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The Multiple Identities of *Vibrio parahaemolyticus*

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Abstract

***Vibrio parahaemolyticus* is a ubiquitous marine bacterium and human pathogen. The organism possesses multiple cell types appropriate for life under different circumstances. The swimmer cell, with a single polar flagellum, is adapted to life in liquid environments. The polar flagellum is powered by the sodium motive force and can propel the bacterium at fast speeds. The swarmer cell, propelled by many proton-powered lateral flagella, can move through highly viscous environments, colonize surfaces, and form multicellular communities which sometimes display highly periodic architecture. Signals that induce differentiation to the surface-adapted cell type are both physical and chemical in nature. Surface-induced gene expression may aid survival, whether attached to inanimate surfaces or in a host organism. Genetic rearrangements create additional phenotypic versatility, which is manifested as variable opaque and translucent colony morphotypes. Discovery that a LuxR homolog controls the opaque cell type implicates intercellular signaling as an additional survival strategy. The alternating identities of *V. parahaemolyticus* may play important roles in attachment and detachment, how bacterial populations adapt to growth on surfaces, form structured communities, and develop biofilms.**

Introduction

Occupying a variety of niches, *Vibrio parahaemolyticus* is a common bacterium in marine and estuarine environments. It can exist planktonically or attached to submerged, inert and animate surfaces, including suspended particulate matter, zooplankton, fish and shellfish (Kaneko and Colwell, 1973, 1975). This organism is recognized as a major, worldwide cause of gastroenteritis, particularly in areas of the world where seafood consumption is high such as Southeast Asia (Joseph *et al.,* 1982). It is an emerging pathogen in North America. In 1997, a large outbreak of *V. parahaemolyticus* food poisoning, attributed to raw oyster consumption, occurred along the Pacific coast (CDC, 1998). Thus, *V. parahaemolyticus* seems suited to multiple lifestyles: a planktonic, free-swimming state and a sessile existence within a microbial community attached, for example, to shellfish in a commensal relationship, to the bottoms of boats or other surfaces in the ocean (biofouling), or in a host organism (pathogenesis). What are the survival strategies that allow this bacterium to adapt to life in dilute liquid environments and to life on surfaces or in biofilms?

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The Swimmer Cell

The free-living form of *V. parahaemolyticus*, the swimmer cell (Figure 1), is well-suited for locomotion in liquid environments. The rod-shaped bacterium is efficiently propelled by a single polar flagellum. Energy to power the flagellum is derived from the sodium motive force (Atsumi *et al.,* 1992). Possession of sodium energetics has an advantage in the marine environment because the pH of seawater is approximately 8.0 (Kogure, 1998). Sodium-driven flagellar motors are remarkably fast. In liquid medium with 300mM NaCl, the swimming speed of the bacterium is approximately 60μm per sec. Sodium-powered rotation of the polar flagellum of the closely related bacterium *Vibrio alginolyticus*, which swims at an equivalent speed in liquid, has been clocked using laser-darkfield microscopy at rates as fast as 1,700 r.p.s. (Magariyama *et al.,* 1994).

The polar organelle is a complex flagellum. There are six polar flagellin genes, organized in two loci (McCarter, 1995; GenBank Accession U12816 and U12817). Moreover, the polar flagellum is sheathed by what appears to be an extension of the cell outer membrane (Allen and Baumann, 1971). The mechanism of how a sheathed flagellum rotates has not been elucidated. Potentially, the flagellar filament could rotate within the sheath, or the sheath and filament could rotate as a unit (Fuerst, 1980). The flagellum plays a key role in initial adsorption of bacteria to surfaces. A plethora of studies with a variety of bacteria have shown that motility is important for adhesion as well as pathogenesis (O'Toole and Kolter, 1998; Otteman and Miller, 1997; Pratt and Kolter, 1998). As a propulsive organelle, it brings the bacteria into close proximity with surfaces and perhaps aids in overcoming negative electrostatic interactions. In fact, studies have shown that the faster the swimming, the greater the adhesion to glass (Kogure *et al.,* 1998). Flagellar sheaths allow specific interaction between a bacterium and a surface (Sjoblad and Doetsch, 1982; Sjoblad *et al.,* 1983). Certainly, it seems clear the sheath extends the surface area of the bacterium. Cell-surface components, including potential adhesins, may be differentially distributed or available on the flagellum and cell body. The flagellum in Figure 1 is studded with colloidal gold particles, after immunogold-labeling with rabbit antiserum prepared against whole cells. In contrast, there is little gold-labeling of the cell body, suggesting that antigens are presented differently by the cell body and the sheathed flagellum.

The Swarmer Cell

Growth on surfaces or in viscous environments induces differentiation to the swarmer cell. Septation ceases and the cell elongates, usually up to 30 μm in length. Induction of a second motility system, the lateral system, leads to elaboration of numerous peritrichously arranged flagella. The swarmer cell is adapted for movement on surfaces or

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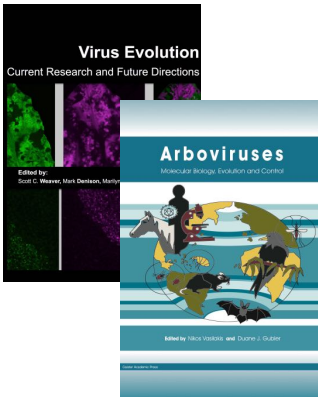

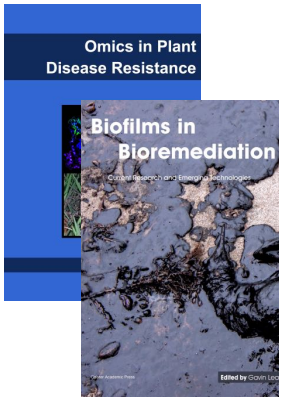

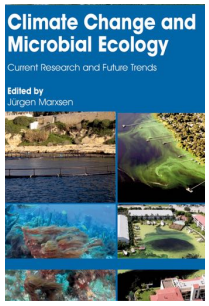

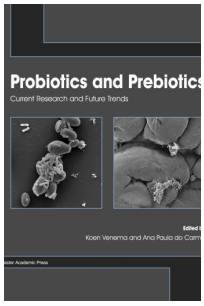

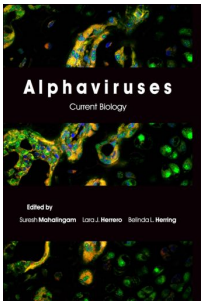

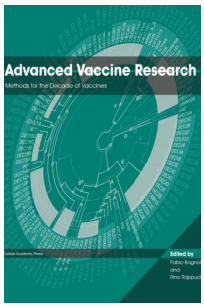

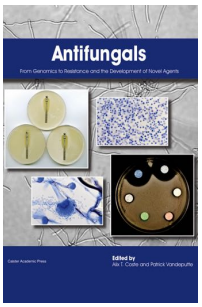

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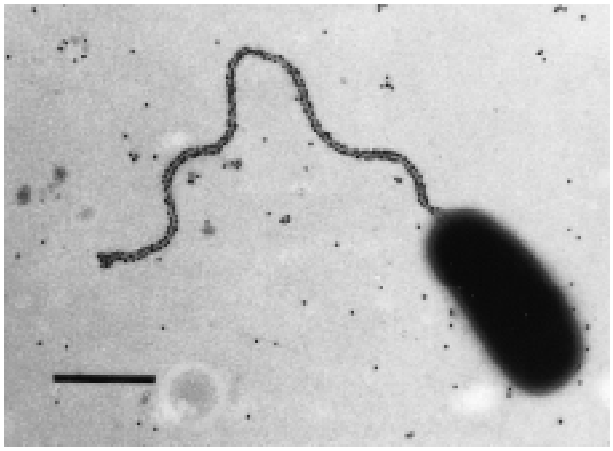

Figure 1. Electron Micrograph of a Swimmer Cell
Electron micrograph of a swimmer cell reacted with rabbit antiserum prepared against whole cells and 15nm protein A-bound colloidal gold particles. Bar indicates approximately 1 μm.

through highly viscous environments. The polar flagellum performs poorly in medium of high viscosity while lateral flagella efficiently propel the bacterium in highly viscous environments. The speed of laterally flagellated cells is unaffected, remaining constant at about 25μm per second, on addition of the long branched-chain polymer polyvinylpyrrolidone (10% PVP-360) which increases viscosity to 10 centipoise (cP). In comparison, polarly propelled swimming motility decreases from 60 to less than 15 μm per second under this condition (Atsumi *et al.,* 1992). It has been shown for *V. alginolyticus* that lateral flagella can work at viscosities as high as 100 cP (Atsumi *et al.,* 1996).

Lateral flagella are not sheathed, and the filament is polymerized from a single flagellin subunit (McCarter and Wright, 1993). The flagella are very sensitive to mechanical shearing (Allen and Baumann, 1971). So many flagella are produced per cell and sloughed, or broken off, that detached flagellar aggregates are readily visualized in the light microscope as giant, coiled bundles (Belas *et al.,* 1986; Ulitzur, 1974). These flagella are entirely distinct from the polar flagellum. Genetic experiments suggest that there are no shared structural components among the two motility systems. Mutants unable to swarm retain swimming motility and vice versa (McCarter *et al.,* 1988). Flagellar motility systems are typically encoded by at least 40 genes; therefore, two very large, distinct gene sets encoding the motility systems exist. When grown on a surface, the organism assembles two distinct types of flagella. What this means in terms of signals for flagellar export and assembly will be interesting to elucidate. Not only do the lateral and polar flagella differ at the structural level, but also are powered by different energy sources. Although in each case, energy is derived from the electrochemical transmembrane potential, the coupling ions differ. Lateral flagella are powered by the flow of protons through the motor in contrast to the sodium-driven polar motors (Atsumi *et al.,* 1992).

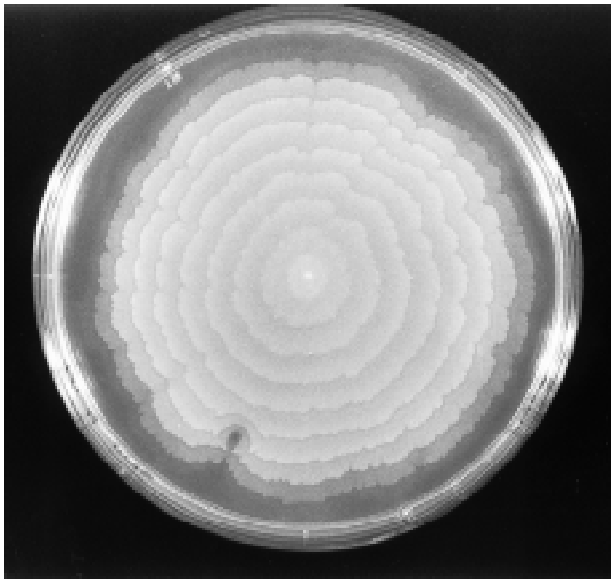

Figure 2. Swarming Colony on Rich Medium after Overnight Incubation at 30 °C.

When inoculated on solidified medium, differentiation to the swarmer cell allows the bacterium to swarm, or move, over surfaces or through viscous environments, leading to colonization of surfaces (Figure 2). The movement is very vigorous. The percent agar over which swarming will occur is high (up to 2.0%), and the rate of radial expansion is rapid, for example, on Heart Infusion agar at 30ºC swarming will progress at a rate of 5mm/hour. A number of bacteria are known to swarm. Although some show mixed flagellation like *V. parahaemolyticus* with distinct polar and peritrichous organelles (*e.g.*, *V. alginolyticus, Rhodospirillum centenum,* and *Azospirillum brasilense*; Allen and Baumann, 1971; Jiang *et al.,* 1998; Moens *et al.,* 1996), others possess single, peritrichous flagellar systems. In general the extent of hyperflagellation of the swarm cell correlates with successfulness of swarming. *V. parahaemolyticus* and *Proteus mirabilis* are superior swarmers and can have hundreds of flagella per cell. In comparison, *Serratia* species, *Escherichia coli,* and *Salmonella typhimurium* swarm under a more restricted set of conditions with agar concentrations at 0.5 – 0.8 % (Harshey, 1994; Harshey and Matsuyama, 1994). Movement for some of these swarming organisms has been shown to also require production of extracellular molecules capable of altering surface tension. Surfactant-like lipopeptides are produced by *S. marcesans* and *S. liquefaciens* (Matsuyama *et al.,* 1992; Lindum *et al.,* 1998). Rapid spreading of *P. mirabilis* requires production of extracellular capsular polysaccharide (Gygi *et al.,* 1995). Extracellular agents acting as swarming facilitators have not been demonstrated for *V. parahaemolyticus.* It may be that the copious production of fragile flagella substitutes to reduce frictional drag.

The concentric ring patterns or terraces, which are characteristic of the periodic swarming of *Proteus* species (Rauprich *et al.,* 1996), develop as a result of alternating cycles of active swarming and consolidation. During the consolidation period, swarmer cells divide to produce short cells, which eventually differentiate to the swarmer cells that initiate a new wave of swarming. Although the phenomenon has been studied extensively in *P. mirabilis*, this type of behavior is not restricted to *Proteus* species.

Under certain conditions (specifically, swarm plates non-optimally conducive to swarming such as those made with Luria broth rather than Heart Infusion broth), certain *V. parahaemolyticus* strains produce the highly developed, periodic architecture shown in Figure 2. Detection of pattern formation may require a special balance between the rates of reproduction and movement of the cells as well as the particular surface properties of the substratum (i.e., surface tension).

Physical Signaling: The Polar Flagellum as Tactile Sensor

In addition to its role as a propulsive organelle and its potential for aiding attachment, the polar flagellum acts as a sensor. Polar flagellar function is coupled to expression of the swarmer cell gene system. The polar flagellum is produced constitutively, irrespective of liquid or surface-associated growth. All conditions that slow down polar flagellar rotation, lead to swarmer cell induction. Such conditions include increasing viscosity or using antibodies to inhibit flagellar function (Belas *et al.,* 1986, McCarter *et al.,* 1988). Physical conditions that impede flagellar rotation seem to act as a signal. Polar flagellar function can be perturbed in other ways. Genetic interference with polar function affects swarmer cell gene expression (McCarter *et al.,* 1988). All swimming-defective, transposon-generated mutants constitutively express swarmer cell genes when grown in liquid (Figure 3). The motor itself can be slowed down by using the sodium-channel-blocking drug phenamil (Kawagishi *et al.,* 1996). Just as increasing viscosity leads to swarmer cell differentiation, increasing concentrations of phenamil lead to decreasing swimming speed and concomitant induction of swarmer cell gene expression. Thus, the polar flagellum seems to act as a mechanosensor: interference with flagellar rotation signals swarmer cell differentiation. How signaling is transduced to program gene expression is not known. Current work involves dissecting the architecture of the sodium-driven motor of *V. parahaemolyticus* (McCarter, 1994a, 1994b; Jaques *et al.,* 1999).

Cell Division and the Long Cell Phenotype

One of the initial events after transfer to a surface is inhibition of cell division, and as a consequence, swarmer cells are characteristically very long. Differentiation is transient. Since, swarmer cells must grow, i.e., divide, for the swarm colony to expand, the cell must have a mechanism to escape from the block to cell division. Thus, the inhibition of cell division during the swarm cell cycle must be carefully regulated for prolonged repression of cell division would be a terminal event.

Strains in one class of swarming constitutive mutants possess defects in *lonS*, a gene that codes for a homolog of the *E. coli* Lon protease (Stewart *et al.,* 1997). *E. coli lon* mutants were originally isolated as a class of UV-sensitive mutants (Gottesman, 1996). The role of Lon in *E. coli* is multifunctional, e.g., it targets the degradation of a transcriptional regulatory protein controlling production of extracellular polysaccharide (*cps*) as well as the cell division inhibitor SulA. SulA is induced by UV exposure as part of the SOS DNA repair response. Wild-type cells form long filaments after UV exposure, but with time SulA is degraded by Lon and the filaments resolve. Mutants with defects in *lon* cannot recover from elongation.

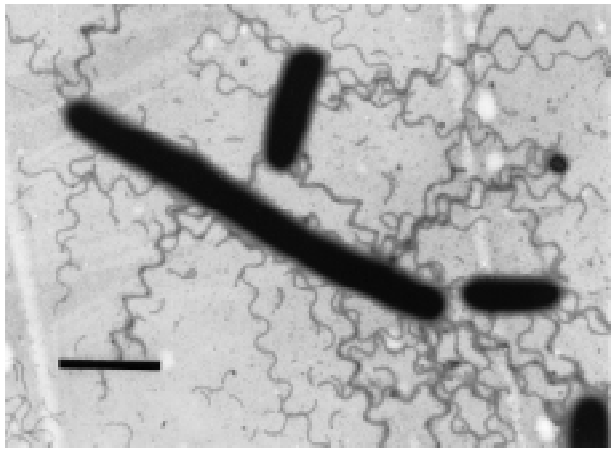

Figure 3. Electron Micrograph of a Constitutive Swarmer Cell
Grown in liquid and negatively-stained with 0.5 % phosphotungstic acid. Bar indicates approximately 3 μm.

The *V. parahaemolyticus* counterpart closely resembles *E. coli lon* and substitutes functionally, by complementing *E. coli* mutants to restore UV resistance and *cps* regulation. In addition, *lonS* mutants in *V. parahaemolyticus* are more UV sensitive than the wild-type strain. It is attractive to hypothesize the existence of swarmer-cell specific cell division inhibitor. LonS could act as a policeman to keep in check the important cell division and regulatory proteins that mediate surface sensing. Potential regulatory targets for LonS could include transcriptional activators controlling the lateral flagellar gene system or extracellular polysaccharide.

Chemical Signaling

Differentiation to the swarmer cell requires considerable commitment in terms of cellular economy, including energy expenditure and gene expression. As a result, the developmental switch seems tightly controlled and multiple environmental stimuli are essential for cueing development. One cue that has been determined is iron starvation. Starvation signals seem an appropriate cue because the availability of nutrients or the diffusion of nutrients may be limiting for cells in a community attached to a substratum. Development to the swarmer requires iron limitation and perturbation of flagellar function (McCarter and Silverman, 1989). Nutrient deprivation and surface signaling may ensure detection of the specific conditions for which swarming is appropriate, e.g., a dense community of sessile cells.

Although others kinds of signals may also be important, *V. parahaemolyticus* can be induced to swarm on minimal medium (Figure 4; McCarter, 1998). This has not been shown to be the case for other bacteria where amino acids are implicated as swarming signals. Glutamine has been reported as essential to induce swarming of *P. mirabilis* on minimal medium (Allison *et al.,* 1993). Casamino acid supplementation is necessary for *S. liquefaciens* (Eberl *et al.,* 1996b); however, failure to swarm on minimal medium could also represent a growth rate barrier and not the lack

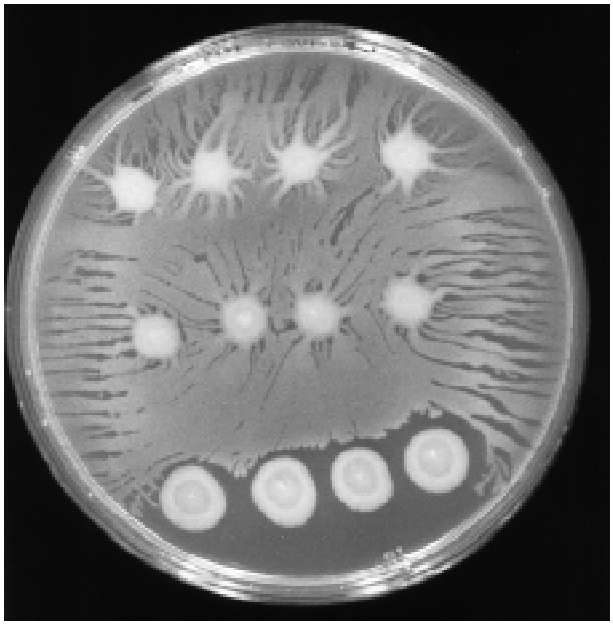

Figure 4. Swarming Colonies on Minimal Medium
Galactose was the carbon source, 5-day incubation at 30°C. The strain inoculated in the bottom row carries a defect in the lateral motility system; therefore, it is unable to swarm.

of a specific inducing signal.

In Figure 4, the swarming colonies in the top and middle row do not interfere with each other's radial expansion, while there is inhibition of swarming proximal to the strain in the bottom row which is a nonswarming mutant defective in lateral flagella formation. Swarm colonies are chemotactic. Mutants with defects in *V. parahaemolyticus che* genes, although fully flagellated, fail to expand and make coherent progress on swarm plates (Sar *et al.,* 1990). Presumably strains migrate towards attractants and away from repellents. Usually swarming colonies will merge seamlessly; however occasionally strains, e.g., spontaneous variants or mutant strains, will fail to converge. In this case, sharp lines demarcating zones where growth is inhibited are formed. A similar phenomenon was observed (but not explained) many years ago with strains of *Proteus* species and a compatibility screen, the Dienes test, was used for strain typing (Dienes, 1946; Senior *et al.,* 1977). What allows some colonies to fuse and not others? Perhaps defects or differences in small-molecule signaling or reception or incompatibility of cell surface moieties leading to interference with coordination of the swarm. Chemotactic control of movement is integrated in the sense that some of the *che* genes are shared by both motility systems. It remains to be discovered if there are unique liquid- and surface-specific components to chemotaxis.

Intercellular Communication

The complex growth patterns observed for colonies on plates suggest modes of cooperative behavior. Colony dynamics must accommodate diffusion of nutrients, motility, cell division and intercellular communication (Ben-Jacob *et al.,* 1994; Shapiro, 1998). Budrene and Berg (1991) have shown complex pattern formation by motile cells of *E. coli* as gradients of attractants are established by the cells themselves. Other effects may take place at the level of gene expression. Density-dependent sensing has been postulated to be an important component of bacterial colonization and growth in communities (Batchelor *et al.,* 1997; McLean *et al.,* 1997). Biofilm architecture can be profoundly influenced by cell-to-cell signaling (Davies *et al.,* 1998). Small molecule signaling and intercellular communication may provide the cell one method for discrimination between a free-living, low cell density environment and an attached, high cell density environment (Fuqua *et al.,* 1996). *S. liquefaciens* produces two extracellular signaling molecules belonging in the N-acyl homoserine lactone (AHL) family of autoinducers (Eberl *et al.,* 1996b). Mutants unable to produce autoinducers show defects in swarming. Experiments suggest that defects in autoinducer production lead to an inability to produce the surfactant required for swarming (Lindum *et al.,* 1998).

Much of what is known about autoinducer signaling in *V. parahaemolyticus* derives from cross-talking between *V. parahaemolyticus* and the closely related *Vibrio harveyi*. Density-dependent regulation of luminescence is well-studied in *V. harveyi* (Bassler *et al.,* 1994; Miyamoto *et al.,* 1996). The two *Vibrios* both produce two distinct autoinducer molecules, one belonging in the AHL family while the nature of the second seems unique and has not yet been determined. Although most *V. parahaemolyticus* strains are not luminescent, supernatants of cultures from these strains will induce luminescence of *V. harveyi* (Greenberg *et al.,* 1979; Bassler *et al.,* 1997). Light production in *V. harveyi* is controlled by the dual signaling systems: two autoinducers uniquely interact with cognate receptors to activate phosphorelay cascades that ultimately affect transcription of *luxR*. The product of *luxR* is a transcriptional activator of the *lux* operon, which encodes the enzymatic activities necessary for luminescence. This is an either/or system: loss of function of one signaling pathway does not eliminate luminescence or regulation of luminescence. Thus, the ultimate transcriptional activating component of the autoinducer signaling pathways in *V. harveyi* is LuxR. When the function of the gene encoding the LuxR homolog is eliminated *in V. parahaemolyticus*, swarming is unaffected (McCarter, 1998). Such mutants expand at rates comparable to the wild-type strain on rich or minimal swarm media. This suggests that autoinducer signaling mediated through LuxR is not a requirement for swarming in *V. parahaemolyticus*.

Opaque/Translucent Variation in Colony Morphology

If the LuxR homolog is not directly implicated in swarming, then what is its function in *V. parahaemolyticus*? Expression of this gene does dramatically affect other attributes of the organism. Introduction of a clone carrying the *luxR*-like locus into *V. parahaemolyticus* converts colony morphology from translucent to opaque (McCarter, 1998).

In addition to the swimmer/swarmer dimorphism, *V. parahaemolyticus* exhibits another kind of phenotypic switching. It is manifested in variable colony morphology. Descendants of a single colony can have multiple colony morphotypes. The variants are described as opaque (OP) and translucent (TR) as a result of differences in the transmission of light by the colony (Figure 5). The switching

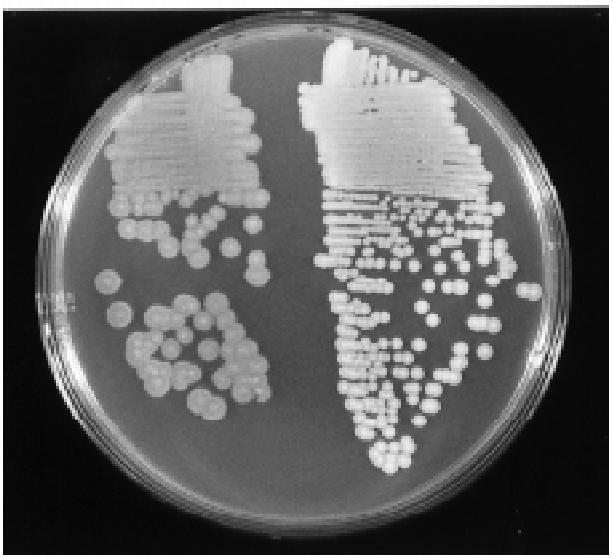

Figure 5. Opaque (OP) and Translucent (TR) Colonies are Shown on Right and Left, Respectively

event is slow enough so that it is possible to obtain essentially uniform populations with less than 1 alternate form per 1000 cells. Properties of OP and TR are distinct and multiple traits are affected. For example, OP cells aggregate in certain kinds of liquid media, possess a thick, ruthenium-red staining capsular material, display a different array and distribution of outer membrane proteins, and swarm very poorly compared to TR cells. It is postulated that differences in cell surface characteristics lead to differential cell packing within the colony, which determines the opaque/translucent properties.

Evidence suggests that the LuxR homolog is a global regulator controlling opacity in *V. parahaemolyticus*, and so the gene has been named *opaR.* Expression of *opaR* correlates with opacity. The gene is transcribed in OP but not TR cells. When the coding sequence for *opaR* is placed under control of the pTac promoter, opacity becomes inducible by isopropyl-ß-D thiogalactopyranoside. Furthermore, disruption of the gene by transposon insertion converts an OP strain to TR. Due to the extremely high homology of the gene and its promoter region with the *V. harveyi* locus, it seems likely that *opaR* expression will be responsive to autoinducers; however, this remains to be determined as does the nature of the signaling inputs, which are also unknown in *V. harveyi*.

DNA rearrangements occur in the *opaR* locus and this determines one basis for variation in colony morphology. Physical alterations in the DNA preclude transcription of *opaR* in the TR state. Whether the recombinational switch itself is controlled, *i.e.* responsive to environmental signals, or is a result of spontaneous genetic rearrangement is not known. So, there is potential in the system for multiple levels at which this variable phenotype can be determined. OP/TR switching alternates between expression competent and incompetent states. Transcription, in the competent state, may be regulated by environmental and or intercellular signals, and the switching event itself may be spontaneous or responsive to specific cues.

Multiple Identities of *V. parahaemolyticus*

In order to survive in changing environments, bacteria possess enormous adaptive capabilities that allow them to modulate their behavior and program gene expression in response to environmental and intercellular cues. Figure 6 illustrates potential roles for the multiple identities of *V. parahaemolyticus* and is consistent with many observations on bacterial survival in the real world (Costerton *et al.,* 1995). Motility and chemotaxis have obvious advantages for the lifestyle of the free-living swimmer cell. Moreover, swimming may allow a bacterium to find and closely approach a surface or viscous layer. Initial contact with surfaces may be facilitated by specific adhesins on the cell body or the sheathed flagellum. In liquids and/or on surfaces, cell types switch reversibly between OP and TR. Switching may occur randomly, so that a subset of the population is preadapted, or it may be responsive to specific environmental conditions. The OP and TR forms, having different cell surface characteristics, may adhere preferentially to different surfaces or selectively autoaggregate and thus facilitate detachment. Once the cell makes initial contact with the surface, performance of

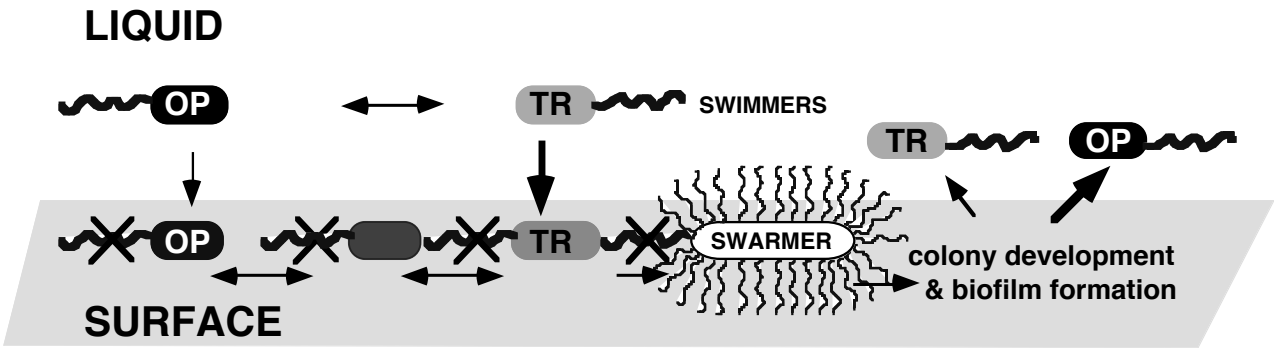

Figure 6. Multiple Identities of *V. parahaemolyticus*
Swimmer, Swarmer, Opaque (OP) and Translucent (TR) cell types allow adaptation and survival under different circumstances, for example planktonic survival in liquid versus growth in viscous environments, on surfaces, or in biofilms. OP and TR forms interconvert and may preferentially attach, or detach, to different surfaces (indicated by thick versus thin arrows). Immobilization of polar flagellar rotation (indicated by "X") signals a surface and leads to induction of surface-induced genes, including specific adhesins, potential virulence factors and the lateral motility system. Swarmer cell differentiation, colony development and biofilm formation is also influenced by chemical and cell-to-cell signaling.

the polar flagellum is impeded. This constitutes surface recognition, and the cell synthesizes new molecules appropriate for life on surfaces. What are these molecules? Some may aid adherence or allow protection. For other bacteria known to swarm, virulence factors are clearly produced in response to growth on surfaces (Mobley and Belas, 1995). Chemical signals, such as iron starvation, are required in addition to mechano-inactivation of flagellar rotation, to induce the swarmer cell developmental program. Differentiation to the swarmer cell allows movement over surfaces and through viscous environments. Cells recognize each other, and movement is coordinated and social in nature, resulting in complex multicellular behavior and growth in organized communities.

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