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Glycerol Elicits Energy Taxis of *Escherichia coli* and *Salmonella typhimurium*

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***Escherichia coli* and *Salmonella typhimurium* show positive chemotaxis to glycerol, a chemical previously reported to be a repellent for *E. coli*. The threshold of the attractant response in both species was 10^{-6} M glycerol. Glycerol chemotaxis was energy dependent and coincident with an increase in membrane potential. Metabolism of glycerol was required for chemotaxis, and when lactate was present to maintain energy production in the absence of glycerol, the increases in membrane potential and chemotactic response upon addition of glycerol were abolished. Methylation of a chemotaxis receptor was not required for positive glycerol chemotaxis in *E. coli* or *S. typhimurium* but is involved in the negative chemotaxis of *E. coli* to high concentrations of glycerol. We propose that positive chemotaxis to glycerol in *E. coli* and *S. typhimurium* is an example of energy taxis mediated via a signal transduction pathway that responds to changes in the cellular energy level.**

Chemotaxis has been extensively investigated in the enterobacteria *Escherichia coli* and *Salmonella typhimurium* (reviewed in references 6, 23, 24 and 42). Four chemotaxis receptors (methyl-accepting chemotaxis proteins [MCPs]) recognize most attractants and some repellents and transmit a signal to the interior of the cell (reviewed in references 13 and 30). In *E. coli* and *S. typhimurium*, adaptation to an attractant stimulus occurs when the CheR methyltransferase transfers methyl groups from *S*-adenosylmethionine to the γ-carboxyl group of glutamyl residues in the cytoplasmic domain of the receptor (39).

In contrast to methylation-dependent chemotaxis, chemotaxis to oxygen and redox molecules, and to substrates of the phosphoenolpyruvate:sugar phosphotransferase transport systems in *E. coli* and *S. typhimurium*, does not require an MCP or methylation of a transmembrane receptor for adaptation (5, 27). Chemotaxis to proline in *E. coli* is another energy-dependent (10), methylation-independent (26) process. The phosphotransferase signaling pathway and the MCP signaling pathway converge at the CheA protein, the central chemotaxis regulator (22, 35). Adaptation to phosphotransferase sugars may occur by restoring the prestimulus level of unphosphorylated enzyme I or by stimulating CheA to offset enzyme I inhibition of CheA (22). Convergence of the aerotaxis signaling pathway and the MCP signaling pathway also occurs at the level of CheA (35). Aerotaxis and related responses, such as electron acceptor taxis (44) and redox taxis (5), are dependent on the sensing of changes in electron transport and proton motive force (5, 37, 38). However, the mechanism of adaptation in energy-dependent behavioral responses remains unknown.

Glycerol is a widely used carbon and energy source for growth of *E. coli* and *S. typhimurium*. Early studies of chemotaxis reported that glycerol is not an attractant for *E. coli* (1, 2). Later, it was demonstrated that high glycerol concentrations repel *E. coli* cells (28). Negative glycerol taxis requires any one of the MCPs, and demethylation of MCPs is observed upon addition of 1 M glycerol to cells (29).

We now present evidence that glycerol is an attractant for de-energized *E. coli* and *S. typhimurium* and confirm that glycerol is a repellent for *E. coli* when used at high concentrations. *S. typhimurium* also shows negative taxis to high glycerol concentrations.

(A preliminary report of these studies was presented at the 91st General Meeting of the American Society for Microbiology [34].)

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used are listed in Table 1. All strains were grown at 30°C for optimal motility or at 37°C for optimal growth. The growth media, used as specified later in this report, were nutrient broth, Luria-Bertani medium, Vogel-Bonner citrate medium E (VBC) (46) with 0.7% (wt/vol) glucose (VBC-glucose) or 1.0% (wt/vol) glycerol (VBC-glycerol) added as a carbon source, or VBE, a modified VBC in which citrate was replaced with 10 μM EDTA. All media were supplemented with thymine (25 μg/ml), and VBC and VBE were supplemented with thiamine at 0.5 μg/ml and each of the required amino acids at 45 μg/ml.

Chemicals. Glycerol (99.5% purity, reagent grade) was obtained from Sigma (St. Louis, Mo.). Tetraphenylphosphonium chloride was obtained from Aldrich (Milwaukee, Wis.).

Behavioral assays. Chemotactic responses to amino acids, sugars, and glycerol were measured on VBC- or VBE-semisoft agar plates containing 0.25% (wt/vol) agar (Difco Laboratories, Detroit, Mich.), thiamine, required amino acids, a carbon source, and a chemoeffector (if different from the carbon source). Chemical-in-plug assays were performed essentially as described previously (45). Cells were mixed with semisoft agar (0.275%) in 10 mM potassium phosphate, pH 7.0.

For temporal gradient assays, cells were grown in VBC-glycerol or VBE-glycerol medium, washed three times, and resuspended in 10 mM potassium phosphate buffer containing 10 μM EDTA. A 9-μl drop of a diluted bacterial suspension (2×10^7 cells/ml) in 10 mM potassium phosphate buffer was placed on a microscope slide. The compound to be tested (1 μl) was added to the suspension, and changes in motility were recorded (40). Tumbling frequency was determined by computerized motion analysis with a VP110 video processor (Motion Analysis Corp., Santa Rosa, Calif.) and a program developed by using EXPERTVISION software (21). The time base display was from a Panasonic WJ 810 video time generator (Matsushita Electrical Industries). The responses to oxygen were measured in a microchamber ventilated with humidified nitrogen or oxygen as described previously (18).

Methylation of MCPs in vivo. Methylation of MCPs in vivo was performed as described by Kort et al. (16), with L-[*methyl*-^{3}H]methionine. The proteins were separated by electrophoresis (17) on 8% polyacrylamide Tris-glycine slab gels, immersed in En^{3}Hance (Du Pont, Boston, Mass.), dried, and exposed to X-ray film (X-Omat AR; Eastman Kodak Co., Rochester, N.Y.) at −70°C for 3 to 7 days. For cold-chase experiments, L-methionine at 30 μg/ml was added after incubation with L-[*methyl*-^{3}H]methionine. Aliquots were removed from the sam-

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TABLE 1. Bacterial strains used in this study

Bacterium	Relevant phenotype	Reference or source
E. coli strains		
MM335	Wild type	7
RP437	Wild type	31
RP1075	CheB	49
RP4971	CheB	J. S. Parkinson
AW655	Tsr	15
AW656	Tar	15
AW701	Trg	15
Lin227	GlpR GlpK	*E. coli* Genetic Stock Center
CS8asp/23	Asp	9
S. typhimurium strains		
ST1	Wild type	3
ST171	CheB	4

ples every 5 min for 30 min. The fluorograms were scanned on a Beckman DU-8 spectrophotometer, and the areas under the peaks were integrated (33).

Measurement of membrane potential. Membrane potential was determined by using a tetraphenylphosphonium ion-selective electrode essentially as previously described (5, 50). The final cell concentration was 8×10^9/ml. Membrane potential was calculated by using the Nernst equation and a cell volume for *S. typhimurium* of 0.9×10^{-15} liter (14).

RESULTS

Positive chemotaxis to glycerol in *E. coli* and *S. typhimurium*. Strains of *E. coli* and *S. typhimurium* that are wild type for chemotaxis produced chemotactic swarms when grown on minimal semisoft agar plates containing 1 mM glycerol (Fig. 1), indicating that glycerol may be a chemoattractant for both species. The swarms were shaped like an inverted saucer, and the outermost ring was near the bottom of the agar. An inner ring was usually present at the upper surface of the agar. In addition to swarming in response to a glycerol gradient, three alternative hypotheses for the mechanism of swarming were considered. (i) The cells might swarm toward an optimal oxygen concentration. *E. coli* and *S. typhimurium* are repelled by higher concentrations of oxygen and attracted to a lower concentration of oxygen that supports optimal growth (36). This could explain the slower progression of the swarm ring at the surface, where the oxygen concentration is highest, compared to the faster progression of the swarm near the bottom of the agar. (ii) The cells might be attracted to aspartate excreted into the medium and not to glycerol itself. (iii) The cells might swarm in response to a contaminant in the glycerol or agar. A temporal gradient assay was used to distinguish among these possibilities.

Addition of 1 mM glycerol to suspensions of free-swimming *E. coli* and *S. typhimurium* cells resulted in suppression of tumbling by the cells and caused a smooth-swimming response that is typical of responses to attractants (Table 2). The possibility that the smooth-swimming response was elicited by oxygen in the glycerol solution that was added to the cell suspension was eliminated by a control in which buffer alone was added. The response times of strains ST1 and MM335 to glycerol were within the range observed for attractants that are detected by the methylation-dependent chemotaxis pathway (aspartate [11]), the phosphotransferase-dependent chemotaxis pathway (mannitol), or the energy-dependent pathway (sodium borohydride [5]) (Table 2). Cells of *S. typhimurium* ST1 exhibited a smooth-swimming response to glycerol over a wide concentration range, with a response threshold of approximately 1 μM glycerol (Fig. 2). Similar results were obtained with *E. coli* MM335 (data not shown).

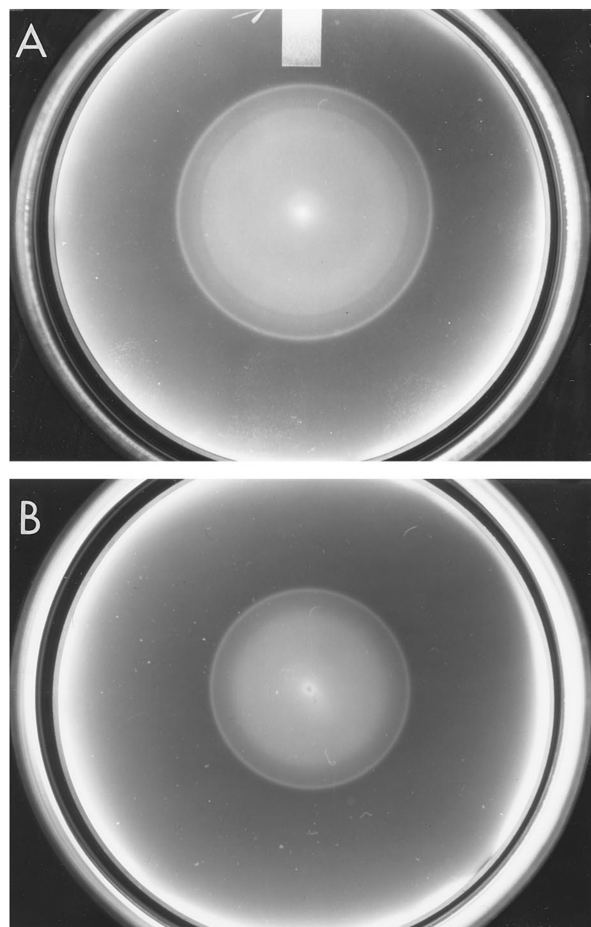

FIG. 1. Chemotactic rings formed by wild-type strains of *E. coli* (MM335) (A) and *S. typhimurium* (ST1) (B) on semisoft minimal (VBE) agar containing 1 mM glycerol. Plates (10-cm diameter) were incubated at 35°C for 16 h.

E. coli may form symmetric colony patterns in semisoft agar in response to aspartate that is secreted by the bacteria (9). To verify that aspartate release is not involved in chemotaxis to glycerol, *E. coli* CS8asp/23, a strain that does not excrete aspartate (9), was inoculated into glycerol semisoft agar. This

TABLE 2. Temporal responses of *E. coli* and *S. typhimurium* to chemoattractants[a]

Chemoattractant (concn)	Mean smooth-swimming response time (s) ± SD	
	E. coli MM335	*S. typhimurium* ST1
None (buffer control)	NR[b]	NR
Glycerol (1 μM)	16 ± 4	19 ± 5
Glycerol (1 mM)	91 ± 10	99 ± 12
Aspartate (0.5 μM)	34 ± 4	31 ± 3
Mannitol (10 μM)	17 ± 3	19 ± 3
Sodium borohydride (0.2 μM)	15 ± 4	15 ± 3

[a] Cells were grown in VBC-glycerol, washed three times, and resuspended in 10 mM phosphate (pH 7.0)–10 μM EDTA. The assay was performed as described in Materials and Methods.
[b] NR, no response detected.

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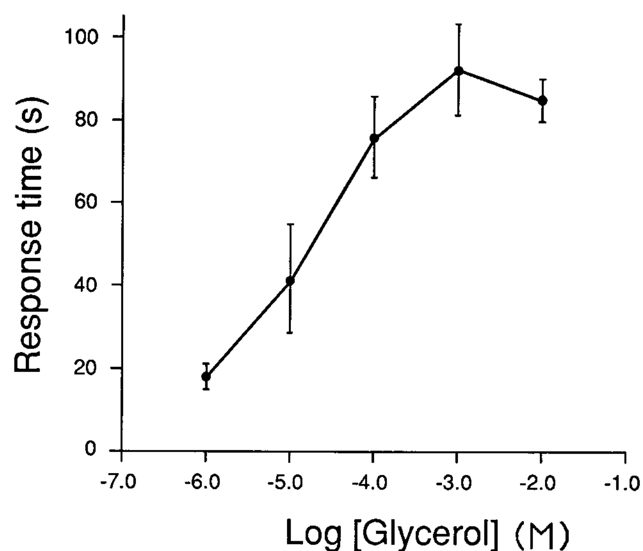

FIG. 2. Dose-response curve of the behavioral response of *S. typhimurium* ST1 to glycerol in a temporal gradient assay. Cells were challenged with various concentrations of glycerol, and the mean smooth-swimming response times were determined. Cells were grown in VBC-glycerol medium, washed three times, and resuspended in 10 mM potassium phosphate, pH 7.0, supplemented with 10 μM EDTA. Glycerol solutions were prepared in the same buffer.

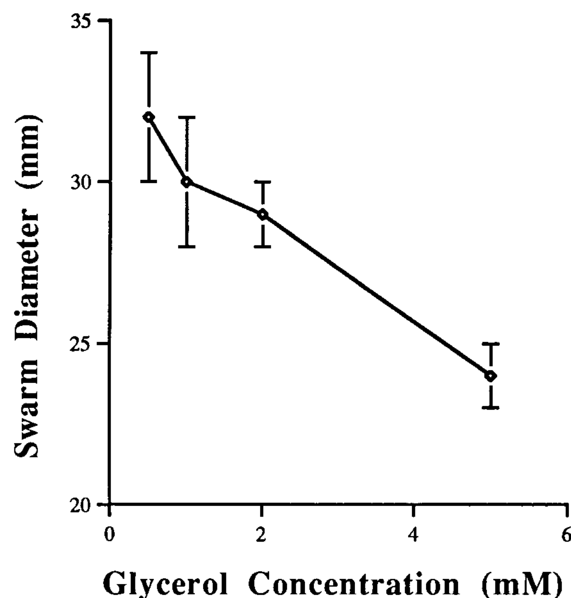

FIG. 3. Swarming of *E. coli* MM335 cells on glycerol minimal (VBE) semisoft agar as a function of the glycerol concentration. Cells (10 μl, 10^8 cells/ml) grown in VBE-glycerol were inoculated in the center of petri dishes containing VBE semisoft agar (0.3%) with different concentrations of glycerol. Plates were incubated for 16 h at 30°C, and swarm diameters were measured.

strain formed normal chemotaxis rings on the glycerol plates (data not shown).

To verify that the bacteria were responding to glycerol and not to fatty acids, which are the major contaminant in commercial glycerol (8), we used temporal assays to compare chemotaxis responses to various grades of glycerol, in which the maximum concentration of impurities varied from 0.5 to 13%. Although there was a 26-fold range of impurity concentrations in the glycerol, the responses and the response thresholds were similar for all of the glycerol samples tested. This suggested that glycerol per se was the attractant.

Further experiments conclusively established that glycerol was an attractant. The concentration of glycerol in semisoft agar was varied between 0.5 and 5 mM (Fig. 3). The concentration of oxygen remained constant. The rate of swarming increased as the concentration of glycerol decreased. Swarming increases at lower concentrations of an attractant because the bacteria quickly exhaust the surrounding attractant (as a carbon source) and swarm out in response to the resulting attractant gradient. Swarming on glycerol semisoft agar is dependent on consumption of glycerol to generate a glycerol gradient. Chemotaxis to glycerol was also observed for *E. coli* MM335 and RP437 in a spatial glycerol gradient formed independently of glycerol metabolism. Plugs of firm agar that contained glycerol or serine were inserted into semisoft agar that contained motile bacteria but no chemoeffector (45). Chemoattractant diffusion from the plugs creates an attractant gradient, and a ring of bacteria is visible within 10 to 30 min around plugs that contain an attractant. No band formed around a plug from which attractant was omitted. Dense rings of bacteria formed around plugs that contained 1 mM glycerol or 10 mM serine (data not shown).

Metabolism of glycerol is required for behavioral response. *E. coli* Lin227, which is constitutive for expression of the *glp* operon but deficient in the *glpK* gene, which codes for glycerol kinase, is able to take up glycerol but does not metabolize it. Lin227 cells did not show a chemotactic response to glycerol in swarm plates or in temporal gradient and chemical-in-plug assays but showed normal chemotaxis to serine (data not shown).

Positive chemotaxis to glycerol in MCP-deficient strains. Previously, glycerol was reported to be a repellent for *E. coli*. Addition of glycerol caused a tumbling response and demethylation of the methyl-accepting chemotaxis proteins (29). The threshold of the tumbling response was reported to be 1 mM, whereas the threshold of MCP demethylation was 300 mM (29). In the present study, we observed a smooth-swimming response in both *E. coli* MM335 and *S. typhimurium* ST1 when 1 mM glycerol was added to the cells in chemotaxis buffer. Strains defective in individual MCPs, AW655 (Tsr), AW656 (Tar), and AW701 (Trg), all showed a smooth-swimming response to 1 mM glycerol in a temporal assay and formed chemotactic rings on glycerol plates, suggesting that these MCPs are not required for positive glycerol taxis. In *S. typhimurium* ST1, there was no accompanying change in MCP methylation in response to 1 mM glycerol, whereas aspartate (1 mM) and serine (1 mM) caused a substantial increase in methylation (data not shown).

Glycerol response in *cheB* mutants. Strains of *E. coli* (RP1075 and RP4971) and *S. typhimurium* (ST171) with mutations in *cheB* (41) showed no response to glycerol over a wide range of concentrations (1 μM to 1 M). The MCP-dependent attractants aspartate and serine cause a smooth-swimming response in *cheB* mutants, but the cells do not adapt to these stimuli (49). MCP-independent chemoeffectors, such as oxygen and redox molecules, cause inverted responses in *cheB* mutants (5, 12). That is, addition of an attractant causes a tumbling (repellent) response and addition of a repellent causes a smooth-swimming (attractant) response. The absence of the anticipated smooth-swimming response to glycerol in *cheB* strains led us to hypothesize that the response to glycerol was inverted. An inverted response (tumbling) to an attractant would not be seen in constantly tumbling *cheB* strains. We used

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a strategy developed previously, therefore, to study inverted responses in *cheB* strains. The *cheB* cells were preincubated with 10 μM 1,4-benzoquinone to induce prolonged smooth swimming (5). Glycerol (1 mM) was then added, and a 1- to 2-min tumbling response was observed. All *cheB* mutants of *S. typhimurium* and *E. coli* behaved similarly.

Glycerol-stimulated changes in cellular energy. The inversion of glycerol chemotaxis in *cheB* strains suggested that glycerol, like oxygen, is sensed via an alternative signal transduction pathway that is mediated by changes in electron transport and proton motive force (43). To test this hypothesis, we measured changes in membrane potential at neutral pH, where the proton motive force consists mainly of an electrical component (38). Addition of 1 mM glycerol to a suspension of *S. typhimurium* cells caused an increase in membrane potential (Fig. 4A). Calculated membrane potentials were −120 mV (inside negative) in unstimulated cells and −160 mV in cells stimulated with 1 mM glycerol. Under the same conditions, a similar increase in membrane potentials was observed in *E. coli* cells upon addition of glycerol (Fig. 4B). When 10 mM lactate or succinate was present in the medium, no significant changes in the swimming behavior of *E. coli* MM335 and *S. typhimurium* ST1 cells were observed on addition of glycerol in the temporal assay. However, cells were able to respond chemotactically to aspartate, serine, and mannitol in the presence of lactate or succinate. In the presence of 10 mM lactate or succinate, no significant changes in membrane potential in *S. typhimurium* and *E. coli* cells were observed upon addition of glycerol (data not shown). Similarly, no changes in membrane potential were observed upon addition of lactate to the cells that were stimulated with glycerol (Fig. 4), whereas lactate caused an increase in membrane potential when added to unstimulated cells (data not shown). Addition of lactate (1 mM) to MM335 cells in phosphate-EDTA buffer elicited positive chemotaxis (smooth swimming) (data not shown).

High concentrations of glycerol repel *E. coli* and *S. typhimurium*. By using a temporal assay, we confirmed the previous findings by Oosawa and Imae that, at high concentrations, glycerol acts as a repellent for *E. coli* (28, 29). However, the threshold for negative chemotaxis to glycerol in *E. coli* in our study (50 mM) was significantly higher than that reported previously (1 mM) (28, 29). We observed similar responses to high concentrations of glycerol in *S. typhimurium* ST1, for which the threshold of negative chemotaxis in a temporal gradient assay was 70 mM.

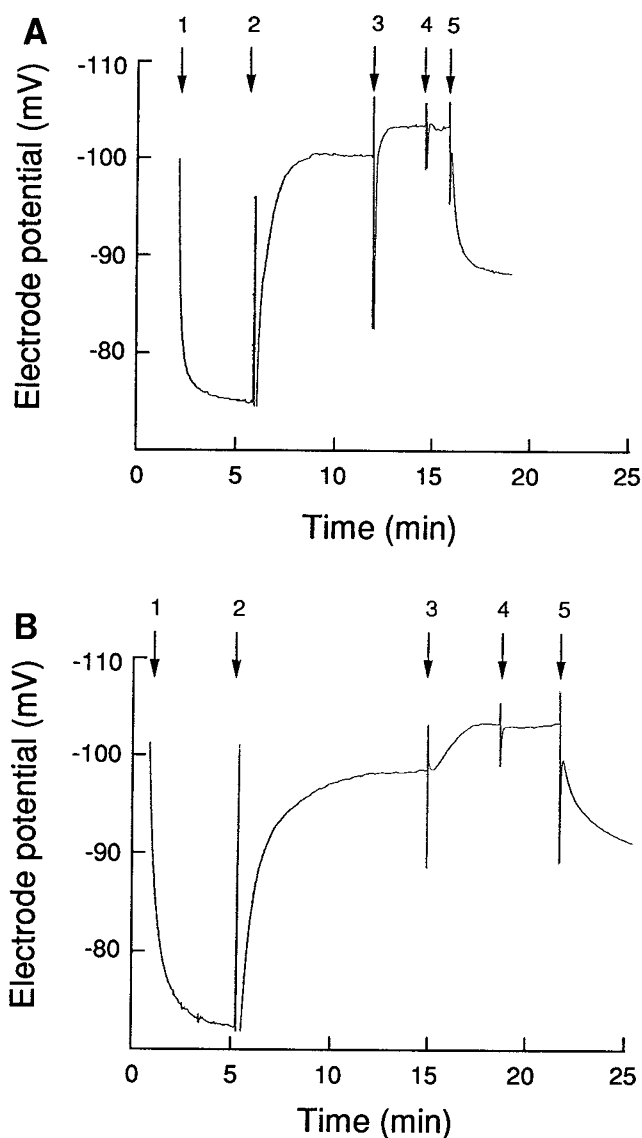

FIG. 4. Changes in membrane potential of *S. typhimurium* ST1 (A) and *E. coli* MM335 (B) upon stimulation with 1 mM glycerol. Membrane potential was monitored at pH 7.5 by using a tetraphenylphosphonium ion-selective electrode. The electrode traces are shown. A 9-ml aliquot of 100 mM potassium phosphate buffer (pH 7.5) was placed in a vessel and equilibrated at 30°C with stirring. The arrows indicate the times at which the following additions were made (all concentrations are final): 1, 4 μM tetraphenylphosphonium ion; 2, bacterial cells (8×10^9/ml); 3, 1 mM glycerol; 4, 1 mM lactate; 5, 0.5 μM valinomycin to collapse membrane potential.

DISCUSSION

By using both spatial and temporal behavioral assays, we have demonstrated that glycerol is an attractant for both *E. coli* and *S. typhimurium* at concentrations of up to 10 mM in the absence of an alternative energy source. In the temporal assay, both *E. coli* and *S. typhimurium* showed a typical attractant response to the addition of glycerol, with a threshold of 1 μM and a smooth-swimming response for up to 100 s when 1 mM glycerol was added (Table 2). Swarming on glycerol semisoft agar was not the result of aerotaxis. It was not elicited by a contaminant in the glycerol, nor was it a response to aspartate excreted by the cells. Glycerol taxis was defective in strains that do not metabolize glycerol, indicating that in contrast to most other chemoeffectors, glycerol must be metabolized to cause the behavioral response.

Our conclusions differ from previous reports that glycerol is not an attractant for *E. coli* (1, 2, 28, 29, 47), and some of these reports can be explained by our findings. The semisoft agar used by Wolfe and Berg (47) had a glycerol concentration of 25 mM. We observed swarming at concentrations of glycerol between 0.5 and 5 mM (Fig. 3) but little swarming at concentrations higher than 20 mM (data not shown). Oosawa and Imae (28, 29) investigated glycerol chemotaxis in the presence of lactate, which abolished attraction to glycerol in our experiments also. It is more difficult to explain the capillary assays of Adler, in which he systemically varied the concentration of glycerol between 10^{-7} and 10^{-1} M and observed no attraction to glycerol (1). We observed under a microscope the accumulation of bacteria around the mouth of a capillary filled with 1 mM glycerol. The bacteria aggregated in a "halo" around the mouth of the capillary, with a clear zone that contained few

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bacteria between the halo and the capillary. For most other attractants, the bacteria form a cloud at the mouth of the capillary and bacteria can be seen accumulating in the capillary. For additional studies of glycerol taxis in a spatial glycerol gradient, we used the chemical-in-plug assay.

The increase in membrane potential in both *E. coli* and *S. typhimurium* cells upon addition of glycerol (Fig. 4) is consistent with the notion that glycerol is an efficient energy source for these bacteria. There was no significant increase in membrane potential upon the addition of glycerol when lactate was present in the medium as a carbon source. The 40-mV increase in membrane potential obtained upon stimulation of de-energized *S. typhimurium* cells with 1 mM glycerol was similar to the increase in membrane potential (50 mV) observed when anoxic cells were exposed to 21% oxygen (38). In both cases, an increase in membrane potential coincided with a smooth-swimming response (positive taxis). In the presence of lactate, there was no glycerol taxis or glycerol-stimulated increase in membrane potential. This suggests that glycerol taxis is an energy taxis in which the cells respond to a change in cellular energy (e.g., the proton motive force). The absence of positive glycerol taxis in *glpK* mutants that cannot metabolize glycerol is further evidence that glycerol elicits an energy taxis.

The inverted-saucer shape of the swarms in glycerol semisoft agar is consistent with this hypothesis. The proton motive force in *E. coli* is maximal when the concentration of oxygen is about 20% of the concentration in air-saturated media (51). Consequently, in regions where glycerol has not been depleted, bacteria near the bottom of the agar would have a higher proton motive force than bacteria at the surface. Positive taxis to glycerol occurs when bacteria swim from a glycerol-depleted zone to a zone with a higher glycerol concentration, where they experience an increase in proton motive force. The proton motive force in glycerol-depleted cells is essentially independent of the oxygen concentration. Consequently, the increase in proton motive force will be greatest in bacteria that swim from an oxygen-depleted zone to a zone that has both glycerol and the optimal concentration of oxygen. The larger the increase in bacterial proton motive force, the stronger the tactic stimulus (43) and the higher the velocity at which the cells migrate up the glycerol gradient since cell speed is a function of the proton motive force (25). This hypothesis is consistent with a higher velocity for the swarm ring near the bottom of the glycerol semisoft agar.

Other examples of energy taxis in *E. coli* and *S. typhimurium* include aerotaxis (18, 37), electron acceptor taxis (44), proline chemotaxis (10), and redox taxis (5). It is likely that other rapidly metabolized carbon sources can also elicit energy taxis. The smooth-swimming response to lactate demonstrated in this study is consistent with this hypothesis. It has been proposed that aerotaxis, redox taxis, and other forms of energy taxis are mediated by a common signal-transducing protein that monitors cellular energy levels by sensing proton motive force or electron transport (5, 43). Recent studies done in our laboratory (32) have identified the Aer and Tsr proteins as independent transducers that monitor cellular energy levels in *E. coli*. An *aer tsr* double mutant is deficient in aerotaxis, redox taxis, and glycerol taxis.

Our results are in agreement with the previous findings that high concentrations of glycerol result in negative taxis in *E. coli* (28), and we demonstrated similar negative responses in *S. typhimurium*. The concentrations of glycerol that elicit negative taxis in *E. coli* and *S. typhimurium* are known to cause osmotic behavioral responses in bacteria (19, 20, 48). However, our results demonstrate that, at physiological concentrations, glycerol is an attractant for both *E. coli* and *S. typhimurium*.

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