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Evidence Against the Involvement of Chemotaxis in Swarming of *Proteus mirabilis*

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Nonswarming and nonchemotactic mutants of *Proteus mirabilis* were isolated after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or ultraviolet light. These mutants were used in experiments to determine if chemotaxis is involved in the swarming of *P. mirabilis*. Nonchemotactic mutants failed to form chemotactic bands in a semisolid casein hydrolysate medium, yet they swarmed on the same medium containing 1.5% agar. Nonswarming mutants were attracted towards individual amino acids and components of tryptose. In cross-feeding experiments, no evidence was obtained to indicate the production of a diffusable chemical repellent. In studies with the wild-type *P. mirabilis*, no clear-cut negative chemotaxis was seen even though three different assays were used and numerous chemicals were tested. Additional evidence against the involvement of chemotaxis in swarming comes from finding that dialysis does not interfere with swarming; swarm cells will swarm immediately when transferred to fresh media, and swarm cells will swarm on an agar-water medium supplemented with a surfactant. These data indicate that chemotaxis is not involved in the swarming of *P. mirabilis*.

Two members of the genus *Proteus*, *Proteus mirabilis* and *Proteus vulgaris*, will readily swarm on common laboratory media. This phenomenon results in the bacterium completely covering the surface of a solid medium, usually within 24 h. Swarming of *Proteus* is always accompanied by morphological changes in the cells. Nonswarming cells are about 2 to 4 μm in length, whereas swarm cells reach lengths of up to 80 μm and possess many more flagella per unit of surface area than do the short forms (9). Groups of swarm cells actually move over the agar surface and produce the swarming phenomenon. Frequently, periodic swarming occurs in that swarming proceeds for a period of time and then stops. The cessation of swarming is accompanied by the swarm cells undergoing division into short cells, a process called consolidation. The resulting short cells undergo a period of growth, and then new swarm cells are formed at the periphery of the growth zone and swarming starts again. This produces a zonation effect, with concentric rings of swarming giving a "bulls eye" appearance on the agar surface.

Since the original description of swarming by Hauser (8), investigators have attempted to explain the swarming phenomenon. The most widely accepted theory is the negative chemotaxis hypothesis of Lominski and Lendrum (12). Although based upon weak experimental evidence, this hypothesis accounts for the morphological changes in the cells and the periodicity frequently associated with swarming. Briefly, they propose that during growth of *Proteus* on a solid surface the cells excrete one or more toxic waste products that inhibit cell division, thus leading to the formation of swarm cells. The toxic agent diffuses through the agar medium, out from the region of growth, establishing a gradient of the toxic substance. The swarm cells detect the gradient and move over the agar surface down the gradient towards a lower concentration of the toxic agent (negative chemotaxis). When the cells have moved to a subtoxic region, the inhibition of division is relieved and the swarm cells stop swarming and start dividing. The growth of short forms results once again in the production of the toxic substance, swarm cells are again produced, and swarming starts again.

The Lominski and Lendrum hypothesis can be easily modified into one that proposes positive chemotaxis for swarming. This theory would resemble the depletion of nutrients hy-

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pothesis of Moltke (13). Growth of a central colony depletes the available nutrients in the immediate area, resulting in the formation of a gradient of nutrients. Swarm cells are formed due to the depletion of nutrients, and, once formed, they move up the nutrient gradient (positive chemotaxis) to richer regions.

Because of the attractiveness of the Lominski and Lendrum hypothesis and the recent advances in our understanding of bacterial chemotaxis (3), we felt that we were in a position to test whether chemotaxis is involved in swarming by using several different experimental approaches. Through the use of nonchemotactic and nonswarming mutants we felt that any requirement for chemotaxis could be detected, and if chemotaxis is required for swarming a chemical gradient of either an attractant or repellent could be implicated. This report describes the results of studies leading to the conclusion that chemotaxis is not involved in the swarming of *Proteus*.

MATERIALS AND METHODS

Organisms. The wild-type *P. mirabilis*, strain IM47, used in these studies was obtained from the culture collection of the Bacteriology Department, Iowa State University. All of the mutants of *P. mirabilis* produced in this study were derived from IM47. Cultures of *Proteus morganii*, *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Escherichia coli* K-12 were obtained from the same culture collection. Additional nonswarming mutants of *P. mirabilis* were obtained from W. M. Bain (University of Maine, Orono) and C. D. Jeffries (Wayne State University, Detroit, Mich.). All cultures were maintained on slants of either nutrient agar or Trypticase soy agar and stored at 4 C.

Culture media. Nutrient broth with NaCl (NB) consisted of nutrient broth (GIBCO) supplemented with 0.5% NaCl. Tryptose broth contained 2.0% tryptose (Difco) and 0.5% NaCl. Trypticase soy broth with yeast extract (TSYE) consisted of 0.5% yeast extract added to Trypticase soy broth (BBL). Casitone phosphate broth (CP) was composed of 1% Casitone (Difco); 10^{-2} M potassium phosphate, pH 7.0; 10^{-3} M $MgSO_4 \cdot 7H_2O$; 0.001% nicotinic acid; and 0.5% NaCl. Casein hydrolysate (CH) medium was prepared with a basal salts solution modified from that of Jones and Park (10). It contained, per liter: K_2HPO_4, 11.2 g; KH_2PO_4, 4.8 g; $(NH_4)_2SO_4$, 0.54 g; NH_4Cl, 0.54 g; $Fe(NH_4)_2(SO_4)_2 \cdot 6\ H_2O$, 20 mg; $MgSO_4 \cdot 7\ H_2O$, 40 mg; nicotinic acid, 2.4 mg; NaCl, 4.8 g; vitamin-free Casamino Acids (Difco), 3.0 g. The $MgSO_4$, nicotinic acid, NaCl, and Casamino Acids were autoclaved as separate solutions and added aseptically. The $Fe(NH_4)_2(SO_4)_2$ was prepared in 0.02 N HCl, membrane filtered (0.45-μm pore size, Millipore Corp.), and added aseptically.

Semisolid media contained 0.2% agar (Difco) and solid media contained 1.5% agar (Difco). Petri plates containing a solid medium were always dried overnight at 35 C before use to remove droplets from the agar surface.

The chemotaxis medium described by Adler (2) was used in the quantitative chemotaxis assay for washing the cells and preparing the attractant solutions. It contained 10^{-2} M potassium phosphate buffer (pH 7.0) and 10^{-4} M ethylenediaminetetraacetic acid. The ethylenediaminetetraacetic acid was added as the free acid and was neutralized with potassium hydroxide. The medium was prepared with deionized water, filter sterilized, and stored at room temperature.

Isolation of nonswarming mutants. Cells of *P. mirabilis* (IM47) from 10 ml of a 14-h, agitated culture in TSYE were collected by centrifugation (900 $\times$ *g* for 10 min) at room temperature. The pellet was suspended in 10 ml of a 0.85% sodium citrate solution, containing 500 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml (Aldrich Chemical Co.), adjusted to pH 5.0 with HCl. This suspension was incubated at 35 C for 1 h and was then centrifuged, and the cells were suspended in cold NB. This procedure normally resulted in a 99 to 99.9% reduction in viable cell numbers. While the suspension was refrigerated, viable cell counts were determined on the mutagenized suspension by using spread plates of nutrient agar. Nutrient agar without NaCl fails to support swarming, so that discrete colonies are produced. On the basis of the viable cell count, the suspension was diluted to a concentration of 500 to 750 viable cells/ml.

Two methods were used for the selection of nonswarming mutants from the diluted suspension. The first method involved plating 0.1 ml of the suspension on TSYE or nutrient agar (GIBCO) with 0.5% NaCl (NA); both media support normal swarming of IM47. The plates were incubated at 35 C until the individual colonies began to show evidence of swarming. Nonswarming colonies were picked and reinoculated onto the same medium to allow confirmation of their inability to swarm. The second selection method involved a type of "blind selection" in which 0.1 ml of the diluted suspension was plated on nutrient agar without NaCl and incubated for 16 h at 35 C. Normal swarming does not occur under these conditions. Individual colonies were then picked and reinoculated onto individual sectors of quadrant plates (Quad-Petri, Lab-Tec Products) containing TSYE. These plates were then incubated at 35 C for 16 h and examined for the presence of nonswarmers.

Isolation of nonchemotactic mutants. A 200-ml NB culture of *P. mirabilis* IM47 was grown to midlog phase at 35 C with shaking and then was harvested by centrifugation at 10,000 $\times$ *g*. The cells were resuspended in cold 0.5% sterile NaCl and kept at 0 C before use. A 25-ml sample was dispensed into the bottom of half of a glass petri plate (100 mm) and exposed to a 15-W germicidal lamp (Champion G15T8) for 20 to 55 s with a dose rate of 12 ergs/mm^2. During ultraviolet irradiation the culture was constantly agitated on a lateral shaking machine. Samples were removed at 10-s intervals for the determination of viable cell numbers and inoculation of enrichment plates. Care was taken to avoid photo-

reactivation. To enrich for nonmotile and nonchemotactic mutants, each irradiated sample was inoculated onto the center of five CH soft-agar plates, which were incubated at 35 C until the outermost chemotactic band of cells reached the edge of the plate. At that time a transfer was made from the center of the plate to a fresh plate of the same medium. This procedure was continued for up to 16 serial transfers. To isolate mutants, 0.1 ml was removed from the center of a soft-agar plate, diluted, and pour plated in CH soft agar. After incubation for 14 to 16 h at 35 C, each plate was examined for the presence of nonchemotactic and nonmotile colonies. These were detected as colonies without chemotactic bands of cells or colonies with sharp distinct edges in the soft agar. Those that were found were picked and transferred to individual plates of soft agar for the verification of defects in chemotaxis or motility. Plates with between 10 and 20 colonies proved optimal for detecting mutants.

Confirmed mutants were cloned and then examined for their ability to give typical reactions of *P. mirabilis* in a number of biochemical tests. Each mutant was further characterized for motility, swarming, and chemotaxis. Motility tests were made in NB and soft-agar media at 25 and 35 C by the direct microscopic observation of wet mounts or hanging-drop preparations. Swarming was tested on NA, tryptose agar, TSYE agar, and CH agar at 25 and 35 C. After 24 h each plate was examined for swarming, and if no swarming was observed the central colony was examined microscopically for the presence of motility and swarm cells at the colony edge. Qualitative tests for chemotaxis towards amino acids were made by looking for the formation of chemotactic bands in tryptose soft agar or CH soft agar at 25 and 35 C.

Several different techniques were used to test for negative chemotaxis. The method of Doetsch and Seymour (7) was used to test the wild-type *P. mirabilis* IM47 and control cultures of *S. marcescens*, *P. aeruginosa*, and *P. morganii*. In this test a suspension of bacteria fills the inside of a flat capillary tube plugged with agar at each end. One agar plug contains the test agent; the other plug serves as a control. The capillary is observed microscopically for the presence of a dense band of cells moving away from the test agent. All cultures were grown at room temperature (20 to 22 C) in either CP broth or Trypticase soy broth (BBL) and washed before use. Each culture was examined for motility before use, and the suspension was used only if at least 60% of the cells displayed normal motility. The following compounds were tested in the chemotaxis assay: hydrochloric acid, pH 1.0 to 5.0; $FeCl_3$, 10^{-1} and 10^{-2} M; $FeSO_4$, 10^{-1} and 10^{-2} M; $ZnCl_2$, 10^{-2} M; 2-methylbutylamine, 10^{-2} M; isoamylamine, 10^{-2} M; isobutylamine, 10^{-2} M; NaOH, 1 N; indole, 10^{-2} M; succinic acid, 10^{-1} M; sodium acetate, 10^{-1} M; and hydrogen peroxide, 20%.

Two methods of Tso and Adler (15) were also used to test for negative chemotaxis. In the first method (agent in the plug), bacteria were suspended in soft-agar chemotaxis medium in a petri plate. Hard-agar plugs containing the test compound were then placed into the soft agar in the petri plate. Chemicals tested by this method included: sodium acetate, indole, L-leucine, old overswarmed CP agar, and $FeCl_3$. The concentration range tested for all compounds was between 10^{-6} and 1.0 M. During the incubation, samples were removed from areas near the test plugs and away from the test plugs for use in microscopic determinations of relative bacterial motility. A dense band of motile cells migrating away from the test plug constituted negative chemotaxis.

Another method referred to as the "cells in the well assay" (15) was also used. Wells (8 mm) were cut in the solidified test agar plates, and these wells were then filled with a solution of bacteria suspended in 0.3% soft chemotaxis agar. The plates were allowed to stand at room temperature for 30 min before being observed. Chemotactic cells in this experiment would be those that formed a ring in the center of the well.

The quantitative chemotaxis assay described by Adler (2) was used to characterize the chemotactic response towards amino acids of IM47 and those nonswarming mutants derived from it that were motile. This method quantifies the number of bacteria that swim into a capillary tube filled with an attractant. Cells from a hard-agar surface were suspended in chemotaxis medium and then harvested by centrifugation at 900 × *g* for 10 min at room temperature. The pellet was gently resuspended in the small amount of liquid that remained after decanting, and 5 ml of chemotaxis medium was added. This process was repeated a second time to wash the cells before use. The final cell suspension was adjusted to a density of 5×10^7 viable cells/ml and then examined microscopically by using a hanging-drop technique to judge the degree of motility in the suspension. Cells harvested in this manner would normally retain their motility throughout the wash procedure and for over 2 h after the last wash. Chemotactic assays were conducted at 30 C for 50 min.

Cross-feeding. Three types of cross-feeding experiments were performed to test for the presence of a diffusable substance that would promote swarming of a nonswarming mutant or bring about premature swarming of a wild-type swarmer.

The first method involved mutant-mutant crosses in which the ability of one mutant to stimulate swarming of a second mutant was examined. Plates of TSYE agar were inoculated with the two cultures to be tested as two divergent streaks that approached each other at one side of the plate but did not touch. The plates were incubated at 35 C and examined over a period of 48 h for evidence of swarming.

The second method involved crosses between wild-type swarmers and nonswarming mutants, in which the ability of a wild type to stimulate swarming of a mutant was examined. Plates of TSYE agar were prepared with a strip of membrane filter, 0.8 cm wide and 8 cm long (cut from 142-mm filter disks; 0.45-μm pore size, Millipore Corp.), embedded in the agar to divide each plate in half. One side of the plate was inoculated with a wild-type strain, and the plate was incubated at 35 C for 3 h. The other

side of the plate was then inoculated with a mutant, and the plate was reincubated at 35 C and examined over a period of 48 h for evidence of swarming by the mutant. In both of these methods, mutants that showed any sign of swarming were checked for reversion by inoculating cells from the swarm band onto fresh TSYE agar.

The third method involved crosses between mutants and wild types in which the ability of the mutant to stimulate premature swarming of the wild types was examined. At the beginning of the experiment, three sectors of individual quadrant plates containing NA were inoculated with a different mutant as a single streak. The plates were incubated at 35 C for 2 h and were then inoculated with a single wild type on each sector. In the three sectors with the mutants, the wild type was inoculated as the second leg of a "V" so that the mutant and wild type were in direct contact at the vertex. In the fourth quadrant, the wild type was simply inoculated as a single streak, and this served as a control. The plates were reincubated at 35 C and examined microscopically to determine exactly when the wild type began to swarm. Mutants that showed any ability to induce premature swarming of the wild type, as compared with the control, were examined in a duplicate experiment.

Isolation of swarm cells. Swarm cells were isolated comparatively free of short forms by washing the cells from the outermost swarm band from either CP agar or tryptose agar using the wash medium. The cells were washed twice before use, and microscopic examination showed 80 to 90% swarm cells.

Dialysis experiments. A plastic chamber (2.5 cm in diameter and 5 mm in depth), open at the top and having two ports entering the chamber at opposite sides, was used for dialysis. The chambers were made from tissue culture chambers (Research Plus Laboratories Inc.) in which the central pillar had been flattened out, leaving a slightly rounded bottom in the well of the chamber. CP agar was aseptically pipetted onto an 8- by 5-cm piece of sterile dialysis tubing and allowed to dry as a thin film at room temperature overnight in a sterile petri dish. The CP agar-covered dialysis tubing was laid on top of the dialysis chamber so that it came into contact with the dialyzing medium. A uniform flow of dialysis medium into and out of the chamber was regulated by a variable-speed polystaltic pump (Beckman). The flow rate was between 3 and 4 ml/min. *P. mirabilis* IM47 was grown overnight in CP broth and washed, and an inoculum was placed onto the center of the agar-covered dialysis tubing that had already been placed onto the dialysis chamber to equilibrate. CP agar-covered dialysis tubing was also placed onto CP agar plates and inoculated to serve as a control. Dialysis was allowed to continue for 30 h at room temperature. If no swarming occurred on the dialyzed agar, the agar-covered dialysis tubing was removed from the dialysis chamber and placed onto a plate of CP agar and observed.

RESULTS

Nonswarming mutants. A total of 23 nonswarming mutants were obtained. In addition, 10 other nonswarming mutants of *P. mirabilis* were obtained from other investigators (these were derived from other wild-type strains). The mutants isolated in this laboratory were assigned the designation Nsw and a 3-digit number. Those mutants obtained from other laboratories have the strain designation used in that laboratory.

When the mutants were examined under different culture conditions, a number of them demonstrated the ability to swarm. Table 1 summarizes the responses of the 33 mutants on three different media at two temperatures. The ability to form swarm cells and the motility of the cells under nonswarming conditions are also given. Of the Nsw mutants, those with a number between 100 and 200 were selected on nutrient agar with salt, and 5 of the 12 demonstrated the ability to swarm on a richer medium. Those mutants with a strain designation between 200 and 300 were selected on TSYE agar. Five of the Nsw mutants (Nsw202, -203, -206, -211, and -214) were obtained by using the blind selection technique by which they were selected on the basis of their response in an isolated environment. No information was available on the selection methods of the mutants from other laboratories.

Cross-feeding experiments were performed between nonswarming mutants whereby individual mutants were examined for their ability to stimulate swarming of other mutants. Initially, crosses were made between potential "producers" (organisms that form swarm cells) and "detectors" (organisms that do not form swarm cells), but eventually nearly every mutant was crossed with every other mutant. No evidence for a diffusable agent that would stimulate a nonswarming mutant to swarm was obtained. Several cases of swarming by the mutants were observed, but, in nearly all cases, cells removed from the swarm band retained the ability to swarm when placed on fresh medium. The orientation of these apparent reversions seemed random, and the movement of the swarm bands did not appear to be affected by the other mutant. Occasional stimulation of nutritional and temperature-sensitive mutants was observed, but these results were not reproducible.

Using the second type of cross-feeding method involving the plates with membrane filters, 26 of the nonswarming mutants were crossed with 23 different wild-type swarmers. The membrane filter embedded in the agar prevented the wild type from swarming into the mutant colonies for 24 h. A significant number of reversions were noted, and the number appeared to increase as the mutant cultures were

TABLE 1. *Characteristics of nonswarming mutants*

Mutants	Ability to swarm						Swarm cell formation[a]	Motility[a]
	TSYE		TA[b]		NA			
	25 C	35 C	25 C	35 C	25 C	35 C		
Nsw104, Nsw107, Nsw202, Nsw206, Nsw211, Nsw215, Nsw221, Nsw235, 75	−	−	−	−	−	−	−	−
Nsw115, Nsw231	−	−	−	−	−	−	+	−
Nsw109, Nsw214, Nsw228	−	−	−	−	−	−	−	+
Nsw203, Nsw122, 2NS, 4NS, 60, 91b, DA, MI	−	−	−	−	−	−	+	+
Nsw110	+	−	+	−	+	−	+	+
Nsw227, Nsw101	+	−	+	−	+	−	−	+
Nsw112, Nsw114, Nsw106, Nsw108	+	+	+	−	−	−	+	+
77NS, 73NS	+	+	+	+	−	−	+	+
Nsw127	+	−	+	−	−	−	+	+
91NR	+	−	−	−	−	−	+	+

[a] Under nonswarming conditions.
[b] TA, Tryptose agar.

serially transferred in broth through the course of the experiments. When fresh cultures were prepared from the stock slants, however, the number of reversions decreased significantly. No evidence was obtained for the presence of a diffusable agent, produced by a wild type, that would stimulate swarming of a mutant.

In the third type of cross-feeding experiment, 7 different wild-type swarmers were crossed with 32 nonswarming mutants. Again, no evidence was obtained for the presence of a diffusable agent, produced by a mutant, that would stimulate premature swarming of a wild type. The incubation period, required before swarming of the wild type occurred, ranged between 3.5 and 6 h for the different strains, but it remained essentially constant for a single wild type crossed with the various mutants. Cases of slight stimulation were re-examined using the same procedure, but no reproducible stimulation could be detected.

Chemotaxis of nonswarming mutants in soft agar. The positive chemotactic responses of the wild-type swarmers and the nonswarming mutants were determined by using tryptose soft agar; Adler (1) used a similar medium to demonstrate the response of *E. coli*. He found that when a soft-agar plate was centrally inoculated, well-defined bands of cells emerged from the colony edge and moved out through the soft agar as concentric rings. The cells in each ring utilized a particular nutrient in the medium and responded chemotactically in the gradient that was established. The cells in each subsequent band utilized a different component of the medium.

The chemotactic response of the different swarming strains of *P. mirabilis* were variable, as indicated by the pattern of bands that formed. The chemotactic rings normally became evident after 2 to 3 h of incubation at 35 C and were well formed over most of the plate after 8 to 10 h.

When the nonswarming mutants were examined in this way and compared with their respective wild type, three different responses were noted. A large number were nonmotile and, therefore, failed to respond chemotactically. Mutants of this type demonstrated a very distinct colony edge with no sign of spreading. Mutants of the second type were motile and demonstrated a spreading colony with diffuse edges, but no chemotactic bands formed. Microscopic examination of cells from this type of colony showed that they were uncoordinated, with few cells showing any translational movement. Mutants of the third type were motile and demonstrated chemotactic bands, but in the cases where the wild-type parents were available for comparison the patterns of bands were different. Table 2 summarizes the responses of the absolute nonswarming mutants tested.

The quantitative chemotactic responses of IM47, two mutants that were chemotactic in soft agar (Nsw109 and Nsw228), and the two mutants that were motile but nonchemotactic in soft agar (Nsw203 and Nsw 214) were deter-

TABLE 2. *Chemotactic responses of nonswarming mutants on tryptose soft agar*

Mutant	Motility[a]	Chemotaxis[b]
Nsw104	−	−
Nsw107	−	−
Nsw115	−	−
Nsw202	−	−
Nsw206	−	−
Nsw211	−	−
Nsw215	−	−
Nsw221	−	−
Nsw231	−	−
Nsw235	−	−
75	−	−
Nsw203	+	−
Nsw214	+	−
Nsw109	+	+[c]
Nsw122	+	+[c]
Nsw228	+	+[c]
2NS	+	+[c]
4NS	+	+[c]
60	+	+[d]
DA	+	+[d]
Ml	+	+[d]

[a] As indicated by movement of the colony edge and microscopic examination.

[b] As indicated by the formation of bands of cells moving away from the central colony.

[c] Positive chemotaxis but pattern of bands was altered when compared to the wild type.

[d] Wild types were not available for comparison.

mined on cells removed from hard-agar medium that would support swarming of the wild type. Figure 1 illustrates the responses of IM47, Nsw109, and Nsw228 towards 14 amino acids. The response of IM47 to six other amino acids (isoleucine, leucine, proline, threonine, tyrosine, and valine) was determined to be negligible, and the mutants were, therefore, not tested towards those amino acids. The two mutants that failed to respond chemotactically in soft agar also failed to respond in the quantitative assay, so the values for these two organisms are omitted from the figure.

Chemotaxis of swarm cells. When swarm cells were harvested by the procedure described above, a suspension judged to be at least 90% swarm cells was obtained. Motility of the swarm cells was sluggish, but a high proportion of them showed translational movement. This suspension was assayed using the quantitative method towards alanine, asparagine, glutamate, histidine, methionine, phenylalanine, serine, and a 2% solution of tryptose. No significant response was obtained for alanine, asparagine, or glutamate, and the net response for the remaining five attractants ranged between 2,800 for serine and 7,200 for histidine. Whereas microscopic examination of the suspension indicated that the swarm cells were motile, these cells failed to undergo the rapid direction changes demonstrated by the short forms.

Nonchemotactic mutants. Numerous nonchemotactic mutants were isolated by using the enrichment procedure. Because it is very likely that all the mutants isolated from a particular enrichment series are identical, we have grouped the mutants to indicate that they were obtained from identical enrichment plates (see Table 3). The 17 groups of mutants isolated were examined for their positive chemotactic abilities on CH soft agar at two different temperatures. Table 3 lists the results of this study as well as motility determinations performed during the progress of the experiment; nonmotile mutants, temperature-sensitive mutants, and positive chemotaxis mutants were isolated with the mutagenesis procedure used.

The 17 different groups of mutants were characterized as to their swarming ability on four different media at two different temperatures. Table 4 summarizes the results of this experiment and also lists information on motility and swarm cell formation. The majority of those mutants capable of motility showed an ability to swarm at both temperatures. To test for the possibility that swarming was caused by revertants, cells were isolated from a swarm band and rechecked for chemotaxis in CH soft agar. All isolates showed impaired chemotaxis identical to the mutant phenotype.

Motility studies of mutants. In overnight NB cultures (12–18 h), those mutants that were deficient in positive chemotaxis demonstrated a distinctive type of motility. Whereas the wild-type *P. mirabilis* IM47 showed a pattern of tumbles and short runs similar to that described by Berg and Brown (5), positive chemotaxis mutants manifested a greater tendency to tumble. An extreme example of this trait was shown by mutant Nch9 after incubation at room temperature. When viewed microscopically, the majority of the motile cells from this culture tumbled almost unceasingly and behaved rather like tiny "pinwheels."

Those mutants that were earlier found to be nonmotile in CH soft agar (Nch18 and Nch39) were likewise nonmotile when cultured in NB.

Negative chemotaxis. With the capillary tube method of Doetsch and Seymour no negative chemotactic responses were observed for *P. mirabilis*, whereas all the control organisms exhibited negative chemotactic responses to most chemical agents tested (Table 5). The control organisms responded to the chemical gradients by forming dense bands of motile bacteria, which migrated along with the diffusing chemicals down the capillary tubes away from

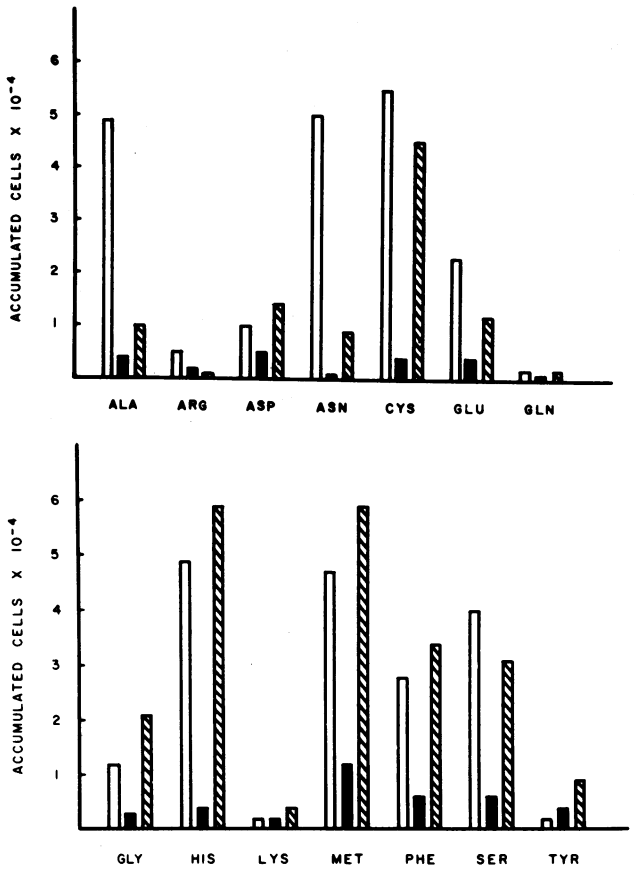

FIG. 1. *Quantitative chemotactic responses of IM47 (open bars), Nsw109 (closed bars), and Nsw228 (crosshatched bars) to seven amino acids. The cells were harvested from the surface of a tryptose hard-agar plate. All values represent the averages of three determinations. Cysteine was used at a concentration of 10^{-2} M; the remaining amino acids were used at a concentration of 10^{-1} M.*

the source. Bacteria within a band were highly motile and appeared to be trapped within the band. Rapid directional changes were exhibited by the bacteria within the band, whereas bacteria outside the band, on the side away from the diffusing chemical, traveled longer distances between directional changes. Only nonmotile bacteria were observed in the region between the test agar plug and the band of cells. Those bacteria that moved out of the band into the more concentrated region of the gradient (towards the test agent) were observed to quickly change directions, eventually returning to the band. Likewise, bacteria that moved out of the band into the less concentrated region of the gradient (towards the control end) also rapidly changed directions and returned to the band. The result of the activities of many motile bacteria appeared to confine the bacteria within a narrow region of the gradient, and this confinement of motile bacteria was visualized and interpreted as a distinct band of motile chemotactic bacteria. The numbers of motile bacteria within the band increased because of the entrance of new bacteria as the band moved down the capillary tube.

P. mirabilis IM47 never formed a band in response to any of the chemicals tested. As a toxic chemical diffused down the capillary tube, more and more of the bacteria became immobilized. Under microscopic observation (×400), motile cells of *P. mirabilis* were seen entering the toxic region and becoming immobilized before any avoidance response was initiated. A line of demarcation was seen moving down the capillary tube. On the proximal side (test agent), nonmotile bacteria were seen, whereas on the other side (control) normal motility was observed. Motile cells were not observed to enter the lethal region of the diffusing gradient and reverse direction, as was seen with the control organisms. Swarm cells of *P. mirabilis*

IM47 also failed to demonstrate a chemotactic response to the compounds tested. These cells also swam into the lethal region of the gradient and quickly became immobilized. Swarm cells did not tumble or initiate any rapid directional changes in response to the diffusing gradient.

When the chemical in the plug technique of Tso and Adler (15) was used to assay negative chemotaxis with *P. mirabilis*, some apparent negative chemotactic responses were observed. Zones of clearing surrounded by dense bands of bacteria were observed around agar plugs containing sodium acetate, indole, and $FeCl_3$. The threshold concentration for each was: sodium acetate, 10^{-1} M; indole, 10^{-2} M; and $FeCl_3$, 10^{-2} M. A 1-mm or greater zone of clearing, measured from the edge of the agar plug to the inner edge of the band of bacteria encircling the zone of clearing, was considered a positive test. Upon microscopic examination of the zones of clearing around agar plugs of sodium acetate and indole, motile bacteria were found, whereas no motile bacteria were observed in the clear zones around $FeCl_3$ agar plugs, which abolished motility. It was impossible to determine whether or not band formation was caused by the chemotactic response of motile bacteria, because both motile and nonmotile bacteria were found in the band. The number of motile bacteria within the band was not significantly greater than the number of motile bacteria observed within the zones of clearing around the test agar plugs. The presence of as many motile bacteria in the clear zones as in the band suggested that many of the motile

TABLE 3. *Characteristics of positive chemotaxis mutants on CH soft agar*

Mutant[a]	Chemotaxis		Motility	
	24 C	35 C	24 C	35 C
Nch18	−	−	−	−
Nch39	−	−	−	−
Nch2-7	+	−	+	+
Nch22-26	+	−	+	+
Nch9-11	−	−	+	+
Nch12	−	−	+	+
Nch15-17	−	−	+	+
Nch19-21	−	−	+	+
Nch27-28	−	−	+	+
Nch29-32	−	−	+	+
Nch33-34, 36-37	−	−	+	+
Nch38	−	−	+	+
Nch40-42	−	−	+	+
Nch13	−	+[b]	+	+
Nch8	+[b]	+[b]	+	+
Nch14	+[b]	+[b]	+	+
Nch35	+[b]	+[b]	+	+

[a] Mutants listed on the same horizontal line were isolated from the same enrichment plate.

[b] Showed positive chemotactic bands of smaller diameter than those produced by the wild type (IM47).

TABLE 4. *Swarming responses of positive chemotaxis mutants*

Mutants[a]	Ability to swarm								Swarm cell formation[b]	Motility[b]
	TSYE		TA[c]		CH[d]		NA			
	24 C	35 C	24 C	35 C	24 C	35 C	24 C	35 C		
Nch18	−	−	−	−	−	−	−	−	−	−
Nch39	−	−	−	−	−	−	−	−	−	−
Nch2-7	+	−	+	−	+	−	+	−	+	+
Nch8	+	+	+	+	+	+	+	+	+	+
Nch9-11	+	+	+	+	+	+	+	+	+	+
Nch13	+	+	+	+	+	+	+	+	+	+
Nch14	+	+	+	+	+	+	+	+	+	+
Nch15-17	+	+	+	+	+	+	+	+	+	+
Nch19-21	+	+	+	+	+	+	+	+	+	+
Nch22-26	+	+	+	+	+	+	+	+	+	+
Nch29-32	+	+	+	+	+	+	+	+	+	+
Nch35	+	+	+	+	+	+	+	+	+	+
Nch38	+	+	+	+	+	+	+	+	+	+
Nch40-42	+	+	+	+	+	+	+	+	+	+
Nch12	+	+	+	−	+	−	+	−	+	+
Nch27-28	+	+	+	+	+	+	+	−	+	+
Nch33-34, 36-37	+	+	+	+	+	+	+	−	+	+

[a] Mutants listed on the same horizontal line were isolated from the same enrichment plate.

[b] Determined microscopically with samples taken from the edge of colonial growth.

[c] TA, Tryptose agar.

[d] CH, Casamino Acids agar.

TABLE 5. *Results of the Doetsch and Seymour assay for negative chemotaxis*

Organism	Test agent eliciting a negative chemotactic response[a]
P. aeruginosa	HCl, $FeCl_3$, $FeSO_4$, $ZnCl_2$, MBA, IAA, NaOH, H_2O_2 (not tested: IBA, succinic acid, acetate)
P. morganii	HCl, $FeCl_3$, $FeSO_4$, MBA, IAA, IBA, NaOH, Ind, succinic acid, H_2O_2 (not tested: acetate)
S. marcescens	HCl, $FeCl_3$, $FeSO_4$, MBA, IAA, NaOH, H_2O_2 (not tested: IBA, AMC, Ind, succinic acid, acetate)
P. mirabilis IM47	None (all agents tested)

[a] Abbreviations: MBA, 2-Methylbutylamine; IAA, isoamylamine; IBS, isobutylamine; Ind, indole; AMC, acetylmethylcarbinol.

bacteria were nonchemotactic in the assay. Old CP agar, on which swarming had been allowed to completely cover the agar surface, was tested, and no bands or zones of clearing were formed in response to this agar medium. A nonmotile mutant (Nsw202) was tested in the assay system as a control. Zones of clearing were observed around the 1 M sodium acetate agar plugs and around the 10^{-1} M $FeCl_3$ agar plugs, whereas no zones were observed around 10^{-1} M indole agar plugs or around old, overswarmed agar plugs. The diffusion of sodium acetate caused a 1-mm (radius) zone of clearing to appear around the agar plug, whereas $FeCl_3$ caused a 3- to 4-mm zone to appear. The responses of motile *P. mirabilis* IM47 to sodium acetate and $FeCl_3$ at the same concentration were 2 mm and 4 to 5 mm, respectively.

No negative chemotactic responses were observed using the cells in the well assay system. If a response had occurred, a ring of bacteria would have formed in the center of a well in response to a gradient of the diffusing compound. No rings were observed with indole, sodium acetate, or L-leucine. Overswarmed CP agar and $FeCl_3$ were not tested.

Dialysis experiments. *P. mirabilis* was harvested from CP broth (overnight growth) and either inoculated directly or washed first and then inoculated onto the dialysis chamber and the control CP agar plates. Swarm cells were seen at 7 h on the dialysis chamber and plates, and swarming commenced at 8 h and continued for 2.5 h. The distance covered by the first swarm band for both the dialyzed colony and the control was 10 mm (width measured from edge of primary colony to edge of first swarm band). During the dialysis experiments the pH of the medium was monitored with pH paper and found not to change, in contrast to nondialyzed control plates with which the pH rose to 8.5 to 9.0.

Experiments with swarm cells. Swarm cells were collected from the second or third swarm band of *P. mirabilis* IM47 grown on CP agar (18 h at 30 C), by either washing the swarm cells off the surface of the agar with wash medium and then washing the swarm cells by the standard wash procedure or by scraping the swarm cells directly off the agar with an inoculating loop. When the collected swarm cells (either procedure) were inoculated onto fresh CP agar plates, immediate swarming was observed. The migration of rafts of swarm cells and the motion of the swarming was similar to the swarming originating from a primary colony. The distance swarmed (width of swarm band) appeared to be related to the number of swarm cells inoculated onto the agar; however, no quantitative studies were made. Also, the distance swarmed (width of swarm band) varied depending on when the swarm cells were harvested. Swarm cells harvested after 1 h of swarming tended to swarm further when transferred to fresh medium than did swarm cells harvested near the end of the swarming period. The maximum distance swarmed for transferred IM47 swarm cells was a width of 15 mm. Swarm cells harvested near the end of the swarm period swarmed an average of 5 mm. Swarm cells taken from other sources, such as the defined agar medium, swarmed within 2 min, but not immediately, when inoculated onto CP agar. Immediate swarming was observed only between similar media, for example, CP agar to CP agar or TSYE to TSYE agar, but between different media swarming was delayed about 2 min.

When CP agar-grown swarm cells were inoculated onto a CP agar film on the dialysis tubing and dialyzed, immediate swarming was observed on both the dialyzed agar and the nondialyzed control plates.

When washed swarm cells of IM47 were placed onto a medium consisting of 1.5% agar in water, the cells were motile but did not swarm. When the agar-water medium was supplemented with 200 to 500 μg of Triton X-100, Tween 80, or Tergitol per ml, swarming occurred out to a radius of 3 to 5 mm. The surfactants appeared to be toxic to the swarm cells, because the cells stopped swarming sooner

when exposed to higher concentrations and motility was lost when swarming ceased.

DISCUSSION

These studies were originated to examine experimentally the negative chemotaxis hypothesis proposed by Lominski and Lendrum (12) to explain the swarming of *Proteus*. The isolation of a number of nonswarming mutants seemed to present a direct approach for testing the production of the waste metabolite and for the detection of the hypothetical gradient. Our cross-feeding experiments attempted to detect those mutants defective in the production of the waste product or defective in the detection of the waste products. We assumed that some nonswarming mutants would be defective because they no longer produced the negative chemotactic agent; we presumed, however, that they still had the ability to respond to the agent if it were produced by another organism. Similarly, some mutants were assumed to be defective in the detection of the waste product, but they might still produce the chemical repellent. One might also assume that most nonmotile mutants still produced the repellent and would represent an excellent source of the negative chemotactic agent. The experimental results of the cross-feeding studies with nonswarming mutants strongly contradict the presence of a readily diffusible metabolite that stimulates swarming by nonswarming mutants or that promotes premature swarming by the wild type. If the production of this metabolite also results in a toxic environment that leads to the formation of swarm cells, some of these mutants certainly should be producers because they still form swarm cells, although swarming does not follow. It therefore seems that the possibility that all of the mutants were nonproducers is very unlikely. First, the wild-type culture would certainly be a producer and we found no evidence that the wild type would promote swarming by nonswarmers. Secondly, the blind selection technique used to isolate five of the nonswarming mutants was employed to avoid the criticism that all of the mutants were nonproducers, because the original selection technique had these nonproducing mutants being fed by neighboring colonies, and they appeared as swarming colonies and were missed. Although we only isolated five mutants using the blind selection approach, these mutants were not phenotypically different from the mutants isolated using the conventional selection technique. Thus, we feel that the cross-feeding studies strongly argue against the Lominski and Lendrum hypothesis by finding no evidence for a readily diffusible metabolite that can be detected by the organism. According to the negative chemotaxis hypothesis, it is the swarm cells that move down the gradient of repellent, and perhaps the short cells do not respond to the hypothetical metabolite. Several of the nonswarming mutants and the wild type form swarm cells on agar. If only these types of cells respond to a repellent, we should have detected swarming by those mutants that form swarm cells and premature swarming by the wild type in the cross-feeding experiments. This was not observed.

The phenotypes of the nonswarming mutants suggest, at the very least, that the theory of negative chemotaxis must be modified. The large number of mutants that are both nonmotile and non-swarm cell producers (Table 1) indicates that the formation of elongated cells is not simply the result of a toxic product. If it was, one would expect that a mutant with defective motility would still form swarm cells unless it also had a block in the production of the toxic agent. The relatively large number of mutants of this type obtained casts doubt upon the possibility that they represent double mutations.

Further evidence against the negative chemotaxis hypothesis comes from the negative chemotaxis assays using the wild-type *P. mirabilis* IM47. In our studies three different assay systems were used. With the Doetsch and Seymour system we used control organisms (*P. aeruginosa*, *S. marsescens*, and *P. morganii*), and these organisms responded to a number of agents as reported by others (7), but we observed no response by *P. mirabilis* IM47 to any test agent. Close microscopic examination of both the short forms and the swarm cells revealed that the cells became immobilized before a negative chemotactic response could be elicited. A negative chemotactic response would be characterized by a series of tumblings followed by net movement of bacteria away from the toxic region (14, 15). The short forms tumble briefly when they enter the lethal region of the gradient, whereas the swarm cells do not. Neither move out of the lethal region, as was observed for the control organisms. According to the negative chemotaxis theory for swarming, it is the swarm cells that recognize the subtle differences in a gradient and, since this study indicates that these cells are insensitive to potentially lethal compounds, it is unlikely that swarm cells swarm because of a recognition of a gradient of toxic metabolites.

In the Tso and Adler chemotaxis assay system, *P. mirabilis* appears to respond in a negative chemotactic manner. As described by Tso

and Adler using *E. coli* (15), zones of clearing appeared around test agar plugs and a dense band of cells encircled the zone. The threshold responses for *P. mirabilis* are 10 to 100 times higher than those reported for *E. coli*, and also the zones of clearing around the agar disks are smaller for *P. mirabilis*. Tso and Adler tested nonchemotactic motile mutants and found that the diffusion artifact described by Clayton (6) could not account for the negative chemotactic results from their chemotactic strains of *E. coli*. With the higher concentration of compounds (10 to 100×) required for eliciting chemotactic responses for *P. mirabilis*, the diffusion artifact must be considered. The study with nonmotile mutants of *P. mirabilis* indicates that a zone of clearing, which would ordinarily be labeled a negative chemotactic response, is a diffusion artifact for many of the compounds tested. This is an important point for *P. mirabilis*, since the numbers of motile bacteria composing the band are as numerous as the motile bacteria found within the zone of clearing. If negative chemotaxis exists for *P. mirabilis*, the response is 10 to 100 times less than the negative chemotactic responses reported for *E. coli* (15) and *Salmonella* (14), and this weak response is certainly not supportive of the Lominski and Lendrum negative chemotaxis hypothesis. We were unable to obtain sufficient numbers of swarm cells to test them in the Tso and Adler assay systems.

With a minor change, the Lominski and Lendrum negative chemotaxis hypothesis can be changed into a positive chemotaxis hypothesis. The presence of a waste product gradient can be replaced by a gradient of nutrients produced by the main colony during growth. This hypothesis is similar to that proposed by Moltke (13).

The results of the studies with nonchemotactic mutants and the quantitative chemotaxis studies with the nonswarming mutants contradict a theory involving positive chemotaxis. Of the nine groups of motile, nonchemotactic mutants, six showed swarming on all media tested at either of two temperatures. Of the three groups of mutants that did not show normal swarming, they only showed this abnormality on NA at 35 C. Mutant Nch12 was somewhat more hampered in that at 35 C it failed to swarm on any of the media tested except TSYE agar. Even with the exceptions, it can be stated that the majority of motile, chemotaxis mutants swarmed, indicating that positive chemotaxis is not required for swarming. The nonchemotactic mutants isolated were probably the generally nonchemotactic type described by Armstrong et al. for *E. coli* (4), because they were selected on a complex medium. Although they were not tested for their chemotactic response towards other classes of chemicals, they did lose the ability to form chemotactic bands on the semisolid CH medium (a medium that supports swarming when prepared with 1.5% agar). In all probability these mutants were also defective in negative chemotaxis as well; however, we were unable to demonstrate a clear-cut negative chemotactic response with the wild-type culture and consequently were unable to test these mutants for negative chemotaxis. The *E. coli* mutants described by Tso and Adler (15) that lacked all positive chemotaxis had also lost the negative chemotactic response; this might be the case with our mutants. If this assumption is correct, the nonchemotactic mutants provide strong evidence that neither positive nor negative chemotaxis is required for swarming.

The quantitative examination of chemotaxis towards amino acids was examined in two nonswarming mutants that showed modified chemotaxis in tryptose soft agar. The results indicated that, although Nsw109 does show impaired chemotaxis, Nsw228 shows near normal chemotaxis; both mutants show the ability to move towards attractants, yet neither swarms. When these results are combined with those from the study of nonchemotactic mutants, it would seem that chemotaxis is not involved in swarming. Furthermore, swarm cells of the wild-type culture failed to show a significant chemotactic response towards amino acids, and since the swarm cell suspension also contained short cells the weak response that was observed could be due to those short cells in the suspension.

A number of experiments attempted to eliminate the formation of a chemical gradient (attractant or repellent). When *P. mirabilis* IM47 was growing on the surface of a solid medium that was being dialyzed from beneath, the organism still swarmed. The results of pH measurements indicated that the dialysis was efficient and that the formation of a gradient of dialyzable substances was unlikely. Even better evidence against the requirement of a gradient for swarming comes from the observation that swarm cells removed from one agar surface will swarm almost immediately when placed onto a fresh agar surface. A similar observation was made by Kvittingen in 1949 (11). We have subsequently found that no nutrients need be present in the second medium; swarm cells swarmed on agar-water when any of a number of detergents was present. Should either negative chemotaxis or positive chemotaxis be involved in swarming, the establishment of a gradient of the attractant or repellent would be

essential for any chemotactic response. It is difficult to explain the immediate swarming of swarm cells when transferred to fresh medium if a gradient is required. Time is just too short. That they will swarm on the agar-water-detergent medium rules out the formation of an attractant gradient and a positive chemotactic response. Furthermore, a negative chemotactic response is unlikely because washed swarm cells move almost immediately after being placed onto fresh medium.

The results presented here essentially rule out the involvement of chemotaxis in the swarming of *P. mirabilis*. It is now clear that swarming can be subdivided into at least three stages: the formation of swarm cells; the movement of swarm cells across the agar surface; and the division of swarm cells back to the short forms (consolidation). Our current work is directed at determining the conditions that lead to swarm cell production.

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