Friend or Foe: Analyzing Biofilm Behavior and the Role of Exopolysaccharide in Bacterial Interactions

by Ryan Keane and Dr. James Berleman
Saint Mary’s College of California Biology Department

**Background**

Bacteria, whether we appreciate it or not, live everywhere from the dirt we walk on to the lining of our colon. In nature, bacteria often favor the formation of microbial communities and biofilms to increase their chance of survival (Hanson *et al.*, 2012). These biofilms are also capable of competing with other communities of microbes. Biofilm structure and behavior is particularly important to the field of medicine as they are more resistant to antibiotic treatment, and are often opportunistic pathogens. These communities can either consist of a single species, such as *Pseudomonas aeruginosa* films that form in the lungs of patients suffering from cystic fibrosis, or multiple species, such as the thousands of bacteria that reside in our large intestine, including *E. coli*. Understanding the structural and behavioral characteristics of these microbial populations is key in understanding how to promote beneficial microbial communities.

The two bacterial species of focus in this study are *Myxococcus xanthus* and *Bacillus subtilis*, two common soil bacteria particularly adept at biofilm formation. They are model species for studying biofilm behavior and structure. *M. xanthus* has recently been shown to be a predatory bacterium that “hunts” in “wolf packs,” releasing antibiotics and digestive enzymes to feed on other bacterial species such as *E. coli* (Berleman *et al.*, 2009). Bacillus species are also known to produce antibiotics, such as Bacitracin, and commonly outcompete other soil microbes in routine bacterial isolations (Vlamakis *et al.*, 2013). In addition, both organisms are amenable to genetic studies to determine the molecular components responsible for competitive and predatory behaviors (Berleman *et al.*, 2011). These include the FrzS pathway that regulates exopolysaccharide secretion in *M. xanthus* (Berleman *et al.*, 2011) and the Spo0A
regulatory pathway in *B. subtilis* that regulates the *eps* operon (Vlamakis *et al.*, 2013).

In this study, single species colonies of *B. subtilis* and *M. xanthus*, as well as interactions between the two species, were analyzed with fluorescent dyes that can differentially label live and dead bacteria (Molecular Probes), with the aim of attaining qualitative and quantitative measures of cell death during interspecies interactions. To do this, we prepared competition assays and appropriate controls analyzing 4 strains of each species. **Figure 1** shows a complete list of *M. xanthus* and *B. subtilis* strains used in this study. In addition to wildtypes of both species, two *B subtilis* *eps*- strains and one *M. xanthus* *eps*- strain were analyzed. *B subtilis* 662 is an *eps*- mutant missing a critical component in the Spo0A pathway, a regulatory pathway for the expression of extracellular matrix components including exopolysaccharide, and is thus unable to synthesize EPS. *M. xanthus* *eps*- and *B. subtilis* 76 are both unable to secrete EPS. *M. xanthus* HPO5 is a predation deficient strain unable to kill other Gram negative bacteria.

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**Figure 1:** Table of *M. xanthus* and *B. subtilis* wildtypes and mutants. *M. xanthus* and *B. subtilis* mutants used in this study are listed and described above. Wild types and *eps*- mutants were used for both species.

A secondary focus of this study was the correlation between *M. xanthus* predation and exopolysaccharide secretion. As mentioned above, *M. xanthus* is a primarily predatory species that lyases and digest bacterial species for nutrients. In this
study, *M. xanthus* wildtypes and mutants were diluted down and colonized on top of *E. coli* *dh5α* wildtype colonies to test the formation of *M. xanthus* fruiting bodies, a sign of digested *E. coli*, at varying dilution levels. Preliminary work was performed in this study, and will be followed by a future study focusing on bacterial resistance in *E. coli*.

**Materials and Methods**

**Competition Assays**

From a single colony, each species of *M. xanthus* and *B. subtilis* was cultured in 3mL of Casitone-Yeast Extract (CYE) broth at 32°C. *M. xanthus* was cultured for 48 hours, and *B. subtilis* for 24 hours. 1.5mL of each liquid culture was spun down at 8,000 rpm for 5 minutes, and resuspended in 250µL of sterile water. 2 µL of *B. subtilis* and *M. xanthus* were plated onto CYE agar approximately 1mm from each other. These competition assays then were incubated for 48 hours at 32°C, and observed and photographed.

**Fluorescent Analysis of Mono and Co-Cultures**

In order to quantify the levels of cell life and death in both single and multi-species bacterial communities, the "BacLight Live/Dead" stain from Molecular Probes was used to fluorescently distinguish between live and dead cells. The "Live/Dead" stain is a two-component mixture of two nucleic acid stains: SYTOX 9 (green) and propidium iodide (red). The green stain is able to penetrate the membrane of both live and dead bacteria and cause the interior of the cell to fluoresce green. The red stain is only able to pass through looser, damaged bacterial membranes, a quality prevalent only in dead bacterial cells. From a single competition assay, three samples were taken using an inoculating loop: one from the interaction zone, and two from the edges of both colonies. These samples were suspended in MgSO₄/MOPS buffer, incubated at room temperature, washed, and then stained with the Live/Dead stain. Each sample was analyzed under a fluorescent microscope, and three photographs were taken of the same area at 40x: one in phase contrast, one in green fluorescence (for live bacteria), and one for red fluorescence (for dead bacteria). These three images were then overlapped using ImageJ, and live, dead, and unstained cells were manually counted (n= ~ 200) (**Figure 2**).
Analysis of M. xanthus Plaque Formation

The four M. xanthus strains (Figure 1) were serially diluted from 0 to 10⁹ fold and incubated on top of colonies of E. coli for five days. Plaque formation was recorded daily, and the experiment repeated three times.

Wild type DZ2, wild isolate spsm1, eps- mutant, and predation deficient HPO5 mutant were cultured for 48 hours in 3mL of CYE culture at 32°C. E. coli was cultured in CYE at 32°C for 24 hours. 1.5mL of each culture was washed and resuspended in ~250µL of sterile water.

Concentrated E. coli was inoculated in 10µL aliquots on CF minimal media, and allowed to air dry at room temperature for approximately 10 – 20 minutes. All four M.
xanthus strains were serially diluted. Wild isolate spsm1 was serially diluted from ten to a billion fold in powers of ten (ten total dilution steps). Wild type DZ2 and eps- mutant were diluted down to $10^7$ fold, and predation deficient HPO5 mutant was diluted down to $10^5$ fold.

For each dilution factor of the M. xanthus strains, 10µL was plated on top of the dried E. coli. The plates were allowed to air dry, and incubated over 5 days at 32°C. The plates were analyzed and photographed each day, and plaque formation was quantified using plaque forming units (PFUs).

Each dilution of the M. xanthus mutants was also plated alone of CYE plates to measure colony formation. Each plate was incubated for five days at 32°C, and colony formation was recorded in colony forming units (CFUs) and photographed every day.

Results

The data presented in the Mono Culture Analysis and both Co-Culture Analysis sections is based on one data set; time constraints prevented this experiment from being repeated for precision. Thus, this is preliminary data that in the future will be followed up by a more accurate technique and repeated multiple times for consistency.

Mono Culture Analysis

Single species colonies of all M. xanthus and B. subtilis mutants were fluorescently stained and analyzed as controls for this study. Surprisingly, large differences were observed in the viability of strains under conditions thought to be most amenable to growth: high nutrient availability with no interspecies competition. Significant differences were observed, particularly with the EPS – mutants. The B. subtilis wild type, 3610, had the lowest cell death at 13%, and the extracellular enzyme deficient mutant 215 had the second lowest level of cell death at 17%. The two EPS- mutants, 76 and 662, had the highest cell death at 32% and 48%. This experiment was repeated once more, but there was high variability between the two data sets. For instance, while there was 13% cell death in the 3610 wildtype in the first data set (Figure 3A), there was only 3% cell death in the second culture of 3610 (data not shown). While there was, on average, an 18% difference in the quantity of cell death between the two data sets, the eps- mutants both exhibited approximately 3.5 times the level of cell death as the wild type.
The four *M. xanthus* types displayed a similar trend. Just as with *B. subtilis*, the wild types displayed lower levels of cell death than the eps- mutants. Only 0.48% of the *DZ2* wild type colony was dead in the first data set. The predation-deficient mutant *HPO5*, which is unable to kill other Gram-negative bacteria, had 1.77% cell death. The wild isolate *spsm1* colony had 9.71% cell death, and the eps- mutant had the highest level of cell death at 19.62% (Figure 3K). This assay was also repeated once more. Between the two data sets, the *DZ2* cultures had a 4% difference, and the eps- cultures had a 9% difference in cell death (data not shown). While high variability was observed with *M. xanthus*, the eps- mutant had more cell death than the wildtype.

*M. xanthus*, *B. subtilis*, and *E. coli* wild type mono-cultures grown on CF minimal media were also analyzed for cell viability. *M. xanthus DZ2* had 9.94% cell death, over a 9% increase compared to rich media colonies. Conversely, *B. subtilis* experienced a large decrease in cell death, from 13.00% to 3.05%. In minimal media, *B. subtilis* was also observed to sporulate, which may explain the decrease in cell death compared to rich media (Figure 4B & 4C). In comparison, *E. coli* had 5.99% cell death (Figure 4B).
**Co-Culture Analysis in Rich Media**

*M. xanthus* and *B. subtilis* were incubated side-by-side in competing co-cultures on rich media, by adding 3 µL of each culture ~2 mm apart on a CYE agar plate. The wildtype of one species was grown in competition with four strains of the other species. *M. xanthus* wildtype DZ2 was grown side-by-side with *B. subtilis* wildtype 3610, as well as mutants 215, 662, and 76. This assay allowed us to observe what happens as the two organisms encounter competition. Figure 6F-I shows the result of 48 h growth at the colony level. Both species claim territory, but under theses conditions, *B. subtilis* is much more proficient in colonization than any of the *M. xanthus* strains. Closer analysis reveals that while a sharp divide arises in the interaction zone between two wild types, the mutants 76 and 662 have a fuzzy interaction zone, where cells of each organism blend together. Wildtype *B. subtilis* 3610 was also put in competition with *M. xanthus* strains DZ2, wild isolate spsm1, and mutants HPO5 and eps-. *B. subtilis* again exhibited a higher proficiency for colony formation, compared to *M. xanthus*.

For fluorescent analysis, samples were taken from three regions: the far away edges of the two bacterial species to act as secondary controls, and one from the...
interaction zone, containing both bacterial species.

**Colony Edges**

Samples were taken from the colony edges to serve as controls for interacting species. Very low cell death occurred in the edge of the 3610 colony grown with the eps- and HPO5 *M. xanthus* mutants (1.33% and 3.09%, respectively), while higher cell death occurred in the colonies grown next to *M. xanthus* wildtype DZ2 (9.38%) and the *M. xanthus* wild isolate spsm1; 9.38% and 12.81%, respectively (*Figure 5A*).

When the *M. xanthus* wildtype DZ2 was pitted against the *B. subtilis* types, the lowest cell death occurred in the DZ2 colony competing against the two eps- mutants, although the DZ2 colony facing *B. subtilis* 76 had a much lower level of cell death (1.72%) than the DZ2 colony facing *B. subtilis* 662 (15.38%), even though both are eps-mutants. The DZ2 colony grown next to the *B. subtilis* wildtype had the second highest

![Cell Viability of B. subtilis Against M. xanthus DZ2](image)

![Cell Viability of M. xanthus Against B. subtilis 3610](image)

*Figure 5: Cell Viability in Interacting Wildtype Colonies.* Samples were taken from the far edges (away from interaction zone) of wildtype colonies competing with various mutants, including eps-. The controls from *Figure 3* were included for comparison purposes. A shows the viability of *B. subtilis* wildtype 3610 against *M. xanthus* types, and B shows the viability of *M. xanthus* wildtype DZ2 against *B. subtilis* types. Both graphs show that the lowest cell death occurred in “wildtype vs. eps-” interactions with the exception of the 3610 control in A.
Figure 6: Cell Viability in the Interaction Zones Between B. subtilis Wild Type 3610 and M. xanthus. Wildyptes of M. xanthus were pitted against B. subtilis strains (A-D), and the B. subtilis wildtype was pitted against the M. xanthus strains (F-I) for eight total interactions. Samples were taken from the interaction zone and fluorescently stained to identify live and dead bacteria, and cells were counted as live or dead. In the M. xanthus wildtype vs. Bacillus set, the eps-/-wildtype had the lowest level of cell death at 1.60% (E). In the Bacillus wildtype vs. M. xanthus set, the eps-/-wildtype interaction had the second and third highest levels of cell death (16.42% and 14.66%) (J).
level of cell death at 35.19%, and the highest cell death occurred in the DZ2 culture fighting B. subtilis 215 (Figure 5B).

**Interaction Zones**

In the interaction between B. subtilis wildtype and M. xanthus wildtypes and mutants, 22.29% cell death was observed in the interaction zone between the M. xanthus wild isolate spsm1 and the B. subtilis wildtype, the highest among the four interactions. In the wildtype vs. wildtype (DZ2 vs. 3610) interaction, 9.44% cell death was observed. The interaction zone between predation deficient HPO5 M. xanthus mutant and B. subtilis 3610 had 7.76% cell death, and the eps- vs. 3610 zone had the lowest level of cell death at 1.60% (Figure 6E).

Between M. xanthus wildtype DZ2 and the B. subtilis wildtype and mutants, the interaction between B. subtilis 215 (the “eps ++” mutant) and M. xanthus DZ2 had the highest cell death: 21.28%. The interactions between DZ2 and the two eps- B. subtilis strains 76 and 662 had the next highest levels of death at 16.42% and 14.66%, respectively. The “wildtype vs. wildtype” interaction, in this set of interactions, had the lowest level of cell death at 9.04% (Figure 6J).

**Co-Culture Analysis in Minimal Media**

Wildtype co-cultures were also grown on CF minimal media to analyze bacterial interactions in low nutrient media. M. xanthus DZ2 wildtype was grown side-by-side with B. subtilis 3610 and E. coli wildtype.

When B. subtilis and M. xanthus interacted, B. subtilis sporulation occurred, although the B. subtilis spores still took up the fluorescent stain. Only 3.48% of the B. subtilis spores in the control colony were dead. The M. xanthus colony edge had 2.14% dead cells. The interaction zone showed only 1.77% dead cells. (Figure 7).

When M. xanthus DZ2 was grown in proximity to a colony of E. coli, the M. xanthus colony swept over the E. coli colony, causing the E. coli cells to lyse. M. xanthus fruiting bodies formed at sites of E. coli lysis and digestion (Berleman 2009, et al.) Only 2.29% of the E. coli cells were dead in the colony edge; in the M. xanthus colony edge, 20.0% of the cells were dead. In the interaction zone, 25.50% of the cells were dead (Figure 7).
Analysis of M. xanthus Plaque and Colony Formation

Colonies of four M. xanthus mutants were grown at various ten-fold dilutions on both rich media and on top of E. coli colonies on minimal media.

The four mutants were: DZ2, the laboratory raised wildtype; spsm1, a wild isolate of M. xanthus; HPO5, a mutant unable to kill Gram negative bacteria, and an eps-mutant. After five days, spsm1 was able to form 1 x 10^2 colony forming units (CFU). eps- was able to form 1 x 10^3 CFU, and HPO5 was able to form 2 x 10^4 CFU. The lab wild type DZ2 had the highest CFU number at 2 x 10^5 CFU (Figure 8).

A different trend was observed for plaque formation. While spsm1 struggled to
form colonies past a 100-fold dilution, it had $1 \times 10^7$ plaque forming units (PFU). DZ2 had $2 \times 10^5$ PFU, the second highest and the same as its CFU number. eps- reached $1 \times 10^3$ PFU, and HPO5 had $1 \times 10^2$ PFU, the lowest PFU number (Figure 9).

**Figure 8: M. xanthus Colony Formation on CYE agar.** Four *M. xanthus* mutants were serially diluted in ten fold steps from 0 to $10^7$ fold dilutions and plated on CYE agar for five days (Figure 6A-E). Figure shows the growth of an spsm1 colony at a 100 fold dilution over five days. The number of individual colonies is multiplied by the dilution factor to obtain the colony forming unit (CFU). Figure 6F tracks the CFU progress of all four *M. xanthus* mutants over five days. DZ2 had the highest CFU at $2 \times 10^5$; HPO5 had the next highest at $2 \times 10^4$. eps- and spsm1 had the two lowest CFU values at $1 \times 10^3$ and $1 \times 10^2$ CFU, respectively.
Exopolysaccharide Plays a Role in Both Colonization and Predation

The fluorescent analysis of the mono cultures of *M. xanthus* and *B. subtilis* show that cells that are unable to secrete exopolysaccharide (like *M. xanthus* eps- and *B. subtilis* 76) or synthesize EPS (like *B. subtilis* 662) have higher rates of cell death throughout the colony. Since EPS is critical for biofilm structure, this confirms that *B. subtilis* and *M. xanthus* fare far better in a biofilm community than individually.

Interestingly, when the mono-culture analysis was repeated, the quantity of cell death...
was far different, but the trend persisted: eps- mutants fared worse that wildtypes. This indicates that there is high variability in biofilm viability among communities of the same strain.

The same trend does not hold true in the interaction zone analysis. When *M. xanthus* eps- was grown next to *B. subtilis* wildtype, only 1.60% cell death was observed in the interaction zone. This was one of the lowest percentages of cell death throughout the entire study. However, when the two eps- *B. subtilis* mutants were pitted against the *M. xanthus* wildtype, high cell death (approximately 15% in both cases) was observed. Even more puzzling, the interaction zone between the “eps++” *B. subtilis* mutant and *M. xanthus* wildtype had an even higher level of cell death (21.28%). A possible explanation for this observation is that the eps ++ mutant was unable to avoid contact with *M. xanthus*, which could trigger *M. xanthus* predation that the *Bacillus* strain was unable to defend itself against.

These inconsistencies can be somewhat explained by the technique used in the fluorescent analysis and the properties of *M. xanthus* and *B. subtilis* colonies. Live and dead cells were visually counted from microscope photographs, and while live and dead cells were well distinguished with the stain, it was nearly impossible to safely discern between *M. xanthus* and *B. subtilis* cells. They are both rod-like, and their shape and size can be highly variable (*Figure 2*). This means that the percentages of cell death in the interaction zones include both *M. xanthus* and *B. subtilis* cells, and are not inherently equal in proportion. *B. subtilis* grows nearly twice as fast as *M. xanthus*, and thus the colony grows to a far larger size than the *M. xanthus* colony in 24 hours, and was far easier to remove from the agar plate for the fluorescent assay than *M. xanthus*. Thus, it is highly probable that the majority of the cells in the interaction zone are *B. subtilis* cells since they are easier to remove from the agar plate. This helps explain why cell death is so low in the samples between *M. xanthus* eps- and *B. subtilis* wildtype, but is much higher in the *B. subtilis* eps- and *M. xanthus* wildtype interactions: the *M. xanthus* eps- colony is unable, because of its lack of EPS, to perform the same predatory or defensive functions as wild colonies, and was unable to successful attack and harm the advancing *B. subtilis* colony. Exopolysaccharide may also play an important role in *B. subtilis* defense, which would explain the high levels of cell death.
between \textit{eps-} \textit{B. subtilis} and \textit{M. xanthus} wildtype. Ultimately, the current staining technique we employed in this study is unable to conclusively answer our original questions, but has instead provided us with a preliminary theory to pursue with a more accurate technique.

Originally, it was suspected that the low level of cell death in the interaction zones between \textit{M. xanthus} and \textit{B. subtilis} was due to rich media, and thus abundant nutrients, allowing both species to co-exist. The minimal media interaction between \textit{M. xanthus} and \textit{B. subtilis} was performed with the intent of forcing the two species, particularly \textit{M. xanthus}, to have to compete for food, or use their neighbors as a nutrient source. However, perhaps as a response to the low nutrient, \textit{B. subtilis} sporulated, protecting it from any possible attack from the \textit{M. xanthus} colony. In turn, \textit{M. xanthus} cells may have either died from starvation or been forced to turn on their neighbors for nutrients, a quality occasionally observed in \textit{M. xanthus} colonies (Berleman \textit{et al.}, 2009).

\textbf{Exopolysaccharide Is Utilized Differently for Colonization and Predation}

For colony formation, the \textit{M. xanthus} mutants were ranked from strongest to weakest based on their CFUs; for plaque formation, the rank was based on PFUs (\textbf{Figure 10}).

In every trial, \textit{spsm1} was unable to form colonies beyond a 100 fold dilution, but was consistently able to form plaques at the lowest dilution for all four mutants, $10^7$ fold. In fact, \textit{spsm1} didn’t even form truly individual colonies; cells only thrived in larger, denser communities. However, the ability of the wild isolate and lab wild type to form plaques after being highly diluted demonstrates that in its natural environment, \textit{M. xanthus} both prefers to stay in large packs, but is able to hunt individually if forced. For both \textit{eps-} and \textit{DZ2}, their PFU and CFUs were equal, unlike \textit{HPO5} and \textit{spsm1}. \textit{HPO5}, since it was unable to kill Gram negative bacteria like \textit{E. coli}, was far more adept at forming colonies than plaques, and was able to form colonies at dilutions than both wildtypes and the \textit{eps-} mutant. This shows that while \textit{M. xanthus} is an accomplished bacterial predator, it is also able to live in smaller communities if it is forced to do so, as long as it has a alternate nutrient source. The fact that \textit{eps-} formed the same number of CFU as PFU, but less units than wildtype \textit{DZ2}, indicates that exopolysaccharide is not
only key for colony formation, but may also play a role in predation. Why DZ2 formed
the same number of CFU as PFU, but the wild isolate did not, is unclear, although the
fact that that the spsm1 strain is the wild isolate while the DZ2 strain is the “laboratory
wildtype” may be a factor.

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**Figure 10: M. xanthus ranking for colony and plaque formation.** M. xanthus colony formation was measured in colony forming units (CFU). The rank of M. xanthus types’ colony forming ability, from highest to lowest, is: wildtype DZ2, predation deficient HPO5, eps- mutant, and lastly, wild isolate spsm1. M. xanthus’ predation abilities were based upon each types’ ability to form plaque forming units (PFU). Wild isolate spsm1 had the highest PFU level at 1x10^7. The laboratory wildtype DZ2 had the second highest number of PFU, eps- the third, and the predation deficient mutant HPO5 had the lowest level.

**Conclusions**
- While the Live/Dead stain did not provide as accurate as results as we originally believed it would, it does indicate that exopolysaccharide plays a key role in biofilm behavior and survival, although possibly through different mechanisms for *M. xanthus* and *B. subtilis*. A lack of exopolysaccharide secretion seems to inhibit predation capabilities in *M. xanthus* and defensive capabilities in *B. subtilis*. 


• *M. xanthus*, in its natural environment, prefers to stay together in its large “wolf pack” formation but is more than capable of performing its predatory functions in smaller colonies. These experiments doubly confirm that exopolysaccharide plays a role in *M. xanthus* predation.

**Future Work**

**A More Accurate Staining Technique**

Since *M. xanthus* and *B. subtilis* cannot be visually separated from each other in the interaction zone, a new staining technique will be needed to provide a clearer picture of cell viability in the interaction zone. A new staining technique called the “ViaGram Red+ Bacterial Gram Stain and Viability Kit,” developed by Molecular Probes, fluorescently labels both live and dead cells, as well as Gram positive bacteria. Fortunately, while *B. subtilis* is Gram positive, *M. xanthus* is Gram negative, so this stain can be used to compare the level of cell death of *B. subtilis* to the level of cell death of *M. xanthus* in the interaction zone. This technique will be used on the eight interactions shown in this study, starting with the “wildtype vs. wildtype” interaction.

**Can Bacteria Become Resistant to Other Bacteria?**

The colony/plaque formation data was able to give us a clear ranking of *M. xanthus’* predation abilities, which will aid us in our future study of *E. coli’s* ability to form resistance to other bacteria, primarily *M. xanthus*.

**Acknowledgments**

We would like to thank the Saint Mary’s College of California Biology Department and Summer Research Program, the Auer Labs at Lawrence Berkeley National Laboratories, and Mal and Sylvia Boyce.

**References**


