Proteus mirabilis Mutants Defective in Swarmer Cell Differentiation and Multicellular Behavior†

ROBERT BELAS,1,2* DEBORAH ERSKINE,1 AND DAVID FLAHERTY1

Center of Marine Biotechnology, The University of Maryland, 600 East Lombard Street, Baltimore, Maryland 21202;1 AND Department of Biological Sciences, University of Maryland Baltimore County, Baltimore, Maryland 212282

Received 21 June 1991/Accepted 24 July 1991

Proteus mirabilis is a dimorphic bacterium which exists in liquid cultures as a 1.5- to 2.0-µm motile swimmer cell possessing 6 to 10 peritrichous flagella. When swimmer cells are placed on a surface, they differentiate by a combination of events that ultimately produce a swarmer cell. Unlike the swimmer cell, the polyploid swarmer cell is 60 to 80 µm long and possesses hundreds to thousands of surface-induced flagella. These features, combined with multicellular behavior, allow the swarmer cells to move over a surface in a process called swarming. Transposon Tn5 was used to produce P. mirabilis mutants defective in wild-type swarming motility. Two general classes of mutants were found to be defective in swarming. The first class was composed of null mutants that were completely devoid of swarming motility. The majority of nonswarming mutations were the result of defects in the synthesis of flagella or in the ability to rotate the flagella. The remaining nonswarming mutants produced flagella but were defective in surface-induced elongation. Strains in the second general class of mutants, which made up more than 65% of all defects in swarming were motile but were defective in the control and coordination of multicellular swarming. Analysis of consolidation zones produced by such crippled mutants suggested that this pleiotropic phenotype was caused by a defect in the regulation of multicellular behavior. A possible mechanism controlling the cyclic process of differentiation and dedifferentiation involved in the swarming behavior of P. mirabilis is discussed.

Proteus mirabilis is a motile gram-negative bacterium, similar in many aspects of its physiology to other members of the family Enterobacteriaceae, such as Escherichia coli and Salmonella typhimurium. It was originally described and named by Hauser in 1885 for the character in Homer's Odyssey who "has the power of assuming different shapes in order to escape being questioned" (quoted from reference 18). P. mirabilis is considered to be an opportunistic pathogen and is one of the principal causes of urinary infections in hospital patients with urinary catheters (32, 34). Its ability to colonize the surfaces of catheters and the urinary tract may be aided by the characteristic first described more than a century ago and currently referred to as swarmer cell differentiation.

When grown in suitable liquid media, P. mirabilis exists as 1.5- to 2.0-µm motile cells with 6 to 10 peritrichous flagella. These bacteria, called swimmer cells, display characteristic swimming and chemotactic behavior, moving toward nutrients and away from repellents (36). However, a dramatic change in cell morphology takes place when cells grown in liquid are transferred to a nutrient medium solidified with agar. Shortly after encountering an agar surface, the cells begin to elongate. This is the first step in the production of a morphologically and biochemically differentiated cell, referred to as the swarmer cell. The process of elongation takes place with only a slight increase in cell width and is due to an inhibition in the normal septation mechanism, although the molecular mechanism of inhibition is not known. Elongation of the swarmer cell can give rise to cells 60 to 80 µm in length. During this process, DNA replication proceeds without significant change in rate from that in the swimmer cell (15). Not surprisingly, the rate of synthesis of certain proteins, e.g., flagellin (FliC, the product of the fliC gene), the protein subunit of the flagellar filament, is altered markedly in the swarmer cell (3, 5, 14). The result of this process is a very long, nonseptate, polyploid cell. The number of chromosomes in the swarmer cell is roughly proportional to the increase in length, such that a 40-µm swarmer cell has about 20 chromosomes (6). Eventually septation and division do take place at the ends of the long swarmer cells, producing a microcolony of differentiated cells.

Concurrent with cellular elongation, changes take place in the rate of synthesis of flagella on the swarmer cell. Although swimmer cells have only a few flagella, the elongated swarmer cells are profusely covered by hundreds to thousands of new flagella (Fig. 1A) synthesized specifically as a consequence of growth on the surface (4, 19, 20). The term "flagellin factories" was first used by Hoeniger (19) to describe the tremendous synthesis of new flagella in swarmer cell differentiation. The newly synthesized surface-induced flagella are composed of the same flagellin subunit as the swimmer cell flagella, indicating that the same flagellar species is overproduced upon surface induction (6). The result of the surface-induced differentiation process is a swarmer cell, which differs from the swimmer cells by having the unique ability to move over solid media in a translocation process referred to as swarming (17). However, individual swarmer cells by themselves do not have the ability to swarm (10). Swarming is the result of a coordinated, multicellular effort of groups of differentiated swarmer cells (10). The process begins when a group of differentiated swarmer cells move outwards as a mass and continues until the swarming mass of bacteria is reduced in number as a result of loss of constituent cells which fall behind on the surface or when the mass reverses direction.

Swarming of P. mirabilis is cyclic. Once swarmer cells have fully differentiate, the swarming colony moves outwards in unison from all points along the periphery for a
FIG. 1. Swarmer cell morphology and swarming motility of *P. mirabilis*. (A) Electron micrograph of a wild-type swarmer cell taken from the periphery of a swarming colony grown on L agar at 37°C for 6 h and negatively stained with uranyl acetate. Bar, 5 μm. (B) Swarming colony and resulting consolidation patterns of BB2000 grown on an L agar plate at 37°C for 16 h. Oblique lighting directed at the underside of the agar plate was used to accent the consolidation zones, which show up as lighter concentric rings in the swarming colony.
period of several hours and then stops (10, 12). This cessation of movement is accompanied by a dedifferentiation of the swarmer cell back to swimmer cell morphology, in a process referred to as consolidation (see reference 37 for a review). The cycle of swarming and consolidation is then repeated several times until the agar surface is covered by concentric rings formed by the swarming mass of bacteria (12). This cycle of events gives rise to the characteristic bull’s-eye appearance of \textit{P. mirabilis} colonies (Fig. 1B). The swarmer cell requires continuous contact with the surface to maintain the differentiated state. When removed from the surface of an agar plate and suspended in liquid medium, cells quickly begin to separtate and divide into short cells, and the synthesis of flagella returns to the level observed in swimmer cells (21). Thus, the differentiation process is reversible both as a result of the consolidation process and as a consequence of removal of the inducing stimulus from the surface.

Although many attempts have been made to explain the mechanism of the swarming in \textit{Proteus} species (37), including slime production (33) and chemotaxis (25, 36), the process of swimmer cell differentiation and multicellular swarming motility remains elusive. However, swimmer cell differentiation is not unique to \textit{P. mirabilis}. Other bacteria, most notably \textit{Serratia} (1) and \textit{Vibrio} (9, 29) strains, have also been reported to swarm as a result of a surface-induced differentiation from swimmer to swimmer cell. The regulation of swimmer cell differentiation is best understood for \textit{V. parahaemolyticus} (29). The polar flagellum in \textit{V. parahaemolyticus} serves in a sensory capacity by monitoring external conditions which cause the inhibition of flagellar rotation (9, 27). When such conditions are encountered, inhibition of rotation of the polar flagellum sends a signal into the cell which triggers a chain of events ultimately producing a differentiated swimmer cell. In addition to the control exerted by monitoring the inhibition of polar flagellum rotation, a secondary signal is evidently sensed when iron becomes limiting (28). The combination of conditions which inhibit the rotation of the polar flagellum and limit the iron concentration is essential for initiation of transcription of the \textit{lfq} genes (29).

Until recently (2), in-depth studies by modern techniques have not been applied to understanding the genetic regulation and sensory transduction mechanisms of swimmer cell differentiation and multicellular swarming of \textit{P. mirabilis}. Since little is known about the genetic regulation of \textit{Proteus} swarming, we wanted a means of analysis which would be independent of the mutant phenotype under investigation. The objective of this investigation was to use \textit{Tn5} mutagenesis to develop a bank of \textit{Proteus} mutants defective in wild-type swimmer cell differentiation and multicellular swarming motility. The present report is concerned with our analysis of the resulting mutants. By a combination of assays, \textit{Proteus} mutants defective in swarming were grouped into two general categories. Mutants completely defective in swarming motility were in large part due to mutations in genes affecting flagellin synthesis. The second group of mutants produced non-wild-type swarming and are most probably due to mutations in genes necessary for intercellular signaling. Analysis of the second class of mutants suggests that a cascade of signal molecules may be required for the cycles of differentiation and dedifferentiation associated with swimmer cell motility.

**MATERIALS AND METHODS**

**Bacterial strains.** The strains used in this study are listed in Table 1. BB2000 is a spontaneously occurring rifampin-resistant mutant of PRM1 (7) and is used as the wild-type strain throughout this study.

**Media and growth conditions.** \textit{E. coli} and \textit{P. mirabilis} strains were grown as described in the accompanying paper.

### Table 1. Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype or phenotype*</th>
<th>Derivation</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{E. coli}</td>
<td>trp lacZ strA thi recA FliC$^-$</td>
<td>Spontaneous from PRM1</td>
<td>J. Shapiro</td>
</tr>
<tr>
<td>CSH4</td>
<td>supE44 supF38 hsdS3 (r$^+$ mB$^-$) dapD8 lacY1 glnV44 Δgal-</td>
<td>pUT/mini-Tn5 Cm x BB2000b</td>
<td>This study</td>
</tr>
<tr>
<td>DH5a</td>
<td>supF38 hsdS3 (r$^+$ mB$^-$) dapD8 lacY1 glnV44 Δgal- srlC5748 tyrT388 gyrA29 tonA53 Δ(ThyA57)</td>
<td>pUT/mini-Tn5 Cm x BB2000x</td>
<td>This study</td>
</tr>
<tr>
<td>\textit{P. mirabilis}</td>
<td>Wild type</td>
<td>BB2000</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>PRM1</td>
<td>Rif$^R$</td>
<td>BB2002</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>BB2022</td>
<td>swr-2022: Tn5 Cm Swr$^{+}$ Elo$^{-}$</td>
<td>pUT/mini-Tn5 Cm x BB2000</td>
<td>This study</td>
</tr>
<tr>
<td>BB2029</td>
<td>swr-2029: Tn5 Cm Swr$^{+}$</td>
<td>pUT/mini-Tn5 Cm x BB2000</td>
<td>This study</td>
</tr>
<tr>
<td>BB2035</td>
<td>swr-2035: Tn5 Cm Swr$^{-}$ Mot$^{-}$</td>
<td>pUT/mini-Tn5 Cm x BB2000</td>
<td>This study</td>
</tr>
<tr>
<td>BB2040</td>
<td>swr-2040: Tn5 Cm Swr$^{-}$ Elo$^{-}$</td>
<td>pUT/mini-Tn5 Cm x BB2000</td>
<td>This study</td>
</tr>
<tr>
<td>BB2075</td>
<td>swr-2075: Tn5 Cm Swr$^{-}$ Che$^{-}$</td>
<td>pUT/mini-Tn5 Cm x BB2000</td>
<td>This study</td>
</tr>
<tr>
<td>BB2076</td>
<td>swr-2076: Tn5 Cm Swr$^{-}$ Elo$^{-}$</td>
<td>pUT/mini-Tn5 Cm x BB2000</td>
<td>This study</td>
</tr>
<tr>
<td>BB2105</td>
<td>swr-2105: Tn5 Cm Swr$^{+}$ Elo$^{+}$</td>
<td>pUT/mini-Tn5 Cm x BB2000</td>
<td>This study</td>
</tr>
<tr>
<td>BB2128</td>
<td>swr-2128: Tn5 Cm Swr$^{-}$ Mot$^{-}$</td>
<td>pUT/mini-Tn5 Cm x BB2000</td>
<td>This study</td>
</tr>
<tr>
<td>BB2178</td>
<td>swr-2178: Tn5 Cm Swr$^{+}$ Che$^{-}$</td>
<td>pUT/mini-Tn5 Cm x BB2000</td>
<td>This study</td>
</tr>
<tr>
<td>BB2180</td>
<td>swr-2180: Tn5 Cm Swr$^{-}$ Elo$^{+}$</td>
<td>pUT/mini-Tn5 Cm x BB2000</td>
<td>This study</td>
</tr>
<tr>
<td>BB2202</td>
<td>swr-2202: Tn5 Cm Swr$^{+}$ Flab</td>
<td>pUT/mini-Tn5 Cm x BB2000</td>
<td>This study</td>
</tr>
</tbody>
</table>

* Che$^{-}$, produces rotating flagella but defective in chemotactic response; Elo$^{+}$, swimmer cell elongation constitutive; Mot$^{+}$, lacking elongation of swimmer cell; Flab, no 36.7-kDa flagellin produced; Swr$^{+}$, produces nonrotating flagella; Swr$^{-}$, absence of swarm on agar media; Swr$^{+}$, non-wild-type or crippled swarming and unusual consolidation patterns.

† \textit{P. mirabilis} mutants defective in swimmer cell differentiation and swarming motility. Produced from conjugation of \textit{E. coli} harboring pUT/mini-Tn5 Cm and BB2000 (7).
Swarming motility was observed on L agar (7), whereas LSW\(^{-}\) agar (7) was used to inhibit swarming. For selection of Tn\(^5\) Cm inserts, chloramphenicol was added to a final concentration of 150 \(\mu\)g/ml of medium. Swarming motility in plates was determined by using a semisolid agar medium containing 10 \(g\) of tryptone, 5 \(g\) of NaCl, and 3.5 \(g\) of agar per liter. This medium is referred to as Mot agar. Swarming motility in liquid was assessed by microscopic examination of cultures grown in Mot broth (Mot agar lacking agar).

**Mutagenesis with Tn\(^5\) derivatives.** Derivatives of transposon Tn\(^5\) carried on a suicide vector (11) were used to mutagenize *P. mirabilis* as described by Belas et al. (7). A bank of 13,036 chloramphenicol-resistant (Cm\(^{\text{r}}\)) mutants was produced from conjugal matings of *E. coli* with recipient *P. mirabilis* (7).

**Analysis of swarming and swimming motility.** In all swarming tests, L agar plates were used after thorough drying at 42°C (45 to 60 min) to provide uniform conditions for testing swarming motility. To test swarming motility of the *Proteus* mutants, we replica plated colonies onto L agar. After 4 to 6 h of incubation at 37°C, swarming motility was scored and potential nonswarming mutants were transferred to a set of submaster plates. The swarming analysis was repeated again until no colonies demonstrated active swarming during the 6-h incubation. The putative nonswarming colonies were then transferred to L agar and incubated at 37°C for 16 h. After overnight incubation, many of the putative nonswarming colonies produced swarming motility on L agar, although none demonstrated wild-type swarming when compared with BB2000. Mutants that did not show any swarming motility were designated as Swr\(^{-}\) to distinguish them from mutants showing non-wild-type swarming, which were called Swr\(^{\text{cr}}\). Swr\(^{\text{cr}}\) refers to the crippled nature of these mutants defective in wild-type swarming motility and consolidation.

Swimming motility was assessed by both a semisolid agar analysis and light-microscopic examination of the motility of cells grown in Mot broth. As a preliminary test, the bank of Swr\(^{-}\) and Swr\(^{\text{cr}}\) *Proteus* mutants was transferred to Mot agar. Following overnight growth at 37°C, nonmotile bacteria were identified as those that failed to swim through the Mot agar. This was repeated, and the resulting nonswimming (Swm\(^{-}\)) bacteria were identified. Since Swm\(^{-}\) mutants could be defective in either synthesis of flagella (Fla\(^{-}\)), flagellum energetics (Mot\(^{-}\)), or chemotaxis behavior (Che\(^{-}\)), the motility of the Swm\(^{-}\) bacteria was further analyzed by light microscopy of individual strains grown in Mot broth for 4 to 6 h at 37°C to a cell density of 10\(^{10}\) to 10\(^{10}\) cells per ml. Strains that displayed active swimming motility in liquid but failed to show migration through Mot agar were designated Che\(^{-}\). The remaining strains were all nonswimming in both liquid and semisolid media and could be either defective in producing flagella (Fla\(^{-}\)) or defective in rotating the flagella (Mot\(^{-}\)). Fla\(^{-}\) strains were separated from Mot\(^{-}\) strains by Western immunoblot analysis with rabbit anti-flagellin antisera. In all swimming assays, in addition to the wild-type *P. mirabilis*, *E. coli* DH5\(\alpha\) (FlIC\(^{\text{r}}\)) and CSH4 (FlIC\(^{-}\)) served as positive and negative controls, respectively, for motility assays.

**Protein analysis of flagellin and western hybridization techniques.** Purified flagella from strain BB2000 were prepared as follows. An overnight culture of cells was spot inoculated in the center of each of 10 L agar plates. Following incubation at 37°C for 10 h (the time required for the swarming cells to cover the surface), cells were scraped off the agar surface with an L-shaped glass rod and suspended in 50 ml of phosphate-buffered saline (PBS; 20 mM sodium phosphate [pH 7.5], 100 mM NaCl). After vigorous vortexing for 2 min, whole cells were removed from the supernatant, which contained free flagella, by centrifugation in a Beckman J-21 rotor at 4°C for 10 min at 10,000 rpm. Remaining whole cells were removed by a second centrifugation under identical conditions. Cell debris and fragmented cells were further removed by centrifugation of the supernatant in a Beckman J-A-20.1 rotor for 20 min at 15,000 rpm and 4°C. Suspended flagella contained within the supernatant were then pelleted by centrifugation in a Beckman SW27.1 rotor at 25,000 rpm for 60 min at 4°C. The resulting pellet was suspended in 100 to 250 \(\mu\)l of PBS. The crude preparation of flagella was layered on a 25 to 75% sucrose step gradient (in PBS). Flagella were separated from membrane components and other cellular debris by centrifugation at 20,000 rpm for 3 h at 4°C in a Beckman SW27.1 rotor. A single band of >95% pure flagellin was extracted from the 30% sucrose step, extensively dialyzed against distilled water and lyophilized to dryness. The flagella were suspended in distilled water and used as a flagellin standard in immunoblots.

To analyze the production of flagella in Tn\(^5\)-generated mutants, we grew bacteria overnight in 2 ml of L broth containing chloramphenicol. The cells were concentrated by centrifugation, and the pellet was suspended in ice-cold PBS to an optical density at 600 nm of 0.35. A 1-ml sample of each culture was then pelleted in a microcentrifuge at 14,000 rpm for 20 min at 4°C. The supernatant was removed, the pellet was suspended in 50 \(\mu\)l of distilled water, and the sample was stored at −20°C until needed. Wild-type whole-cell preparations were treated in an identical manner.

The presence of flagella on various Swr\(^{-}\) and Swr\(^{\text{cr}}\) mutants was determined by Western blotting (immunoblotting) with antisera directed against the 36.7-kDa flagellin protein of *P. mirabilis*. Immunoblotting was done by standard methods with only minor modifications (16). The protein concentration of purified preparations of flagella and whole cells was determined by using the bicinchoninic acid (BCA) protein assay reagent (Pierce Chemical). For whole-cell preparations, 3 \(\mu\)g of protein was loaded per lane, whereas for purified flagella, 0.8 \(\mu\)g was analyzed. Briefly, proteins in sample buffer were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% polyacrylamide resolving gel according to the procedure of Laemmli (23). Electrophoretic transfer of proteins (35) to Immobilon-P membranes (Millipore) was done at 4°C for 1 h at 100 V in a buffer consisting of 0.096 M glycine, 0.125 M Tris base, and 20% methanol. Western blots were blocked with saturated (5% nonfat dry milk in 0.9% NaCl–20 mM Tris (pH 7.5)). The immobilized proteins were then reacted for 2 to 12 h with antiserum directed against the purified 36.7-kDa flagellin protein. Antiserum 497 (anti-flagellin antiserum) was used throughout this study at a dilution of 1:1,000. At this concentration a small amount of background hybridization to three unidentified nonflagellin proteins was observed. This background did not hamper the analysis of flagellin and was subtracted from the analyses. Unbound anti-flagellin immunoglobulin G (IgG) was washed away, and goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma Chemical) was then reacted with the immobilized proteins for 2 h at room temperature. The secondary antibody was removed by washing in 0.9% NaCl–20 mM Tris (pH 7.5). To visualize the bound IgG conjugate, we reacted the blot with 5-bromo-4-chloro-3-indolylphosphate and Nitro Blue Tetrazolium substrate (BCIP-NBT substrate) until the protein bands were suitably dark (usually 30 min). The reaction was stopped by rinsing the blot in PBS containing 20...
mM EDTA. Once dried, blots were photographed and stored at room temperature. A silver-staining procedure (16) was used to visualize proteins from whole-cell extracts when comparisons between immunoblots and total whole-cell protein samples were required.

**Antibody production.** Polyclonal antiserum were prepared against the purified and denatured Proteus 36.7-kDa flagellin protein. Purified flagellin was separated by SDS-PAGE as described above. Proteins were visualized by Coomassie blue staining by standard procedures (13). The 36.7-kDa flagellin protein was excised from the stained gel, minced to homogeneity with Freund’s incomplete adjuvant, and subcutaneously administered to New Zealand White rabbits. Initially, 200 µg of purified protein was injected. Booster shots of an additional 50 µg of flagellin were given every 2 weeks. Antibody titer was tested 1 week following an antigen boost and empirically determined by using immunoblots to purified flagellin. Administration of the antigen continued until the titer peaked, usually 45 to 60 days after initial injection. At the time of maximum titer, blood was withdrawn from the ear artery and collected in a glass tube. Following clotting, the serum was removed and stored at −20°C until needed.

**Swarmer cell elongation analysis.** The ability of P. mirabilis Swr− and Swr+ mutants to elongate as a result of surface-induction was analyzed as follows. An overnight broth culture of P. mirabilis cells grown in L. broth plus chloramphenicol was inoculated on fresh L agar containing chloramphenicol. After incubation at 37°C for 6 h, a loopful of cells from the edge of the colony was removed aseptically, placed in 1 mL of PBS, and vortexed briefly. A sample was removed and immediately examined by phase-contrast light microscopy. Wild-type P. mirabilis served as a control for elongation comparison. A strain was considered to be Elo+ (surface-induced cell elongation) if more than 10% of the population had an average length of at least 20 µm (10 swarmer cell lengths). To test for constitutive elongation (Elo−) mutants, a 16-h broth culture of P. mirabilis was inoculated in fresh L. broth containing chloramphenicol. The culture was incubated with shaking for 6 h at 37°C, after which a sample was removed for light-microscopic analysis. A strain was designated Elo− if, in this noninducing environment, at least 10% of the cells were 20 µm or longer. BB2000 served as a control and typically had less than 0.5% swarmer cells in an uninduced population.

**Materials and reagents.** All reagents were of the highest purity available. Components of bacteriological media were purchased from Difco. The indicators BCIP and NBT, used to assay alkaline phosphatase, were purchased from Boehringer Mannheim Biochemicals and Sigma Chemical Co., respectively.

**RESULTS**

**Analysis of mutants defective in swarmer cell differentiation and swarming motility.** Our interest in P. mirabilis concerns the genetic regulation of swarmer cell differentiation and the mechanisms of sensory transduction controlling multicellular swarming behavior. To accomplish these goals, we undertook an analysis of mutants defective in wild-type swarming. As described in the accompanying paper (7), a derivative of Tn5 was used to produce a bank of 13,036 P. mirabilis Cm' mutants. Initially, the bank of mutants was maintained on agar plates which prevented swarming motility (LSW− agar). To detect strains which were defective in the swarming phenotype, we replica plated the bank of mutants onto L agar medium, which permitted swarming motility after incubation at 37°C for 4 to 6 h. Wild-type swarming-proficient colonies, designated Swr+ (Fig. 1B), were distinguished from those completely lacking swarming (Swr−, Fig. 2A) and those showing abnormal or crippled multicellular swarming behavior (Swr+ [cr for crippled]; Fig. 2B to D) by examining the zone immediately around the replicated colony. When strong backlighting was used, a thin film of swarming bacteria could be observed to move outward from the point of initial inoculation in Swr+ strains, whereas Sw− and Swr+ strains showed no bacteria past the point of inoculation after 6 h of growth at 37°C. Of the 13,036 original Cm' mutants, 70 were found to be completely defective in swarming motility (Swr−) and 142 produced non-wild-type swarming. This latter category contained pleiotropic Swr− mutants, demonstrating a wide variety of swarming behaviors including changes in the rate of swarming motility and variations in consolidation pattern formation from the wild type. Although it was possible to distinguish wild-type swarming from Swr− or Swr+ mutants at 6 h or earlier, the distinction between Swr− and Swr+ was not seen until these mutants had been grown for 16 to 24 h at 37°C. After such incubations, Swr− colonies were observed to show no growth past the point of inoculation (Fig. 2A), whereas Swr+ strains translocated outward from the inoculation point (Fig. 2B to D). However, although this group of Swr− mutants did manifest swarming motility, their movement and multicellular behavior was not wild type. The group of 142 Swr− mutants included strains which showed changes in the spatial organization of consolidation zones (Fig. 2B and C), as well as mutants whose consolidation zones were indeterminate or ill defined (Fig. 2D).

Analysis of the frequency of acquiring Swr− and Swr+ in the population suggests that the regulation of swarmer cell differentiation and multicellular swarming motility must involve a complex set of genes. Although these experiments did not specifically address the question of how many genes are involved in swarming, our data show that approximately 1.6% (212 Sw− and Swr+ mutants + 13,036 total mutants) of all Cm' mutants were defective in some aspect of swarming. If the chromosome size and coding capacity of P. mirabilis is roughly equivalent to that of E. coli, the data suggest that roughly 60 genes ([(212 Sw− and Swr+ mutants + 13,036 total mutants) × 4,000 genes per chromosome]) may play a role in the process of swarming motility. If only Swr− mutants are considered in this type of analysis, the number of genes involved in swarming and swarmer cell differentiation drops to around 20. These estimates compare favorably to the 40-gene estimate reported by others (2). Since the development of swarmer cells and multicellular swarming behavior is a complex phenotype, most probably involving between 20 and 60 genes, we analyzed the group of 212 Sw− and Swr+ mutants for other characteristics associated with swarmer cell differentiation and multicellular swarming motility in an attempt to further define the nature of each mutation.
rotate the flagella. Fourth, multicellular interactions and signaling must occur between swarmer cells to allow them to
move out on the agar surface in groups and to consolidate (dedifferentiate) in unison. If any one of these events is
defective, the end result will be either complete loss of
swarming motility or abnormal swarming behavior. Thus, at
least four possible phenotypic categories should be observed
in our analysis of \textit{Proteus} Swr^- and Swr^{cr} mutants.

As mentioned above, one of the principal defects leading
to a Swr^- strain is the failure to synthesize flagella. \textit{P. mirabilis}
produces only one type of flagellum, whose expression is surface induced such that cells become hyperflagel-
lated when swarmer cell differentiation occurs (4, 6). There-
fore, Fla^- \textit{Proteus} mutants would be expected to not only be
defective in swarming motility, but also to have defects in
their swimming motility. To test for the presence and func-

FIG. 2. Colony morphology of \textit{P. mirabilis} mutants defective in wild-type swarming motility. A loopful of an overnight culture grown in
\textit{L} broth was inoculated as a 1.5- to 2-cm line at the center of an \textit{L} agar plate, and the cells were incubated for 16 h at 37°C. (A) BB2202 (Swr^- Swm^- Fla^-) is an example of a mutant totally devoid of swarming motility. (B to D) Strains BB2180, BB2022, and BB2029, respectively, show normal swimming and flagellar synthesis (Swm^- Fla^-), but are crippled in wild-type swarming motility (Swr^{cr}) because of loss of spatial or temporal control of consolidation (BB2180 and BB2022) or as a result of uncoordinated multicellular migration (BB2029), resulting in an indeterminate consolidation pattern.
tioning of flagella in the Swr− and Swr+ strains, we analyzed swimming motility, chemotactic behavior, and the ability to synthesize flagellin. Overnight broth cultures or cells suspended from plates were prepared as described in Materials and Methods. Except where noted, all preparations were from cells grown in L broth. Lanes: 1, purified flagellin; 2, BB2000 (wild type, broth); 3, BB2000 (wild type, agar); 4, BB2035 (Swr− Swm− Fla−); 5, BB2128 (Swr− Swm− Fla−); 6, BB2075 (Swr− Swm− Che− Fla−); 7, BB2178 (Swr− Swm− Che− Fla−); 8, BB2040 (Swr− Swm− Elo− Fla−); 9, BB2076 (Swr− Swm− Elo− Fla−); 10, BB2105 (Swr+ Swm+ Elo+ Fla+); 11, BB2029 (Swr+ Swm+ Fla+); 12, BB2202 (Swr− Swm− Fla−). (A) Silver stain of whole-cell preparations and (B) Western blot with rabbit polyclonal antiserum 497 which is directed against the P. mirabilis 36.7-kDa flagellin (FliC) subunit. The blot was subsequently developed with goat anti-rabbit IgG-alkaline phosphatase secondary antiserum and NBT-BCIP substrate. The arrow indicates the presence of the 36.7-kDa FliC subunit. Molecular size markers in kilodaltons are indicated on the left.

4 to 6 h, and the zone of swimming migration was measured and compared with that of the wild type. Since lack of migration through Mot agar can be attributed either to a defect in flagellar synthesis (Fla−), or in the ability to rotate the flagellum (Mot−) or to a defect in chemotactic sensing (Che−) of the attractant gradient established by the growing cells (24), we conducted tests to distinguish among the three possibilities. Strains that failed to migrate in Mot agar were grown for 4 to 5 h at 37°C in Mot broth and then examined for swimming motility by using light microscopy. If motile cells were observed by light microscopy but motility was not seen on Mot agar, the strain was considered Che−. If no motility was seen in either case, the cells were considered either Fla− or Mot−. To distinguish between these two possibilities, we determined the presence of flagellin in whole-cell preparations by using a Western analysis with rabbit polyclonal antisera elicited against the purified 36.7-kDa flagellin protein of BB2000 (Fig. 3). The presence of a flagellin band was used to confirm the existence of flagella. It is presumed for the purposes of this assay that the only detectable flagellin present in the cell exists in the assembled flagellar filament, since a pool of unassembled flagellin monomers has not been found to exist in P. mirabilis (6). Strains possessing paralyzed flagella (Fla+) were designated as Mot−.

The results of the motility, chemotaxis, and flagellin testing are shown in Table 2. Not surprisingly, the loss of swimming motility was consistent with the loss of swimming motility. The predominant defect (47 of 70) in Swr− mutants was due to a failure to synthesize flagellin. The frequency of Swr− Fla− mutants in this analysis is comparable to the frequency of P. mirabilis Swr− Fla− mutants reported elsewhere (2). These results support the idea that the same flagella used for swimming motility are overexpressed during swarmer cell differentiation in P. mirabilis. As seen in Fig. 3, Western analysis with polyclonal antiserum directed against the 36.7-kDa flagellin protein indicated that the amount of

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>No. of mutants</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swr− Swm− Fla−</td>
<td>47</td>
<td>Nonswarming; do not synthesize flagellin</td>
</tr>
<tr>
<td>Swr− Swm− Mot−</td>
<td>2</td>
<td>Nonswarming; paralyzed flagella</td>
</tr>
<tr>
<td>Swr− Swm+ Che−</td>
<td>6</td>
<td>Nonswarming; defective in chemotaxis</td>
</tr>
<tr>
<td>Swr− Swm+ Elo−</td>
<td>7</td>
<td>Nonswarming; defect in elongation</td>
</tr>
<tr>
<td>Swr− Swm+ Elo+</td>
<td>4</td>
<td>Nonswarming; constitutive elongation</td>
</tr>
<tr>
<td>Swr− Swm+ Elo+ ??</td>
<td>4</td>
<td>Nonswarming; unknown mutation</td>
</tr>
<tr>
<td>Swr+ Swm−</td>
<td>0</td>
<td>Crippled swimming; do not swim</td>
</tr>
<tr>
<td>Swr+ Swm+ Che−</td>
<td>13</td>
<td>Crippled swimming; defect in chemotaxis</td>
</tr>
<tr>
<td>Swr+ Swm+ Elo−</td>
<td>26</td>
<td>Crippled swimming; defect in elongation</td>
</tr>
<tr>
<td>Swr+ Swm+ Elo+</td>
<td>10</td>
<td>Crippled swimming; constitutive elongation</td>
</tr>
<tr>
<td>Swr+ Swm+ ??</td>
<td>93</td>
<td>Crippled swimming; unknown mutation</td>
</tr>
</tbody>
</table>

* ???, undefined mutants, defective in a currently unknown locus. For other definitions, see Table 1, footnote a.
flagellin synthesized per cell was dependent on the type of swimming defect. For example, Mot− strains BB2035 and BB2128 (Fig. 3, lanes 4 and 5) appeared to produce less flagellin than did the wild type (lane 2) or Che− mutants (BB2075 and 2178 [lanes 6 and 7]), which produce slightly more flagellin than the wild type. The cause of this variation in flagellar synthesis and its effect on multicellular swimming motility is not fully understood, but possible explanations are discussed below.

Loss of swimming motility was not observed in Swr− mutants. Since Swr− mutants are strains that are defective in multicellular behavior but display swimming motility, it is not surprising that they have rotating flagella. Interestingly, 13 of the 142 Swr− mutants (and 6 of 70 Swr− mutants) were defective in chemotaxis behavior in semisolid and liquid media. The interpretation of this result is that a functioning chemotaxis sensory system is necessary for swimmer cell differentiation and/or multicellular swimming motility. This agrees with the evidence on the involvement of chemotaxis in V. parahaemolyticus swimming motility (31), but is contradictory to the evidence accumulated for P. mirabilis (36). Possible explanations for this contradiction are discussed below.

The second general defect that can give rise to a Swr− or Swr+ mutant pertains to the set of genes controlling swimmer cell elongation. Because elongation appears to be intimately involved in the process of swimming and differentiation, we examined the 212 Swr− and Swr+ mutants to determine whether defects in surface-induced elongation (Elo−) were present. Two criteria were used to assess swimmer cell elongation: (i) the percentage of the induced population whose length equaled or exceeded 20 μm, and (ii) the average length of the swimmer cell population. BB2000 grown on agar was used as a control and typically showed >80% swimmer cells at 40-μm average length under inducing conditions. As shown in Table 2, Elo− mutants accounted for 10% (7 of 70) of the Swr− mutants and 18% (26 of 142) of the Swr+ mutants. Some of the Swr− Elo− mutants (15 of 26) produced tightly arranged consolidation zones with limited swimming motility, such as demonstrated by BB2022 (Fig. 2C). The remaining Swr− Elo− mutants in this group, although producing unusual, non-wild-type consolidation zones, were pleiotropic, showing a range of swimming rates and consolidation patterns. The Swr− Elo− mutants did not swim on agar, although swimming motility in this group appeared to be wild type.

An interesting category of swimming-defective mutants appeared while we were examining the strains for swimming motility. Some mutants from both Swr− and Swr+ groups constitutively expressed the elongated swimmer cell (EloO). Both groups had wild-type swimming motility and were Che+. Flagellin synthesis in these cells was unusual. It was anticipated that a constitutive swimmer cell would have greatly increased flagellin synthesis, typical of surface-induced BB2000; however, the Western analysis (Fig. 3, lanes 9 and 10) indicated that flagellin synthesis in broth-grown Swr− EloO and Swr+ EloO mutants was slightly lower or just equal to that seen in broth-grown wild-type cells (lane 2). Flagellin synthesis on Swr− EloO and Swr+ EloO mutants grown under inducing conditions was wild type, and the cells appeared to have no other defects. One possible explanation for this is that these EloO mutants are constitutive only for elongation and not the entire pathway leading to the differentiated swimmer cell. Thus, EloO mutants are partially defective in swimmer cell differentiation.

Many of the Swr+ mutants (more than 65%) and a few of the Swr− mutants (4 of 70) were wild type for swimming (Fig. 3, lane 11) and elongation phenotypes. Although our analysis does not provide definite answers about the identity of these mutants, many of the Swr− mutants in this category produced very unusual swimming zones. As shown in Fig. 2D, this atypical swimming migration was unlike wild-type swimming (Fig. 1A) and was very different from the tight consolidation zones seen in other Swr− mutants (Fig. 2C).

The ruffled or frilled appearance of these indeterminate mutants suggests that the cells in this swimming colony are defective either in synthesizing or in sensing the intercellular signals which coordinate the processes of swimmer cell differentiation and consolidation (or dedifferentiation).

DISCUSSION

It is intuitively evident that swimmer cell differentiation and swimming motility are the result of complex sensory transduction and global control mechanisms. Since alternative genetic methods, such as those for E. coli, are not currently available for use in P. mirabilis, we have developed transposon mutagenesis techniques and applied them to our long-term study of the genetic regulatory mechanisms controlling swimmer cell differentiation and multicellular swimming motility in this organism. More than 1.5% of all chloramphenicol-resistant mutants were in some way defective in the swimming phenotype. Since this phenotype is complex, involving recognition of a surface, elaboration of a multiflagellated, elongated, polyploid swimmer cell, and intercellular communication necessary for swimming motility, it is not unreasonable to expect this frequency of Swr− and Swr+ mutants. Our data indicate that upward of 60 genes might be involved in some aspect of swimming in P. mirabilis. Other reports of P. mirabilis mutants defective in swimming (2) agree closely with our own estimate of 60 genes, in this case placing the value at around 40 genes. Furthermore, for another swimming bacterium, V. parahaemolyticus, estimates of genes essential for its swimming behavior fall in the range of 25 to 50 genes (8), further supporting the data for P. mirabilis. Thus, approximately 40 to 60 genes are involved in P. mirabilis swimming behavior, suggesting that this system is as complex as other global regulatory systems (30).

Genetic analysis of Proteus strains showing non-wild-type swimming resulted in the identification of two general classes of mutants. Bacteria in the first class of swimming mutants manifested no outward swimming motility when placed on a substrate (L agar) known to induce swimmer cell differentiation and swimming motility in the wild type. This phenotype is referred to as Swr−. P. mirabilis synthesizes only one type of flagellin and flagella, although the synthesis is surface induced (Fig. 3, lanes 2 and 3). This statement is supported by the following observations. (i) In the present analysis, all Fla− mutants were also Swr−. (ii) Only one flagellin band was observed by PAGE and Western hybridization. (iii) Null mutants (such as BB2202 [Fig. 3, lane 12]), which are completely defective in flagellin synthesis, are defective for both swimming and swimming. (iv) The Proteus flic (flagellin) gene has recently been cloned (6) in our laboratory. Hybridization of cloned flic sequences to Proteus chromosomal DNA does not indicate the presence of multiple genes (6). Because the same flagellin is used for swimming and swimming motility, it is not unusual to find that all mutants defective for swimming motility (Swm−) were also Swr−. Thus, the flagellin genes which encode the constituent parts of the “swarming” flagellar structure are the same genes
encoding the “swarming” flagella in *P. mirabilis*. This is similar to the case in *S. marcescens* (1), but is in contrast to swarmer cell differentiation in *V. parahaemolyticus*, in which the constitutively synthesized polar flagellum genes are apparently unique and not shared in the production of the surface-induced lateral flagella, whose synthesis is encoded by a different set of genes (29).

Western blots with rabbit polyclonal antisera directed against the 36.7-kDa flagellin protein from *P. mirabilis* were used to detect the presence of flagellin in whole cells grown under noninducing (liquid) conditions. These analyses were used principally to confirm that cells were synthesizing flagella and to qualitatively measure the amount of flagellin synthesized in uninduced cultures. *P. mirabilis* flagellin is a good indicator of swarmer cell induction, since its synthesis is surface induced. For example, lanes 2 and 3 in Fig. 3 show the difference in the amount of flagellin produced by wild-type cells grown under noninducing conditions (lane 2, broth-grown cells) and inducing conditions (lane 3, agar-grown cells). Densitometric measurements of the stained 36.7-kDa protein indicate that there is at least a 40- to 50-fold increase in flagellin synthesis in cells grown on agar compared with that in cells grown in liquid medium (Fig. 3).

Silver stains of whole-cell preparations also reveal that many other proteins are differentially regulated as a consequence of surface induction (lanes 2 and 3). The identity of these other surface-induced proteins is currently not known, but they may be essential for the formation of cell walls or flagellar components in the differentiated cell. This evidence coincides with that in other reports showing similar differences in enzymatic activity and protein composition when swimmer and swarmer cells are compared (3, 5, 14).

Immunoblots also demonstrated that although many of the Swr− and Swr+ mutants produced flagellin, the synthesis of this protein was lower than that in the wild type. The reason for this lowered production is not clear from our data, but may reflect the nature of the mutation. For instance, both Mot− strains examined in Fig 3 (lanes 4 and 5) showed decreased flagellin synthesis, whereas both Che− strains produced wild-type amounts of flagellin. This correlation is not yet fully understood, however, when extended to other phenotypes, particularly in Elo− and Elo+ mutants (lanes 8 to 10). In this case, Swr− Elo− and Swr− Elo− strains produced decreased level of flagellin (compared with the wild type), but a Swr+ Elo+ mutant appeared to synthesize normal amounts of flagellin. One possible explanation for this result is that the transposon has inserted in genes at different points in the regulatory hierarchy controlling swimmer cell differentiation. Such a possibility is currently being explored.

Some of the Swr− (as well as Swr+) mutants were defective in chemotaxis behavior. These mutants possessed functioning flagella, but had lost the ability to detect the presence of attractants or repellents in the surrounding environment. Sar et al. (31) have shown that in *V. parahaemolyticus*, the same chemotaxis system which controls the rotational bias of the polar flagellum functions to regulate the direction of rotation of the lateral flagella. This conclusion supports the work presented here and indicates that mutations in *che* genes give rise to defects in swelling behavior in *P. mirabilis*. However, Williams et al. (36), in an attempt to substantiate the hypothesis that chemotaxis is the driving force in *Proteus* swarming (25), failed to find any evidence to suggest that chemotactic behavior was necessary for the signal initiating swarming motility. The current data cannot resolve the obvious contradiction between the earlier work with *P. mirabilis*; however, the fact that both Swr− and Swr+ phenotypes shared defects in chemotaxis behavior suggests that the role of chemotaxis in *Proteus* swarming may have to be reevaluated. Moreover, our data implicate chemotaxis behavior not in the initial onset of swarmer cell differentiation, but in the latter stages of swarming motility, specifically the stages involving multicellular coordination of the consolidation process.

Although most of the Swr− mutants were also Swm−, the remaining 30% of Swr− strains were wild type in their ability to synthesize and rotate their flagella (Fla− Mot+) and in chemotaxis (Che+). These Swr− Swm− mutants fell into at least three distinct groups. Some of the mutants were defective in wild-type swarmer cell elongation (Table 2, Elo− and Elo+ groups). Unlike the wild-type cells, which do not separte properly when grown on a surface, Elo− mutants septated normally, and did not elongate, when grown under inducing conditions. This may be due to a defect in a global surface induction response or in a specific gene responsible for regulating septation. In contrast, Elo+ mutants were constitutive for swarmer cell elongation and demonstrated inhibited septum formation at all times. Although such defects in *P. mirabilis* have not been analyzed, possible candidates for such mutations might be analogs of the *ftsZ* genes of *E. coli*. In *E. coli*, these genes regulate normal septation and, when mutated (26), can cause a phenotype similar to that of the elongated *Proteus* swarmer cell. The *E. coli ftsZ* genes are not known to be regulated by growth on surfaces, so if *Proteus* analogs of *ftsZ* are involved in swarmer cell elongation, they must be under a different control mechanism from their *E. coli* counterparts.

The majority of swarming mutants isolated in this study were not completely defective in swimmer cell differentiation and swarming motility (null mutants [Fig. 2A]); rather, they displayed pleiotropic phenotypes, suggesting that many, the mutation might be in a gene affecting multicellular behavior. In this report we have opted to designate mutants that show non-wild-type swimming as Swr+ mutants to distinguish them from mutants which fail to swim at all (Swr−). These Swr+ mutants failed to produce wild-type consolidation zones (Fig. 2B to D). Mutants in the Elo+ class of mutants were classified into either narrow or broad consolidation zones, nonuniform consolidation zones, or indeterminate consolidation zones. The first phenotypic class appeared to have a defect in the spatial or temporal control of the multicellular behavior presumed to control the sequence of events involving differentiation and dedifferentiation. It may be that in such mutants the signals regulating swarming behavior are either synthesized more rapidly than in the wild type, giving rise to faster consolidation and narrower zones (Fig. 2B to C), or synthesized more slowly, resulting in broad zones due to a diminished consolidation process. The class of Swr+ mutants which shows nonuniform consolidation zones is depicted in Fig. 2B. These mutants form swimming colonies that do not have the equal spacing between these zones typical of the wild type. The last class of Swr+ mutants found in *P. mirabilis* manifests no discrete pattern of consolidation zones (Fig. 2D). Only a few such mutants (9 of 142) were isolated; however, they are perhaps the most interesting of all our mutants because of the presumed nature of the defect. The swarming pattern produced by these indeterminate mutants suggests that the swarming colony fails to either produce or sense the presence of the signals needed for consolidation. The result is a swarming colony with many ruffles or frills that are produced as a result of uncoordinated differentiation and dedifferentiation cycles. We hypothesize that the mutations
producing this phenotype are localized in genetic loci which encode proteins necessary for the production or recognition of putative signal molecules whose primary activity is to coordinate each cycle of differentiation and dedifferentiation. Such Proteus mutants have been observed by others (2), implying that this phenotype is real and not the result of a double mutation or some spurious event. Genetic analysis in Myxococcus xanthus has defined at least four signal molecules (csgA, csgB, csgC, and csgD) that apparently control the multicellular differentiation leading to fruiting-body formation (22). Although merely speculative at this juncture, it is nevertheless plausible that Proteus swarmer cell differentiation and swarming motility share some of the features of the sensory transduction pathways observed in the myxobacteria. The use of transposon insertions to produce mutations in swr genes will facilitate our efforts to understand the nature of these defects, since a selectable marker (the chloramphenicol resistance gene) has now been physically linked to the Proteus gene. We are currently using this approach to clone the flanking Proteus DNA into E. coli for further analyses aimed at understanding cell-to-cell communication in P. mirabilis.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant AI-27107 from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health.

We thank Roger Chien for his contribution to some of the experiments described in this paper; Philip Rutledge for assistance with the electron microscope; and Paul Lovett, Harold Schreier, and James Shapiro for useful discussions.

REFERENCES