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In Situ Mutagenesis and Chemotactic Selection of Microorganisms in a Diffusion Gradient Chamber

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ABSTRACT

A new method has been developed to rapidly generate and select microbial strains having increased resistance to an inhibitory compound. The method combines *in situ* mutagenesis with use of a continuous gradient of the inhibitor to sort cells according to their resistance levels. Microbial chemotaxis is induced to accelerate the selection process. The method was used to develop a strain of *E. coli* having a feedback-resistant DAHP synthase enzyme. An unsteady-state mathematical model of the process has been developed. The model, that can reproduce key trends observed experimentally, was used to explore the effects of chemotaxis on the efficiency of the selection process.

Index Entries: Chemotaxis; selection; *E. coli*; mutagenesis; DGC.

INTRODUCTION

In many applications, it is desirable to increase a microbe's tolerance of an inhibitory substance. In biocatalysis, cells may need to function either in high concentrations of a toxic substrate or product, or under extreme conditions (e.g., low pH). Metabolic engineering involves alteration of the microbe's natural patterns of metabolic regulation so as to increase carbon flux through a desired biosynthetic pathway. Mechanisms for metabolic regulation include inhibition of DNA transcription and inhibition of enzymes. Pathway substrates, intermediates, or products are common inhibitors in such cases. As an example, the first committed step in the common aromatic biosynthetic pathway of *Escherichia coli* is the condensation between erythrose 4-phosphate (E4P) and phosphoenolpyruvate (PEP) to form 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP). Feedback in-

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hibition has been demonstrated to be the dominant regulatory mechanism in controlling carbon flow into this pathway *(1)*. The enzyme catalyzing the reaction, DAHP synthase, is strongly inhibited by the products of the pathway. The isozyme of DAHP synthase designated AroF is strongly inhibited by L-tyrosine *(2)*. Also, transcriptional repression of the gene encoding the AroF enzyme by tyrosine can lead to a 20-fold reduction in DAHP synthase activity *(3)*.

Enzyme-level inhibition may be ameliorated by changing the amino-acid sequence of the enzyme, so as to improve the enzyme's kinetic properties. Both random and site-specific mutagenesis of the structural gene for the enzyme have been used for this purpose. Transcription-level regulation may be altered by changing promoters, increasing the copy number of the structural gene, or modifying the regulatory gene *(4,5)*.

Regardless of which approach is taken, an improved strain must be selected after the genetic transformation. Selection can be a challenging task; in many instances, there is no rapid method to screen a large number of cells for improved properties in the presence of the inhibitor. The traditional, brute-force approach is to screen isolates for growth in medium containing high concentrations of the inhibitor *(6)*. One difficulty with this method is that the optimal inhibitor concentration for the screening is typically not known in advance. Too high a concentration may prevent even the best mutant from growing, whereas too low a concentration allows too many strains to grow. Consequently, experiments are generally repeated at different inhibitor concentrations. This redundancy makes the brute-force method inefficient and time intensive.

We have developed a new method that combines *in situ* mutatagenesis with selection based on each strain's relative tolerance to the inhibitor. The method uses a diffusion gradient chamber (DGC) to generate a continuous concentration gradient of the inhibitor across a slab of semisolid agarose. Motile cells can rapidly swim through the agarose into regions of having high inhibitor concentrations. Because only cells tolerant of the inhibitor can grow in these regions, this method sorts cells according to their relative tolerance to the inhibitor.

The new method also uses chemotaxis to enhance the selection efficiency. Chemotaxis is the ability of a microbe to sense a concentration gradient of a chemoattractant and migrate in the direction of the gradient. Many common microbes, including *E. coli*, are chemotactic *(7)*. By overlaying a chemoattractant gradient on the inhibitor gradient, cells can be rapidly drawn by chemotaxis into increasingly higher inhibitor concentrations, thus accelerating the sorting process.

This paper describes the new method and its successful application to isolate *E. coli* strains having a tyrosine-resistant AroF enzyme. A mathematical model of the DGC is presented and used to investigate how the interplay of diffusion and cell transport results in cell selection, and how the selection efficiency is enhanced by chemotaxis.

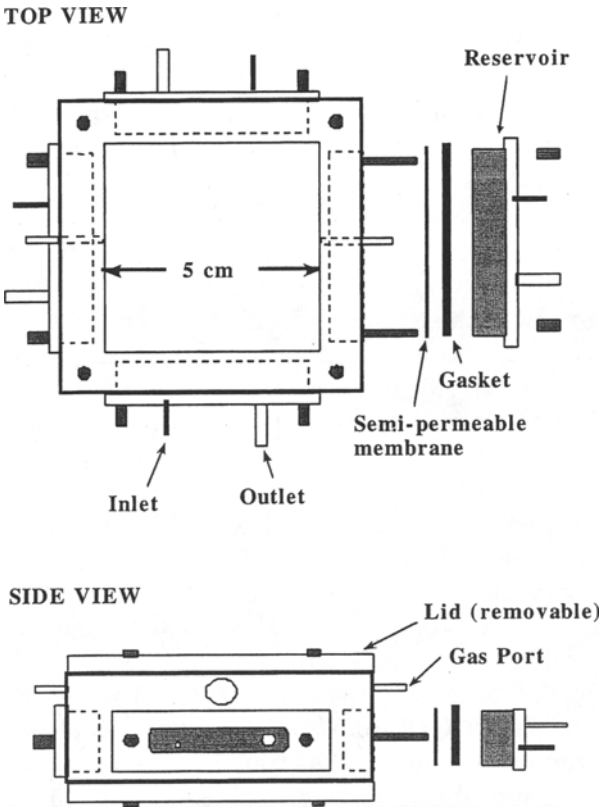

Fig. 1. Schematic diagram of the diffusion gradient chamber

METHODS

E. coli Strain

The *E. coli* strain was provided by John Frost. It was developed from host AB3248, which lacks all DAHP-synthase activity *(8)*, by the chromosomal addition of a single *aroF* gene. The AroF is 50% inhibited by 20 μ*M* tyrosine *(9)* and is strongly inhibited by the nonmetabolized tyrosine analog *m*-fluorotyrosine (m-FT) *(10)*.

Diffusion Gradient Chamber

The DGC, shown schematically in Fig. 1, has been previously described *(11)*. It was fabricated by Koh Development (Ann Arbor, MI). The polycarbonate body of the DGC contains a slab-shaped layer of dilute (0.15%) agarose. The agarose prevents convective liquid movement within the DGC yet still allows cell swimming. The agarose is surrounded on four sides by liquid reservoirs. A 0.05-μm pore-size polycarbonate membrane allows exchange of small molecules between the agarose and the reser-

voirs. When solute exchange is not wanted, a silastic silicone sheet (Dow Corning, Midland, MI) is used in place of the membrane. The stainless-steel inlet and outlet ports of the reservoir allow fresh liquid feed to be pumped through the reservoirs to maintain a constant solute concentration. When different solute concentrations are maintained in reservoirs on opposite sides of the DGC, diffusion creates a solute concentration gradient across the agarose.

The experimental protocol was patterned after Emerson et al. *(11)*. A minimal salt medium (M63) supplemented with 5 m*M* glycerol and 40 mg/L of the amino acids histidine, isoleucine, proline, arginine, valine, and serine was used in the liquid reservoirs and to prepare the agarose. The *E. coli* strain was auxotrophic for these amino acids, as well as the aromatic amino acids. The glycerol served as the carbon source for growth. Two Erlenmeyer flasks, each containing 800 mL of this supplemented M63 medium, were used to provide fresh medium to two liquid reservoirs on opposite sides of the DGC. One of the flasks (designated the source flask) was also supplemented with 125μ*M* m-FT as an inhibitor and/or 5 m*M* glucose as a chemoattractant *(12)*. The other was designated the sink flask. The remaining two reservoirs were sealed off using silicone sheets. This arrangement created a one-dimensional glucose gradient that spanned 0 to 125 μ*M* m-FT and/or 0 to 5 m*M* glucose from the sink reservoir to the source reservoir. The DGC, liquid-feed flasks, and agarose solution were all steam sterilized prior to use. Sterile transfer operations, such as pouring the molten agarose into the DGC, were carried out in a laminar flow hood.

To generate the gradient, liquid medium from the two Erlenmeyer flasks was continuously pumped through the source and sink reservoirs of the DGC. A flow rate of 2.5 mL/h was maintained using a dual-channel peristaltic pump (LKB Bromma Microperpex, Sweden). The effluent from the reservoir was carried by tubing to an effluent chamber. The elevation of the effluent chamber relative to the DGC had to be adjusted carefully, because it regulated the back pressure in the chamber reservoirs. Excessive back pressure causes flooding of the agarose, and insufficient back pressure causes agarose shrinkage because of liquid siphoning. Flow of liquid through the reservoirs was started 6 h before inoculation to initiate the gradients.

A starter culture of *E. coli* was grown in 5 mL of sterile LB medium from a single colony and then incubated at 37°C overnight with shaking. One mL of the starter culture was added to 100 mL of supplemented M63 medium containing 10 m*M* glycerol. This culture was grown (37°C and 250 rpm shaking) to stationary phase, and four 1-mL aliquots were concentrated by a factor of 16 by microcentrifugation. The center point of the DGC was inoculated with 15 μL of the 16X concentrated culture using a micropipet to disperse the cells evenly throughout the depth of the agarose as the pipet was withdrawn from the gel.

After inoculation, the DGC was stationed on a transilluminator box (TB) located in a 30°C warm room. Inside the TB, two 30-cm fluorescent

lights (single 8 W, cool white bulbs) provided diffuse illumination from about a 45° angle beneath the DGC. The bottom of the TB was covered with black felt to provide a dark background. A portion of the light was diffracted by the cells toward a CCD camera (Pulnix TM-7CN, Sunnyvale, CA) mounted directly above the DGC. Consequently, regions of the agarose containing cells appeared bright against a dark background. The TB light was turned on only when an image was being recorded. The images were recorded on a dedicated personal computer using Photofinish software (Zsoft, Marietta, GA).

In Situ Mutagenesis

The removable lid of the DGC allowed mutagenesis by ultraviolet (UV) radiation to be performed *in situ*, while the cells were growing. The method for UV mutagenesis was adapted from Miller *(13)*. Conditions giving the desired survival rate (approx 0.1%) were determined by manipulating the UV-radiation exposure time. *E. coli* cells grown on M63 agar plates were exposed to the UV radiation (Sylvania germicidal 8W lamp) from a distance of 60 cm for a duration of 0 (control), 5, 10, 15, 30, 60, 80, or 100 s immediately after plating. The optimal exposure time was found to be 10 s, which resulted in a 0.6% survival rate. An identical exposure time was used to mutagenize *E. coli* cells growing in the DGC. To confirm that UV radiation would penetrate the agarose gel, the absorbance spectrum of the agarose was determined using a UV spectrophotometer. The gel did not absorb light significantly in the wavelength range of 200 to 800 nm.

Mathematical Model

The mathematical model of the DGC system developed by Widman et al. *(14)* was modified and used to predict the performance of the DGC method for strain selection. The original model consisted of two-dimensional, unsteady-state, material balance equations for a single species of cells, a nutrient (H), and two chemoattractants. One of the chemoattractants (S) is the applied gradient (e.g., glucose), and the other (Q) is oxygen. For this study, a second cell balance was added to account for both the original (A) and the mutant (B) strains. Also, a balance was added to account for diffusion and cellular uptake of the inhibitor (P) by both strains:

$$\frac{\partial P}{\partial t} = D_P \nabla^2 P - \left(\frac{v_{aP} P}{C_{aP} + P}\right) u_a - \left(\frac{v_{bP} P}{C_{bP} + P}\right) u_b \qquad (1)$$

where D_P is the diffusion coefficient of the inhibitor; v_{aP} and v_{bP} are the specific consumption coefficients for populations A and B consuming P;

and C_{aP} and C_{bP} are the saturation constants for consumption of P by populations A and B, respectively.

The chemotactic sensitivity coefficients for both populations responding to the chemoattractants S and Q, the random motility coefficients, and the maximum specific growth rates were modified to incorporate the inhibition effects. The modified chemotactic sensitivity is given by

$$\chi'_{0ij} = \frac{\chi_{0ij}}{\left[1 + \left(\frac{P}{K_{Iij\chi}}\right)\right]} \tag{2}$$

where χ'_{0ij} is the inhibited chemotactic sensitivity for the the i^{th} population ($i = a$ or b) responding to the j^{th} chemoattractant ($j = S$ or Q), χ_{0ij} is the uninhibited chemotactic sensitivity, and $K_{Iij\chi}$ is the inhibition constant for the chemotactic sensitivity of the i^{th} population to the j^{th} chemoattractant. The modified random motility coefficient is given by

$$\mu'_i = \frac{\mu_i}{\left[1 + \left(\frac{P}{K_{Ii\mu}}\right)\right]} \tag{3}$$

where μ'_i is the inhibited random motility coefficient for the i^{th} population, μ'_i is the uninhibited random motility coefficient, and $K_{Iij\mu}$ is the inhibition constant for the random motility of the i^{th} population. The inhibited maximum specific growth rate is given by

$$v'_{iH} = \frac{v_{iH}}{\left[1 + \left(\frac{P}{K_{Iiv}}\right)\right]} \tag{4}$$

where v'_{iH} is the inhibited maximum specific growth rate for the i^{th} population growing on H, v_{iH} is the maximum specific growth rate, and K_{Iiv} is the inhibition constant for the specific growth rate of the i^{th} population. In terms of the variables defined above, the two cell balance equations are given by

$$\frac{\partial u_i}{\partial t} = \mu'_i \nabla^2 u_i - \sum_{j=S}^{Q} \chi'_{0ij} \nabla \cdot \left[\left(\frac{K_{Dij}}{(K_{Dij} + j)^2}\right) u_i \nabla j\right] + \left[\frac{(v'_{iH}H)}{(C_{iH} + H)}\right] u_i \tag{5}$$

where u_i is the i^{th} cell population (either *a* or *b*). The nutrient balance was also modified to include the inhibited maximum specific growth rates:

$$\frac{\partial H}{\partial t} = D_H \nabla^2 H \left\{\left[\frac{v'_{aH}H}{(C_{aH} + H)}\right]\left(\frac{u_a}{Y_{aH}}\right) - \left\{\left[\frac{v'_{bH}H}{(C_{bH} + H)}\right]\left(\frac{u_b}{Y_{bH}}\right)\right\} \quad (6)$$

where D_H is the diffusion coefficient for H, C_{iH} is the half-saturation constant, and Y_{iH} is the yield coefficient. Values for the modeling constants were taken from the literature *(14)* insofar as possible. Values for the inhibition constants included in *equations (2–5)* were chosen that were physically realistic and gave reasonable agreement with the experimental trends.

RESULTS

Chemotaxis Toward Glucose

In this experiment, the source flask contained glucose but not m-FT. Flow of liquid was initiated through the source and sink reservoirs 6 h before the DGC was inoculated. After 72 h, there was a clear chemotactic bias in the cells' growth pattern; at that time, cell growth extended to within 6 mm of the source reservoir, but was no closer than 18 mm from the sink reservoir.

The mathematical model was able to reproduce this chemotactic bias. Fig. 2 shows a time sequence of contour plots depicting cell concentration as a function of position. The chemoattractant (glucose) gradient is indicated by gray shading, with lighter gray indicating a higher glucose concentration. The source reservoir is on the side corresponding to the top of each figure. Bias of the cell's migration in this direction is comparable to that observed experimentally.

Generation and Selection of Feedback-Resistant *E. coli* Mutant

To select for mutants resistant to tyrosine inhibition, a similar experiment was conducted using simultaneous, parallel gradients of glucose and m-FT. The glucose gradient was intended to draw the cells into increasing concentrations of m-FT. Because the growth medium contained no tyrosine, and the native AroF enzyme was strongly inhibited by m-FT, no growth was expected near the m-FT source. After inoculation, the DGC was incubated for 3 d, and the image shown in Fig. 3A was captured. (In Fig. 3, the arrows indicate the source of the glucose and m-FT. The growth pattern is indicative of severe inhibition by m-FT, with no growth occurring in the vicinity of the m-FT source. After Fig. 3A was recorded, UV mutagenesis was performed. As shown in Fig. 3B, putative feedback-resistant

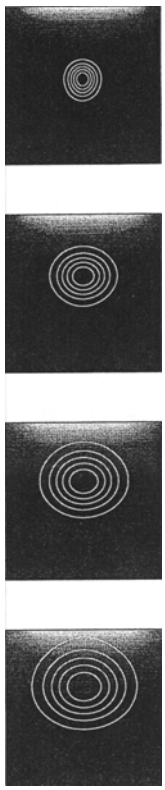

Fig. 2. Simulation of the wild-type population responding to a chemoattractant gradient. No inhibitor is present. The contour lines indicate the cell population, and the shaded gray area represents the chemoattractant diffusion gradient. The whiter areas correspond to higher chemoattractant concentrations. Each graph represents a single time-point, with the earliest time at the top of the figure.

mutants migrated into regions of higher m-FT concentration 7 d after mutagenesis. Nine days after mutagenesis (Fig. 3C), the cell pattern reached the source reservoir, indicating a high level of resistance to m-FT inhibition.

Cell samples taken from regions showing growth in high m-FT concentrations were streaked onto agar plates. One of the colonies tested expressed an AroF enzyme whose activity was unaffected by tyrosine over the range of 0 to 330 μM. Details of the methods for characterizing the mutants have been given elsewhere *(15)*.

The ability of the mathematical model to reproduce the experimental trends shown in Fig. 3 was evaluated. Figure 4 shows the predicted growth-pattern evolution in which gradients of both a chemoattractant and an inhibitor are applied, and only the population sensitive to the inhibitor is present. The effect of chemotaxis, which was significant in Fig. 2,

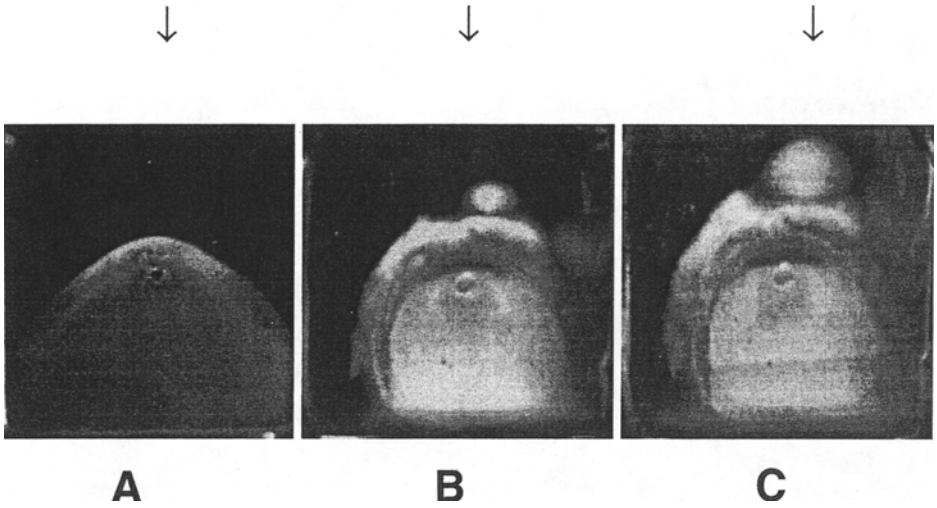

Fig. 3. **(A)** Cell-growth pattern in the DGC prior to mutagenesis (3 d of growth); **(B)** cell growth pattern in the DGC 7 d after mutagenesis; **(C)** cell growth pattern in the DGC 9 d after mutagenesis.

is overwhelmed by the effect of the inhibitor. As the concentration gradient of the inhibitor becomes established, cell growth occurs predominantly in the sink end of the DGC. This trend was observed experimentally in Fig. 3A.

Figure 5 is a simulation of a mutation event giving rise to a mutant (population *B*) that is insensitive to the inhibitor. As in Fig. 4, population *A*, which is sensitive to the inhibitor, grows preferentially near the sink. Population *B* takes longer to appear in significant concentration, because it was initiated well after population *A*, and at a lower concentration. The beneficial effect of chemotaxis in separating the two populations is evident, as population *B* moves preferentially toward the source reservoir, whereas population *A* remains predominantly near the sink reservoir. These trends are similar to those observed experimentally in Figs. 3B and 3C.

Figure 6 shows a simulation of the case where the mutation occurs in the middle of the growth pattern of population *A*, and the mutant (population *B*) was not chemotactic to *S*. In this case, the two populations were still able to be separated, but the separation is based predominantly on the influence of the inhibitor gradient and spreading caused by chemotaxis to *Q*. To estimate the benefit obtained by chemotaxis toward *S*, this simulation was then repeated with chemotaxis to *S* reinstated. The results, shown in Fig. 7, indicate that chemotaxis toward *S* does further enhance the rate of separation. Presumably, the more potent the chemoattractant (i.e., the higher the χ_{Oij} value) the greater would be the enhancement.

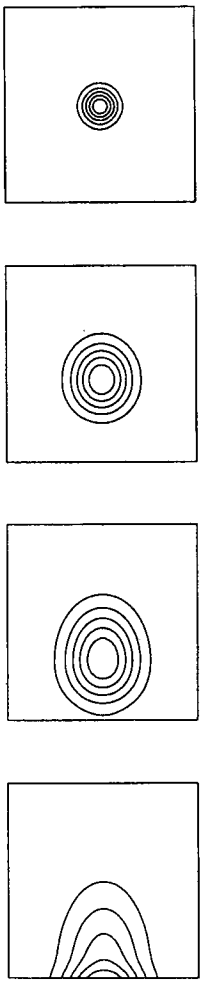

Fig. 4. Simulation of the wild-type population responding to a chemoattractant gradient and an inhibitor gradient. The contour lines indicate the cell population.

DISCUSSION

This new method for *in situ* mutagenesis and strain selection offers several advantages over traditional approaches. First, it allows different strains to be physically sorted according to their maximum tolerances of the inhibitor. The mutant having the highest tolerance would be able to grow closest to the inhibitor source, and could thus be easily selected. In the traditional methods, a fixed inhibitor concentration is used. However, the optimal inhibitor concentration for the screening is not known in advance, so some experiments would be expected to give too many isolates and others none at all.

Second, the method uses chemotaxis to draw cells toward the source of the inhibitor, thus accelerating the sorting process. Although, cell move-

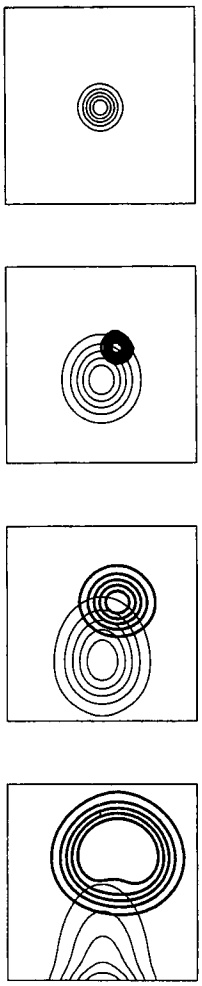

Fig. 5. Simulation of the experiment shown in Fig. 3. Simultaneous gradients of glucose and m-FT are diffusing in from the top. The wild-type strain, represented by the thin contour lines, is initially present. The mutant strain, represented by the thick contour lines, appears later near the upper right hand edge of the wild-type growth pattern.

ment by random motility could eventually result in cell sorting in the presence of the inhibitor gradient, cellular fluxes caused by chemotaxis have been shown to be orders of magnitude higher than those caused by random motility *(14)*. The mathematical model confirmed the beneficial effect of chemotaxis in selection.

Third, because the mutagenesis is performed *in situ* in a nondestructive fashion, multiple rounds of mutagenesis, or even continuous mutagenesis, may be used. As the cells become more tolerant of the inhibitor, they migrate into regions having higher concentrations. In this way, the selection pressure is automatically increased to continue challenging mutants

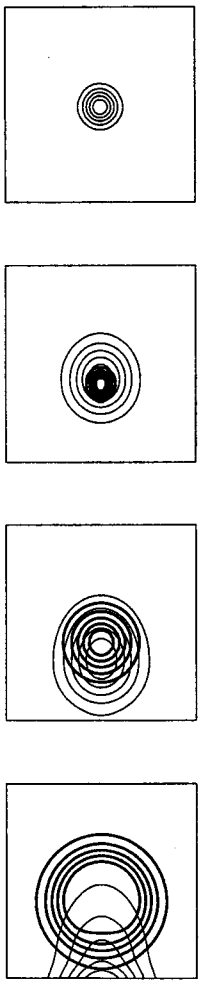

Fig. 6. Same as Fig. 5, except the mutant strain appears in the carbon-source depleted region in the center of the wild-type population, and there is no chemotaxis toward glucose.

as the evolution process continues. As a result, increasingly more tolerant strains could be developed rapidly over time with minimal additional effort.

This new method was developed because a more traditional method consisting of chemical mutagenesis followed by selection on agar plates containing m-FT failed to produce an *E. coli* mutant with a feedback-resistant AroF enzyme. The new method succeeded on the first attempt. To investigate the reproducibility of the method, the process was repeated, and was successful again. The method was found to be reasonably simple to implement, and should thus find numerous applications where enhanced tolerance to an environmental challenge is desired. Examples include metabolic engineering, in which reactants, metabolic intermediates,

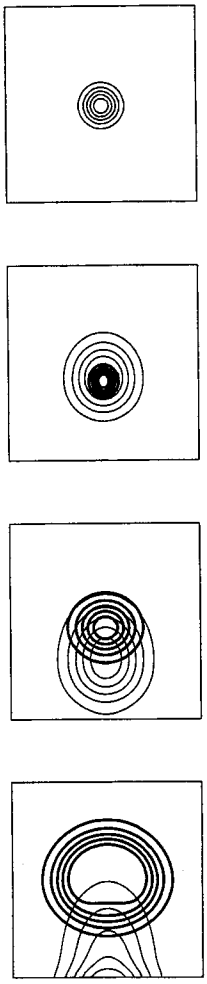

Fig. 7. Same as Fig. 6, except chemotaxis toward glucose has been reinstated.

or products may be inhibitory; environmental engineering, in which tolerance to toxic chemicals is needed; and biocatalysis using extremophiles, in which cell survival under unusual conditions (e.g., high or low pH) is desirable.

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