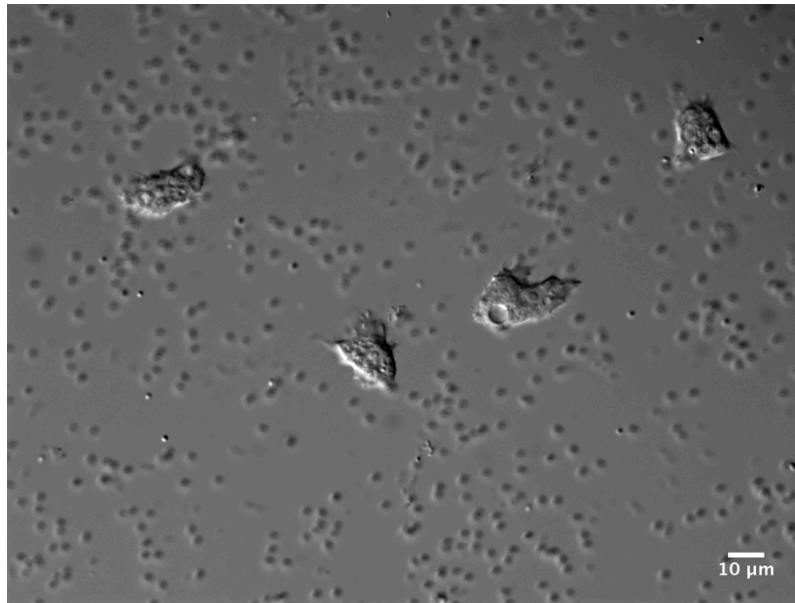


Predator-Prey Interactions Between an Amoeba and a Bacterium from Cedar Swamp



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Abstract:

Protozoa-bacteria interactions in the environment represent a dynamic evolutionary system in which each is constantly adapting to the other. These adaptations can allow bacteria to escape predation, or allow protozoa to find preferred prey or avoid pathogenic prey. This study attempted to characterize the interaction between an amoeba and bacterium enriched from Cedar Swamp to look for any directional movement or lysis in response to compounds secreted in a biofilm.

Introduction:

Consumption by protozoa is a major source of bacterial mortality, especially for non-motile bacterial communities, such as biofilms, where bacteria cannot easily escape. There are several adaptations that can improve bacterial survival under the selective pressure of predation. Quorum-sensing-dependent upregulation of toxins in dense bacterial communities can kill protozoans. For example, conserved virulence factors like rhamnolipids and ExoU in *Pseudomonas aeruginosa* biofilms have been shown to kill the amoeba *Dictyostelium discoideum*. *Salmonella enterica* also shows expression of the *rfb* virulence locus under predation. *Pseudomonas fluorescens* produces lipopeptides that are induced upon grazing by *Naegleria americana*¹. Predation-mediated variation in virulence genes indicates that protozoan grazing may have, in part, driven the development of bacterial pathogenicity. Many of the targets of these toxins are universal eukaryotic features found in human cells as well². Bacteria can also adapt to become intracellularly pathogenic once ingested. The soil nematode *Caenorhabditis elegans* exhibits lawn avoidance of the highly pathogenic *Serratia marcescens*. The main cue is the biosurfactant serrawettin W2 that is sensed by two AWB chemosensory neurons. Actually, *B. subtilis*-produced surfactin also induced lawn avoidance even though *B. subtilis* is not a *C. elegans* pathogen, likely because of the structural similarity between these lipopeptide surfactants. On the other hand, *C. elegans* shows variation in its response to different *B. thuringiensis* isolates from around the world, illustrating that host-pathogen interactions are highly dynamic over ecological differences³. *C. elegans* are adapted to recognize and choose their food sources carefully out of the variety of soil bacteria they encounter⁴. In their pressure for developing careful feeding selection in a diverse microbial world, nematodes and protozoans are similar. However, the mechanisms of protozoan feeding selection are not well understood since most studies focus on how grazing affects the distribution of bacterial taxa present. For example, it is known that gram-negative bacteria generally take less time to digest than gram-positive bacteria, but it is unknown how exactly digestion of prey may regulate protozoan prey selection⁵. One way to study prey selection is to look at chemotaxis either by attraction or avoidance to a given prey.

Chemotaxis is essential for survival, and gradient sensing involves many interconnected signaling networks that allow for directional sensing and polarization. Chemoattractant receptors activate heterotrimeric G proteins that regulate chemotaxis⁶. When amoebae are treated with a microtubule inhibitor, nocodazole, the pseudopod dynamics are unaffected, but amoebae are no longer able to perform chemotaxis. Microtubules allow for pseudopod selection in the appropriate direction during the reorientation process. Chemoattractants may signal to microtubules to preferentially stabilize pseudopods⁷. While most work has focused on chemoattractants, I am interested if there exists a mechanism for avoidance based on chemosensory cues similar to that in *C. elegans*.

Many studies on protozoa-bacteria interactions focus on easily culturable taxa for both predator and prey, and there is likely much more diversity in these interactions than what we understand. I isolated an endospore-former from Cedar Swamp that could be pathogenic, or release toxins as a chemical defense that kill or induce avoidance in an amoeba also isolated from Cedar Swamp. Endospore-forming gram-positive bacteria are also likely more difficult to digest, which may also induce avoidance based on feeding preferences. Chemorepellents have not been extensively studied in protozoans, although they are known to exhibit chemoattraction and selectively feed. On the other hand, there

could be biofilm-associated compounds that act as canonical chemoattractants such as cAMP and folic acid. In my project, I wanted to investigate how the amoebae responds to biofilm-associated compounds of the endospore-forming bacterium.

Methods:

Media composition:

5YE: 5g/L yeast extract

5YE-MES: 5g/L yeast extract, 5mM MES pH 6.15

5YE plates: 5g/L yeast extract, 15g/L agar

1x Freshwater Base (FWB): 17.1mM NaCl, 1.97mM MgCl₂·6H₂O, 0.68mM CaCl₂·2H₂O, 6.71mM KCl)

Amoeba isolation medium (AIM) agar: 1x FWB, 5mM MES pH 6.15, 0.5g/L yeast extract, 15g/L agar

Amoeba growth medium: 0.1g/L yeast extract in 1x FWB

Isolation and culture:

Unidentified amoeba was enriched from Cedar Swamp using an unidentified non-motile endospore-forming prey bacterium also from Cedar Swamp by plating serial dilutions of the environmental sample in overnight-grown liquid culture of the prey in 5YE-MES medium on amoeba isolation medium agar plates. Plaques were observed after about one week. Agar plugs of the plaques were picked using a P1000 pipette and transferred to petri dishes with amoeba growth medium. Amoebae were maintained by exchanging medium every 3-4 days. Pellicle-forming bacterium was also enriched from Cedar Swamp in the Endospore-forming *Bacillus*-related enrichment. Approximately 1g soil was pasteurized in 4.5ml sterile 1x FWB buffered with 5mM MOPS, pH 7.2 at 86°C for 30 minutes before plating serial dilutions on 5YE plates. One of the purified isolates was grown overnight in 5YE medium shaking at 37°C before incubating at room temperature without shaking for 2-4 days until a visible pellicle film appeared at the air-liquid interface.

Microscopy:

All imaging was done on the inverted Zeiss Axio Observer using 35mm MatTek coverslip bottom dishes and ibidi μ -Slide VI 0.4 6-well slides for the chemotaxis assay.

Amoebae membranes were visualized using thermofisher FM4-64 lipophilic dye at 5 μ g/ml concentration diluted in 2ml 1x FWB and added to MatTek dishes with seeded amoebae to be imaged immediately.

Chemotaxis assay:

The bottom of the petri dish was scraped with a cell scraper to resuspend amoeba into 2ml solution. 30 μ l of cell suspension was seeded into each well of the ibidi μ -Slide VI 0.4 6-well slide (well length is 17mm). Pellicle was collected using a P1000 pipet to touch the pellicle and attach some of the biofilm to the pipet tip, which was resuspended in 100 μ l of 1x FWB and vortexed for 1 minute. The suspension was centrifuged at 6000rpm for 5min. 2 hours after seeding, the ibidi slide was placed on the stage of the microscope, and 30 μ l of pellicle extracted supernatant was added to the first three wells of the ibidi slide to one reservoir, and 15 μ l was aspirated from the opposite reservoir to move pellicle extract into the

channel to create a gradient. At approximately 15 minutes, 1 hour, 2 hours and 4 hours, time lapse images were taken every 5 seconds over 5 minutes at the pellicle extract side of the gradient, and also at the control side for 1 hour, 2 hours, and 4 hours, all for the 3 pellicle gradient channels. Cells were also counted for individual frames at 10x magnification along the gradient for all 6 wells at 0 hours and 6 hours after gradient formation.

Image analysis:

Chemotaxis assay movies (over only 1 minute) were analyzed using the Fiji plug-in Quimp⁸, which was developed at the University of Warwick. Track data on segmented amoeba cells was transferred to MATLAB, where x- and y- coordinate data was centered to start at the origin before plotting as line tracks.

Sequencing and clone library:

I extracted genomic DNA using the Zymo Quick-DNA Microprep Plus Kit and amplified the 18S rRNA gene using PCR with 360F and 1391R primers. I ligated 3 μ l PCR product into the Promega pGEM-T easy vector according to the manufacturer's instructions. 2 μ l of the ligation reaction was transformed into 50 μ l JM109 competent *E. coli* cells, which were grown at 37°C shaking for 1 hour before plating on LB+100ug/ml ampicillin. Unfortunately, the colony PCR using T7 and M13 vector primers was not successful in amplifying an insert of the expected size from the initial 18S rRNA PCR (~1500bp).

Results:

The amoeba was isolated from Cedar Swamp using endospore-forming prey bacteria also from Cedar Swamp. Spores are seen in the amoeba in vacuoles, and are exocytosed periodically, indicating they cannot be digested. The indigestion of the spores does not seem to deter the amoeba from feeding on endospore-forming bacteria when it is the dominant prey available.

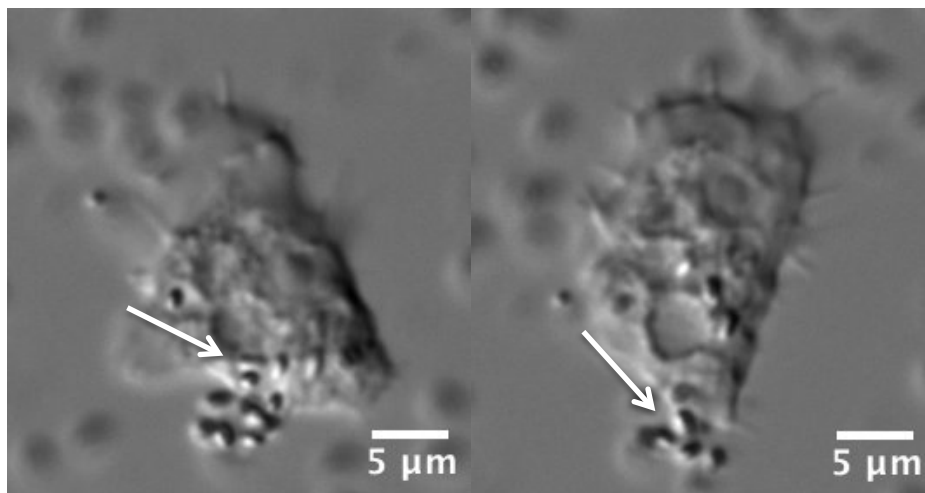


Figure 1: Spore exocytosis from back of amoeba (shown with arrows). Right image is 25 seconds after left image.

The amoeba has clear pseudopodia, which are seen to extend and retract all around the membrane as the cell moves directionally by selection and maintenance of some extensions over other (Figure 2). There is also often a tail-like collection of pseudopodia at the back of the amoeba.

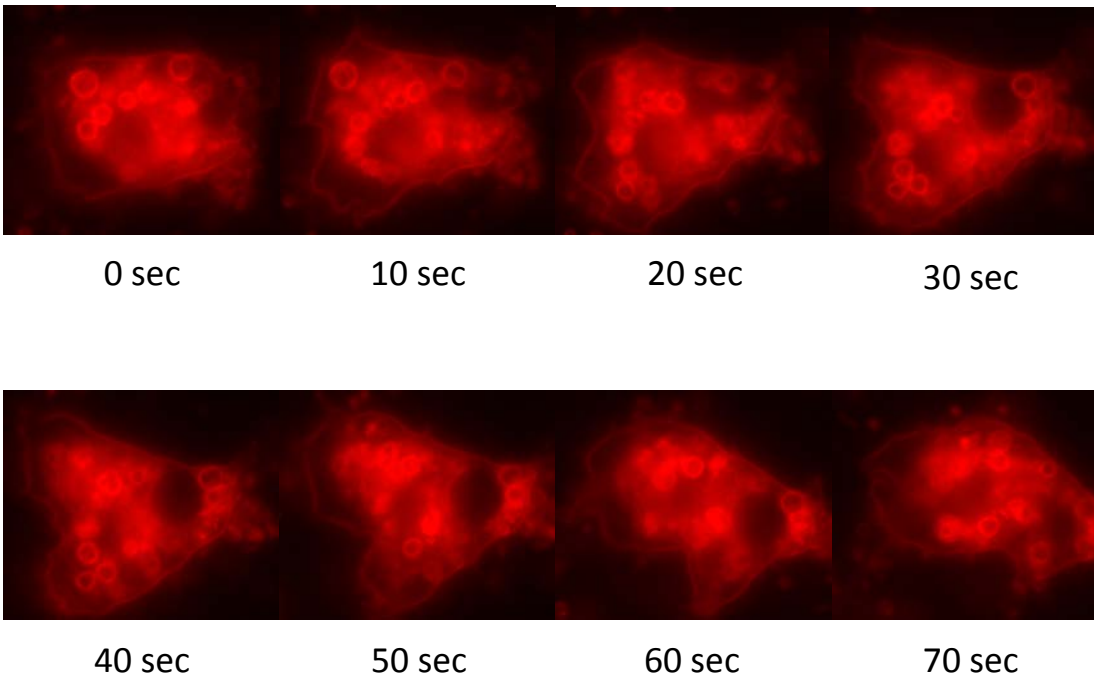


Figure 2: Time lapse over 70 seconds of FM4-64 membrane-dyed amoeba that shows directional motility through selection of one extension over the other. Note: dye is also visible in endocytic vesicles inside amoeba.

Another endospore-former was enriched from Cedar Swamp with an interesting colony morphology (umbonate shape with mucoid center and fimbrous periphery), shown in Figure 3 along with the cell morphology on the left. There is also pellicle biofilm formation after a few days in liquid culture (Figure 3). One way to look for lipopeptide surfactant production is a drop collapse assay. When some of the pellicle biofilm extract (cell-free) is dropped onto parafilm, the drop collapses more than water or 5YE medium (Figure 3). This indicates there may be a lipopeptide or other surfactant molecule that is associated with biofilm formation. Such molecules have been shown to have broad toxic effects, be necessary for biofilm formation and swarming, and induce avoidance in *C. elegans*. I was interested to see if the amoebae would also exhibit avoidance or experience toxic effects and lysis. I expected there might also be other biofilm-associated compounds that act as chemoattractants so that the amoebae can find dense bacterial areas for grazing.

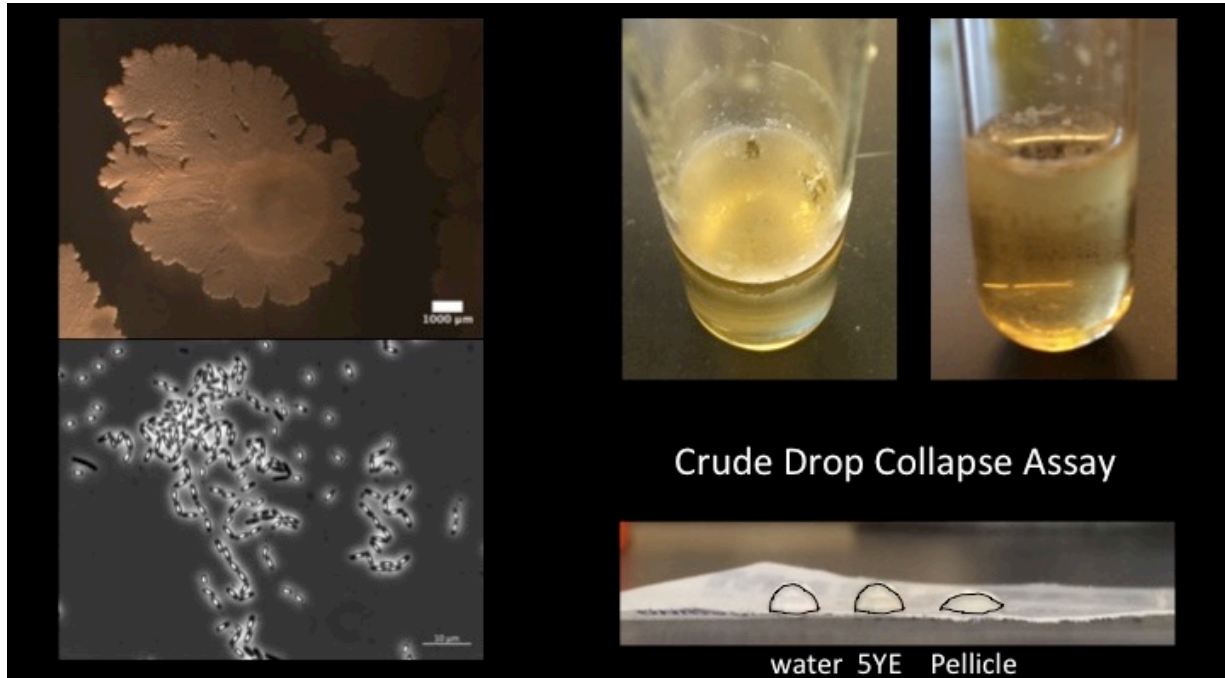


Figure 3: Top left: colony morphology, Bottom left: cell morphology, Top right: pellicle biofilm formation from top and side, Bottom right: drop collapse assay on parafilm where drops have been outlined by hand to better visualize shape difference.

To look into these different possibilities, I used the 6-channel ibidi slide to seed amoeba and then set up 3 gradients with extract from the pellicle, as well as 3 control wells. I counted cells along the gradient at 0 and 6 hours, and the average differences in these counts are shown in Figure 4.

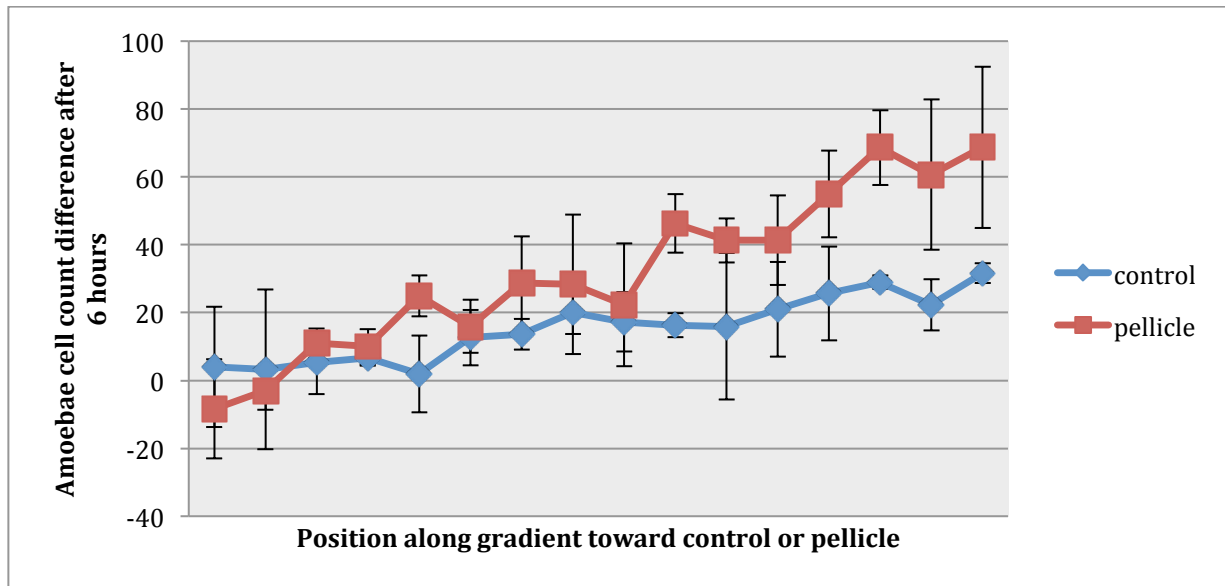


Figure 4: Cell counts of individual frames at 10x magnification along the gradient where the extract was added at the right side. Amoebae were also seeded on the right side 2 hours previously. Counts 6 hours after gradient formation are normalized to starting counts with standard deviation error bars across three replicates. Differences between control and pellicle are not statistically significant.

There appears to be an increase in the cell count for both the pellicle gradient and control on the right side. This is likely the result of seeding the amoeba on that side, as there may have been more amoeba that attached in the reservoir rather than flowing into the channel, and these migrated into the channel over the 6-hour period. While the differences between control and pellicle gradient are not statistically significant, the trend of the pellicle gradient having more amoebae was consistent across the replicates. They do not seem to be moving from the opposite side of the channel since there is roughly no change in count on that side. Another hypothesis is that more amoebae moved from the reservoir into the channel with the pellicle extract added. Movement in this direction could be driven by avoidance, but there is insufficient supporting data. The fact that counts do not seem to decrease in the pellicle extract channel indicates little to no toxicity of any pellicle-associated compounds.

I then analyzed videos of amoeba movement at each side of the channel to look for movement in predominantly one direction, either away from or towards the pellicle extract.

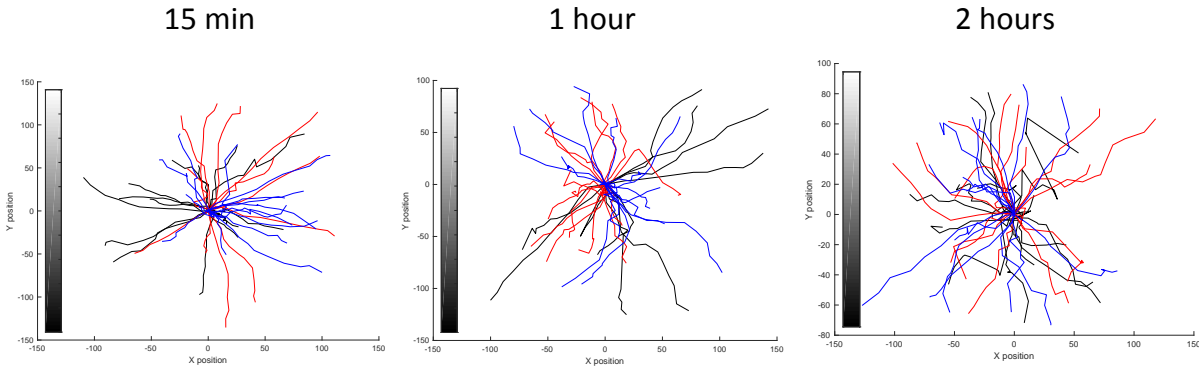


Figure 5: Centered cell tracks of amoebae over 1 minute at the end of the gradient where the pellicle extract was added (gradient shown along y-axis based on extract being added below) 15 minutes, 1 hour, and 2 hours after addition. The three colors correspond to different biological replicates in different wells. Data generated using Quimp.

At 15 minutes, 1 hour, and 2 hours after gradient formation, the amoebae near the pellicle extract seem to be moving in all directions as opposed to predominantly in one direction (Figure 5). There is no indication of any avoidance or chemotaxis.

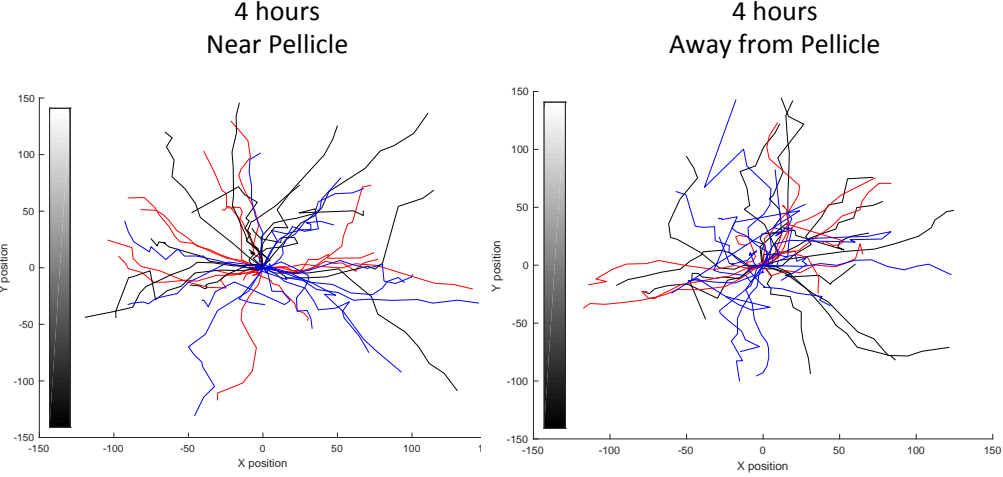


Figure 6: Cell tracks of amoebae over 1 minute at both ends of the gradient (gradient shown along y-axis based on extract being added below) 4 hours after addition. The three colors correspond to different biological replicates in different wells. Data generated using Quimp.

At 4 hours, the amoeba seem to be generally moving more away from than towards the pellicle extract at the pellicle side of the channel. This is not apparent in the amoebae on the side father away from the pellicle extract. While this may be a result of avoidance, the cell tracks are too short and well-distributed to strongly support this conclusion. It is also unclear how avoidance would occur only at 4 hours and not before, especially since the

channels are not made for chemotaxis assays, and therefore likely do not maintain the gradient that long. Further study is required to characterize the response, if there is one.

Discussion:

The main finding of this study is that the amoebae do not seem to experience toxicity or move one way or another in the early time points. While there may be an interaction here based on later time points and distribution of amoebae after 6 hours, more experiments would be needed to reach any conclusion on a potential avoidance response.

First, I would want to add a known chemoattractant to the slide, and maybe use a different ibidi slide that has additional channels for gradient maintenance. I would also grow the biofilm in the presence of amoeba since this signal is often required to induce toxin expression in the prey. It would be interesting to test the pathogenicity of the bacterium, which would mean there is selective pressure for adaptation of an avoidance response. Even if there is no pathogenicity, I would look closer at feeding preferences against other bacteria (possibly with a fluorescent marker to distinguish between prey) since these prey have endospores and more peptidoglycan, and may be avoided when other, more easily digestible prey are available at the same level.

This study has shown that for analysis of amoeba movement, the Quimp plugin for Fiji is valuable. To look more at chemotaxis, I would like to go deeper into the analysis on pseudopod extension and selection in response a more stable gradient. I would also add microtubule-inhibiting drugs like nocodazole to see if chemotaxis is then inhibited with the same stimulus.

Finally, I think it is important to continue to follow up on environmental isolates and characterize their relationships to expand our understanding of protozoa-bacteria interactions and adaptations.

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Appendix – Videos

<https://youtu.be/YTZiSVQ8TQU>

<https://youtu.be/HX5cExICLTM>

<https://youtu.be/DcSy7nx8qpQ>

<https://youtu.be/LXpbtlvLar0>

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