Surface sensing, swarmer cell differentiation, and biofilm development

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INTRODUCTION

Bacteria are versatile organisms that can quickly adapt to a changing environment which aids these cells in survival. A major adaptive strategy, and perhaps the predominant mode of growth for bacteria in nature, is biofilm formation. The formation of a biofilm often requires the interaction of groups of bacteria which exhibit a multicellular behavior reminiscent of the coordinated development associated with higher organisms. Predominant in these events are carefully orchestrated responses by the bacteria to cues derived from the surface and other bacteria. These signals are used to optimize bacterial survival and perpetuate life in the developing biofilm.

Bacteria in biofilms live a different existence than in the planktonic state, and this is reflected in their morphology and metabolic functions. These bacteria are often resistant to reagents that kill their planktonic counterparts and are thought to be the pathogenic forms of many disease-causing bacteria. This is demonstrated by the genus *Proteus*, in which swarmer cell differentiation and swarming motility has been correlated to expression of virulence genes[1, 2] and urinary tract pathogenesis[3]. In *P. mirabilis*, biofilm formation is initiated after bacteria encounter a solid substratum. Upon this encounter *P. mirabilis* cells differentiate into elongated, hyperflagellated cells that can move collectively on the surface (Figure 1). The phenomena of cell elongation, hyperflagellation and swarming has also been demonstrated in other bacteria such as *Vibrio*[4] and *Serratia*[5] and is referred to as swarmer cell differentiation and swarming behavior. Swarming is a multicellular behavior used by these bacteria not only

[1] C. Allison, N. Coleman, P. L. Jones ,and C. Hughes, *Infect Immun.***60**, 4740 (1992).
[2] C. Allison, C. H. Lai ,and C. Hughes, *Mol Microbiol.***6**, 1583 (1992).
[3] S. I. Bidnenko, E. P. Bernasovskaia, N. A. Iu, A. B. Iu ,and E. V. Mel'nitskaia, *Mikrobiol Zh.***47**, 81 (1985).
[4] L. McCarter and M. Silverman, *Mol Microbiol.***4**, 1057 (1990).
[5] L. Alberti and R. M. Harshey, *J Bacteriol.***172**, 4322 (1990).

to seek new energy sources but also to move away from a crowded center before it becomes metabolically deleterious.

Unlike chemotactic behavior, which is mediated through the sensing of chemical gradients in the surrounding environment, swarmer cell differentiation is mediated through a physical sensing of a surface by an individual bacterium. This surface sensing is mechanical and mediated through the inhibition of flagellar rotation as the cells get near to a submerged surface. Surface sensing is only the first component in swarming, which also requires active multicellular, coordinate movement of the cells across the surface to form a biofilm. Control of swarming migration requires additional signals to initiate morphological and physiological adaptation to a solid surface. For example, the amino acid glutamine is essential for *P. mirabilis* cell differentiation[6], whereas, in *Vibrio parahaemolyticus* iron limitation appears to be required for induction of differentiation[7]. Swarming behavior is also regulated by cell density sensing mechanisms that appear to be a common form of communication among bacteria in biofilms. In *Serratia liquefaciens*, the *swrl* gene codes for a putative *N*-acyl-homoserine lactone (AHL) and is required for expression of swarmer cell differentiation and function[8, 9]. While in *P. mirabilis*, transposon inactivation of *rsbA*, a member of a two-component regulatory system, results in precocious swarming mutants that do not require surface contact or glutamine for expression of swarmer cell phenotype[10], suggesting that the RsbA protein functions to control migration in this species.

[6] C. Allison, H. C. Lai, D. Gygi ,and C. Hughes, *Mol Microbiol.***8**, 53 (1993).
[7] L. McCarter and M. Silverman, *J Bacteriol.***171**, 731 (1989).
[8] M. Givskov, J. Ostling, L. Eberl, P. W. Lindum, A. B. Christensen, G. Christiansen, S. Molin ,and S. Kjelleberg, *J Bacteriol.***180**, 742 (1998).
[9] P. W. Lindum, U. Anthoni, C. Christophersen, L. Eberl, S. Molin ,and M. Givskov, *J Bacteriol.***180**, 6384 (1998).
[10] R. Belas, R. Schneider ,and M. Melch, *J Bacteriol.***180**, 6126 (1998).

P. mirabilis swarming behavior is punctuated by cycles of differentiation, migration and dedifferentiation (consolidation) that give rise to a "b -eye" colony on nutrient agar media. In a liquid environment, the undifferentiated vegetative or "swimmer" cells are 1.5-2 μm long and possess 6 to 10 peritrichous flagella. When these cells are transferred to a solid substrate, i.e., a nutrient agar surface, they sense the surface and begin differentiating into cells that are 60-80 μm in length that are hyper-flagellated, possessing between 10^3 and 10^4 flagella per cell[11]. After the cells have differentiated, a multicellular phase begins in which cooperation through cell-to-cell contact leads to migration of the colony, as a collective entity, on the surface of the substrate. Finally, after a period of migration on the surface, the cells stop and begin to dedifferentiate to swimmer cells and then multiply. This cycle repeats itself until the entire agar surface is covered by the colony.

The signals that control swarmer cell differentiation and behavior can be divided into those that induce the cellular differentiation process and those that control multicellular migration. Investigations into the molecular nature of these signals and the regulatory mechanisms underlying their control have led to exciting discoveries. It is now known that the signal each individual cell senses is mediated through the inhibition of its rotating flagella. The signal initiated by the tethered polar flagella is conveyed to the transcription machinery and leads to synthesis of new flagella, inhibition of cell wall septation, and other events that change the phenotype into elongated hyperflagellar form. Thus, the first stimulus sensed by these cells as they form a biofilm is physical in nature, occurs at the level of the individual cell, and is transmitted into the cytoplasm via the inhibition of flagellar rotation.

[11] J. F. Hoeniger, *Can J Microbiol.***12**, 113 (1966).

In contrast, the signals controlling swarming migration occur at the level of the colony and function to coordinate multicellular behavior. Cell-to-cell contact and extracellular signaling by amino acids and other small molecules appear to play major roles in multicellular swarming behavior. These findings have come about through experiments that have employed a myriad of genetic and molecular techniques. In this chapter, we will describe some of the methods developed to investigate the molecular mechanisms of surface sensing in gram negative bacteria.

Methodology

Transposon mutagenesis for the construction of mutants defective in swarming and transcriptional fusions to promoterless reporter genes

Transposon mutagenesis in *V. parahaemolyticus*

Transposon mutagenesis has been used to elucidate the role of different genetic loci and different environmental signals in the development of swarmer cells and swarming behavior in *V. parahaemolyticus.* The transposable phage derivative mini-Mu is very practical in studies of regulation of gene expression. The coding sequence for a reporter gene, but not its regulatory sequences, have been incorporated into mini-Mu at the right end and is flanked by only few hundred base pairs. This arrangement allows transcription to proceed from genes outside of transposon into the reporter gene. If the mini-Mu inserts in the correct orientation within a gene, it will generate a transcriptional fusion in which the promoter of the mutated gene controls the expression of reporter gene. Mini-Mu, therefore, can be used to investigate the expression of the target gene under different conditions (Figure 2).

Transposon mini-Mu (dI1681 Kan^R *Bam*HI *cts*) is derived from the Mu dI*lac* phage by deletion of the internal *BamH*I fragment[12]. This transposon carries the structural genes for the *lacZ, Y, A* at the right end and retains transposition replication genes *A* and *B* and the Mu repressor gene *c* on its left end. The *c* allele is heat-sensitive and thus replication and transposition can be induced by temperature elevation. However, since mini-Mu lacks the structural genes for forming infective particles, a helper phage is required for packaging of mini-Mu DNA. *E. coli* POI1681 (*araD araB*::Mu *cts lac recA rpsL*) strain carries a Mu lysogen which can provide the helper functions. After introduction of mini-Mu into *E. coli* POI1681 and temperature induction, the help of resident Mu lysogen forms infective mini-Mu particles.

Another derivative of the mini-Mu transposon harbors a promoterless bacterial luciferase gene cassette and is referred to as mini-Mu *lux*. Mini-Mu *lux* is derived from transposon mini-Mu *lac* (see above) by replacing the *lac* genes with the *lux* genes (*luxCDABE*) from *Vibrio fischeri*[13]. This transposon carries all the genes necessary to produce light in a variety of microorganisms. Mini-Mu *lux* mutagenizes by transposing into the target gene and because of its large size will generally cause a null mutation. Furthermore, insertion in the correct orientation will result in a transcriptional fusion in which the promoter of the target gene will control the expression of luminescence genes. The luminescence production can be easily detected in dark in bacteria that do not naturally produce light. The mutants can be isolated by selection on tetracycline plates and the region flanking the transposon insertion site (target gene) can be cloned.

[12] B. A. Castilho, P. Olfson ,and M. J. Casadaban, *J Bacteriol.***158**, 488 (1984).
[13] J. Engebrecht, M. Simon ,and M. Silverman, *Science.***227**, 1345 (1985).

Mini Mu *lux* is a powerful tool to study regulation of gene expression during swarmer cell differentiation and multicellular swarming behavior because bioluminescence resulting from the transcriptional fusion to *lux* genes can be measured in intact cells. This transposon has been successfully used to mutate and construct fusion strains in *V. parahaemolyticus.* Elegant studies to monitor reporter gene activity and swarmer cell differentiation under different conditions have indicated that lateral flagellar gene (*laf*) expression is critical in differentiation of swarmer cells. Furthermore, the signal for swarmer cell development is mediated by impairing the ability of the bacterium to move relative to its surroundings. Antibodies to the cell surface components of *V. parahaemolyticus* caused agglutination and subsequent tethering of polar flagella and result in the abnormal expression of swarmer cell differentiation[14]. Increasing the viscosity of nutrient broth to 40 cP by the addition of polyvinylpyrrolidone (PVP-360) to 10% (w/v) also resulted in swarmer cell differentiation and in the induction of luminescence in *laf*::*lux* fusion strains[15]. Similar studies have demonstrated that a group of genes that encode the structural proteins of the *V. parahaemolyticus* polar flagellum play significant role in coupling the signal initiated from polar flagella to expression of *laf* gene[16]. Taken together, these data, derived from work with mini-Mu *lux* transcriptional fusions, point out the pivotal role played by the polar flagellum in sensing the physical cue of viscosity and resulting inhibition of flagellar rotation.

In the following paragraphs we provide a protocol for the use of mini-Mu *lac* to mutagenize*V. parahaemolyticus* and generate *lacZ*-fusion strains. The same strategy may be used to construct transcriptional fusions using other derivatives such as mini-Mu *lux*.

[14] L. McCarter, M. Hilmen ,and M. Silverman, *Cell.***54**, 345 (1988).
[15] R. Belas, M. Simon ,and M. Silverman, *J Bacteriol.***167**, 210 (1986).
[16] L. L. McCarter, *J Bacteriol.***177**, 1595 (1995).

Mutagenesis of *V. parahaemolyticus* with mini-Mu (Tet^R)

To mobilize mini-Mu from *E. coli* into *V. parahaemolyticus* coliphage P1 (P1 *clr*-100 CM) is used. Transducing phage P1 is capable of packaging mini-Mu *lux* DNA and infects but does not replicate in *V. parahaemolyticus*. The repression of P1 and that of mini-Mu *lux* transposon are both inactivated at 42^{o}C. Therefore, lytic cycle of P1 and replication of mini-Mu *lux* can be co-induced in an *E. coli* strain that contains mini-Mu *lux* and is lysogenized by P1.

1. Transform a Mu *cts* lysogen of *E. coli* strain ED8654 (*supE supF met hsdR*) with pBA104[17] which carries mini-Mu (Tet^R). Select the transformants on L agar containing 100μg of ampicilin and 20 μg of tetracycline per ml.
2. Infect the cells carrying pBA104 with phage P1 (P1 *clr*-100 CM) and heat-induce the infected cells at 42^{o}C to produce infecting lysate of P1 that carries min-Mu (Tet^R).
3. Transduce the tetracycline resistant gene to *E. coli* strain MC4100 [F$^-$ *ara*D Δ(*lacIOPZYA*) *U169 rpsL*].
4. Screen the transductants for ampicilin-sensitive colonies to obtain colonies that carry mini-Mu (Tet^R) in their chromosome with the concomitant loss of the plasmid pBA104.
5. Grow *E. coli* MC4100 harboring mini-Mu (Tet^R) overnight in L broth in (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter) containing 20 μg of tetracycline per ml. Infect with phage P1 (P1 *clr*-100 CM) to obtain a P1 lysogen of MC4100.
6. Grow wild type *V. parahaemolyticus* strain BB22 over night at 30^{o}C in Difco marine broth 2216 at 75% the recommended concentration (28 g of Difco 2216 in 1000 ml dH_2O)

or heart infusion broth supplemented with 20 g of NaCl per liter supplemented with 10 μg of tetracycline per ml.

7. Temperature-induce *E. coli* MC4100 (mini-Mu *lac* (Tet^R), P1 *clr*-100 CM) at 42°C to prepare infecting lysate.
8. The infecting lysate are incubated with *V. parahaemolyticus* for 2 hours at 30°C and transductants are plated on heart infusion agar (heart infusion broth containing 20 g of agar per liter) supplemented with 15 g NaCl per liter and containing 10 μg/ml of tetracycline. Bacterial colonies do not swarm on this medium thus allowing the selection of single clones of Tet^R-transductants.
9. After over night incubation at 30°C, Tet^R colonies are picked onto a master array. To identify non-swarming colonies replica plate the transductants from the master array onto hear infusion agar containing 20 g of NaCl and 15 g of agar per liter. Measure swarming after 8 hours of incubation at 30°C and save the non-swarmer colonies for further analysis.

Measurement of β-galactosidase activity in *laf*::*lac* fusion strains

Mutant strains of *V. parahaemolyticus* that display β-galactosidase activity (Lac^+) are detected by growing colonies on agar medium containing 20 μg/ml of chromogenic substrate X-gal and 10 μg/ml of tetracycline. To test the surface expression of *lacZ*, harvest cells from agar and liquid medium, adjust the optical densities to give equal readings at 600 nm and perform ONPG assay as described by Miller[18].

[17] R. Belas, A. Mileham, M. Simon ,and M. Silverman, *J Bacteriol.***158**, 890 (1984).
[18] J. H. Miller Experiments in molecular genetucs; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1972.

Measurement of luminescence in mutants with mini-Mu *lux* transcriptional fusion

The *laf*::*lux* fusion strains are sensitive tools for investigating the environmental signals that lead to *laf* gene expression and consequently to swarmer cell differentiation. Mutants that can produce light on agar but not in liquid can be detected in a dark room or by allowing the light producing colonies to expose to an X-ray film in a dark room. The promoter of the target gene (*laf*) that controls expression of the *lux* genes is induced only upon contact with the surface of agar but not in liquid. This activity can be quantified by measuring the level of luminescence. The following protocol describes the measurement of luminescence in *laf*::*lux* fusions of *V. parahaemolyticus* grown on a solid substrate and in liquid.

1. Grow *V. parahaemolyticus laf*::*lux* fusion strains at 30^{o}C overnight in 2216 marine broth at 75% recommended concentration supplemented with 10 μg of tetracycline per ml.
2. Dilute the culture 1 to 2000 in fresh 2216 medium (75% concentration) without tetracycline and incubate at 30^{o}C with shaking until optical density of 0.05 at 600 nm is reached (about 2 hours). More dense sample do not produce light as well in response to surface contact in these fusion strains.
3. Inoculate 5 μl of the sample in 500 μl of liquid medium in a plastic 1.5 ml microcentrifuge tube. Inoculate another 5 μl of the sample on the surface of a cylindrical core of agar medium 1 cm in diameter and 1 cm in depth.
4. Place the fusion strain in liquid (microcentrifuge tube) and on solid substrate (agar core, or membrane filters) in different scintillation vials. Place the vials in a scintillation counter programmed to count each sample repetitively at 30 minute-intervals.

5. Measure luminescence as the output of the chemiluminescence channel of the scintillation counter in terms of light units.
6. To obtain values of light units per cells, remove the sample from the vial, dilute in 2216 broth and spread on 2216 agar containing 10 μg/ml of tetracycline. After over night incubation at 30^{o}C, count the number of colonies and calculate the average light unit per cell by dividing the number of light units obtained for the sample by the number of CFU on the agar multiplied by the dilution factor.
7. A microtiter dish luminometer may be used in place of a scintillation counter and the relative light units recorded accordingly.

Transposon mutagenesis of *P. mirabilis*

Mini-Tn*5* Cm mutagenesis in *P. mirabilis*

The genetic regulation and signal transduction events that control swarming cell differentiation and multicellular swarming behavior of *P. mirabilis* has been investigated in our laboratory by transposon mutagenesis. Tn*5* has proven to be very efficient in generating random mutagenesis in a variety of gram negative bacteria and, unlike mini-Mu, does not require temperature induction or multiple phage systems for delivery and insertion. We have successfully utilized a suicide vector developed by de Lorenzo, et al[19] to generate random insertions in *P. mirabilis* chromosome[20]. This vector is based on pUT plasmid carrying a mini-Tn*5* cassette and a chloramphenicol resistance gene (pUT/mini-Tn*5*/Cm). Plasmid pUT

[19] V. d. Lorenzo, M. Herrero, U. Jakubzik ,and K. N. Timmis, *J Bacteriol.***172**, 6568 (1990).
[20] R. Belas, D. Erskine ,and D. Flaherty, *J Bacteriol.***173**, 6289 (1991).

is a suicide vector derived from pGP704[21] and carries the π protein-dependent origin of replication from plasmid R6K[22]. Only bacteria that carry the *pir* gene encoding this protein can maintain an R6K plasmid. These strains include *E. coli* SM10 (λ*pir*) and S17-1 (λ*pir*) which are both capable of conjugal transfer of *mob*-containing vectors, such as pGP704. The successful insertion and maintenance of mini-Tn*5* into the chromosome DNA is achieved following conjugal transfer when the plasmid fails to replicate and is attacked by host restriction-modification mechanisms. At that point, the Tn*5* transposase gene carried on the pUT plasmid facilitates transposition of the mini-Tn*5* into the target chromosome. The following protocol is used in our laboratory to mutagenize *P. mirabilis* BB2000 (rifampin-resistant) using mini-Tn*5*.

1. Grow donor *E. coli* S17-1 (λ*pir*) cells harboring pUT/mini-Tn*5*-CM over night at 37^{o}C in L broth containing 40 μg chloramphenicol per ml.
2. Grow the recipient *P. mirabilis* BB2000 over night in L broth containing 100 μg rifampin per ml.
3. Place a sterile cellulose membrane filter (diameter, 45 mm; pore size, 0.2 μm) on the surface of LSW^{-} agar (10 g of tryptone, 5 g of yeast extract, 5 ml of ultrapure glycerol, 0.4 g of NaCl, and 20 g of agar per liter). Spot 100 μl of donor cells (ca. 4.5 X 10^{8} bacteria) and 200 ml of recipient bacteria (ca. 1.0 X 10^{9} cells) onto the membrane. LSW^{-} agar is used to prevent the phenotypic expression of swarming motility.
4. Let the culture fluid adsorb and then incubate the membrane at 37^{o}C over night.

[21] V. L. Miller and J. J. Mekalanos, *J Bacteriol.***170**, 2575 (1988).
[22] R. Kolter, M. Inuzuka ,and D. R. Helinski, *Cell.***15**, 1199 (1978).

5. Remove the filter from the agar surface and suspend the bacteria by vortexing in 1 ml of phosphate-buffered saline (20 mM sodium phosphate (pH 7.5), 100 mM NaCl).
6. Spread 100 µl on LSW$^-$ agar supplemented with rifampin and chloramphenicol and incubate at 37^{o}C for 18 to 36 hours.
7. Transfer the *P. mirabilis* Cm^R conjugants to a master plate for further analysis.

Motility and differentiation analysis

The swarming cell differentiation and multicellular behavior of *P. mirabilis* mutants generated by mini-Tn*5* mutagenesis may be examined as follow. In all tests L agar is used after thorough drying at 42^{o}C to provide uniform conditions for swarming assays.

1. Replica plate *P. mirabilis* colonies from the master plate to L agar and incubate at 37^{o}C for 4 to 6 hours.
2. Score the potential nonswarming mutants and transfer them to a submaster plate.
3. Repeat the swarming analysis until no colonies exhibit swarming after 6 hours and designate them as Swr$^-$.
4. Test the Swr$^-$ mutants for swimming behavior on Mot semisolid agar (10 g of tryptone, 5 g of NaCl, and 3.5 g of agar per liter). Colonies that fail to move outward from the center of the Mot agar, after over night incubation at 37^{o}C, are swimming mutants and are designated Swm$^-$.
5. Grow the Swm$^-$ mutants in Mot broth to a cell density of 10^7 to 10^8 cells per ml and examine them under a light microscope. Colonies that swim in the broth but fail to move outward on semisolid agar are chemotaxis mutants and are designated Che$^-$.

6. The remaining colonies that fail to swim in broth or move on semisolid agar are defective in producing flagella (Fla^-) or defective in flagellar rotation (Mot^-). The Fla^- strains can be separated from Mot^- strains by Western immunoblot analysis using anti flagellin antisera.

Examination of Swr^- mutants

1. Inoculate an over night culture of *P. mirabilis* mutant on fresh L agar containing chloramphenicol and incubate at 37°C for 6 hours.
2. Remove a loophole of cells from the edge of the colony, place in 1 ml of PBS and vortex briefly.
3. Remove a sample immediately and examine by phase-contrast light microscopy. If more than 10% of the population of a strain display an average length of at least 20 μm (the length of 10 vegetative swimmer cells) it is considered to be wild type in elongation phenotype (Elo^+). Constitutive elongation mutants (Elo^c) can be detected if they display Elo^+ phenotype after growth in noninducing conditions (growth in L broth with shaking for 6 hours at 37°C).

Construction of fusion strains for study of multicellular-dependant gene expression

Experiments monitoring lateral flagellar gene expression have indicated that these genes are tightly coordinated with swarmer cell differentiation. For example, gene fusion experiments in *V. parahaemolyticus* indicates that *laf* gene expression is induced immediately after sensing of the surface and the cells require continuous contact with the surface to maintain *laf* transcription. Similarly, in *P. mirabilis* experiments with a *flaA*::*lac*Z fusion on a low copy number plasmid have indicated that *flaA* expression increase by 7.5 fold after

surface contact[23]. These experiments have also indicated that induction or increased expression of flagellar genes is essential for differentiation and initiation of biofilm development.

In *P. mirabilis* transition from swimmer cells to swarmer cells also induces the expression of a host of different factors including the IgA-dependant metalloprotease, ZapA[24]. In a study by Walker et al.[25] a *zapA::lacZ* fusion strain was used to study the pattern of ZapA expression during swarmer cell differentiation and swarming motility. These data indicated an interesting pattern of gene regulation. Although, *zapA* expression is tightly coordinated with swarmer cell differentiation, its expression is not required for differentiation and swarming motility. However, expression of *zapA* appears to be modulated by the cycles of multicellular swarming behavior. Careful analysis of β-galactosidase activity in swarming *zapA*::*lacZ* fusion strains indicates that maximum expression of *zapA* occurs at the boundary before the consolidation zone (zone of dedifferentiation) and is minimal during migration of swarmer cells. This suggests that specific genes involved in biofilm formation are expressed only at critical points during the growth of the biofilm.

In the following protocol, we describe a second approach to construct a transcriptional fusion between chromosomal gene, in this case *zapA,* and promoterless reporter gene, i.e., *lacZ.* This alternative method does not use random transposon mutagenesis which may be a "plus" under certain circumstances, but does require that the target gene has been cloned and partially characterized.

[23] R. Belas and D. Flaherty, *Gene.***148**, 33 (1994).
[24] C. Wassif, D. Cheek ,and R. Belas, *J Bacteriol.***177**, 5790 (1995).

Construction of *zapA'*::*lacZ*::'*zapA* transcriptional fusion strain

The *zapA* gene was cloned from an 8.6 kb insertion of P. mirabilis chromosomal DNA on the recombinant plasmid pCW101 [24] using polymerase chain reaction (PCR), The oligonucleotide primers used in this amplification incorporated engineered *Kpn*I restriction sites at their 5' end. The resulting amplicon, containing the *zapA* ribosome binding site and terminator, was cloned into the *kpn*I site of pBluescript II SK+ (Promega) to produce pKW305. To construct a transcriptional fusion between *zapA* and *lacZ*:

1. Digest pKW305 with *Bgl*II, which cuts the *zapA* but not the plasmid vector.
2. Ligate the digested pKW305 to the 4.5 kb *Bam*HI *lacZ*-Kan^R transcriptional fusion cassette from plasmid pLZK83[26] to generate pKW314 which carries the *zapA*'::*lacZ*-Kan^R::'*zapA* cassette.
3. Digest pKW314 with *Kpn*I to remove the *zapA*'::*lacZ*-Kan^R::'*zapA* fragment and ligate the fragment to the *Kpn*I-digested suicide vector pGP704 (see above) to generate the mutator plasmid pKW350.
4. Transform *E. coli* SM10 λ*pir* with pKW350 and then mobilize the fusion cassette from *E. coli* to *P. mirabilis* BB2000 by filter conjugation as described above.
5. Select the mutants on LSW$^-$ agar with rifampicin, tetracycline and kanamycin. Select the representative strains that display β-galactosidase activity (Lac$^+$) on LB gar containing Xgal.
6. Check the mutant strains for loss of protease activity by streaking on skimmed milk agar (15 g protease peptone, 10 g skimmed milk powder and 15 g agar per liter) and incubating

[25] K. E. Walker, S. Moghaddame-Jafari, C. V. Lockatell, D. Johnson ,and R. Belas, *Mol Microbiol.***32**, 825 (1999).
[26] G. J. Barcak, M. S. Chandler, R. J. Redfield ,and J. F. Tomb, *Methods Enzymol.***204**, 321 (1991).

over night at 37^{o}C. A positive reaction consists of a zone of clearing of the agar around the protease positive colonies.

7. Screen the protease-negative colonies for the ability to degrade IgA by incubating the concentrated supernatant from *P. mirabilis* mutants with human IgA for 13 hours at 37^{o}C. The presence or absence of IgA-degrading protease activity can be detected by subjecting the incubation mixture to 12.5% SDS-PAGE.

Concluding remarks

A great deal remains to be learned about molecular machinery of surface sensing and multicellular swarming behavior leading to the development of a biofilm. We have described many of the methods that have been successfully employed in addressing these questions. Transposon mutagenesis is a powerful tool to generate mutants that are defective in surface sensing, swarming and biofilm formation. Moreover, when coupled with transcriptional fusion to a promoterless reporter gene, transposon insertion into target genes can provide valuable information about the signals required for surface-induced gene expression. One possible limitation in using transposon-reporter gene construct for gene expression studies is the large size of the reporter component. *Lac* genes in mini-Mu *lac* and *lux* genes in mini-Mu *lux* add substantially to the size of the transposon. Integration of these transposons usually leads to insertional inactivation of the target gene and polar effects on downstream genes in an operon. If the functional loss of the target gene is deleterious to the organism, then mutations in this gene will not be detected. This is a limitation of any study in which random mutagenesis is used to identify important genetic elements.

A different reporter system recently developed is a variant of Green Fluorescent Protein (GFPuv)[27] from *Aequorea victoria*, which has been used as a reporter gene in bacteria. Fluorescence production by GFP is species-independent and does not require any substrate, cofactor or other auxiliary genes. Furthermore, it has been possible to construct an N- or C-terminal fusion protein with the target gene while retaining the reporter activity of the GFP

[27] A. Crameri, E. A. Whitehorn, E. Tate ,and W. P. Stemmer, *Nat Biotechnol.***14**, 315 (1996).

and function of the target gene[28, 29, 30]. Bacterial colonies expressing GFPuv can be detected on agar using a UV transilluminator and quantitation of promoter activity of the target gene can be measured using a fluorometer in a 96-well microtiter plate format. This system thus has the advantage of the transposon-generated transcriptional fusions, yet retains the activity of the target gene and lacks polar effects. This and other innovations will certainly further our knowledge of the genes and signals involved in surface sensing and swarmer cell behavior that go into the formation of a swarming biofilm.

[28] J. Flach, M. Bossie, J. Vogel, A. Corbett, T. Jinks, D. A. Willins ,and P. A. Silver, *Mol Cell Biol*.**14**, 8399 (1994).
[29] S. Wang and T. Hazelrigg, *Nature*.**369**, 400 (1994).
[30] T. Stearns, *Curr Biol*.**5**, 262 (1995).