

Territoriality in *Proteus*: Advertisement and Aggression

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Dr. Karine A. Gibbs received her Bachelor's degree in Biochemical Sciences from Harvard University and earned her Ph.D. in Microbiology and Immunology with Dr. Julie Theriot at Stanford University. After receiving her doctorate, she trained as a postdoctoral fellow with Dr. E. Peter Greenberg at the University of Washington in Seattle, where she analyzed the genetic components of territorial behavior and self-identity in bacteria. She recently joined Harvard University as an Assistant Professor in the Department of Molecular and Cellular Biology, where she is building a research group that focuses on the social behaviors of microbes, specifically investigating the molecular mechanisms that underlie the ability of bacterial cells to discriminate self from nonself.



Dr. E. Peter Greenberg received his Bachelor's degree from Western Washington University, a Master's from the University of Iowa, and a Ph.D. from the University of Massachusetts. After a postdoctoral at Harvard, he joined the faculty at Cornell University in 1977, eventually moved back to the University of Iowa (1987), and finally returned to the Pacific Northwest as a member of the UW Medicine Microbiology faculty in 2005. Dr. Greenberg has spent his scientific career uncovering aspects of microbial social behavior. Due in part to his efforts, we now understand that many bacteria communicate by using chemical signals. Bacterial communication controls virulence in a variety of pathogenic bacteria, and it has thus become a target for development of new therapeutic strategies. Bacteria have also become models for studies of selection for and evolution of cooperative behavior.

1. Introduction

*“Thus, principles of evolution are dramatically exhibited by studying strains of *Proteus* on a solid medium, which contains a source of nutrients and oxidizable organic compounds. If the descendants of an individual are to compete successfully in this defined habitat, they must have evolved (i) highly efficient enzyme catalysts to release and trap free energy, (ii) mobile mechanisms to access the site where more free energy is available, and (iii) mechanisms to stake out a claim on free energy—territoriality, one of the fundamental principles of ecology. Microbes did it first.”*¹—Ralph S. Wolfe

Members of the bacterial species *Proteus mirabilis* are capable of rapid swarming over a hard agar surface. Swarming colonies display a striking phenotype in which a visible boundary forms between swarms of different strains. In contrast, boundaries do not arise between two swarms of

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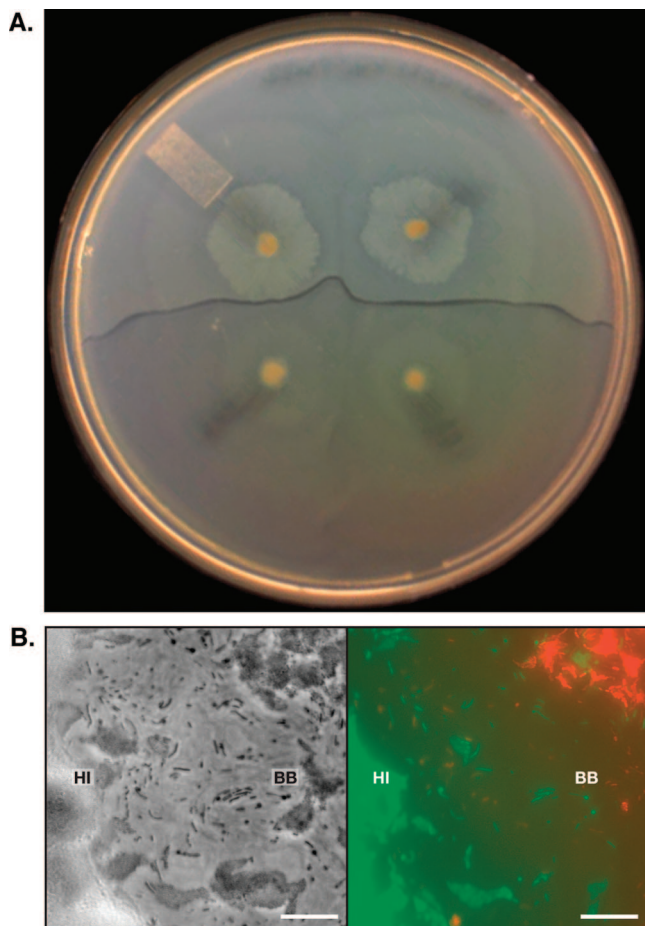


Figure 1. Boundary between *P. mirabilis* strains. (A) A boundary between two different wild-type strains of *P. mirabilis*, HI4320 (HI) and BB2000 (BB). Top, two swarms of HI that have merged with each other; bottom, two swarms of BB that have merged with each other. Swarms of the same strain merge. (B) Higher-resolution phase contrast and fluorescent microscope image of a boundary zone between HI (green) and BB (red). Phase on left, pseudocolored overlay on right. Bar, 10 μm . Reprinted with permission from ref 31. Copyright 2008 American Association for the Advancement of Science.

a single strain (Figure 1). This behavior is a demonstration that *P. mirabilis* swarms are capable of territoriality and self-versus nonself-recognition. Though first described over 70 years ago, the molecular mechanisms underlying boundary formation in *P. mirabilis* have remained stubbornly elusive despite the combined efforts of several research groups. In this article we attempt to give a concise review of the historical and current models for *Proteus* boundary formation. We begin with a brief background on the genus *Proteus* and specifically the species *Proteus mirabilis*, followed by a review of swarm boundary formation. We then analyze the two prominent models for boundary formation: (1) preemptive antagonism and (2) recognition. We conclude with the broader implications of *P. mirabilis* territoriality and self-versus nonself-recognition.

2. The Genus *Proteus*

2.1. General Information about the Ecology of *Proteus*

The genus *Proteus* is a member of the *Enterobacteriaceae* family. *Proteus* can be found in soil and contaminated water and, for some *Proteus* species, in the intestinal tract of

animals. In soil and water, and perhaps healthy animals, *Proteus* species are likely involved in the degradation of organic material. These bacteria are noted for their ability to utilize urea as a source of nitrogen and energy.² The two *Proteus* species most commonly found in humans are *P. mirabilis* and, to a lesser extent, *P. vulgaris*.³ Jacobsen et al. and Manos and Belas recently reviewed *Proteus* pathogenesis in detail.^{2,3} In brief, *P. mirabilis* is an opportunistic pathogen that causes complicated urinary tract infections, especially in people requiring long-term indwelling catheterization.⁴ In hospital and nursing home settings, *Proteus* species can also cause infections in the ear, nose, throat, and skin, among others, especially in immunocompromised individuals. Infections at multiple sites in a single patient are caused by single *P. mirabilis* strains; *P. mirabilis* strains isolated from the catheter, urine, feces, and kidney stones of a single patient are often the same strain, suggesting that *P. mirabilis* infections are clonal.^{5–7}

2.2. *Proteus mirabilis* Virulence Factors

P. mirabilis employs a wide variety of virulence factors during colonization and subsequent infection. One major virulence factor is urease, an enzyme that converts urea to ammonia and carbon dioxide. Activity of urease results in an increase of the local alkalinity and eventually the crystallization of magnesium ammonium phosphate and calcium phosphate.⁸ The alkaline conditions can lead to the formation of kidney stones and of crystalline biofilms that encrust the catheter tubing.⁸ Biofilms often result from the attachment of cells to catheter walls via adhesins.² Other virulence factors include a hemolysin, an IgA-degrading protease, mannose-resistant *Proteus*-like fimbriae (MR/P), mannose-resistant *Klebsiella*-like hemagglutinin (MR/K), *P. mirabilis* fimbriae (PMF), nonagglutinating fimbriae (NAF), and ambient-temperature fimbriae (ATF).^{4,9–11} Production of these virulence factors, including urease, is associated with surface-attached growth.¹²

2.3. *Proteus mirabilis* Life Cycle

P. mirabilis cells undergo a morphologically distinct developmental cycle upon contact with a surface or in viscous liquid environments (Figure 2). In normal liquid growth medium, *P. mirabilis* exists as short (1–2 μm in length), rod-shaped swimmer cells that have 6–10 peritrichous flagella. Upon contact with a surface, swimmer cells differentiate into elongated (10–80 μm), hyperflagellated

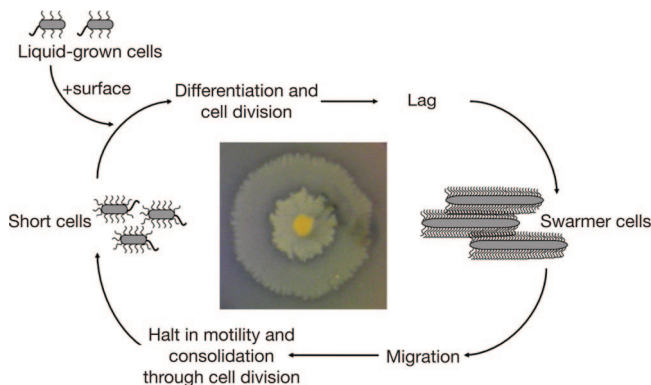


Figure 2. Diagram of the developmental cycle of surface-attached *P. mirabilis*. Swarming colonies form bull's eye patterns (photograph at center) because of the repeated cycles of migration followed by consolidation. This figure was adapted from ref 57.

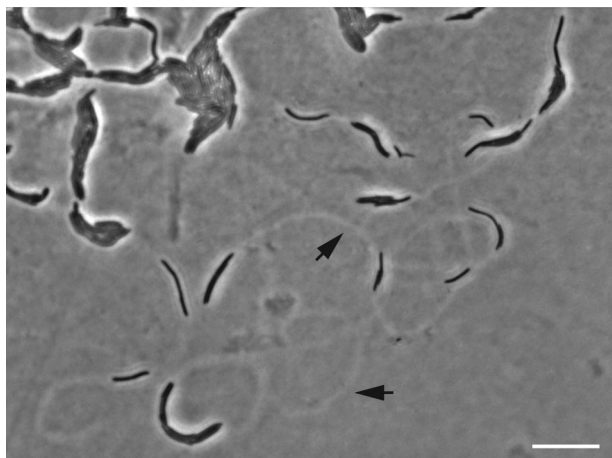


Figure 3. Phase contrast micrograph of the leading edge of a *P. mirabilis* swarm. The cells are black, and the agar is gray. Rafts of cells and individual cells are evident as are tracks (black arrows) on the agar surface. Bar, 10 μ m.

swarmer cells.³ The main component of the flagella, the flagellar filament protein FlaA, is the same for both the swimmer and swarmer morphotypes.³ Swarmer cells have multiple copies of the chromosome yet appear to be a single cell without a division plane or septum. Some of the developmental cues leading to swarmer cell differentiation include increased expression of flagellar genes as directed by the flagellar regulator, FlhDC,^{13–16} inhibition of flagellar rotation,¹⁷ and the accumulation of putrescine.¹⁸ Expression of virulence genes is coincident with the developmental switch from a swimmer cell to the swarmer cell morphotype.^{17,19–22} The initiation of *P. mirabilis* swarmer cell differentiation has been thoroughly reviewed recently.^{3,23}

Upon initial surface contact there is a lag period, and then swarmer cells migrate rapidly across the surface in a coordinated cell-to-cell behavior. The swarming cells migrate as rafts of cells connected by flagella. Cells in rafts are aligned in a parallel manner as rafts move.²⁴ Occasionally an individual cell moves alone near the front of an advancing swarm. Swarming rafts and cells leave visible tracks on the agar surface (Figure 3). Following an initial period of migration, the swarmer cells arrest movement and divide into shorter cells. This results in a macroscopic consolidation ring. Interestingly, the consolidation phase appears to be independent of nutrient availability and metabolite changes.^{25,26} This arrest in migration is puzzling because the medium still provides an excess of nutrients and energy sources. Perhaps an internal (and unknown) factor in swarmer cells dictates the consolidation. After a brief delay, the new short cells differentiate into swarmer cells and population migration resumes. The repeated cycles of migration followed by consolidation result in the characteristic bull's eye pattern of *P. mirabilis* swarm colonies (Figure 2). The ability to swarm has been implicated in pathogenesis, perhaps for migration across for catheters or initial colonization, as evidenced by the fact that nonswarming *P. mirabilis* strains are not able to ascend to the urinary tract in mouse models and exhibit significantly reduced virulence;²⁰ however, swarmer cells are not observed in tissues isolated from infected mice, and nonflagellated *P. mirabilis* strains do not exhibit decreased infectivity when inoculated intravenously into mice.^{27,28} It is likely that *P. mirabilis* cells utilize the swarming behavior to rapidly migrate across catheters and perhaps ascend the urinary tract against the flow of urine.⁴

3. Swarm Boundary Formation and Territoriality

3.1. Discovery of Swarm Boundaries

In 1946, Dienes described the formation of a boundary between clinical isolates of *P. mirabilis* from two patients.²⁹ Dienes observed that colonies of the same isolate merge at the juncture where two swarming populations approach. However, a boundary, visible to the naked eye, forms at the intersection between two colonies of two different *Proteus* strains:

*“Halos starting from colonies of different Proteus strains do not penetrate into each other. Even after several days of incubation a sharp line of demarcation persists between the areas covered by different strains. The halos sometimes stop a short distance from each other without coming into contact, indicating the diffusion of antagonistic substances into the medium. Meanwhile extension proceeds in other directions.”*²⁹

As such, the boundary is often referred to as a Dienes line and the occurrence is referred to as the Dienes phenomenon. *P. vulgaris* exhibits similar territorial behavior.²⁹ Strains that merge are considered members of the same identity group, whereas strains that form boundaries are of different identity groups. Typing by Dienes line formation is a rapid method for discriminating among clinical isolates of *P. mirabilis*, and its discriminatory effectiveness has been confirmed by ribotyping and pulsed-field gel electrophoresis.^{7,30}

3.2. Description of Swarm Boundaries

The Dienes line boundary is marked by the arrest in forward migration of approaching *P. mirabilis* swarms. The boundary can range in diameter from 0.5 up to 3.0 mm. After the initial interaction of swarm fronts, subsequent layers of swarming cells continue to approach the boundary but do not cross, forming a metaphoric canyon in which the stacked layers of cells at the swarm front amass like cliff walls and the boundary lays sparse like a dry riverbed. When observed at higher resolution, boundaries appear to consist of unoccupied regions, cell debris, and round bodies (Figure 1).³¹ It is likely that the debris is due in part to production of *Proteus*-specific antibacterial peptides called proticines.^{32,33} The round bodies in the boundaries are sometimes associated with the filamentous swarmer cells.^{29,34} Dienes described that it is as if the swarmer cells were emptying their cytoplasmic contents into the round bodies.²⁹ These large, round bodies also form when *P. mirabilis* is refrigerated, transferred to tap water, or exposed to the poison mercuric chloride, HgCl₂,^{29,34} indicating that *P. mirabilis* cells may form round bodies under stress conditions. There are suggestions that the round bodies are viable.²⁹ However, it is more likely that the round bodies represent cells that have died.³⁵ Reports have suggested that boundary formation only occurs when *P. mirabilis* cells are in the swarming phase of the developmental cycle; however, the round bodies are produced even when the cells are mixed in liquid culture, i.e., are short swimmer cells.^{29,36}

4. Territoriality

4.1. Territorial Boundaries between *Proteus mirabilis* Swarms

Boundary formation between *P. mirabilis* strains is a vivid example of intraspecific territoriality in bacteria. Territoriality, generally defined as the behavior to establish and maintain an area that the organism occupies exclusively and defends, is commonly associated with larger organisms and is likely based on complex communication strategies, and even perhaps division of labor.^{37,38} In the case of *P. mirabilis*, one strain is able to hold off and inhibit colonization by a competing different strain. It is an act of mutual exclusion. Similar territorial behavior has more recently been reported in other bacteria, for example, *Escherichia coli* and *Myxococcus xanthus*.^{39,40} Territorial behavior in larger organisms is generally considered to result from overt aggression or advertisement.³⁸ Dienes initially hypothesized that the boundary formation could be due to antagonistic substances. Since then, two hypotheses have emerged as the dominant explanations for boundary formation in *Proteus*: (1) preemptive antagonism and (2) recognition (Figure 4). We recently provided evidence that recognition is a key element of boundary formation (discussed below).³¹

4.2. Preemptive Antagonism Model

The preemptive antagonism hypothesis postulates that boundary formation results from an antagonistic signal that is conveyed from one strain to the other. Preemptive antagonism is essentially overt aggression. This is opposed to the recognition hypothesis (discussed in a later section), which postulates that *P. mirabilis* strains are able to recognize and differentiate themselves from each other without aggression or prior to an aggressive reaction through a mechanism that enables advertisement. These hypotheses are not mutually exclusive. Boundary formation might involve a combination of recognition and aggression.

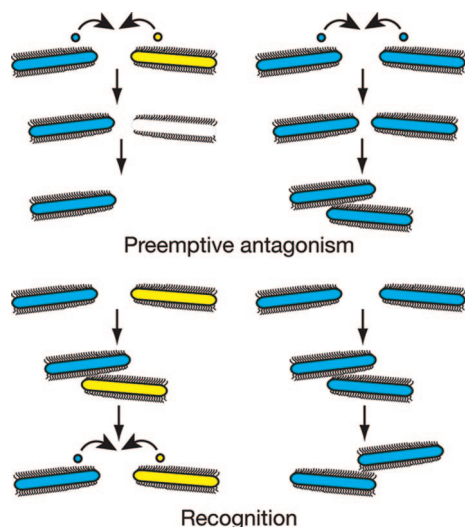


Figure 4. Models for *Proteus* boundary formation. (Top) The preemptive antagonism model, which postulates that secretion of proticines, or other toxins, by the dominant *Proteus* strain into the zone between strains causes cell death in the inferior strain, ultimately resulting in boundary formation. (Bottom) The recognition model, which postulates that, upon interaction with the approaching swarm, *P. mirabilis* cells can recognize and identify identical strains as self and different strains as nonself. This may or may not result in killing.

The preemptive antagonism model infers death of cells in the boundary zone as a result of direct killing mechanisms, or at least growth inhibition. This model predicts that swarming *Proteus* cells secrete toxins that are specific for other *Proteus* strains. The toxins may work as a toxin–antitoxin system and are highly specific to the surface composition of other *Proteus* strains. The preemptive antagonism hypothesis posits that secretion of strain-specific toxins and the resultant cell death caused by the toxins are primarily responsible for boundary formation between *P. mirabilis* strains.^{32,34,35} Interestingly when an Anapore membrane, which allows the relatively free diffusion of small molecules and prohibits the migration of cells, is placed between swarms of two different *P. mirabilis* strains, a boundary will fail to form; in fact, both swarms will move directly to the edge of the membrane before arresting movement, suggesting that a freely diffusible small molecule is unlikely the source of boundary formation.³⁴

As mentioned above, round bodies are evident in Dienes lines.²⁹ Though still debated, cells of each of the strains in approaching swarms appear to form round bodies. Dienes believed that there was a viable subpopulation of round bodies emerging primarily from one of the strains;⁴¹ more recent studies suggest that round bodies are primarily formed by one strain and not the other. Evidence suggests that close cell proximity, if not direct cell–cell contact, is necessary for round body formation,^{34,35} but the data really only support the idea that there is not a diffusible substance inducing round body formation. We favor the idea that round bodies reflect cell death caused by the interactions between incompatible strains.

It is likely that the activity of proticines contributes to the formation of round bodies. As discussed above, proticines are *Proteus*-produced bacteriocins, proteinaceous antibacterial toxins of ~10 components that can inhibit growth and cause cell death of sensitive *Proteus* strains.^{33,42} Though the mechanisms of proticine production are not completely understood, it appears that these toxins function by binding specific cell surface receptors in a manner similar to the action of colicins; different *Proteus* strains probably display different receptors.^{33,42} Senior published an extensive investigation of the link between proticines and boundary formation in *P. mirabilis* and *P. vulgaris* isolates.³² Strains were placed in identity groups based on the formation of boundaries. Those strains that did not form boundaries with each other were placed in the same group. If a boundary formed between two strains, they were placed in different groups. Senior also examined proticine production by each strain and the proticines to which each strain was sensitive, resulting in a proticine production/sensitivity (P/S) type for each strain. Out of 204 *Proteus* isolates, 98 distinct groupings emerged.^{32,43} There were 14 subclasses for proticine production and separately for proticine sensitivity, as well as a classification called atypical boundary formation. For the vast majority but not all strains, boundary formation correlated with proticine P/S types.³²

Of note, there were four strains that neither produced nor were sensitive to proticines. In this subset of isolates, one strain formed a boundary with all of the others, even though none of the strains produced (or were sensitive to) proticines.³² These exceptions indicate that proticine production and sensitivity do not define the basis of boundary formation.³² Proticines likely contribute to the cell death observed in the boundary zones, but they are not required for boundary

formation. Evidence indicates that boundary formation does not involve a diffusible substance, and perhaps cell-to-cell contact is needed for boundaries to develop.^{31,34} However, proticines are diffusible, and they function to inhibit cell growth in liquid media.³³

4.3. Recognition Model

Central to territoriality is the ability for an organism to recognize self, or identity, and discriminate self from nonself. According to the recognition hypothesis, *Proteus* cells at the edge of an advancing swarm can identify cells in an approaching swarm as self or nonself, after which there is an appropriate reaction that leads to a merger of the swarms or boundary formation. Boundary formation might or might not involve or require killing. This sort of recognition behavior is similar to allorecognition in eukaryotic cells. The capability for self- versus nonself-recognition is a common theme in biology.^{39,44–46} We recently identified a genetic locus in *P. mirabilis* that encodes components sufficient for self- versus nonself-recognition and for the definition of strain-specific identity.³¹

5. *ids* Self-Identity Locus

5.1. *ids* Genes

We identified genetic determinants of self- versus nonself-recognition in a screen for *P. mirabilis* mutants that formed a boundary with the wild-type parent.³¹ This was a screen for recognition defects. We isolated one mutant and mapped the mutation to a six-gene locus termed *idsABCDE* for identification of self. Swarms of a mutant in which the entire *idsA–F* region was deleted formed a boundary with the parent, as well as with other wild-type *P. mirabilis* strains.³¹ The recognition defect could be complemented by ectopic expression of the *ids* genes; complemented strains merged with the parent and formed boundaries with the *idsA–F* deletion strain.³¹ These results demonstrate that the *idsA–F* genes are required and sufficient for self–nonself-recognition in *P. mirabilis* swarms.

5.2. Parent and *ids* Mutants Do Not Kill Each Other

Unlike the debris-containing boundary between two wild-type *P. mirabilis* strains, there is little visible debris contained in the boundaries between *ids* mutants and the parent. Furthermore, when mixed in broth, an *idsA–F* deletion strain and the parent grow equally well.³¹ Therefore, the *idsA–F* locus does not appear to be a toxin–antitoxin system. Rather, functional copies of the *ids* genes are needed for self- or nonself-recognition.

5.3. Analysis of *ids* Gene Products

There is essentially nothing known about the biochemistry of the *ids* gene products, but sequence comparisons and genetic analyses have provided insights about possible Ids functions. IdsA shows sequence similarity to the Hcp protein.³¹ Hcp is secreted and forms hexameric rings that can polymerize into tubular structures.^{47,48} IdsB shows sequence similarity to VgrG family members.³¹ Hcp and VgrG are components of Type VI secretion systems, which are widely distributed on *Proteobacteria*. It is believed that

secretion of other proteins is mediated by a surface structure involving Hcp and VgrG.^{49–52} Though *P. mirabilis* appears to have a type VI secretion system, the other necessary components of a complete type VI secretion system, such as the *clpV* ATPase and *icmF*, are not located near the *ids* genes on the *P. mirabilis* genome.⁵³ IdsC, IdsD, and IdsE are each unique to *P. mirabilis*, and IdsF is a conserved hypothetical bacterial protein of unknown function.³¹

The *idsABCDE* locus was present in each of seven *P. mirabilis* isolates examined. These seven isolates all formed boundaries with each other. Among the seven strains, the polypeptides encoded by *idsA*, *idsB*, *idsC*, and *idsF* were >96% identical.³¹ In contrast, IdsD and IdsE contained regions of high variability (72–84% pairwise identity and 32–80% pairwise identity, respectively) flanked by regions of >96% identity.³¹ This sequence pattern of variable regions flanked by conserved regions is reminiscent of alleles encoding antigenic variation in other bacteria.⁵⁴

Genetic analyses have led to a model describing functional roles for the *ids* gene products. First, *idsABCDE* constitute an operon (unpublished). The *idsBCDE* genes are required for self- versus nonself-recognition, and IdsF has a distinct function from the functions of IdsBCDE.³¹ Specific IdsA mutants behave like the parent. IdsA is not required for self- versus nonself-identification. There are three other *hcp* homologues in the *P. mirabilis* genome, and we believe that one or more of them may be capable of substituting for IdsA. The *idsB*, *idsC*, or *idsF* genes code for required self- versus nonself-recognition functions, but *idsB*, *idsC*, and *idsF* are general functions that are interchangeable between strains. In contrast, *idsD* and *idsE* encode strain-specific (or self-) identity and are sufficient to confer a new self-identity when introduced into a heterologous strain.³¹

5.4. Bar Code Model for *ids* Function

The informatics and genetic analyses described above have led to our current bar code and scanner model for *ids* function. IdsD and IdsE function as unique identifiers, or bar codes, for each *P. mirabilis* strain. IdsA, IdsB, IdsC, and IdsF function to display or interpret the information conveyed by IdsD and IdsE; they act as scanners or display equipment. Although we know that the *idsABCDE* operon confers self- versus nonself-identity, we believe that there may be other such determinants.³¹ The available genetic data support a view in which the initial stage of *P. mirabilis* boundary formation encompasses social recognition and signaling between the approaching swarms.

6. Cell-to-Cell Contact Hypothesis and an Alternative Hypothesis

A simple and elegant experiment showing that nonself-recognition is not mediated by a diffusible factor was published recently.³⁴ Two different isolates of *P. mirabilis* were spotted on a plate and separated by a highly porous Anapore membrane. Both isolates swarmed up to the membrane, and a territorial boundary did not form as it did in control experiments where the isolates were not separated by a membrane. This is strong evidence that nonself-recognition is not mediated by a factor that can diffuse through the agar and through the dialysis membrane. It was also taken as evidence that cell-to-cell contact is required for nonself-recognition. But could there be other explanations for the results, an explanation that does not invoke a cell-

to-cell contact requirement? We will present one interesting possibility. At the leading edge of swarms, cells in rafts as well as individual cells exist as a thin layer on the agar surface, and they leave visible trails (Figure 3). It could very well be that materials in the trails, perhaps IdsD and IdsE, serve as signals for the scanning devices and cell-to-cell contact is not required for nonself-identification. There is as yet no evidence to discriminate between the cell-to-cell contact model and a model whereby a nondiffusible signal is laid down on the surface as cells move. Further, the physical mechanisms of boundary formation are still poorly understood: is it that the populations migrate, interact, and then one or both population(s) retreats; is it that one or both population(s) dies after interacting with the other population; is it that, after initial cells meet, a signal is secreted that discourages future migration into the same region? All of these possibilities support a model in which recognition precedes antagonism in *P. mirabilis* boundary formation.

7. Clinical Implications

The prevalence of *P. mirabilis* infections is likely to rise as the U.S. population ages and the number of patients requiring indwelling catheters continues to increase. Clinical isolates of *P. mirabilis* with resistance to a broad range of antibiotics are emerging.⁵⁵ Identical *P. mirabilis* strains are vertically transferred from mother to newborn and, in rare cases, can cause neonatal meningitis and subsequent brain abscesses.⁵⁶ Further, *P. mirabilis* infections are usually clonal.^{5,6} *P. mirabilis* may use self- versus nonself-recognition and territoriality to ensure dominance of a single strain, perhaps during colonization of catheters or the urethra. The increased presence of antibiotic-resistant strains and the ability of clones to establish dominance could have implications for increased occurrences of antibiotic resistance in urinary tract infections of immunocompromised individuals.

8. Biological Significance

At the cellular level, self- versus nonself-recognition is a behavior conserved throughout biology, from bacteria to animals and to vertebrate immune systems. The puzzle of how cells or organisms recognize and differentiate self from nonself is fundamental to fields as far from each other as immunology and ecology. The boundary formation between *P. mirabilis* is clearly a complex interplay of cell-to-cell communication and population development. In boundary formation, there is not only social recognition but also territorial competition between approaching *Proteus* strains. It seems likely that recognition and antagonism both play a role in the overall boundary formation process, but antagonism is not required for boundary formation. Molecular details of the dance leading to boundary formation remain to be determined. How do Ids proteins function to convey strain-specific identity? How is self-identity maintained? How did territoriality emerge in *Proteus*? What are the costs and benefits of this social activity? *Proteus* provides a wonderful tool to dissect the costs and benefits of territoriality. Where else in the bacterial world does territoriality occur?

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