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# Extracellular Slime Associated with *Proteus mirabilis* During Swarming

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Light microscopy, transmission electron microscopy, and scanning electron microscopy were used to visualize the extracellular slime of *Proteus mirabilis* swarm cells. Slime was observed with phase-contrast microscopy after fixation in hot sulfuric acid-sodium borate. Ruthenium red was used to stain slime for transmission electron microscopy. Copious quantities of extracellular slime were observed surrounding swarm cells; the slime appeared to provide a matrix through which the cells could migrate. Swarm cells were always found embedded in slime. These observations support the argument that swarming of *P. mirabilis* is associated with the production of large quantities of extracellular slime. Examination of nonswarming mutants of *P. mirabilis* revealed that a number of morphological changes, including cell elongation and increased flagellum synthesis, were required for swarm cell migration. It is still unclear whether extracellular slime production also is required for migration.

Morphological changes associated with swarming of *Proteus mirabilis* have been described by several investigators (1, 7, 10, 11, 20). Williams and Schwarzhoff (20) separated swarming into three phases: swarm cell formation (differentiation), migration, and consolidation. When nonswarming cells (0.6 by 1 to 2 μm) of *P. mirabilis* are grown in broth and inoculated onto a suitable solid medium, the cells reproduce as short forms for approximately 3 h. Two morphological changes then occur. The cells continue to grow, but because cell division does not occur, highly elongated (0.7 by 10 to 80 μm), multinucleated swarm cells are formed (12, 13). In addition, the number of flagella per unit surface area increases; swarm cells may have 500 to 3,000 flagella per cell rather than the usual 1 to 10 flagella per cell. After approximately 4 h, the swarm cells begin to migrate out from the colony in groups or "rafts." Migration continues for about 1 h, after which the consolidation phase begins. The swarm cells stop migrating and subdivide into short forms. The short forms grow and divide as before, swarm cells again form, and the swarming process is repeated.

Using light microscopy, Fuscoe (9) observed suspected slime trails behind rafts of migrating *P. mirabilis* swarm cells. More recently, scanning electron microscopic observations of *P. mirabilis* swarm cells confirmed the presence of slime trails and also revealed the presence of a slime layer covering rafts of migrating swarm cells (18). We believe, because of these and other observations, that the production and secretion of slime by swarm cells is necessary for migration to occur. Our objective in this study was to use light microscopy and transmission electron microscopy to visualize the slime layer seen previously only with scanning electron microscopy. Also, nonswarming mutants of *P. mirabilis* were examined to determine if a deficiency in slime production could be related to impaired swarming ability.

## MATERIALS AND METHODS

**Organisms.** Wild-type *P. mirabilis* IM47 was obtained from the culture collection of the Department of Microbiology, Iowa State University, Ames, as were the nonswarming mutants of IM47 (NSW203 and NSW110). Other nonswarming mutants of *P. mirabilis* were obtained from W. M. Bain (University of Maine, Orono) and C. D. Jeffries (Wayne State University, Detroit, Mich.). Characteristics of the nonswarming mutants have been described previously (19).

**Culture media.** Trypticase soy agar (TSA; BBL Microbiology Systems) contained Trypticase soy broth (TSB;BBL) and 1.5% agar (Difco Laboratories). TSA plus yeast extract consisted of TSB supplemented with 0.5% yeast extract (Difco) and 1.5% agar. Tryptose broth was composed of 2% tryptose (Difco) and 0.5% NaCl. Tryptose agar contained tryptose broth and 1.5% agar. Before use, all plates were dried overnight at 35°C to remove water droplets from the agar surface.

**Light microscopy.** A loopful of an overnight culture of *P. mirabilis* IM47 in TSB was placed onto a TSA plate. Inoculated plates were incubated at 35°C until swarming began (ca. 3 to 4 h). The cells were fixed by removing the petri plate cover and inverting the culture over a glass petri plate containing approximately 5

ml of 50% glutaraldehyde. The cells were fixed by the glutaraldehyde vapors for 12 to 24 h under a hood. The plates were then removed from the glutaraldehyde vapors, covered, and allowed to dry at room temperature for 3 to 7 days. After drying, a razor blade was used to cut blocks of agar (1 $cm^2$) that contained part of the colony, swarm cells, and agar not traversed by any cells. Impression mounts were made by inverting these agar blocks onto glass slides and allowing them to dry for 30 to 60 min. The agar blocks were then removed, and the cells were heat fixed.

A solution of 0.025 M sodium borate in concentrated sulfuric acid was heated in a boiling water bath, and several drops were added to each impression mount. After approximately 5 min, the slide was gently immersed in distilled water several times and allowed to air dry. This fixation method sometimes removed cells and slime from the slide, so several slides were often prepared. Phase-contrast micrographs were taken with a Zeiss microscope and either Kodak Panatomic-X or Kodak High-Contrast Copy film.

**Transmission electron microscopy and scanning electron microscopy.** Agar blocks (4 $mm^2$) were prepared by the procedure described for light microscopy. Fixation was performed by the method described in Cagle (3). Blocks were fixed in glutaraldehyde and ruthenium red and postfixed in osmium tetroxide and ruthenium red. The blocks were dehydrated by passage through increasing concentrations of ethanol and were embedded in Epon 812 resin. Thin sections were cut with a Reichert OM U2 ultramicrotome. Unstained sections and sections stained with uranyl acetate and lead citrate were observed with a Hitachi HU-12A transmission electron microscope.

Samples were prepared for scanning electron microscopy by the procedure of VanderMolen and Williams (18). Specimens were coated with a layer of carbon, followed by gold, and were examined with a JEOL JSM-35 scanning electron microscope operated at 20 kV.

**Characterization of nonswarming mutants.** Nonswarming mutants were characterized on the basis of swarm cell formation, presence of large numbers of flagella per cell, and slime production. To determine at what time swarm cell formation occurred, we inoculated a loopful of cells from a log-phase TSB culture of each nonswarming mutant onto TSA, tryptose agar, or TSA plus yeast extract at 60-min intervals for 24 h and incubated the cells at 35°C (37°C for NSW110, a temperature-sensitive, nonswarming mutant). The plates then contained several colonies, each with a l-h difference in age. The plates were fixed over glutaraldehyde vapors as described above. The edge of each colony was examined with phase-contrast microscopy for the presence of swarm cells.

Each nonswarming mutant was negatively stained and then examined for the presence of large numbers of flagella with transmission electron microscopy. Modifications of the negative staining method of Brenner and Horne (2) were used. A loopful of swarm cells (determined by phase-contrast microscopy) from the edge of a colony was emulsified in distilled water, layered onto a Formvar-coated grid, and allowed to dry. A solution containing 8 to 10 drops of 2% phosphotungstic acid (neutralized to pH 6.3 to 7.0), 1 drop of 1% bovine serum albumin fraction V, and 2 to 10 drops of distilled water was sprayed from a glass nebulizer onto each grid. The grids were examined with a Hitachi HU-11C transmission electron microscope.

Agar blocks containing cells at the edge of a colony were prepared for scanning electron microscopy as described above and were examined for swarm cell formation, large numbers of flagella, and slime production.

## RESULTS

**Light microscopy.** The hot sulfuric acid-sodium borate fixation procedure revealed many slime paths emanating from the colony. Under 400× magnification (Fig. 1A) it was observed that most slime paths were connected to one another and appeared randomly distributed. At 400× and 1,000× magnifications, swarm cells often could not be distinguished beneath the slime. However, the impression mount and fixation techniques sometimes removed a portion of the slime. When this occurred, many rafts of swarm cells were often observed (Fig. 1B) and were always surrounded by slime.

At the migration front, several millimeters away from the colony, many swarm cells could be distinguished beneath a thin layer of slime. Observations made near the colony and at the migration front indicated that slime was more profuse in the area immediately surrounding the colony than at the migration front.

**Electron microscopy.** Scanning electron microscopic observations of a slime-covered raft of swarm cells (Fig. 2A) closely resembled previous observations (18). The thin slime layer appeared to encase the whole raft of swarm cells rather than individual swarm cells (Fig. 2B). This layer appeared to be fragile and was easily torn during specimen preparation. From micrographs the thickness of the slime was estimated to be 45 nm. Slime-covered agar was observed behind rafts of swarm cells (Fig. 2A and 2C) and was similar in appearance to the slime surrounding rafts of swarm cells.

To substantiate the light and scanning electron microscopic observations of slime surrounding swarm cells, agar blocks were stained with ruthenium red during fixation and prepared for transmission electron microscopy. A thin layer of ruthenium red-positive material covered the tops of rafts of swarm cells (Fig. 3A). This extracellular slime layer had small perforations but did not surround individual swarm cells. Bundles of flagella, cut in cross section, were also observed (Fig. 3A). The average thickness of the slime layer was about 20 nm. Ruthenium red staining also revealed that a slime layer covered the agar surface adjacent to rafts of swarm cells (Fig. 3B). The slime layer could not be seen covering rafts of swarm cells when the preparations were not stained with ruthenium red (Fig. 3C).

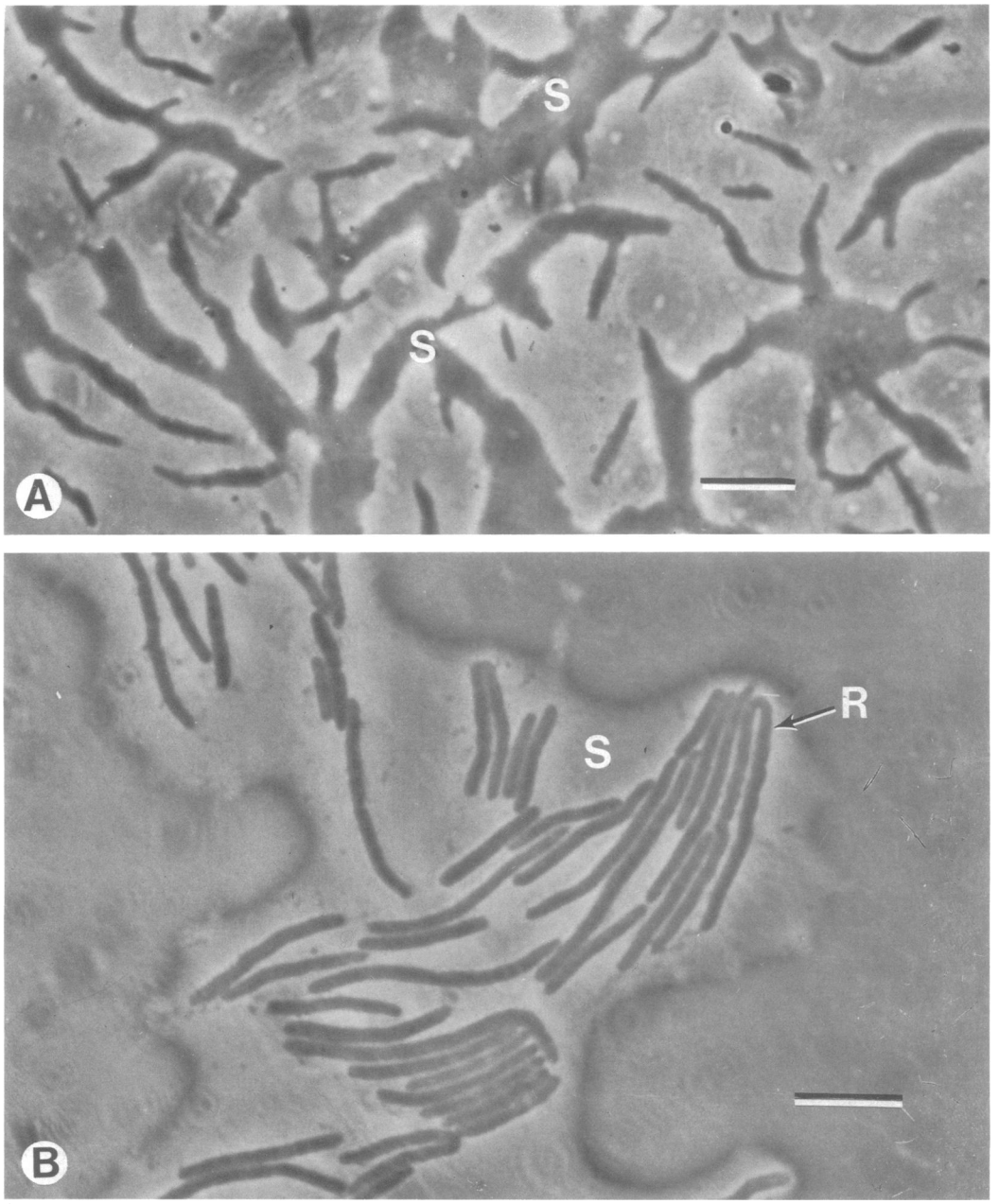


FIG. 1. Phase-contrast micrographs of swarming *P. mirabilis* IM47. Cells and slime were transferred to a glass slide via the impression mount technique and fixed with hot sulfuric acid-sodium borate. (A) Slime paths (S). Bar represents 20 μm. (B) A raft (R) of swarm cells and slime (S) observed when a portion of the slime covering was removed during specimen preparation. Bar represents 5 μm.

To determine at what time the slime layer was produced, we made scanning electron microscopic observations of colonies at 30-min intervals after inoculation of broth-grown cells onto an agar surface (data not shown). Slime was first observed covering cells near the center of the colony 2.5 h after inoculation. As time progressed, the slime appeared to cover more cells, including cells closer to the edge of the colony. When migration began, slime-covered swarm cells were observed.

Characteristics of *P. mirabilis* IM47 and seven nonswarming mutants are presented in Table 1. Interpretations were based on swarm cell mor-

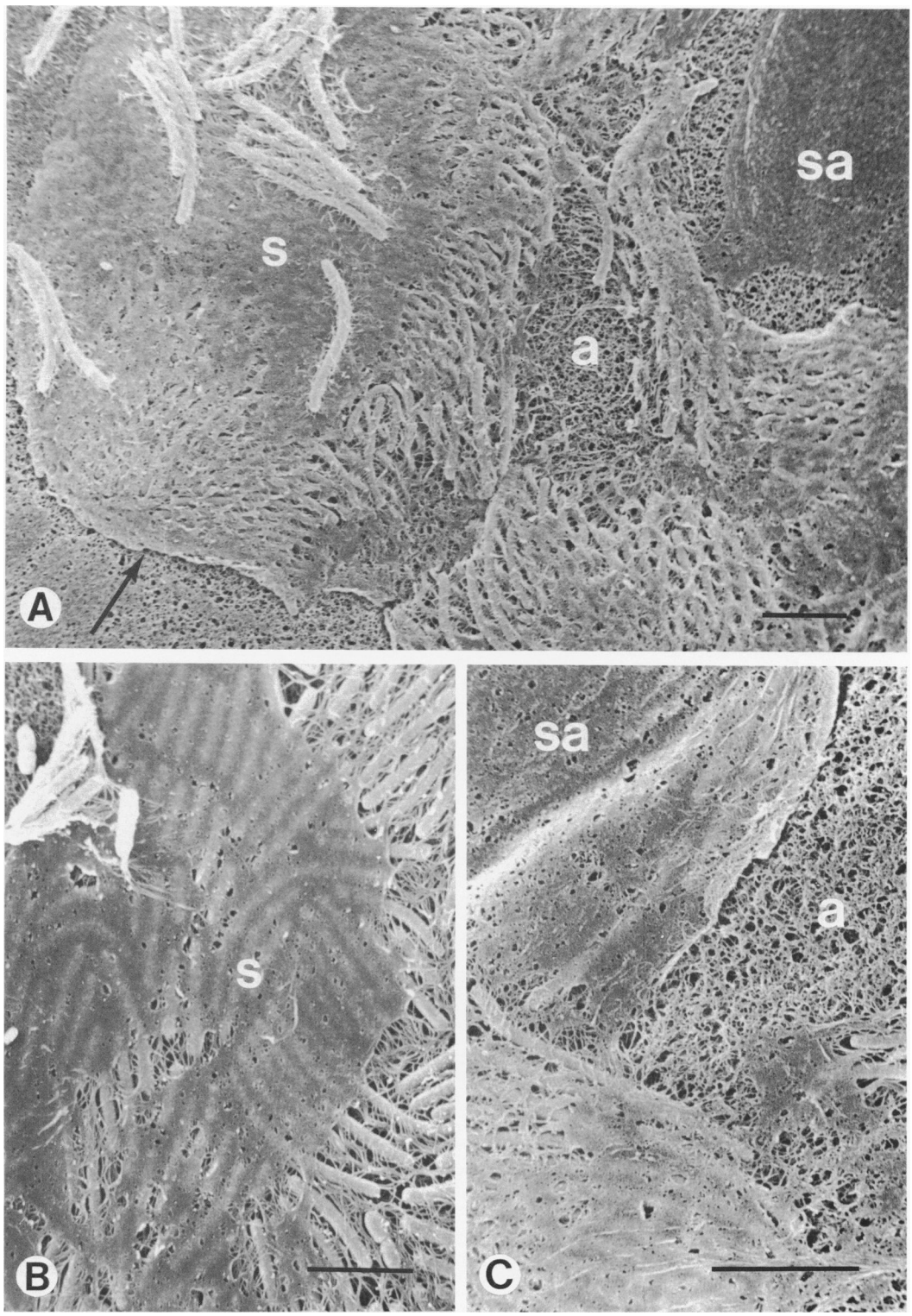


FIG. 2. Scanning electron micrographs of *P. mirabilis* IM47 swarm cells on an agar surface. (A) A typical raft (arrow) of swarm cells covered with slime (s) and slime-covered agar (sa) behind the raft of swarm cells. The agar surface (a) not covered with swarm cells or slime is also visible. (B) A portion of a raft of swarm cells showing the thin slime layer (s) and the highly flagellated cells beneath a tear in the slime. (C) Higher magnification showing the difference between a slime-covered agar surface (sa) and an agar surface (a) not covered with slime. Each bar represents 5 μm.

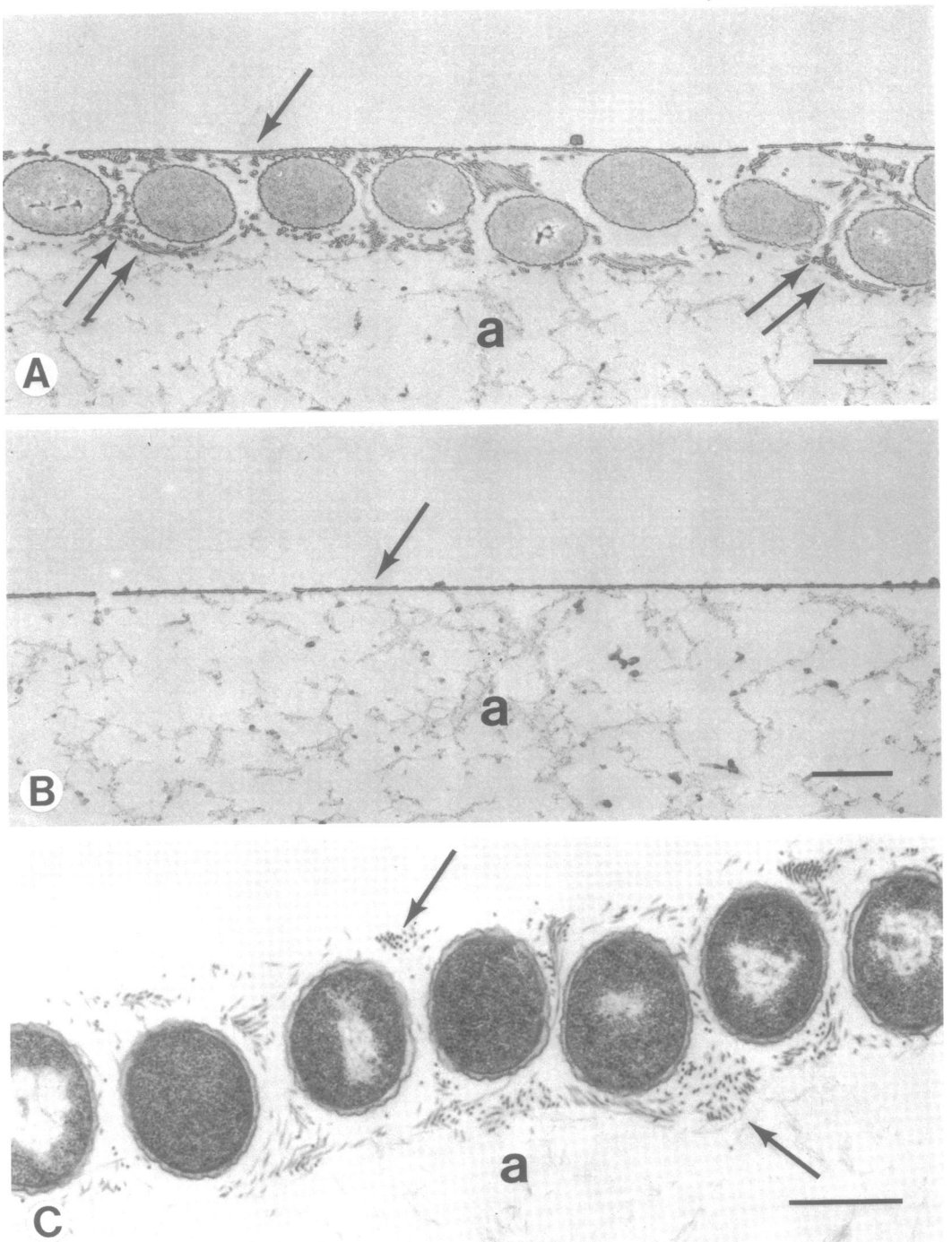


FIG. 3. (A) Transmission electron micrograph of a thin section of *P. mirabilis* IM47 swarm cells on an agar surface (a). Cells were stained with ruthenium red during processing. The ruthenium red-stained slime layers (single arrow) covers the raft of swarm cells, and flagellar bundles (double arrows) surround individual cells. (B) Ruthenium red-stained slime (arrow) covering an agar surface (a). (C) Thin section of a group of swarm cells on an agar surface (a) that was processed without the use of ruthenium red. No slime is visible; flagellar bundles cut in cross section (arrows) are present. Each bar represents 0.5 μm.

TABLE 1. Characteristics of *P. mirabilis* IM47 and seven nonswarming mutants

| *P. mirabilis* strain | Growth medium | Time (h) of swarm cell formation[a] | Presence of large numbers of flagella[b] | Presence of slime[c] |
|---|---|---|---|---|
| IM47 (wild type) | TSA | 3 | + | + |
| NSW203 | TSA | 11 | + | + |
| NSW110 | TSA | 11–12 | + | + |
| 2NS | TSA plus yeast extract | 14–16 | + | + |
| DA | TSA | 8–9 | − | + |
| 91b | Tryptose agar | 13 | − | + |
| 60 | TSA | 4 | − | − |
| ML | TSA | 8–9 | − | − |

[a] Hours represent time elapsed from the inoculation of broth-grown cells onto an agar medium to the production of swarm cells.
[b] Determined from transmission electron microscopic observations of negatively stained preparations.
[c] Determined from scanning electron microscopic observations.

phology, large numbers of flagella per cell, and slime production. Three different groups of mutants became apparent. The first group of nonswarming mutants (strains NSW203, NSW110, and 2NS) formed swarm cells and a slime layer. Large numbers of flagella per cell were seen, as for the wild type. The second group of mutants (strains DA and 91b) were similar to the first group but lacked the large numbers of flagella. Nonswarming mutants 60 and ML comprised the third group. These mutants produced swarm cells that lacked both flagella and a slime layer.

Nonswarming mutants that lacked an extracellular slime layer but otherwise were identical to the wild-type *P. mirabilis* swarm cells were not observed.

## DISCUSSION

Fuscoe (9) was the first investigator to report slime trails by using light microscopy. We have been trying to develop a method of staining the extracellular slime for light microscopy. Stains for capsules and slime layers were found to be relatively ineffective for staining slime. The alcian blue dye of Novelli (16), specific for bacterial polysaccharides, and toluidine blue O, used to visualize uronic acids, sometimes permitted detection of extracellular slime on impression mounts of swarm cells. However, these dyes did not show that slime was present in large quantities, even when much slime was observed with scanning electron microscopy (18). We believe that much slime was lost when preparations were made for light microscopy because the slime was soluble in the dye, was improperly fixed, or was rinsed away during washing. The hot sulfuric acid-sodium borate fixation procedure cemented the slime to the slide so that it could withstand repeated rinsing in distilled water, and the fixative further made the slime visible with phase-contrast microscopy. Without this fixation, slime could not be seen with phase-contrast microscopy. Sodium borate was a necessary ingredient for fixation; when hot sulfuric acid without sodium borate was used, slime was never observed. In the future, we hope to be able to stain this fixed slime with alcian blue dye or toluidine blue O.

An alternate approach to staining slime for light microscopy would be via the interaction of the slime with slime-specific antisera, although this has not yet been investigated.

Several investigators have used ruthenium red to stain extracellular polymers of different types of bacteria for observation with transmission electron microscopy (4–6, 8, 15, 17). The ruthenium red staining technique, combined with our agar block method of preparation, provided a means for slime detection with transmission electron microscopy.

The presence of a ruthenium red-stained slime layer that covered rafts of swarm cells confirmed our light and scanning electron microscopic observations. The similarity between the stained slime layer seen with transmission electron microscopy (Fig. 3A) and the slime layer seen with scanning electron microscopy (Fig. 2B) was apparent. Both layers appeared to be thin and covered rafts of swarm cells, not individual cells. The small breaks in the stained slime layer (Fig. 3B) correlated with the tears in the slime seen with scanning electron microscopy (Fig. 2A). We believe the stained slime on the agar surface (Fig. 3B) was adjacent to rafts of swarm cells. This slime was similar in appearance to the slime-covered agar surface observed with scanning electron microscopy and the slime paths seen when the hot sulfuric acid-sodium borate fixation procedure was used. We believe that this represents slime through which swarm cells have migrated.

Using light microscopy, we observed a differ-

ence in slime thickness between slime found near the colony and slime found near the migration front. At the migration front, swarm cells could be seen through a thin layer of slime. Swarm cells became increasingly difficult to distinguish through the slime layer when the slime paths closer to the colony were viewed. We believe that this represents a difference in slime thickness rather than the presence or absence of swarm cells because large numbers of swarm cells were often seen in slime paths near the colony through tears in the slime covering (Fig. 1B).

The thickness of the slime (45 nm) measured from scanning electron micrographs was slightly greater than the thickness measured from transmission electron micrographs (20 nm). The carbon and gold coatings for the scanning electron microscopic preparations would have increased the actual thickness slightly. The thickness measurements from scanning electron micrographs represent estimates; it was difficult to determine the actual thickness from the micrographs. In addition, we believe that the extracellular slime is composed primarily of water. Dehydration can cause extensive condensation of the hydrated slime, resulting in a condensed remnant. Therefore, our measurements may not be meaningful.

*P. mirabilis* slime is probably an acidic polysaccharide, since the dyes that stain the slime-covered swarm cells, ruthenium red, and, at times, alcian blue, are specific for acidic polysaccharides (14) and bacterial polysaccharides (16), respectively.

The morphological events associated with swarm cell formation (cell elongation and increased flagellum synthesis) begin to occur approximately 3 h after inoculation of broth-grown cells onto an agar surface. The fact that slime was first observed 2.5 h after inoculation suggests that slime production is an additional morphological change associated with swarm cell formation.

Jones and Park (13) suggested that cell elongation was dependent upon uncontrolled flagellum synthesis. Our observations of the nonswarming mutants suggested that the several events associated with swarm cell formation, including slime production, were independent processes, each essential for swarming. Nonswarming mutants DA and 91b, for example, produced swarm cells and slime; the lack of production of large numbers of flagella demonstrated that swarm cell formation and slime production occurred without an increase in flagellum synthesis. Nonswarming mutants 60 and ML produced swarm cells that lacked both slime and flagella; thus, swarm cell formation was not dependent upon the production of either flagella or slime.

The role of slime in the migration of swarm cells could not be definitely established from studies of the nonswarming mutants. For each of the three groups of nonswarming mutants observed, an event other than slime production inhibited swarm cell migration. The mutants in group 1, (NSW203, NSW110, and 2NS) resembled wild-type *P. mirabilis* IM47 morphologically. The fact that these organisms were unable to migrate implied that requirements other than slime production, the presence of large numbers of flagella, and cell elongation were necessary for migration to occur. Nonswarming mutants in group 2 (DA and 91b) possibly were unable to migrate because of the lack of sufficient numbers of flagella. Nonswarming mutants in group 3 (60 and ML) lacked both slime and large numbers of flagella. Both could affect migration of the swarm cells. Since we were not able to obtain a mutant which produced highly flagellated swarm cells, as did the wild type, and lacked only a slime layer, we could not determine positively if slime was one of the necessary requirements for swarm cell migration to occur. Perhaps, in addition to the formation of highly flagellated slime-covered swarm cells, other changes are also needed for migration to occur.

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