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RESEARCH INSIGHT

Developing a qualitative chemotaxis-sensitive assay for bacteriophage-host interactions on *E. coli* BW2511

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ABSTRACT

To better understand the interactions between bacteriophages and host bacteria, one factor to observe is chemotaxis. Chemotaxis describes the biased directional movement of bacteria either towards or away from a substance. This phenomenon is created by the inhibition or potentiation of tumbling events, which randomize the direction of the host every few seconds. The presence of chemoattractants and chemorepellents can modify the number of tumbling events that occur. In order to elucidate the role of bacteriophages in bacterial chemotaxis, this project aimed to optimize an assay to qualitatively observe chemotaxis within E. coli BW2511 (WT) in comparison to a mutant strain with cheA deletion. Three main assay methods were tested: Tris HCl plate assays, Tris HCl microcapillary assays, and TTC Eppendorf assays. Of all the methods tested, only TTC Eppendorf assays with TTC indicator consistently showed chemotaxis. This study continued to use the Eppendorf assay and determined if various bacteriophages could attract E. coli BW2511 to chemotactically move up a layer of 0.4% PBS-based agar and against gravitational pull.

BACKGROUND

With alarming reports of antimicrobial resistance and few new antibiotics on the market, there has been an increased interest in bacteriophage research over the past decade.[1] Bacteriophages, also known as phages, are viruses that can infect and destroy specific bacterial hosts, giving them potential as anti-bacterial interventions.[2] The biased movement of a bacterium towards a certain target is known as chemotaxis.[3] Experiments and techniques previously used to investigate chemotaxis have been unconvincing, with little evidence to suggest unidirectional movement. As a result, there is a need to optimize a known chemotactic assay to distinctly differentiate chemotaxis from random motility of bacteria.

Chemotaxis can be explained as a series of simple switching events between two modes of cellular movement: moving and tumbling.[3] Movement by the bacteria is encouraged by the long flagella, which whips around to create a propulsion motor.[3] In the absence of any chemical gradient, a swimming bacterium moves erratically due to the switching process of tumbling, where the bacterium briefly stops and reorients itself in any random direction before moving again.[3] If the cell is swimming towards an increasing concentration of an attractant, it will suppress the proteins responsible for tumbling, subsequently decreasing the probability of a tumbling event occurring.[3] Alternatively, if a concentration gradient of a repellent is sensed to be near, the bacterium will increase its probability of tumbling as a form of escape.[4] The net effect of this temporal sensing and regulation is the foundation for the biased movement known as chemotaxis.[3]

Within E. coli and other microorganisms, the chemotaxis pathway starts with the binding of ligands (e.g. serine, aspartate, or citrate) to transmembrane receptors.[5] Depending on the type of ligand bound (i.e. chemoattractant or chemorepellent), tumbling will be either encouraged or inhibited.[5] Ligand docking generates an intramolecular conformational change to the receptor that regulates a bound cytoplasmic histidine kinase, also known as chemotaxis protein CheA (CheA).[5] Ligand binding causes CheA to be either upregulated or downregulated, allowing it to phosphorylate itself on a side chain and initiate a signalling cascade that generates the bacterium tumbling state. If an attractant is bound, CheA phosphorylation is downregulated to limit the sudden randomization of movement; the opposite occurs for the binding of a repellent.[6]

PREMISE

In learning more about how phages interact with bacteria, this project developed an assay to clearly differentiate between motility (i.e. random, independent movement) and chemotaxis in E. coli BW2511. The development and optimization of this assay would later be used to better understand whether phages can alter bacterial chemotaxis to improve viral fitness. It was hypothesized that the lysis of a bacterium due to phage infection releases host amino acids and nutrients, which attract other bacterial hosts within the environment. This provides the phage with more opportunities for infection.

In order to determine if bacteriophages play any role in the chemotactic behaviour of bacteria, an accurate and reliable assay had to be developed to differentiate chemotaxis from random motility. Three main assay types were explored: Tris HCl plate assays, Tris HCl capillary assays, and triphenyl tetrazolium chloride (TTC) - based Eppendorf assays.

METHODS AND RESULTS

For every assay type, the bacterial strain E. coli BW2511 (WT) was used along with its non-chemotactic CheA knockout mutant (ΔCheA). In each assay, bacteria were added with either a chemoattractant or chemorepellent, and directional movement was qualitatively observed.

For plate assays, E. coli BW2511 was inoculated in 10 mL of Lysogeny Broth (LB) overnight and then pipetted onto 0.4% 10 mM Tris HCl agar plates (pH 6.8) to stop bacterial growth but allow for chemotaxis. Then, a concentration of either 10 mM, 100 mM, or 1 M of chemoattractant (glucose), repellent (leucine), or nothing (as a negative control) was added at a distance of 1.5 cm away. Plates were left overnight at 37°C and observed for chemotactic movement. Initial results suggested that chemotaxis was not detected, as the diameters of the bacterial circles illustrated between WT and ΔCheA against all conditions were nearly identical. Adjustments made to the concentration, density of agar, and distance of placement all showed no difference in bacterial movement. Glucose was then swapped with serine, which has been demonstrated to be a definitive chemoattractant.[7] It was also suspected that the ineffectiveness of the plate assays was due to the glucose and leucine being too diffuse through the 0.35% agar, causing the concentration gradient to be too weak for chemotaxis. To address this, two layers of 10 mM Tris HCl agar were used, one at a density of 1% and the other at 0.35%. Plates were poured over a period of two days per experiment, with either serine, leucine, or negative control mixed into the 1% agar prior to pouring at a slant. This design yielded no novel findings or affirmations to bacterial chemotaxis. The 1% agar layer, when poured at an angle, generated an artificial concentration gradient for bacteria to swim towards after adding a 0.35% agar overlay (Figure 1). Another set of plates were also built with dual 1% agar gradients of serine and leucine and a 0.4% agar overlay, but they were also ineffective. These plates were the final iteration of the plate assay, as these results suggested that the entire assay design was not appropriate for chemotaxis detection. In parallel with plate assay experimentation, capillary assays (Tris HCl microcapillary assays) were also attempted. Previously, the capillary assay has shown increased attraction of WT to glucose than ΔCheA with this design. The principle behind this assay was to have WT or ΔCheA move up a glass capillary filled with either a chemoattractant, chemorepellent, or Tris HCl (negative control). Using a glass capillary would create a concentration gradient the moment that the capillary came in contact with the liquid within a 96 well plate. Chemotaxis was measured by the concentration of bacteria within the capillary after 30 minutes, which was quantified via a serial dilution in Tris HCl. Serial dilutions of bacteria were pipetted onto 1% standard agar plates and incubated overnight at 37°C. Colony forming units (CFUs) were counted the next day and plotted to create a violin graph (Figure 2). The thickness of each "violin" (purple or gold) represents the degree of consensus of data at that CFU/mL. The thicker the graph, the more data from the capillary experiments was pooled at that CFU/mL value. Although capillary assays were done with comparisons of serine, leucine, and a negative control, the majority of experiments were done with high concentrations of serine to hopefully induce a greater measure of chemotaxis. Ultimately, the majority of results did not reflect any chemotactic behaviour. None of the E. coli BW2511 strains exhibited any directed motility in response to either chemoattractants or repellants. Other changes such as time of incubation and type of chemoattractant also showed inconsistent results. Similar to plate assays, capillary assays were concluded to be an unfeasible methodology for our purposes.

After failures in both plate and capillary assays, triphenyl tetrazolium chloride (TTC) assays were tested to determine if bacterial chemotaxis could be observed up an Eppendorf tube. This assay was pioneered in chemotactic research for the bacterium C. jejuni.[8] TTC is a compound that develops a pink stain in the presence of mitochondrial enzymes from the electron-transport chain,

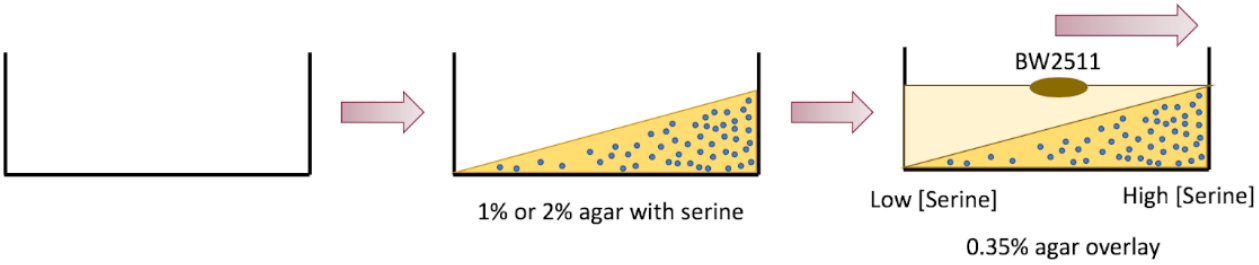

FIGURE 1: Schematic of procedure for layered agar chemotaxis assay. An artificial serine gradient is made (as an example) by allowing the 1% agar to cool at an angle prior to adding the 0.35% agar overlay.

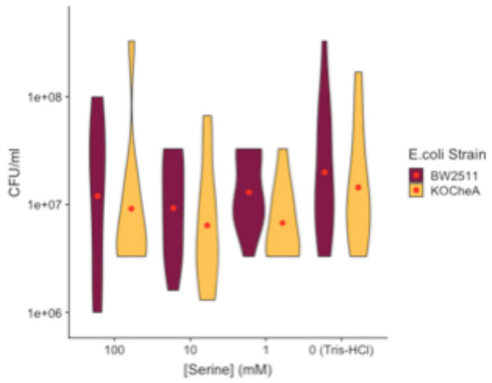

FIGURE 2: Violin graph of capillary assay data, with red dots representing mean values of CFU/mL. It can be seen that the capillary assay has insufficient resolution to distinguish between motility and chemotaxis.

and is often used as an indicator for bacteria within an environment.[9] WT and ΔcheA mutant strains were inoculated in 10 mL LB and grown overnight at 37°C. 1.5 mL of bacteria was transferred into a 2 mL Eppendorf tube and spun down at 1200 RPM to pelletize. The supernatant was decanted and the pellet was resuspended in PBS-based 0.4% agar, a variation on Tris HCl agar to limit growth but not motility. At the very top of the Eppendorf tube, a Whatman filter paper soaked in 50 µL of serine or leucine (between 10 mM and 1 M) was placed to generate a concentration gradient for bacterial chemotaxis. These Eppendorfs were incubated for two days at 37°C, with 0.01% TTC added after to develop colour.

When experimental lines were compared to multiple negative controls (no bacteria and no chemotactic substance), it was clear that the TTC assay qualitatively differentiated randomized motility from chemotaxis. In WT, the addition of serine led to purple colors near the top of the PBS-based 0.4% agar layer, and increasing concentrations of serine correlated with a darker shade of purple in the Eppendorf tube. Furthermore, trials with either the leucine repellent (10 mM to 0.14 M) or negative control were either entirely clear or purple at the bottom of the Eppendorf tube, suggesting that the bacteria demonstrated no upward directional movement. These results affirmed the functionality of the TTC assay to differentiate random motility and directional chemotaxis, giving it the potential to elucidate more details on the interactions of virulent bacteriophages on chemotaxis. However, a key limitation is that the degree to which chemotaxis has occured can be difficult to identify qualitatively, and more quantitative assay methods should be investigated.

CONCLUSION

With more attention being put toward understanding the behaviour and potential of bacteriophages in medicine, there is a rising demand to generate appropriate tools and methodologies to elucidate their complex mechanisms. Here, an assay to detect chemotaxis in E. coli is proven to be accurate and consistent, opening more possibilities to investigate bacteriophage-host interactions.

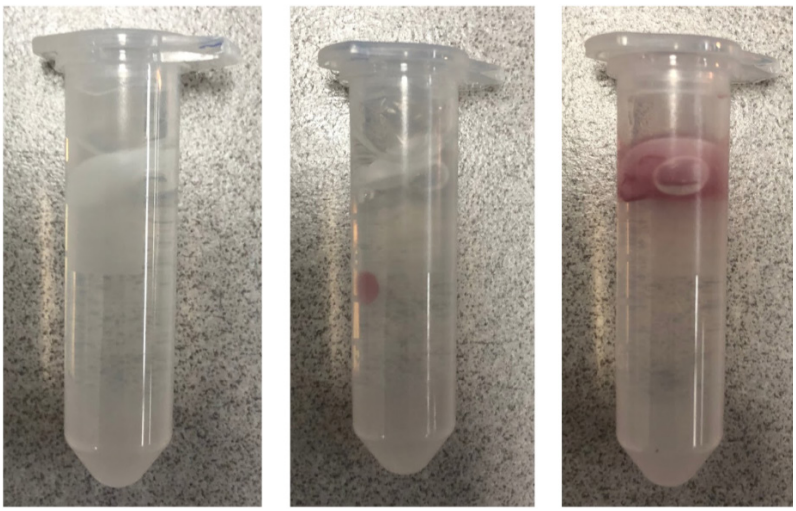

FIGURE 3: Initial results from 0.01% TTC assay on E. coli BW2511 (WT) with Whatman filter paper soaked in (from left to right) nothing, 50 mL of 0.14 M leucine, and 50 mL of 1 M serine.

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