PROJECT PROPOSAL OPEN ACCESS

The role of stathmin microtubule-destabilizing activity in *Shigella flexneri* motility and tunneling

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SUMMARY

Shigellosis, an infection by *Shigella* bacteria, causes many harmful and potentially dangerous symptoms. *Shigella* enter cells that line the human intestinal tract and travel through the cytosol. This environment contains long, thick obstacles called microtubules that form a dense network and provide structural support to the cell. In order to clear a path and tunnel through, *Shigella* use microtubule-destroying proteins. It is possible that the host protein stathmin may be involved in this process, since it is known to destabilize microtubules. This proposal outlines three experiments to determine stathmin's role in tunneling, each involving the infection and comparison of normal and stathmin-lacking hosts. The experiments examine tunnel widths, microtubule densities and bacterial movement patterns in each strain. It is expected that microtubule destabilization and movement will be impaired when stathmin is absent. Since antibiotic resistance in *Shigella* is becoming more common and stathmin may be crucial for movement and subsequent spreading, the findings of this proposed study could provide an important, new treatment target.

ABSTRACT

The infection of the intestinal mucosa by *Shigella* bacteria is a global health issue resulting in a variety of potentially life-threatening gastrointestinal complications. Their unique method of intracellular motility depends on microtubule destabilization to clear the dense host cytoskeletal network in a process called tunneling. It is hypothesized that the host protein stathmin may play a role in this process, due to its tubulin-sequestering capability. This proposal aims to provide potential methodologies to elucidate the function of stathmin with respect to *Shigella flexneri* motility. Three experiments are proposed, involving comparisons between human intestinal epithelial cell strains under varying levels of stathmin expression, each infected with *S. flexneri*. Respectively, the experiments examine tunnel widths via electron microscopy, microtubule densities via imaging fluorescence correlation spectroscopy, and bacterial movement patterns via live fluorescence microscopy. If microtubule destabilization and movement is impaired in null stathmin strains, as predicted, such findings may inform a novel therapeutic target for shigellosis by preventing internal spreading. This is particularly significant in our current landscape, as antibiotic-resistant strains of *Shigella* are growing increasingly prevalent.

Keywords: *Shigella flexneri*, stathmin, microtubule destabilization, tunneling

INTRODUCTION

Shigella encompasses several intracellular bacteria, such as *Shigella flexneri*, that invade human intestinal epithelial cells (IECs) and cause inflammation and destruction of the intestinal mucosa.[1] Resulting symptoms include stomach cramps, ulcers, bleeding, and severe diarrhea.[2] The World Health Organization estimates that approximately 190 million cases of gastroenteritis were caused by shigellosis in 2010, making this infection a very serious global health concern.[3] A unique feature of *Shigella* is their method of movement within infected cells, as they are capable of manipulating host-cell actin dynamics to form a comet tail at one pole (Figure 1).[4] Here, the polymerization of host microfilaments propels them through the cytosol and contributes to dissemination.[4]

The host cytosol contains a dense network of microtubules (MT) that provide structural support to the cell.[4] Intracellular microorganisms, such as *Shigella,* would be unable to navigate this mesh-like architecture if not for specialized proteins that prevent them from becoming trapped.[5] In a process called tunneling, MT-destabilizing proteins destroy immediate MTs and clear a path that *Shigella* subsequently travel through (Figure 2).[4]

There have been several experiments examining *Shi-*

gella motility and its ability to tunnel through cytoplasmic networks. Yoshida et al. demonstrated that *Shigella* in wild type (WT) nocodazole-treated hosts display linear movement patterns, whereas in untreated hosts, they display movement that involves occasional changes in direction and a slight zigzag pattern.[4] Due to the MT-destabilizing activity of nocodazole, the experiment signifies that MTs serve as barriers to intracellular *Shigella* motility. Furthermore, when the same procedure was repeated for null VirA bacteria, *Shigella* movement halted, as VirA is a MT destabilizer.[4] This demonstrates the necessity of tunneling and MT destabilization for adequate *S. flexneri* motility.

The bacterial protease, VirA, modulates host cell cytoarchitectural remodeling during infection.[4] Tunneling may be similarly influenced by other MT destabilizers, including the host protein stathmin, which is known to sequester free tubulin dimers needed for polymerization.[6] Additionally, stathmin is recruited to the bacterial surface during *Shigella* infection, suggesting it as a plausible contributor to tunneling.[4] In the host, the normal function of stathmin is to enhance cell differentiation, growth, and cell mobility.[7]

Listeria monocytogenes is a bacterium with a similar mechanism of infection and motility that also recruits stathmin.[8] Despite not exhibiting tunneling, MT density surrounding *L. monocytogenes* has been shown to increase in stathmin-depleted human colorectal cells.[8] In addition, these bacteria exhibited significantly impeded speed.[8] This provides evidence that stathmin destabilizes MTs and facilitates *Listeria* movement through the cytoskeletal matrix, but whether it is involved in the same process and tunneling during *S. flexneri* infection requires elucidation.

An estimated 164 000 fatalities, associated with shigellosis, are recorded annually.[9] Treatment of bacterial infections rely on antibiotics; we are also acutely aware of growing concerns associated with antimicrobial resistance (AMR). Limitations in treatment exist due to the increasing prevalence of multi-drug resistant strains of *Shigella*.[10] Alternative therapies may be required if AMR renders standard treatments inefficacious. Characterization of stathmin's influence on *S. flexneri* infection may provide an alternative treatment for shigellosis. If expressional differences in stathmin influence bacterial motility and follow-up experimentation provides evidence of associated implications in pathogenesis, stathmin may provide a novel therapeutic target for shigellosis. Specifically, modulation of stathmin activity intracellularly may preclude efficient motility, which is necessary for the dissemination of *S. flexneri* within tissues and the internal spread of infection.[1]

We propose three experiments below to study stathmin's role in tunneling. Each examines factors related to MT destabilization and *S. flexneri* motility under differing levels of stathmin expression. Experiments one and two analyze tunnel widths and local MT density, respectively, to establish stathmin's role in MT clearance and destabilization. Experiment three investigates movement patterns at large, to identify whether stathmin facilitates movement through MT obstacles.

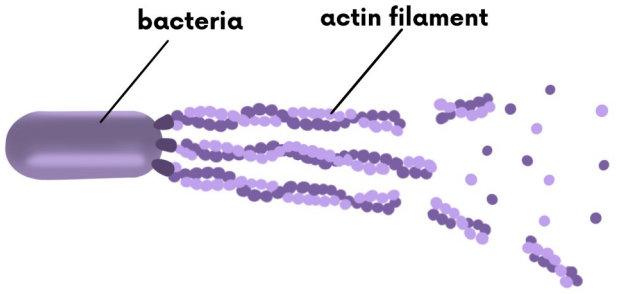

Figure 1. The *Shigella* comet tail. During infection, host actin monomers are recruited and polymerize at one pole of *Shigella*. Movement is facilitated by polymerizing actin filaments that apply a force, propelling the bacterium forward. The collection of trailing filaments is known as the comet tail.

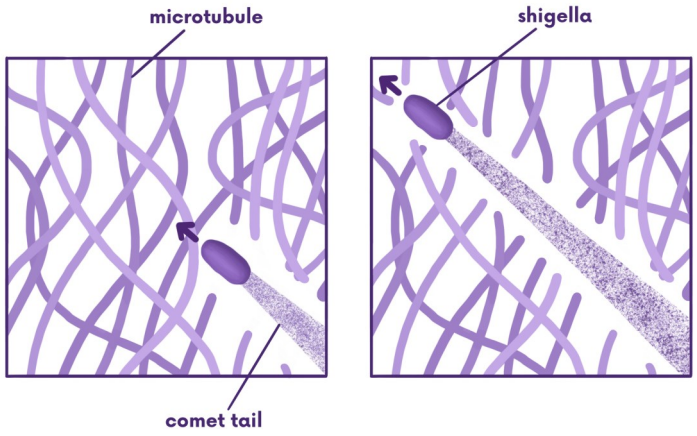

Figure 2. *Shigella* tunnelling through the host MT matrix. Pictured is *Shigella*'s destabilization of the host's dense network of MTs in order to move through the cytosol. MT-destabilizing proteins are used to destroy nearby microtubules and clear a path. This process is referred to as tunnelling.

RESEARCH QUESTION

Does stathmin play a role in microtubule destabilization and tunneling during *S. flexneri* infection?

HYPOTHESIS

Stathmin contributes to microtubule destabilization and tunnel formation during *S. flexneri* infection.

EXPERIMENT 1

Prediction:

Narrower tunnels will form in null stathmin human IECs infected with *S. flexneri*.

Stathmin's influence on MT stability and its broader role in the modulation of host-cell cytoskeletal dynamics is understood; however, its influence on *Shigella* tunneling requires further study. The first method that will be used involves measuring tunnel widths in infected cells under differing levels of stathmin expression, *in vitro*. We predict that narrower tunnels will form in cultured null stathmin human IECs infected with *S. flexneri*.

Three groups of human IECs will be cultured and infected with *S. flexneri*— one control group and two experimental groups. The control group will consist of cultured WT human IECs. The first experimental group will consist of cultured human IEC stathmin null mutants and the second will consist of mutants with a stathmin gene duplication. Gene duplication will represent upregulated stathmin levels. After infection, cultures will be incubated in a sufficient medium at 37°C for five hours, prior to assessment.

After the incubation period, a JEOL 1200EX TEM-SCAN electron microscope will be used to capture images of the cultures. Ten images of each group will be captured during three successive intervals, 20 minutes apart. During each of these intervals, images of a microscopic ruler will be captured at the same magnification to be used to calibrate the image analysis software's measurement tools. Post-image analysis will make use of the software ImageJ to measure the average tunnel widths. ImageJ will be calibrated for each set of images. Using the ten images of each group, collected during each interval, the tunnels' widths will be measured. The average tunnel widths will be calculated for each group during each interval. Comparisons will be made between each of the groups' respective average tunnel widths for each interval and analyzed for statistical significance using Prism GraphPad software. We will make use of a two-tailed Student's t-test between each group and calibrate significance with a p -value less than 0.05.

It is expected that statistically significant differences in average tunnel widths will be observed between all three groups. The ascending order of expected average tunnel widths is: null, WT, duplication. Stathmin destabilizes MTs, therefore, human IEC stathmin null mutants should exhibit narrower tunnels (i.e., less clearance) than cultured cells with a stathmin gene duplication and WT. Conversely, increased expression of a MT destabilizer in the gene duplication group should exhibit wider tunnels (i.e., more clearance) than cultured cells with a stathmin null mutation and WT.

This is a reasonable expectation given previous research involving *S. flexneri* and *L. monocytogenes*. Yoshida et al. demonstrated that the presence of MT destabilizers — VirA and nocodazole — resulted in the clearance of MTs and VirA-implicated tunnel widths in *S. flexneri*-infected cells.[4] Stathmin, with MT-destabilizing properties as well, was theorized to exhibit similar effects. Furthermore, Costa et al. demonstrated that stathmin's sequestration of tubulin leads to depolymerization of MTs surrounding *L. monocytogenes*.[8] Therefore, increased stathmin expression is expected to further destabilize bacterial-surrounding MTs and form wider tunnels.

EXPERIMENT 2

Prediction:

Null stathmin human IECs will have a higher density of MTs near *S. flexneri* during infection, when compared to WT cells.

Stathmin contributes to MT destabilization during *Listeria* infection; however, whether stathmin plays the same role during *Shigella* infection is not well studied. Therefore, examining the MT density near *Shigella* under varying levels of stathmin expression is a valid next step. Higher densities would signify impaired MT -destabilization and implicate stathmin's role in this process. The same control and experimental groups will be used as in experiment one; however this time, all IECs will additionally contain a tubulin-GFP fusion gene construct for MT visualization.

For visualization of the bacteria, direct immunofluorescence will be performed. Each group will be infected with *Shigella* bacteria and treated with a rhodamine-tagged antibody for an O-antigen (O-Ag), a component of lipopolysaccharides found exclusively in gram-negative bacteria such as *Shigella*.[11] All host cultures infected with *Shigella* will be incubated at 37°C for five hours before assessment.

After preparation of the experimental groups, the density of MTs will be measured via imaging fluorescence correlation spectroscopy (ICS), using a Stellaris 5 confocal microscope. A focused laser beam will excite fluorescent molecules near the bacteria and the microscope will capture emitted photons, generating a two-dimensional image to represent the fluctuation of fluorescence intensity.[12] Autocorrelation software will then be used to calculate the MT density from these fluctua-

tions. Statistical analyses to determine significance in the results would be conducted using Prism GraphPad software. A two-tailed Student's t-test and a p-value of less than 0.05 would indicate significance.

If stathmin is involved in MT destabilization during infection, then cells with stathmin will contain a lower density of MTs near *Shigella*, providing evidence for its role in tunneling. It is expected that experimental group two, mutants with stathmin gene duplications, would have the lowest density of MTs. Comparatively, experimental group one, which lacks stathmin, would be expected to have the highest density. The WT control is expected to have a value in between experimental group one and two.

EXPERIMENT 3

Prediction:

In untreated null stathmin IECs, *S. flexneri* will exhibit jagged movement patterns, with more frequent changes in direction compared to the WT.

Dysfunctional movement in null stathmin hosts would provide additional evidence for the hypothesis. Yoshida et al. examined *Shigella* motility in WT hosts and discovered a relatively linear movement pattern upon nocodazole treatment.[4] However, in untreated hosts, there were occasional changes in direction. Since nocodazole is a MT-destabilizer, these findings signify that MTs behave as obstacles and that collisions result in deflections. If stathmin does in fact behave as a MT-destabilizer like nocodazole, then stathmin's absence will result in a zigzag, non-linear movement pattern as *Shigella* collide with MTs more often. This would also demonstrate that stathmin plays a role in tunneling.

The preceding experiments have the objective of elucidating the function of stathmin with respect to MT destabilization during *S. flexneri* infection. However, demonstrating stathmin's activity in this way is not sufficient to determine its implications on motility at large. Therefore, experiment three's examination of *Shigella* movement patterns is necessary. Furthermore, stathmin's overall potential as a therapeutic target may be better understood if motility is truly impaired in null strains, since adequate mobility is required for dissemination and the spread of infection within tissues.[1]

This experiment will include two strains of cultured human IECs to serve as hosts, one of which will be a WT and the other a null stathmin mutant. Additionally, *S. flexneri,* an antibody against an O-Ag tagged with rhodamine, nocodazole, and the Zeiss Axio Imager Z1 fluorescent microscope will be required. Controls will include nocodazole-treated and untreated WT hosts and the experimental groups will consist of nocodazole -treated and untreated null stathmin strains (Figure 3). Nocodazole is a MT-destabilizing drug that significantly reduces the number and density of MTs. Wild-type IECs treated with nocodazole are a control, since previous studies have shown that *Shigella* movement is linear under these conditions from a lack of MT barriers.[4] Null stathmin IECs treated with nocodazole must be compared to the equivalent WT treatment in order to verify that movement patterns are only different due to differences in MT destabilization. This would be evident if no significant differences are observed between the two, as neither will have MT obstacles. Independent variables will include the presence of stathmin and nocodazole. The dependent variable will be the average frequency of direction changes for the *Shigella* in each group.

Throughout the experiment, all incubations and video recordings will occur at 37°C. Initially, a culture of WT and null stathmin IECs will each be treated with a nocodazole solution and incubated for ten hours. This will be followed by the infection of all groups with *S. flexneri* and an additional incubation of five hours. Next, each group will be treated with rhodamine-anti-O-Ag and incubated for a period of 120 minutes. This will be done with the objective of visualizing individual *Shigella* via direct immunofluorescence. Lastly, live *Shigella* motility will be captured and recorded using the Zeiss Axio Imager Z1 fluorescent microscope for 120 minutes under 2500x magnification.

Frequencies of *S. flexneri* direction changes for each group will be recorded using the Manual Tracking ImageJ plug-in, which allows for path tracing and the measurement of angles. Direction changes are to be classified as deviations from the bacterium's course by a minimum of 15° under 2500x magnification. This is to only account for large changes due to collisions with obstacles and ignoring minor random changes in motion. Each direction-change frequency will then be tested for statistical significance using Prism GraphPad software and the two-tailed Student's *t*-test. A p-value smaller than 0.05 will be considered statistically significant.

Due to the MT destabilizing activity of nocodazole, it is expected that both treated groups will have few MTs acting as obstacles. Therefore, they will likely yield linear *S. flexneri* movement and infrequent changes in direction, regardless of whether stathmin is present or not. This would confirm that differences in movement in the other groups are solely due to stathmin's effect on tunneling since movement patterns are not influenced by other factors aside from MT destabilization. The untreated WT control is expected to have slightly more frequent direction changes compared to the nocodazole groups as seen in the study by Yoshida et al.,

but still remain relatively linear due to the presence of stathmin.[4] The untreated null stathmin mutants are expected to have an impaired ability to degrade MTs, thus presenting *Shigella* with zigzag movement patterns and significantly more frequent direction changes compared to all other groups. Movement is not expected to halt completely because *Shigella* are still able to take advantage of other MT-destabilizers, such as the secreted VirA.[4]

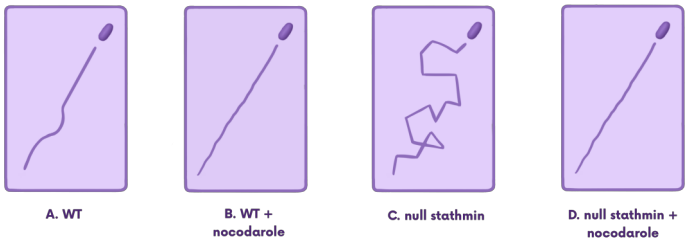

Figure 3. Predicted *S. flexneri* movement patterns for the experiment three groups. (A) WT *Shigella* are expected to exhibit relatively linear movement, with occasional random changes in direction. (B) WT *Shigella* in hosts treated with nocodazole are expected to display linear movement due to the lack of MT barriers. (C) Null stathmin *Shigella* is expected to display a zigzag movement pattern due to impaired MT-destabilization. (D) Linear movement is expected for null stathmin *Shigella* in nocodazole-treated hosts due to the lack of MTs.

LIMITATIONS

In the first experiment, quantification of tunnel widths is associated with a certain level of uncertainty. ImageJ allows users to manually scale its measurement tools using a calibration image. Once calibrated, the user can record measurements of captured images using the scaled measurement tools. The accuracy of calibration and measurements are reliant on the precision of the user's input; therefore, recorded tunnel widths may be inaccurate and implicate experimental findings. Similarly, in the third experiment, the Manual Tracking ImageJ plug-in also requires manual input to measure the angles of *Shigella* movement. Recorded measurements are associated with a given uncertainty and will need to be accounted for during statistical analysis.

In the second experiment, a limitation of ICS includes limited temporal resolution because measurements of density are taken sequentially at each pixel.[13] Autocorrelation analyses are also limited as they are not extracted with high accuracy in *in vitro* cultures.[13] To prevent these potential issues, additional fluorescence correlation spectroscopy methods, such as raster image correlation spectroscopy and single plane illumination microscopy, may be used to validate any findings.

In the third experiment, quantifying direction changes with a minimum deviation angle poses a potential limitation, as this method assumes that collisions with MTs always result in large deflections. A number of smaller diversions from MTs still behaving as barriers to movement may be missed and slightly obscure validity. This is, however, a necessary sacrifice to discount random deviations, as *S. flexneri* seldom travel in a perfectly straight line.[14]

Stathmin has also been found to indirectly contribute to comet tail integrity and movement by activating cofilin.[8] Cofilin is an actin-severing protein that prevents unnecessary growth of actin filaments in the comet tail.[8,15] It has been demonstrated that *Listeria* in null stathmin host cells move slower and have longer comet tails than the WT, due to inactive cofilin.[8] This may be a possible limitation of the study, as it is not known whether a difference in speed will also be present in *Shigella* infection or if it will affect movement patterns, tunneling, or MT destabilization. Regarding movement, similarities in motility between the two nocodazole-treated groups in the third experiment would demonstrate that this is not a significant concern. It is still unclear, however, whether speed might affect the other two processes in a way that cannot be accounted for.

Lastly, as with all *in vitro* experiments, the results of this study cannot be readily extrapolated within the human body, where many additional elements are at play.

CONCLUSION

There are several gaps in our current understanding of *S. flexneri*'s interactions with host cell machinery and the proposed experiments will address these areas. It has been shown that similar to VirA — a bacterial protease — host proteins may influence *S. flexneri* motility and promote pathogenesis. Interactions between stathmin and *L. monocytogenes* influence bacterial movement and dissemination.[8] Yoshida et al. have demonstrated that VirA's MT destabilizing behaviour promotes *S. flexneri* tunneling and dissemination. Therefore, we hypothesize that stathmin may function similarly.[4] Currently, stathmin's interactions with *S. flexneri* remain uncharacterized. Unlike *L. monocytogenes*, *S. flexneri* motility is reliant on tunneling — the clearance of dense MT networks; however, *Shigella* tunneling is not fully understood and requires further investigation. This study seeks to broaden our understanding of *S. flexneri* motility, host-cell interactions, and infection, at large.

With the growing concern of AMR in the treatment of shigellosis, it is imperative that alternative treatments are sought after. The findings of this proposed study can pave the way for a novel therapeutic target in the

form of stathmin, whose inhibition may result in compromised *Shigella* motility. Further research may then suggest that this impairment prevents its spread from cell to cell within tissues, thus limiting infection.

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