. This effect may be mathematically described by using a convection boundary condition:

\[ N_s = -D \nabla S = K_s (S_s - S) \]  

where \( K_s \) is the mass-transfer coefficient, \( S_s \) is the bulk substrate concentration in the reservoir, and \( S \) is the substrate concentration in the gel adjacent to the membrane. The
mathematical model was solved by using a finite-difference approach on a Convex C-220 supercomputer. All calculations were performed with double precision. To verify the computer model, the computer simulations were compared with analytical solutions of equations 3 and 4 for the limiting case of one-dimensional, non-steady-state substrate diffusion without reaction.

RESULTS

Evaluation of gradient formation. Diffusion of glucose into the gradient chamber was measured to evaluate the best means of establishing a gradient and to verify the mathematical model. The diffusion coefficient for glucose used in these calculations was $6.7 \times 10^{-3}$ cm$^2$/s (24). This value has been measured for glucose in deionized H$_2$O at the same temperature, and previous studies have shown that the diffusional flux of small molecules like glucose is virtually unaffected by the presence of highly porous, cross-linked immobilization supports such as 2% calcium alginate (11). The value of $K_s$, $2.2 \times 10^{-3}$ cm$^2$/s, was determined experimentally by fitting the model to the glucose profiles shown in Fig. 2. As is evident from Fig. 2, using these values for $D$ and $K_s$ yields good agreement between predicted and experimental values for the glucose gradient. However, it is important to note that the theoretically predicted curves do not extrapolate to the actual concentration of glucose in the source reservoir (9.3 mM). This indicates that the PC filter membrane between the reservoir and the gel imposes a significant mass-transfer resistance.

The mathematical model was then used to predict the long-term profiles of glucose concentration across the gel. The linear, steady-state profile is approached asymptotically; however, approximately 2 weeks is required for glucose to reach 95% of its steady-state concentration throughout the gel. Thus, for convenience, the experiments reported here were begun with substrate gradients that had not yet achieved steady state. Empirical measurements showed that with 10 mM glucose in the source reservoir, approximately 24 h was required to achieve a detectable concentration of glucose (ca. 20 μM) in the center of the arena (data not shown). About 4 days was required for a detectable amount of glucose (>10 μM) to reach the opposing sink. After 1 week, the gradient from source to sink (5.6 cm) for either glucose or pyruvate spanned approximately 2 orders of magnitude within the region of the arena available to sampling.

Response of E. coli to amino acid gradients. The responses of E. coli to gradients of aspartate and serine are shown in Fig. 3. As expected, the cells grew and migrated more rapidly toward the source (south [S]) of attractant, aspartate or serine, than in the opposing direction (north [N]) toward the sink. However, there were important differences in the growth and migration patterns elicited by the two amino acids. The threshold response concentrations for serine and aspartate, reported to be $1 \times 10^{-7}$ and $3 \times 10^{-8}$ M, respectively (15), suggest that E. coli should perceive, and be attracted to, aspartate at lower concentrations than for serine (the diffusion coefficients of these two amino acids should be nearly the same). Indeed, this appeared to be the case; within 10 h after inoculation, the cells began to show a strong bias toward aspartate, but they did not show a strong bias to serine until about 19 h after inoculation (Fig. 3). In this experiment, the average rate of migration towards aspartate from 10 to 245 h was 1.2 mm/h, and toward serine, it was 0.9 mm/h. The peak rate toward aspartate was 1.6 mm/h versus 1.45 mm/h for serine. Results from separate experiments revealed similar trends: the average rate of migration toward aspartate was 0.94 mm/h (standard deviation [SD], 0.26; n = 6), with a peak rate of 1.3 mm/h (SD, 0.33; n = 6); the average rate toward serine was 0.87 mm/h (SD, 0.19; n = 3), with a peak rate of 1.0 mm/h (SD, 0.19; n = 3).

The migration rates of E. coli toward (up gradient) and away from (down gradient) aspartate in another experiment are plotted in Fig. 4. The migration rate of the front moving up gradient increased rapidly between 9 and 15 h, reaching a plateau of about 1.2 mm/h before decreasing again as the cells approached the aspartate source. However, the rate of migration down the gradient was 0.56 mm/h. This latter value was almost identical to the rate (0.64 mm/h) of cells exposed to a uniform concentration of 2 mM glycerol with no aspartate present, and it was taken to represent the rate of nonbiased migration (however, see Discussion). When only glycerol was present, the cells grew and spread out as a uniform ring from the point of inoculation (results not shown).

The morphology of the cell front moving up a gradient of attractant differed from the front moving down it. Figure 5 shows densitometric profiles made by scanning photographs of cells responding to aspartate as illustrated in Fig. 3. The cell front moving toward the source of aspartate was initially broad and had a density similar to that of the rest of the growth pattern, but with time it took on the appearance of a wave, with a locally high cell density at the leading edge. Cells moving toward the sink (down gradient) also formed a sharp band at the leading edge of the front, but it had a lower cell density and moved more slowly.

Another intriguing component of the growth and migration patterns was the pronounced lateral migration (i.e., the southeast [SE] and southwest [SW] directions of Fig. 3) in response to the strong chemoattractant aspartate. It seemed likely that lateral migration reflects consumption of a potent chemoattractant. In the absence of cells, the gradient perpendicular to the source should be very shallow, probably too shallow for the cells to sense. However, if cells were present to consume the aspartate, they would create a gradient in this orientation to which they might respond. To test this hypothesis, E. coli was exposed to a gradient of α-methyl aspartate, a nonmetabolizable analog of aspartate (15). The results are shown in Fig. 6. In this case, there was a much smaller bias toward the α-methyl...
aspartate source (S) than typically exhibited toward aspartate and no lateral distortion of the front towards the SE or SW. The average rate of migration toward α-methyl aspartate from 16 to 47 h was 0.51 mm/h (about half that exhibited towards aspartate [see above]), whereas in the opposite direction, the rate was 0.46 mm/h. Although the difference was small, the bias towards α-methyl aspartate was distinct and observed repeatedly. These findings are consistent with the hypothesis that

**FIG. 3.** Chemotactic response of *E. coli* HCB 33 to gradients of aspartate and serine. Two gradient chambers were set up in parallel, one receiving 0.1 mM aspartate and the other receiving 0.1 mM serine, both introduced by diffusion from the S reservoir. The N (sink) reservoir received M63 medium containing glycerol but no attractant; the E and W sides were sealed off. The chambers were inoculated in the center with *E. coli* cells 13 h after the gradients were initiated. The numbers in the upper left corners indicate the time (in hours) that the photographs in that row were taken after inoculation. Note that the migration of *E. coli* toward the aspartate source began within 10 h of inoculation, while an obvious bias toward serine was not evident until about 19 h. Moreover, the direction of bias toward serine is almost entirely parallel to the gradient, while the migration toward aspartate displayed prominent lateral biases as well. Also note that in both cases the front moving up the gradient (toward S) was less well defined but appeared to have a higher cell density than the front moving down the gradient.

**FIG. 4.** Migration rates of a front of *E. coli* cells moving up (■; S direction in Fig. 3) and down (○; N direction in Fig. 3) an aspartate gradient. The rates from an experiment similar to that shown in Fig. 3 were plotted.

**FIG. 5.** Densitometric scans of a population of *E. coli* cells responding to aspartate (data from Fig. 3). Note that the front moving toward aspartate is diffuse initially but becomes sharper as the cell density at the front increases with time. The 0- and 50-mm points represent the sink and source, respectively, of the aspartate gradient. Inoculation was at the 25-mm point. The relative density of the cell population is plotted in arbitrary units (0 to 100).
consumption of aspartate by migrating cells increases the steepness of the gradient, thereby stimulating forward and lateral cellular migration compared with that seen with the nonmetabolizable analog.

To demonstrate that both motility and chemotaxis were important to the growth and migration patterns of bacteria in the DGC, *E. coli* cells mutant in chemotaxis or motility were tested for their response to aspartate gradients. The results of these studies are depicted in Fig. 7. *E. coli* RP 2359, which lacks the receptor for aspartate chemotaxis, exhibited wild-type motility but showed no biased response to aspartate (Fig. 7a). Instead, cells migrated radially and symmetrically from the inoculation point at approximately the same rate as the wild type moving without bias. *E. coli* HCB 483, which is gutted for chemotaxis genes but maintains the capacity to run and tumble, also migrated symmetrically from the point of inoculation (Fig. 7b) as would be predicted for cells locked in an unbiased random walk. However, the average rate of migration of this strain was 0.16 mm/h, much lower than the rates displayed by HCB 33 in glycerol alone. Wolfe and Berg (30) observed the same phenomenon for a mutant with a similar phenotype; they suggested the reduced motility of the mutant in agar was due to a difference in the run-and-tumble frequency of the mutant compared with that of the wild type. Strain HCB 437 is also gutted for chemotaxis genes and is motile; however, its flagella spin only counterclockwise. Thus, it executes runs but no tumbles. This strain grew but did not migrate appreciably from the inoculation point (Fig. 7c). This trend was also observed by Wolfe and Berg (30), who suggested that cells locked in a running only mode of behavior may collide with, and become lodged in, pieces of the agarose gel. Since they do not tumble and reposition themselves, most remain entrapped. Strain HCB 137, which is immotile because of a lack of flagella, only grew at the point of inoculation and did not migrate appreciably (Fig. 7d).

**Separation of *E. coli* HCB 33 and *P. fluorescens* with the DGC.** Experiments were conducted to test whether bacteria could be separated with the DGC on the basis of their motility and chemotactic responses. Solutions of valine and aspartate were each fed into separate reservoirs on opposite ends of the DGC. Aspartate and valine are a chemotaxant and a repellent, respectively, for *E. coli* (15). In contrast, we determined in separate experiments with the DGC that *P. fluorescens* was neither attracted to nor repelled by either of these amino acids at the concentrations used. Thus, if a DGC were inoculated with a mixture of these cells, *E. coli* should migrate rapidly toward aspartate but be repelled by valine, while *P. fluorescens* should migrate without bias, unaffected by either amino acid. The results are shown in Fig. 8. As expected, *E. coli* migrated rapidly toward aspartate (source in the S position) at an average rate of 1.0 mm/h, but its migration toward valine (N) was arrested after approximately 20 h. By contrast, *P. fluorescens* migrated uniformly from the point of inoculation at a rate of 0.8 mm/h. *P. fluorescens* also formed two to three concentric bands, an inner band that formed at the surface of the agarose and an outer band(s) that was submerged in the...
FIG. 8. Separation of *E. coli* HCB 33 and *P. fluorescens* in a gradient of aspartate and valine. Three parallel DGCs were set up to contain an aspartate source (0.1 mM) on the S side and a valine source (0.25 mM) on the N side. The E and W reservoirs were sealed off. All chambers were inoculated 18 h after the gradients were initiated. The chamber in the left column of panels was inoculated with *E. coli* only; the chamber in the right column of panels was inoculated with *P. fluorescens* only; and the chamber in the middle column of panels was inoculated with a 1:1 mixture of the two organisms. The numbers in the upper left corners indicate the time (in hours) that the photographs in that row were taken after inoculation. At 27 h, the chamber containing both species was sampled at various sites for organisms (see text).

agarose. Inasmuch as all bands moved at nearly the same rate, their segregation may have been due to a response to an oxygen gradient within the gel or to some undefined surface effect. In any case, when the two species were mixed in a 1:1 ratio and inoculated in the center of the opposing gradients, the resulting pattern looked like the two pure culture patterns superimposed upon one another. As a result of its biased random walk towards aspartate, *E. coli* migrated more rapidly in the S direction and dominated *P. fluorescens* in that region of the gel. In contrast, *P. fluorescens* was not repelled by valine and migrated steadily in the direction of the valine source (N), where it dominated *E. coli*. Differential plate counts at 28 h revealed that only *E. coli* cells (Str⁺, oxidase negative) were present along the southern edge of growth at a concentration of 3.8 × 10⁷ CFU/ml. A sample taken approximately 1 cm S of the center of the chamber yielded 4 × 10⁷ CFU/ml. *E. coli* was predominant there as well, but 2 of 20 colonies tested were *P. fluorescens* (Str⁺, oxidase positive). In contrast, a sample taken from the northern edge of migration yielded 4.2 × 10⁷ CFU/ml and consisted of only Str⁺, oxidase-positive cells, indicating that they were all *P. fluorescens*.

**DISCUSSION**

Chamber design and performance. The DGC is easy to assemble and simple to use, and it is resistant to repeated autoclaving. Moreover, it permits viewing of microbial responses as well as direct sampling for quantitation of bacterial
numbers or chemical analysis. With the DGC, it is possible to establish two-dimensional gradients of various compounds. Theoretically, several substances could be fed simultaneously through the same source reservoir, thus expanding severalfold the number of compounds diffusing into the arena. In addition, because the gas composition of the headspace can be controlled, it is possible to operate the chamber under either oxic or anoxic conditions. Inasmuch as the solutes constituting the gradients are continuously replenished, a wide range of substrate concentrations can be maintained over long periods of time, thus better simulating natural environments. Documentation of bacterial growth and migration patterns in the gel via time-lapse photography allows quantitation of the rates at which bacteria respond to imposed gradients.

**Gradient characteristics.** Solute gradients established in the chamber were continuous and could be predicted on the basis of the laws of Fickian diffusion. Our mathematical model fit the empirical data well. However, an important practical finding was that the PC filter membrane acts as a significant barrier to diffusion, which results in a substantial drop in solute concentration across the membrane between the reservoirs and the gel. Another consideration was that, owing to the relatively long diffusion path length of the chamber, it would take several hours for a steady-state gradient to become established. However, since the approach to the steady state is asymptotic, the rate of concentration change at any given point in the gel decreases exponentially. Within a few days, the change in solute concentration at a given point becomes negligible compared with the range of concentrations to which bacterial sensory and uptake systems are responsive, which can often span orders of magnitude (5). Therefore, and considering that the bacteria themselves may immediately begin to alter any gradients of consumable solutes, it would not be necessary to wait for steady-state gradients to become established before initiating most experiments.

Despite the non-steady-state nature of the gradients, important trends can be established from qualitative and quantitative observations of cell responses in the DGC. Empirical measurements can be made to determine the rate at which bands of bacteria migrate. Plate counts and chemical assays can be done to determine the concentration of viable cells and solutes in various parts of the gel. Furthermore, computer simulation models can reproduce the non-steady-state conditions in the DGC. Incorporation into these models of the appropriate parameters for cell growth and movement should make it possible to accurately model microbial behavior within the DGC. We are currently refining our mathematical model toward this end.

**Chemotactic response of E. coli.** On the basis of a current understanding of its chemotactic response (4, 15, 16), E. coli was found to behave in a predictable manner by biasing its migration in response to imposed gradients of chemotactants in the DGC. In the case of aspartate, growth and movement of the cell population began with little or no visually apparent bias toward the attractant. As the aspartate concentration at the cell front increased, the cells responded with a biased cell migration toward the aspartate source. Thereafter, the rate of migration increased to a plateau and then fell again as the cell front neared the source, where their rate of metabolism of aspartate was probably not rapid enough to reduce the aspartate concentration below the saturation level for the aspartate receptors, approximately $10^{-4}$ M (30). When 0.5 or 1 mM concentrations of aspartate were used to initiate gradients, the distance the cells migrated chemotactically before returning to the unbiased rate of migration was inversely related to the reservoir concentration of aspartate (results not shown).

A note of caution is warranted, however, in estimating the threshold concentration of an attractant that elicits a response in the DGC. On the basis of preliminary calculations using our mathematical model, the reported threshold concentration of aspartate detectable by E. coli, $3 \times 10^{-5}$ M (15), should reach the center of the gel 13 h after initiation of an aspartate gradient from a 0.1 mM source reservoir. For most experiments, the DGC was inoculated 16 to 20 h after initiation, but a visible bias in migration of the cell front was not evident until an additional 4 to 8 h had elapsed (20 to 24 h after gradient initiation). This discrepancy suggests that the DGC may overestimate threshold concentrations compared with the traditional capillary assay for chemotaxis (2), which is independent of growth and which measures the response of individual cells in a population. Of course, it is possible that individual cells in the DGC are responding sooner than we can determine simply because chemotaxis in the DGC is subject to a stimulation response: that is, it requires sufficient growth of the population to be visible. Alternatively, in the DGC, the gradient of chemotactant the cell front initially perceives may be too shallow to elicit a noticeable response until cell growth and metabolism of the attractant create a gradient steep enough for the cells to respond with a noticeable bias. These alternatives are currently being evaluated.

Observations of the migration of E. coli indicated that consumption of the chemotactant affected the rate of biased cell migration and the resultant growth pattern. Apparently, with 0.1 mM aspartate in the source reservoir, metabolism of aspartate was rapid enough to create lateral gradients of attractant sufficient to induce lateral chemotactic migration. When the nonmetabolizable analog of aspartate, α-methyl aspartate, was the attractant, the overall bias was significantly decreased and only parallel to the direction of the gradient. Serine, which can be taken up and metabolized by E. coli, may represent an intermediate case, where there was a strong bias parallel to the gradient but little lateral bias. Whether the apparent lack of lateral bias was due to limited uptake of serine, less sensitivity of the chemosensory apparatus for serine, or a combination of these two effects will require more study.

Substrate consumption and cell growth also affected the migration rate. In all cases, the rates of both unbiased and biased cell migration increased after the first few hours of growth after inoculation (Fig. 4). This was probably a result of an increase in bacterial density, which, in the case of metabolizable attractants, will effectively cause a steeper gradient to form. It is also known that cells in early exponential growth swim more slowly than cells in late exponential growth (3); thus, the phase of growth may also be a factor. The fact that cells migrating up an attractant gradient initially formed a diffuse band, which later coalesced into a much sharper band, is interesting and reminiscent of a propagating wave. Such waves have been observed in other experimental systems and attributed to cellular consumption of a chemotactant leading to locally steep gradients (1, 22). The DGC is well suited for studying the properties of chemotactic waves because it allows the establishment of mathematically quantifiable concentration gradients.

The sharp band of increased cell density that constitutes the front migrating down the attractant gradient requires further study (e.g., see left leading edge of cells in Fig. 5). We have presumed that cells in this front are migrating in an unbiased manner because their migration rate is similar to that of cells in a uniform concentration of glycerol, which is not a chemot-
tractant for E. coli (30). However, we do not wish to imply that this front is moving at a rate governed by completely random motility. It is possible that these cells are responding with a small aerotactic bias to the oxygen gradients that inevitably result from their growth. The increased density of this front may also be due to a localized increase in growth rate supported by glycerol, whose concentration should be higher at the leading edge of the front than just behind it.

Separation of E. coli and P. fluorescens. With the DGC, it was possible to separate two different species on the basis of their differential response to gradients of attractants and repellents. While this experiment was not specifically designed as a competition experiment, it suggests that the DGC may be very useful for assessing the conditions under which chemotaxis provides an organism a competitive advantage. Theoretical calculations have shown that chemotaxis could lead to competitive advantage in some cases (12), but there is a paucity of empirical data to support these claims.

Optimizations for a gradient chamber system. The DGC should prove to be a versatile tool for studying chemotaxis in bacteria. For example, it facilitates the creation of multiple gradients to study how organisms might respond to mixed, potentially conflicting signals or to attractants while undergoing nutritional stress, such as starvation for phosphate or nitrogen. Because the chamber can be run anoxically, it would also be possible to use it to study chemotaxis in strict anaerobes. Another application is in studies of the environmental control of gene expression, with appropriate reporter genes linked to a gene of interest. By using this approach, the response of individual genes, as well as of organisms, to gradients of effector molecules could be studied, and the combined effects of concentration gradients of different effectors could be determined rapidly. Two other types of gradients that we have recently established in the DGC that add to its versatility are temperature gradients and vertical oxygen gradients. The former have been created simply by running hot and cold water through opposing, sealed reservoirs; the latter have been created by substituting a gas-permeable membrane for the bottom plate and placing beneath it a second gas reservoir (9).

An area of particular interest to us is the use of the DGCs for the isolation of organisms with novel properties. Such attempts need not depend strictly on the ability of organisms to migrate but simply on the ability to grow in a portion of the arena containing a suitable microenvironment. For example, we have used DGCs to isolate halotolerant and halophilic bacteria capable of using aromatic hydrocarbons. When a soil sample contaminated with oil brine was inoculated uniformly over the surface of a DGC with perpendicular salt and toluene gradients, distinctive patterns of growth formed in response to the different solute concentrations in various regions of the arena. The chamber provided information about the general community response to these conditions, the synergistic effects of the dual stress of high salt and aromatic compound concentrations, and the response of individual organisms. From such experiments, it has been possible to isolate a number of organisms capable of growth on toluene at salt concentrations of up to 10% (wt/vol) NaCl (9) as well as a new halophilic archaean, Haloferax sp. strain D1227, capable of catabolizing aromatic compounds (10).

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