POLYPHOSPHATE SIGNALING HINTS AT POTENTIAL THERAPEUTICS FOR TUBERCULOSIS

An Undergraduate Research Scholars Thesis

by

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This project did not require approval from the Texas A&M University Research Compliance & Biosafety office.

TABLE OF CONTENTS

ABSTRACT

Polyphosphate Signaling Hints at Potential Therapeutics for Tuberculosis

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Tuberculosis is one of the leading causes of death by any single infectious agent worldwide. The bacterium that causes tuberculosis, *Mycobacterium tuberculosis* (*Mtb*), has evolved mechanisms to bypass host defenses, even when engulfed by macrophages into phagosomes. Phagosomes normally fuse with lysosomes, and this kills ingested bacteria, but *Mtb* prevents this fusion and proliferates in macrophage phagosomes. Understanding how *Mtb* prevents phagosome-lysosome fusion and thus the killing of ingested bacteria may enable us to develop therapeutics for tuberculosis.

Dictyostelium discoideum is a eukaryotic microbe that feeds on bacteria by phagocytosis, like macrophages. We found that chains of phosphates called polyphosphate act as an extracellular signal that prevents phagosome-lysosome fusion and causes *Dictyostelium* cells and human macrophages to retain ingested bacteria alive in the phagosome, analogous to an *Mtb* infection.

We developed an assay to use the power of *Dictyostelium* genetics to characterize the proteins involved in the signal transduction pathway used by polyphosphate to prevent killing of

ingested bacteria. After allowing *Dictyostelium* cells to ingest bacteria, the number of live ingested bacteria in the presence or absence of polyphosphate were counted shortly after ingestion, and at 48 hours.

Wild-type *Dictyostelium* and 20 signal transduction pathway mutants responded to polyphosphate by decreased killing of ingested bacteria. We found three additional mutants that are insensitive to polyphosphate signaling, suggesting that the absent proteins in these mutants are required for *Dictyostelium* to sense polyphosphate. Pharmacological inhibition of any of the human homologs of these proteins might prevent macrophages from sensing the polyphosphate signal from *Mtb,* and thus allow them to kill engulfed *Mtb*. Intriguingly, our findings also led to new biological insights about the chain-length dependence of polyphosphate signaling and allowed us to distinguish differences between polyphosphate and phagocytosis signaling pathways. Due to the conserved biology of polyphosphate across species, future work investigating the fundamental relationships of polyphosphate to other signaling pathways could lead to profound discoveries about the cellular or molecular role of polyphosphate signaling.

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Contributors

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The materials used for *Polyphosphate Signaling Hints at Potential Therapeutics for Tuberculosis* were provided by the Gomer lab and Dr. Ramesh Rijal. Cell strains were obtained from the Dictybase repository. The analyses depicted in *Polyphosphate Signaling Hints at Potential Therapeutics for Tuberculosis* were conducted in part by the Gomer lab and are unpublished. Phagocytic index experiments were conducted and analyzed primarily by a rotating graduate student, Te-An (Ann) Chen. Endogenous polyphosphate assays were conducted by Shiyu Jing.

All other work conducted for the thesis was completed by the student independently.

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NOMENCLATURE

Mtb	*Mycobacterium tuberculosis*
E. coli	*Escherichia coli*
Dicty	*Dictyostelium discoideum*
PolyP	Polyphosphate
OD	Optical Density
LB	Luria-Bertani broth
SIH	Sang-In Hanh synthetic medium
SM/5	Synthetic medium for *Dicty* edge plates
PBS	Phosphate buffered-saline
DAPI	4',6-diamidino-2-phenylindole
PPK1	Polyphosphate kinase 1
PPX1	Exopolyphosphatase 1
MOI	Multiplicity of infection
CFU	Colony forming units
SEM	Standard error of mean

1. INTRODUCTION

1.1 Bacteria and polyphosphate

There is an ancient history to the relationship between bacteria and inorganic chains of phosphates called polyphosphate (PolyP). In fact, it has been proposed that PolyP is a prebiotic molecule that could have played an important part in the origin of life and the use of other "high-energy" phosphoanhydride chains like those of nucleotides (1). The production of polyphosphate by bacteria is mediated by the conserved enzyme polyphosphate kinase 1 (PPK1) (2). When bacteria lack this enzyme or the ability to produce PolyP, they lose essential functions for survival such as cell motility, biofilm formation, and pathogenicity (3). Furthermore, the addition of exogenous exopolyphosphatase (PPX1), which cleaves terminal phosphate groups from polyphosphate, decreases the survival of bacteria in human macrophages (4). However, the mechanisms by which PolyP facilitates these processes are still poorly understood, especially concerning its impact on host-pathogen interactions.

1.2 How do *Mycobacterium tuberculosis* bacteria survive in macrophages?

One way that our bodies fight bacterial infections is with immune cells called macrophages, which ingest and kill the bacteria. This process is called phagocytosis. First, bacteria are engulfed into a membrane-bound compartment called the phagosome. The phagosome then fuses with an acidic compartment called the lysosome, which fully digests the bacteria (5). However, when *Mycobacterium tuberculosis* (*Mtb*) bacteria are ingested by macrophages, they prevent lysosome fusion and the subsequent digestion, and proliferate in the phagosome. This is a primary reason for the virulence of tuberculosis, one of the leading causes of death by a single agent worldwide (6).

The Gomer lab found that long-chain PolyP acts as an extracellular signal that prevents phagosome-lysosome fusion and allows bacteria to survive in macrophages (4). This means that bacteria can still be eaten or engulfed into the phagosome, but PolyP then acts as a "don't kill me" signal by preventing digestion (phagosome-lysosome fusion). Some bacteria like *Mtb* produce detectable levels of extracellular PolyP, which contributes to better survival after phagocytosis (7). Based on these observations, it was hypothesized that the proteins involved in PolyP signaling for bacterial survival could be blocked pharmacologically to induce macrophages to ignore the PolyP "don't kill me" signal from the *Mtb*, and as a result, kill the *Mtb*.

1.3 Quantifying bacterial survival

Phagocytosis is an active area of study with extensive data about protein composition in the phagosome (8, 9). However, working with human macrophages and *Mtb* is both expensive and time-consuming, and there are no mutant strains. As a result, the Gomer lab uses a model system for macrophages called *Dictyostelium discoideum* (*Dicty*), a eukaryotic microbe that also feeds on bacteria by phagocytosis. A considerable amount of research has been conducted about signaling pathways in *Dicty* cells (10). A powerful resource for *Dicty* work is Dictybase, a large collection of mutants that lack a specific signal transduction protein and thus lack a candidate that might be involved in PolyP signaling (11). If a *Dicty* cell was missing a candidate protein in the signaling pathway, it would not respond to the PolyP "don't kill me" signal and continue to kill bacteria. *E. coli* is a well-established bacterial system with many advantages such as the inability to produce detectable levels of PolyP, which allows PolyP levels to be controlled. Therefore, the use of *Dicty* and *E. coli* can allow for an efficient mutant screen of bacterial survival in host cells. Measuring intracellular survival of *E. coli* in *Dicty* in the presence of PolyP

thus models *Mtb* infection. Since most experimental measurements of phagocytosis concern the rate of ingestion of particles, I helped to develop a novel biological assay to quantify long-term bacterial survival in host cells. Using this assay with *Dicty* mutants and *E. coli*, I identified proteins involved in the PolyP and phagocytosis signaling pathways.

2. METHODS

2.1 Cell culture and strains

Dictyostelium discoideum strains were obtained from the *Dictyostelium* Stock Center and were wild-type AX2, *gβ*$^-$ (DBS0236531), *gα3*$^-$ (DBS0235986), *rasC*$^-$ (DBS0236853), *rasG*$^-$ (DBS0236862) *rasC*$^-$*/rasG*$^-$ (DBS0236858) *iplA*$^-$ (DBS0236260), *dagA*$^-$ (DBS0235559), *plC*$^-$ (DBS0267124), *pikA*$^-$ (DBS0350197), *pikB*$^-$ (DBS0350198), *pipkinA*$^-$ (DBS0236779), *ppk1*$^-$ (DBS0350686), *piaA*$^-$ (DBS0349879), *cnrN*$^-$ (DBS0302655), *adcC*$^-$ (DBS0350272), *adcB*$^-$*/adcC*$^-$ (DBS0350445), *racF1*$^-$ (DBS0351505) , *racG*$^-$ (DBS0236849), *racH*$^-$ (DBS0236850), and *pten*$^-$ (DBS0236830) (12-31). Cells were grown at 21°C in liquid culture in SIH defined minimal medium (Formedium, Norfolk, England) and on SM/5 agar (2 g glucose, 2 g bactopeptone, 0.2 g yeast extract, 0.2 g $MgCl_2 \cdot 7H_20$, 1.9 g KH_2PO_4, 1 g K_2HPO_4 and 15 g agar per liter) (http://www.dictybase.org/) on lawns of *E. coli* (DBS0350636). 100 µg/ml dihydrostreptomycin and 100 µg/ml ampicillin were used to kill *E. coli* in *Dictyostelium* cultures obtained from SM/5 agar (32). Cells expressing a selectable marker were grown under selection with the appropriate antibiotics and supplements (5 µg/ml blasticidin, 5 µg/ml neomycin sulfate, 100 µg/ml thymidine, 20 µg/ml uracil, and/or 25 µg/ml hygromycin).

2.2 Bacterial survival – a novel phagocytosis assay

Sodium polyphosphate (Spectrum, New Brunswick, NJ; average chain length 45 monomers) was utilized for the bacterial survival and phagocytic index studies described below. The sodium polyphosphate was dissolved in Sorensen buffer (14.6 mM KH_2PO_4, 2 mM Na_2HPO_4, pH 6) to a final concentration of 15 mg / ml and the pH was verified to be pH ~6.0. This stock was diluted 1:1000 in cultures and defined as 15 µg/ml polyphosphate. Higher

dilutions were used to make lower polyphosphate concentrations. The sodium polyphosphate was also dissolved in Sorensen buffer to 70.4 mg/ml, the pH was checked to be 6.0, and this stock was diluted 100 x in cultures to make 704 µg/ml polyphosphate; higher dilutions were used to make culture concentrations down to 15 µg/ml.

Dictyostelium discoideum cells were cultured on SM/5 agar plates with *E. coli* and then cultured axenically in type 353002 Falcon tissue culture adherence plates (Corning, Big Flat, NY) with 10 milliliters (ml) SIH synthetic media containing 100 µg/ml streptomycin and ampicillin. Adherence plates allow *Dicty* to grow optimally and retain an amoeboid or phagocytic morphology analogous to that of human macrophages. SIH is a minimal nutrient synthetic medium utilized to culture *Dicty* axenically but prevents complete reliance on macropinocytosis (the uptake of nutrients in liquid). In other words, *Dicty* in SIH media can only subsist, and if presented with a more nutritious food source (like bacteria), they will preferentially consume it. The less nutritious conditions of SIH media are vital to the experiment because the primary aim of this assay is to quantify the survival of bacteria that have already been ingested; if the *Dicty* preferred to simply consume the liquid nutrients rather than bacteria, then the assay would not be an effective measure of bacterial consumption and subsequent survival. Additionally, *E. coli* K-12 (BW25113) (CGSC#7636), a non-pathogenic strain of bacteria that do not produce PolyP, were cultured the night before each experiment for approximately 12 hours in 10 ml of Luria-Bertani (LB) broth (BD, Sparks, MD) at 37°C in shaking culture (125 ml Erlenmeyer flasks) (33, 34).

The following morning, *Dicty* cells were first resuspended by removing the old media and rinsing the adherence plates with 10 ml of new SIH media. All 10 ml were collected in 15 ml Falcon conical tubes. A 100 µl aliquot was obtained from these cultures and viable *Dicty* cell

density was quantified by dye-exclusion utilizing a TC20 Biorad automated cell counter (Biorad) with 10 µl from the aliquot and 10 µl 0.4% trypan blue (Invitrogen) dissolved in water. The data was recorded as x * 10^6 *Dicty* cells per milliliter. Only cultures with a starting density above 10^6 cells per ml were used because this density corresponds to the log phase of development, when *Dicty* are amoeboid and possess a phenotype most similar to immune cells.

Dicty cells were then washed by centrifugation at 500 x g and subsequent removal and replacement of the supernatant with new SIH media to remove dead cells, the antibiotics, and any secreted factors.

Following the wash, *Dicty* cells were diluted to a concentration of 10^6 cells/milliliter (ml) by Equation 2.1. 1 ml was added for each experimental condition in the wells of a type 353047 24-well tissue culture plate (Corning, Big Flat, NY), and the cells were allowed to acclimate for 30 minutes.

Equation 2.1:

$$\frac{x\ number\ of\ wells + 1}{cell\ density\ (\frac{10^6\ cells}{mL})} = ml\ needed\ to\ make\ 10^6\ cells/ml \qquad (2.1)$$

10 µl of 1.5 mg/ml PolyP was added to provide a final concentration of 15 µg/ml polyphosphate for experimental conditions. The cells were resuspended by pipetting five times in each corner of the well and the center to ensure that every cell in the experimental wells was exposed to polyphosphate. The *Dicty* cells were then allowed to acclimate for another 30 minutes, ensuring that the polyphosphate was distributed evenly to all *Dicty*.

During the 30 minutes allotted for the *Dicty* to acclimate to polyphosphate, one ml of *E. coli* K-12 in LB broth from the overnight culture was added to an Eppendorf tube and washed three times using the following procedure:

1. *E. coli* were collected by centrifugation at 13,000 x g for one minute in a Hermle Z 223 M-2 centrifuge.
2. The supernatant was removed and replaced with 1 ml of the *E. coli* culture, and the pellet was resuspending by pipetting up and down in the new media.
3. The bacteria were collected by centrifugation again at 13,000 x g for one minute in a Hermle Z 223 M-2 centrifuge.
4. The supernatant was removed and replaced with 1 ml of Sorensen's buffer, taking care not to disturb or remove any of the bacterial pellet. The pellet was resuspended in the new media.
5. A final wash was performed by an identical centrifugation and replacement procedure as steps c) and d).

Following the washing steps above, the optical density of the *E. coli* was obtained via spectrophotometric analysis at 600 nm after diluting the *E. coli* 1:10 in Sorensen's buffer in a cuvette. The amount of solution required to give a multiplicity of infection (MOI) of 5:1 (5 *E. coli* for every *Dicty* cell) is given by Equation 2.2.

Equation 2.2:

$$\frac{0.1}{10 * OD} * 1000 = \mu l\ of\ solution\ added\ to\ each\ well \qquad (2.2)$$

After the 30-minute acclimation period passed, the amount calculated by Equation 2.2 was removed from every well, and then the same amount of *E. coli* K-12 in Sorensen's buffer was added to each solution. The *Dicty* cells were resuspended by pipetting five times in each corner and the center of every well to ensure that all cells were equally exposed to the bacteria. The entire 24-well plate was then centrifuged at 200 x g in a Heraeus Multifuge X3R (ThermoFisher, Rockford, IL) centrifuge for one minute to ensure that all cells began eating

bacteria at the same time and the wells were relatively homogenous. The *Dicty* cells were incubated with the *E. coli* for two hours to allow ingestion of bacteria.

After two hours, the wells were washed by first centrifuging the 24-well plate at 200 x g for one minute as above, and then removing the supernatant from each well and replacing it with 1 ml of SIH media. This centrifugation, removal of supernatant, and addition of SIH was repeated two more times.

10 µl of media were removed and 10 µl of 15 mg/ml Spectrum polyphosphate was added again to the appropriate wells. Similarly, for every well, 4 µl was removed and replaced with 4 µl of 50 mg/ml gentamicin (Sigma, St. Louis, MO). Gentamicin is an antibiotic that cannot be ingested by eukaryotes and was ideal for this assay because it targeted only extracellular bacteria (35, 36). This allowed for a more accurate estimation and comparison of intracellular bacterial survival in PolyP and control conditions.

The wells were then resuspended by pipetting up and down five times to ensure equal distribution of antibiotic in the media, and the 24-well plate was centrifuged for one minute at 200 x g to allow the *Dicty* cells to start digesting the intracellular bacteria at the same time. The *Dicty* cells were given two more hours to clear any remaining extracellular bacteria and allow the antibiotic to eliminate only the bacteria outside the *Dicty* cells.

During this 2-hour incubation period, Eppendorf tubes and LB agar plates were labeled and prepared. Three Eppendorf tubes were labeled for each experimental well for counting the cell density, collecting lysates, and collecting the supernatant of each condition. 8 µl of 10% Triton X-100 (Alfa Aesar, Tewksbury, MA) in phosphate buffered-saline (PBS) (Lonza, Rockland, ME) was added to tubes labeled “L” for lysates to disrupt the membrane of *Dicty*

cells, and thus release the bacteria that survived phagocytosis by the *Dicty*, and 60 µl of SIH was added to tubes labeled "S" for supernatant.

After the two-hour incubation, the wells were washed by the same procedure as before. 10 µl of 15 µg/ml PolyP was added to appropriate wells following the removal of 10 µl from these wells. Every well was resuspended after the washes, and 90 µl were removed and placed into the corresponding Eppendorf tubes for counting. Immediately following this, 200 µl were removed and added to the lysate tubes. The 24-well plate was centrifuged at 200 x g for one minute as above to adhere *Dicty* to the bottom of the wells. 50 µl were removed from every well and placed into the supernatant tubes.

100 µl from the lysate tubes and 100 µl from the supernatant tubes were plated on separate LB agar plates. The added media on the plates were spread by gently wiping the surfaces with serological pipettes to ensure equal distribution. These agar plates were covered and placed in a 37°C incubator for overnight growth of bacteria. The following morning, bacterial colonies growing on the agar plates were counted.

After plating the bacteria, *Dictyostelium* cells in the Eppendorf tubes labeled for counting were resuspended by pipetting up and down and then counted as above with the BioRad cell counter to normalize the number of bacteria present to the number of *Dicty*. The data was recorded as 4-hour *Dicty* cell density. The normalization accounts for some wells that may have varied numbers of *Dicty*, which could inflate or deflate the number of bacteria in the well. For example, if a well contains a particularly high *Dicty* cell density, it is likely that more bacteria would be consumed. As a result, the number of bacteria that remain alive within the *Dicty* could be greater for this well despite the conditions or strain of *Dicty* that are used. In other words, the

number of *Dicty* was identified as a potential confounding variable for the number of bacterial colonies, and as such was controlled for utilizing data normalization.

The 24-well plate was stored at room temperature for 48 hours to allow *Dicty* to digest and kill ingested bacteria. After 48 hours, every well was resuspended by pipetting up and down five times in each corner of the well and the center to suspend the adhering *Dicty* cells in the liquid medium. 100 µl was removed from each well and placed into Eppendorf tubes for counting. The 24-well plate was then centrifuged at 200 x g for one minute to concentrate *Dicty* at the bottom of each well. The supernatant was carefully removed from each well and placed in Eppendorf tubes. The supernatant collected was a control for the number of extracellular bacteria that remain even after adding gentamicin. The supernatant may still contain bacteria because they are much smaller organisms and would not be pushed down to the bottom by 200 x g centrifugation.

150 µl of 0.4% Triton X-100 in PBS was added to each well, lysing the *Dicty* cells. The media was resuspended by pipetting up and down. The 150 µl lysates were then removed from each well and placed in Eppendorf tubes.

100 µl of supernatant and 100 µl of lysates for each well were added to LB agar plates and spread gently by serological pipettes. After drying, the plates were incubated at 37°C overnight. *Dictyostelium* cells in the Eppendorf tubes labeled for counting were resuspended by pipetting up and down and then counted as above. The data was recorded as 48-hour *Dicty* cell density. The following morning, bacterial colonies on the agar plates were counted.

2.3 Phagocytic index as a measure of ingestion

Phagocytic index is a well-established measurement of the uptake of particles by phagocytes (37). To gain more information about the mutant strains tested by the assay described

in the previous section, and the validity of the 4-hour timepoint as a representation of phagocytic index, fluorescence microscopy was used to visualize the uptake of Zymosan A bioparticles by *Dictyostelium* cells.

Prior to beginning an experiment, cells were prepared and cultured in the same manner as the bacterial survival assay to ensure that *Dicty* were in the log phase of development and retained an amoeboid, phagocytic phenotype. First, 15 ml conical tubes were labeled with the appropriate mutant strain name and the media from the *Dicty* cell culture adherence plates was discarded to remove any secreted factors or cell debris. 10 ml of new SIH was added, and a powered pipette was used to suspend the *Dicty* cells in the plates and then transfer them to the 15 ml conical tubes. 100 µl were removed from each tube and counted as above using the Biorad automated cell counter, mixing 10 µl of Trypan Blue, and 10 µl from the Eppendorf tube. This data was then recorded as the initial cell density with units of 10^6 cells / ml of the cell culture and inserted into Equation 2.3 below. The objective was to obtain 1 x 10^6 cells/ml, which allows *Dicty* to be confluent and spread evenly across the surface of a 96-well plate.

Equation 2.3:

$$\frac{1\ x\ 10^6 cells}{cell\ density\ (\frac{10^6\ cells}{mL})} = ml\ to\ obtain\ 10^6 cells/ml \qquad (2.3)$$

The amount calculated by Equation 2.3 for each *Dicty* strain and wild type *Dicty* was added to 15 ml conical tubes and centrifuged at 1500 rpm (roughly 500 x g) for 3 minutes in an IEC Centra CL2 centrifuge (ThermoFisher, Rockford, IL). For each condition being tested, the supernatant was then removed, discarded, and replaced with one ml of new SIH media to eliminate any remaining cell debris or secreted factors that could have accumulated in the original cell culture. This procedure was repeated two more times to wash the cells. After the

washes, *Dicty* were resuspended by pipetting up and down several times, and then 400 µl was added to the wells of a type 353219 96 well, black/clear, tissue culture treated glass bottom plate (Corning, Big Flat, NY).

After 30 minutes, 1.2 µl of 4.6 mg/ml Spectrum polyphosphate was added to half of the wells with a working concentration of 15 µg/ml. The experimental (PolyP containing) wells were then resuspended by pipetting up and down to ensure equal distribution of PolyP to all *Dicty* cells. The cells were given another 30 minutes to incubate with the PolyP and acclimatize. Following the incubation, 5 µl of 1 mg/ml fluorescent Zymosan A Bioparticles (ThermoFisher, Rockford, IL) were added to every well to give a final concentration of 12.5 µg/ml. After allotting 60 minutes for the *Dicty* to ingest the bioparticles, the liquid in the well was gently removed, and then 400 µl of 4% wt/vol of paraformaldehyde (pfa) (Electron Microscopy Sciences, Hatfield, PA) in PBS was added to each well to fix the cells. After fixation for 10 minutes, the wells were washed with 400 µl of Sorensen's buffer solution and then aspirated and stored dry.

Images of the *Dicty* cells were taken with a 40x objective on a Nikon Eclipse Ti2 (Nikon, Melville, NY), and the Richardson-Lucy algorithm was used for deconvolution of images in NIS-Elements AR software. Fluorescence intensity of the Zymosan A Bioparticles and the number of beads per cell were quantified using ImageJ software (ImageJ; National Institutes of Health, Bethesda, MD, USA).

2.4 Endogenous polyphosphate measurements

Long-chain bacterial polyphosphates have long been the central focus of polyphosphate research, especially when studying host-pathogen interactions (38). However, most eukaryotes also produce a measurable amount of short-chain polyphosphate, whose effects on pathogenicity

are still uncharacterized (39). *Dicty* produce PolyP utilizing the enzyme polyphosphate kinase 1 (PPK1), which catalyzes the polymerization of phosphate groups using ATP as a substrate (2). Since *Dicty* produce PolyP and this could have an impact on host-pathogen interactions, endogenous PolyP measurements were conducted for Ax2 wild-type and mutants.

Dicty were cultured as previously described, and experiments were performed over a period of 14 days to observe the production of PolyP during *Dicty* development. On the first day, the liquid media from the adherence tissue culture plate containing the *Dicty* was removed to discard any dead cells. 10 ml of SIH were then used to resuspend the adhered *Dicty* cells and placed in a 15 ml conical tube. 50 µl of the suspended cells was removed and the *Dicty* cell density in x * 10^6 cells/ml was obtained as previously described with the Biorad cell counter. The starting density of each cell culture for these experiments was 500,000 cells/ml, and the volume needed to obtain this cell density from the starting culture in 20 ml of SIH was calculated using Equation 2.4.

Equation 2.4:

$$\frac{0.5\ x \frac{10^6 cells}{ml} x\ 20\ ml}{cell\ density\ (\frac{10^6\ cells}{mL})} = ml\ to\ obtain\ 0.5\ x\ 10^6 cells/ml \qquad (2.4)$$

After adding the calculated volume from Equation 2.4 to a 125 ml Erlenmeyer flask SIH media was added to raise the volume to 20 ml. *Dicty* cells were allowed to grow at 21°C in shaking culture for 14 days. Every 24 hours after the creation of the initial flask, 500 µl were removed and stored in an Eppendorf tube for PolyP measurement. An additional 50 µl were removed and the cell density was quantified every day as previously described using the Biorad cell counter.

The Eppendorf tube for PolyP measurement was centrifuged at 500 x g for 10-15 minutes in the Hermle Z 223 M-2 centrifuge until a dense pellet was formed. After centrifugation, 450 µl

of the supernatant were carefully removed and concentrated by another centrifugation using a 2-kDa spin filter (Pall, Ann Arbor, MI) at 7500 x g for 90 minutes. The PolyP, left on the filter, was resuspended with 200 µl of 10 nM $NaPO_4$ buffer and transferred to another labeled Eppendorf tube. A 96-well, black/clear, tissue culture treated glass bottom plate was obtained and a PolyP standard curve was made utilizing dilutions of a 4.6 mg/ml stock solution of Spectrum polyphosphate dissolved in the same 10 nM $NaPO_4$ buffer. After serial dilutions, 7 wells were brought to a volume of 100 ul with the following final concentrations: 10nM $NaPO_4$ buffer as a control, 4.6 ng/ml PolyP, 46 ng/ml PolyP, 460 ng/ml PolyP, 4.6 µg/ml PolyP, 9.2 µg/ml PolyP, and 18.4 µg/ml PolyP. Additionally, a 100 µl aliquot of the PolyP solution obtained from the spin filter and *Dicty* culture of interest was added to a well. Dilutions were made with technical replicates of the *Dicty* PolyP solution to ensure reliability and that the calculated value lied along the standard curve. A solution of DAPI (4',6-diamidino-2-phenylindole), a widely used fluorescent label for DNA that also interacts with PolyP, was made by diluting 2 mg/ml DAPI 1:10 with sterile water (40). 12.5 µl of the 0.2 mg/ml DAPI solution in water was added to each well and the media was resuspended to equally distribute the DAPI.

The 96-well plate was then loaded onto a Synergy MX microplate reader (BioTek, Winooski, VT) with an Excitation setting of 415/20.0 and an Emission setting of 550/20.0 (415 nm/515 nm). The software was initialized, and the data was exported as an excel. In the excel, the standard concentrations in µg/ml were plotted against the actual fluorescence values, and a linear regression was performed. The fluorescence values of the *Dicty* PolyP wells were divided by the slope obtained by the linear regression of the standard curve, multiplied by the reciprocal of the dilution factor (i.e. a 1:10 dilution was multiplied by 10), and then divided by 1000 to

return the concentration of the *Dicty* PolyP in µg/ml. This PolyP measurement process was repeated every 24 hours for 14 days for each mutant strain and Ax2 wild type.

3. RESULTS

3.1 Results

23 mutants were tested by the bacterial survival assay described above. Data was normalized to *Dicty* cell density by Equations 3.1-3.2. "Sensitivity" to the PolyP "don't kill me" signal was defined as a statistically significant increase ($p < 0.05$) in colony forming units (CFU) in the presence of PolyP compared to the no-PolyP control. An increase in bacterial colonies was consistent with an increase in intracellular bacterial survival like that of *Mtb* infection. The data was analyzed by Mann-Whitney tests, and the number of viable *E. coli* K-12 per 10^6 *Dicty* cells was graphed using GraphPad Prism 9.0.0 (GraphPad Software, San Diego, CA).

Equation 3.1:

$$\frac{CFU\ (4\ hours)}{cell\ density\left(\frac{10^6\ cells}{ml}\right)\ x\ \frac{910\ \mu l}{1000\ \mu l/ml}\ x\ \frac{100\ \mu l}{200\ \mu l}} = CFU\ per\ 10^6\ Dicty \qquad (3.1)$$

Equation 3.2:

$$\frac{CFU\ (48\ hours)}{cell\ density\left(\frac{10^6\ cells}{ml}\right)\ x\ \frac{560\ \mu l}{1000\ \mu l/ml}\ x\ \frac{100\ \mu l}{150\ \mu l}} = CFU\ per\ 10^6\ Dicty \qquad (3.2)$$

At 4 hours, PolyP had no significant impact on bacterial survival in normal *Dicty* cells (called Ax2) or mutants 1, 2, and 3 (these names cannot be disclosed due to pending patents) (Fig. 3.1). This implies that PolyP did not affect the engulfment of bacteria. At 48 hours, PolyP increased bacterial survival in Ax2, but mutants 1, 2, and 3 were still able to kill bacteria at levels comparable to control conditions (Fig. 3.2). The 4-hour timepoint was the earliest initial point at which bacterial survival could be measured and provided a rough estimate of how many bacteria were ingested. The 48-hour timepoint was more indicative of bacterial survival because the *Dicty* had enough time to acidify and fuse bacteria-containing phagosomes with lysosomes.

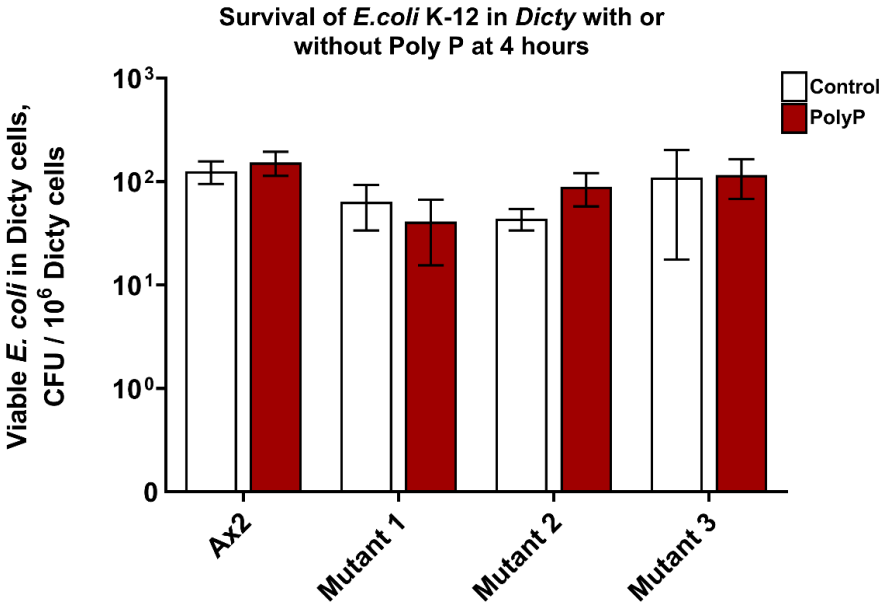

Figure 3.1: Polyphosphate does not affect bacterial survival at 4 hours. Values are mean ± SEM from 5 independent experiments for each mutant and 16 independent experiments for Ax2 wild type.

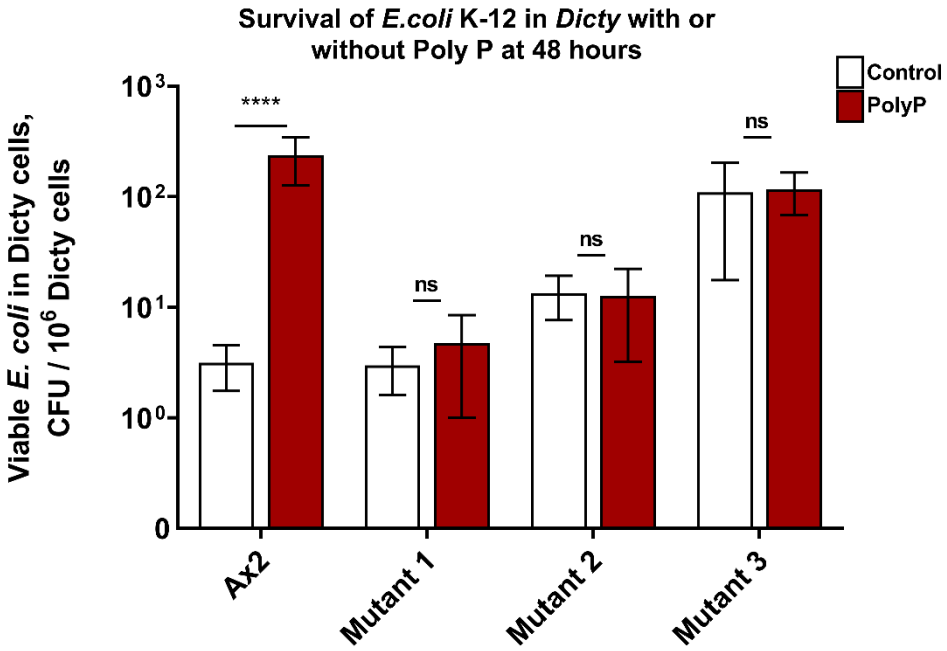

*Figure 3.2: PolyP increases bacterial survival at 48 hours for normal Dicty (Ax2), but not mutants 1, 2, and 3. Values are mean ± SEM from 5 independent experiments for each mutant and 16 independent experiments for Ax2 wild type. **** indicates p<0.0001 by Mann-Whitney Test. ns indicates not significant.*

Twenty other mutants were analyzed and showed varied sensitivity to the PolyP "don't kill me" signal at 48 hours, but generally not at 4 hours (Fig. 3.3 – 3.4). In other words, for most mutants, the engulfment of bacteria was unaffected by PolyP (Fig. 3.3) but the digestion or killing of bacteria was inhibited by the addition of PolyP (Fig. 3.4). The exception was a mutant lacking phospholipase C (*plC*$^{-}$), which showed sensitivity and increased bacterial survival in the presence of PolyP even at 4 hours. Since these twenty mutants (including *plC*$^{-}$) still responded to PolyP at 48 hours, this suggests that the proteins they are lacking may not be necessary for sensing PolyP.

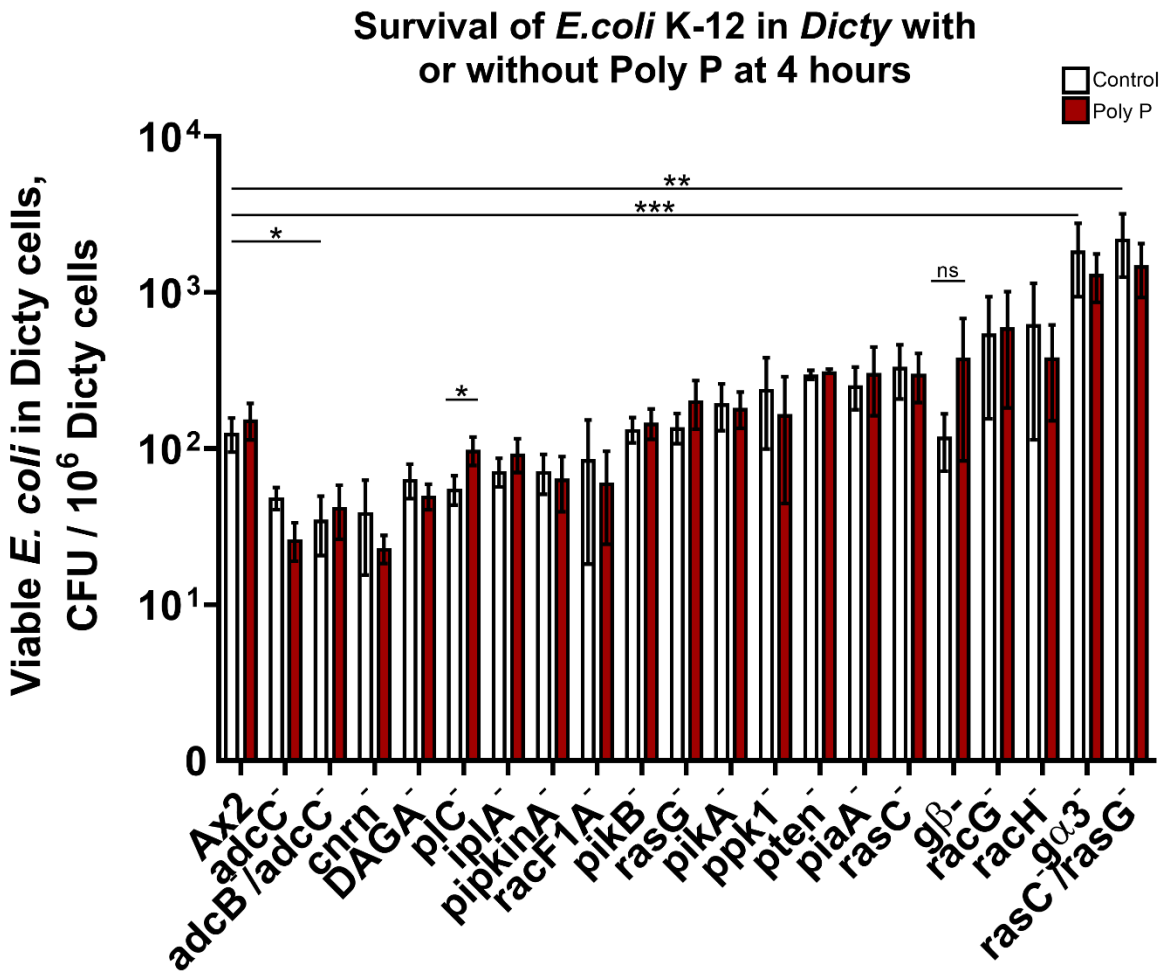

*Figure 3.3: 15 µg/ml polyphosphate did not affect bacterial survival at 4 hours for 19 other mutants. plC^{-} cells had increased numbers of bacteria at 4 hours in the presence of polyphosphate. Values are mean ± SEM from 5 independent experiments for each mutant and 16 independent experiments for Ax2 wild type. * indicates $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ by Mann-Whitney Test. ns indicates not significant.*

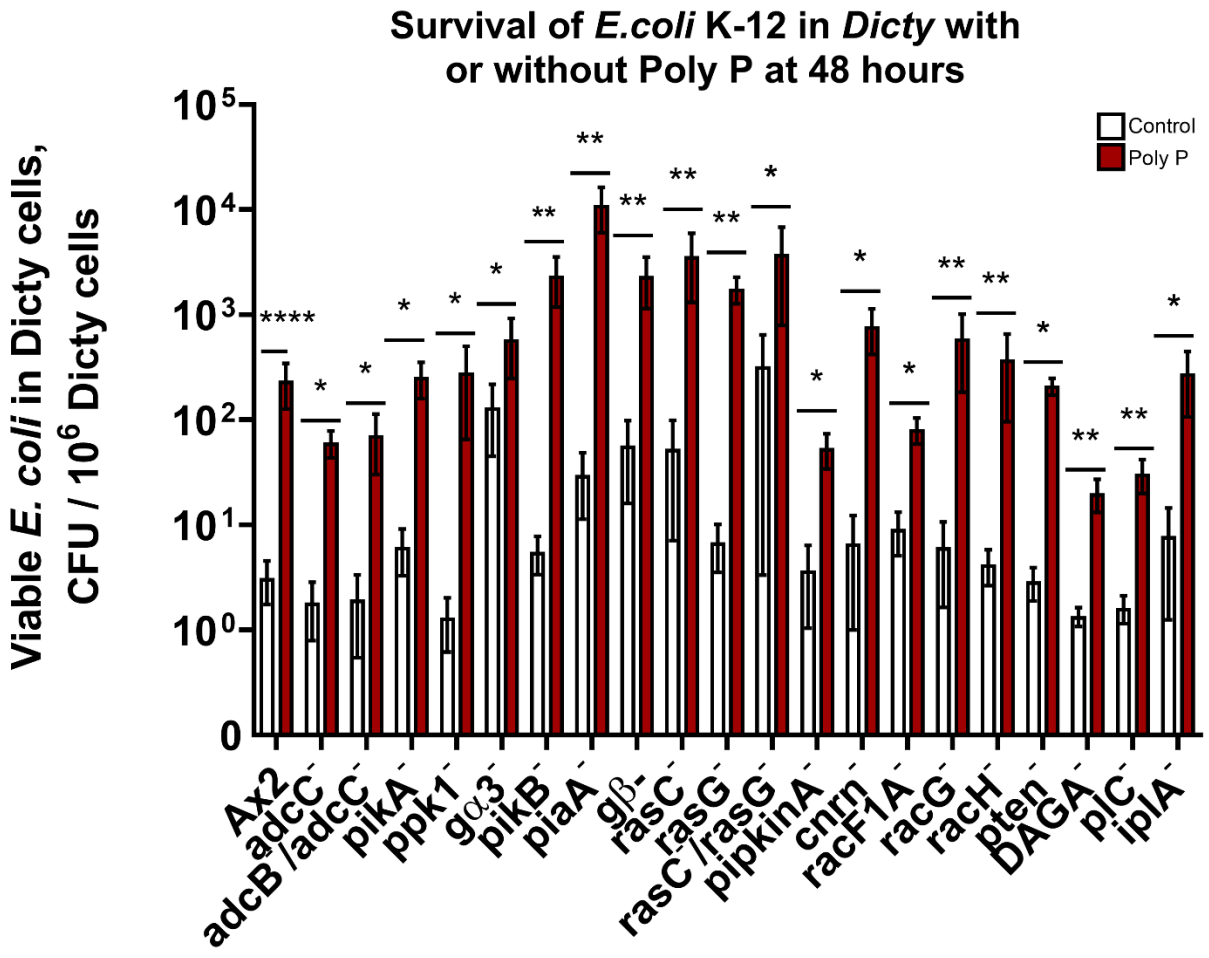

*Figure 3.4: For 20 mutants and Ax2, 15 µg/ml PolyP increased bacterial survival at 48 hours. Values are mean ± SEM from 5 independent experiments for each mutant and 16 independent experiments for Ax2 wild type. * $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$ by Mann-Whitney Test.*

The variation in data at 4 hours, namely that of *gα3*$^{-}$, *rasC*$^{-}$*/rasG*$^{-}$, *adcB*$^{-}$*/adcC*$^{-}$, and *plC*$^{-}$, prompted further investigation of these data. The mutant strains *gα3*$^{-}$ and *rasC*$^{-}$*/rasG*$^{-}$ were of particular interest because they appeared to have an inherent deficiency in the ability to kill bacteria, but an increased ability to consume them. Since there was a statistically significant increase in the number of bacteria without PolyP at 4 hours for both *gα3*$^{-}$ and *rasC*$^{-}$*/rasG*$^{-}$, this implied that these mutants ingested more bacteria. At 48 hours, both *gα3*$^{-}$ and *rasC*$^{-}$*/rasG*$^{-}$ retained significantly more bacteria without PolyP compared to Ax2 wild type. However, the

data obtained at 4 hours is not an established measure of phagocytic index because it has already been shown that the consumption of particles occurs in less than 10 minutes and phagosome-lysosome fusion occurs on the timescale of a few hours (41). For this reason, it was necessary to utilize another assay to determine the actual phagocytic indices of these mutants to assess whether the increase in CFUs at 48 hours was a result of increased consumption of bacteria or a deficiency in digestion.

The particle uptake of Zymosan A Bioparticles by 14 mutants and Ax2 wild type was assessed (Fig. 3.5 – 3.8). Data was recorded and analyzed by ImageJ software by two main metrics: the number of beads per cell in control versus PolyP conditions and the percent of cells that contained any beads in control versus PolyP conditions. The data suggests that the presence of PolyP does not impact the phagocytic index of Ax2 wild type and 11 of the mutants tested. However, the number of particles per cell was increased for *rasC*$^{-}$*/rasG*$^{-}$ cells in the presence of PolyP and the percent of *Dicty* cells that contained bioparticles was decreased by the presence of PolyP for mutants *gβ*$^{-}$ and *pipkinA*$^{-}$. Without PolyP, the phagocytic indices in terms of number of bioparticles per cell for Mutant 1, Mutant 3, *piaA*$^{-}$, *rasG*$^{-}$, *rasC*$^{-}$, *adcC*$^{-}$, *iplA*$^{-}$, and *pikB*$^{-}$ cells were statistically lower than that of Ax2 wild type. Only *rasC*$^{-}$*/rasG*$^{-}$ cells ingested significantly more bioparticles per cell than Ax2 wild type, and there was no statistical difference in the number of bioparticles per cell between Ax2 and *gα3*$^{-}$, *gβ*$^{-}$, *plC*$^{-}$, *pipkinA*$^{-}$, and Mutant 2.

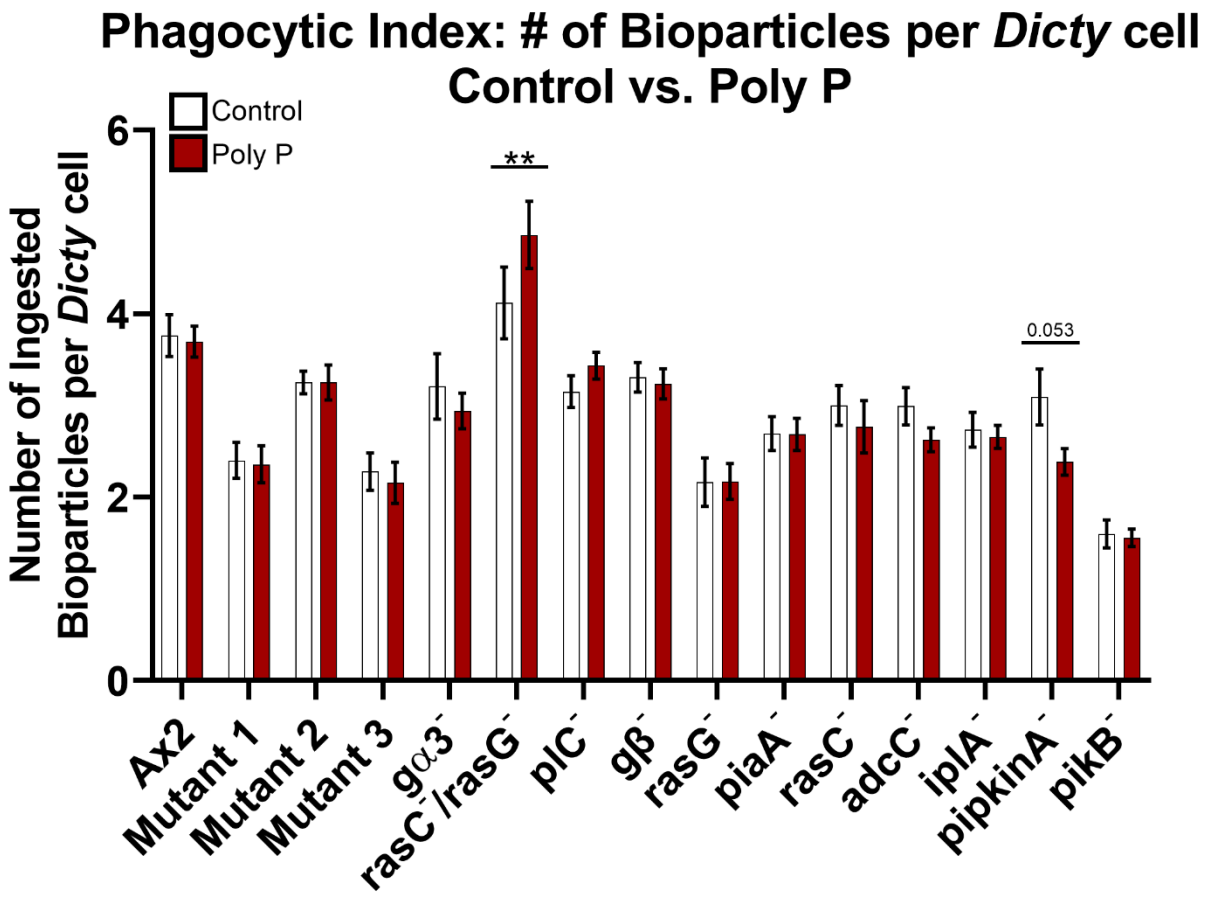

*Figure 3.5: The average number of Zymosan bioparticles per Dicty cell was determined utilizing ImageJ software. rasC^{-}/rasG^{-} cells ingested more particles in the presence of 15 µg/ml PolyP. pipkinA^{-} cells had a p value of 0.053 when comparing PolyP and control conditions. Values are mean ± SEM from 3 independent experiments. *p<0.05, **p<0.01 and ***p<0.001 by Mann-Whitney test.*

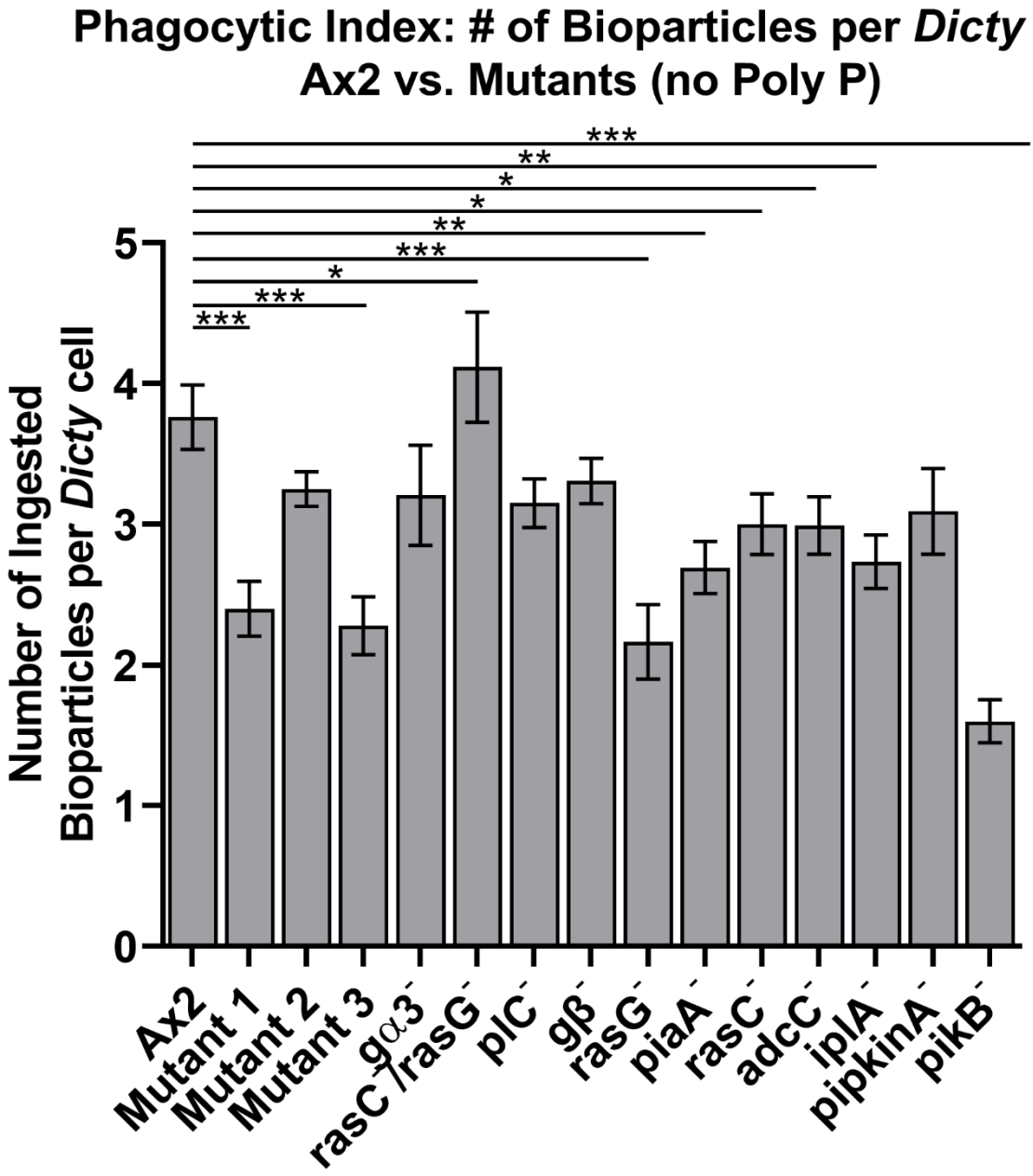

*Figure 3.6: The average number of Zymosan bioparticles per Dicty cell was determined utilizing ImageJ software. The figure illustrates statistical comparisons between Ax2 and mutants without PolyP. Values are mean ± SEM from 3 independent experiments. *p < 0.05, **p < 0.01, and *** p < 0.001 by Mann-Whitney test.*

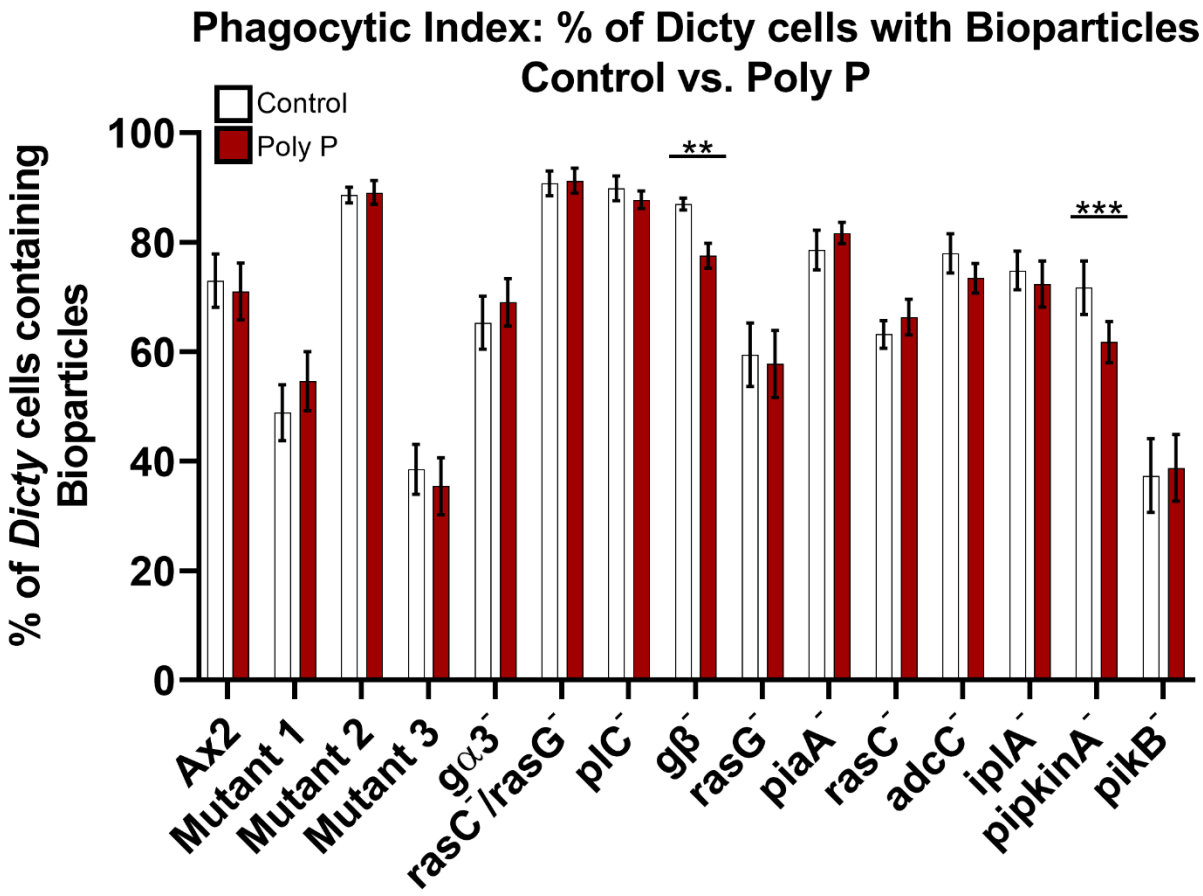

*Figure 3.7: The percent of Dicty cells containing Zymosan bioparticles was determined utilizing ImageJ software. Both gβ$^-$ and pipkinA$^-$ cells showed a decreased number of cells containing particles in the presence of 15 µg/ml PolyP. Values are mean ± SEM from 3 independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ by Mann-Whitney test.*

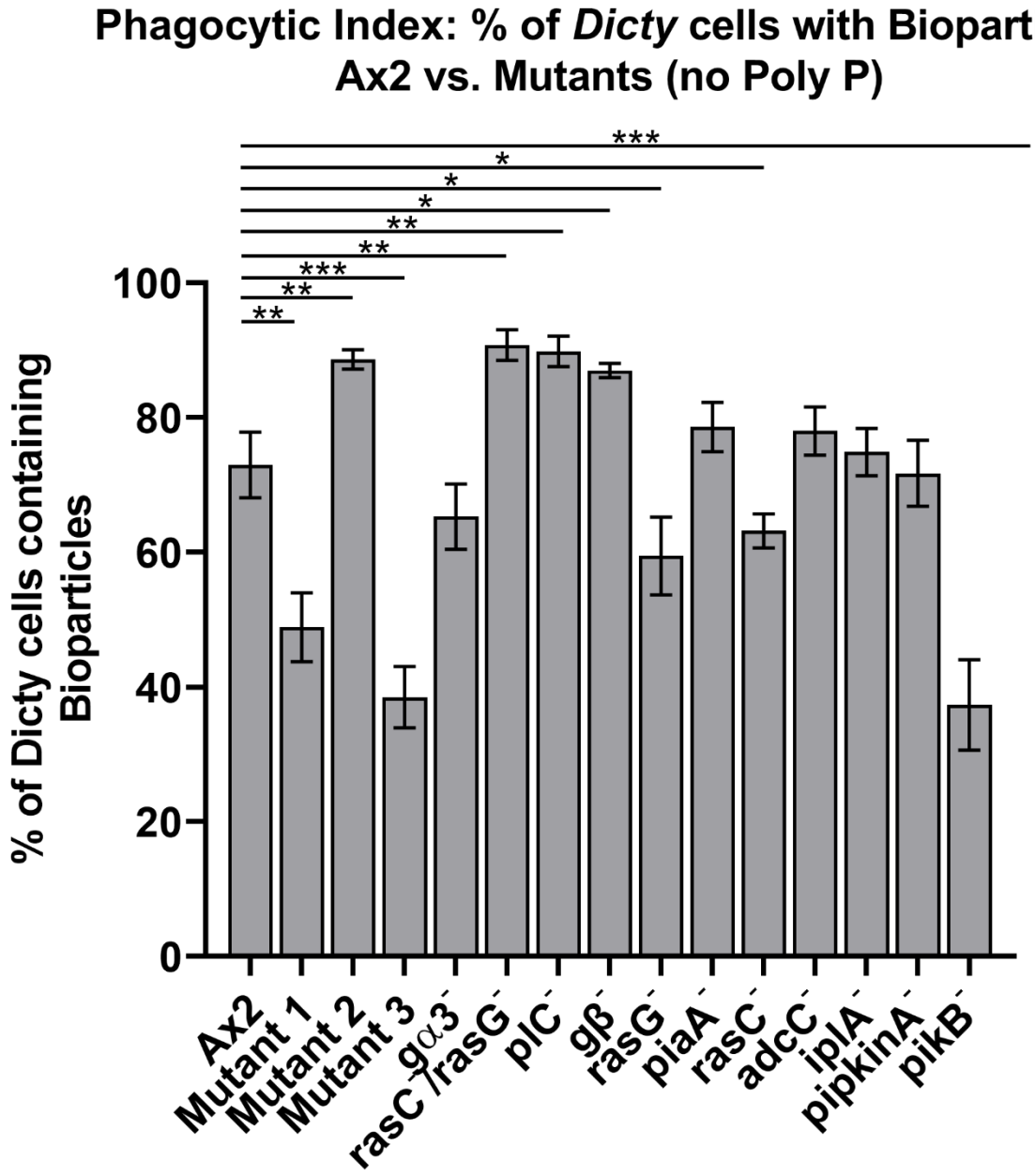

*Figure 3.8: The percent of Dicty cells containing Zymosan bioparticles was determined utilizing ImageJ software. The figure illustrates comparisons between Ax2 and mutants without PolyP. Values are mean ± SEM from 3 independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ by Mann-Whitney test.*

A comparative analysis was conducted by plotting the 4-hour bacterial survival data against the 1-hour phagocytic index data (number of bioparticles per cell). If the two measurements were analogous for the ingestion of particles/bacteria, a simple linear relationship between the 4-hour CFUs and the 1-hour number of beads per cell would be expected. After

conducting a simple linear regression, no statistically significant ($p<0.05$) correlation was discovered and the R^2 value was found to be 0.1938, implying that the two measurements were not dependent on each other.

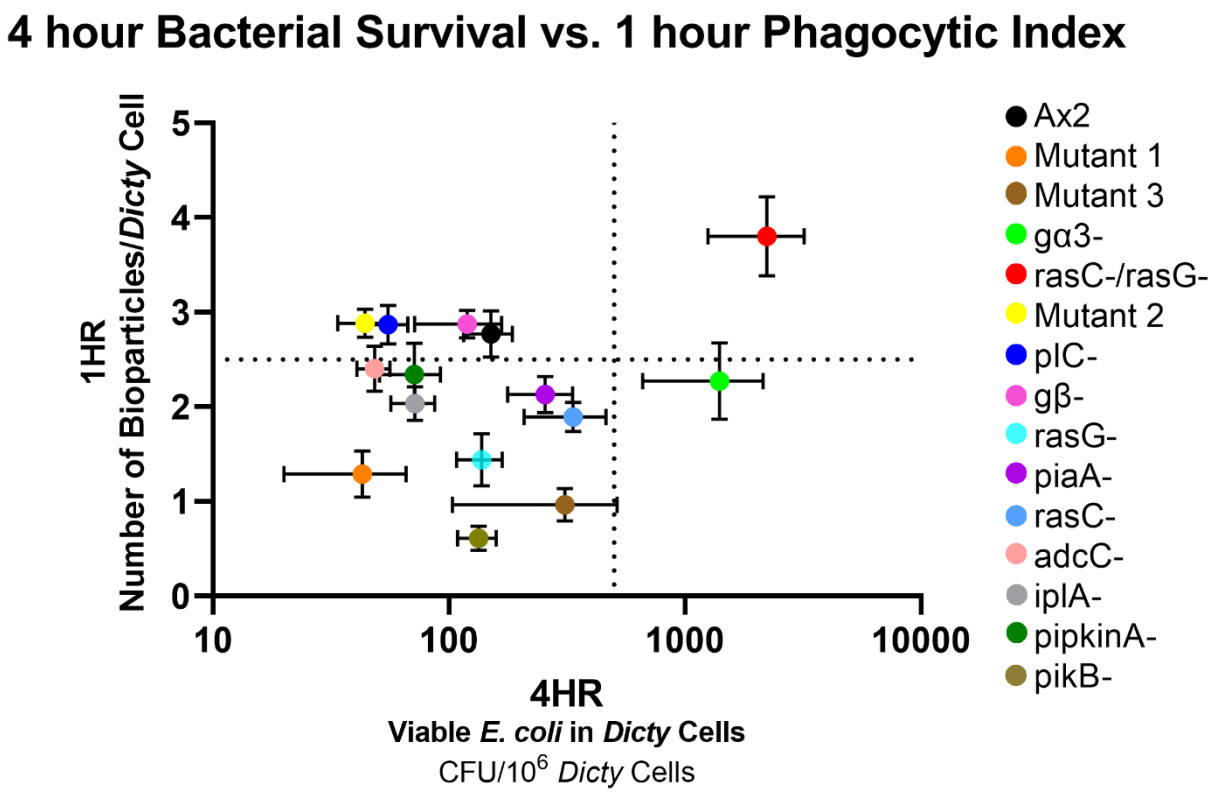

Figure 3.9: The number of bioparticles per Dicty cell was determined utilizing ImageJ software and plotted against the number of colony-forming units (CFU) per 106 Dicty. Data are from figures 3.3 and 3.6. The figure illustrates comparisons between Ax2 and mutants without PolyP.

Based on the data, it was evident that 4-hour data did not always perfectly align with the measurement of phagocytic index. Additionally, the effect of polyphosphate on ingestion of particles could have confounded the data that suggested polyphosphate truly inhibits bacterial digestion. To quantify the impact of PolyP on particle uptake, additional phagocytic index assays were conducted (Fig. 3.9). To eliminate any doubt that PolyP is an extracellular "don't kill me" signal and exclude the effect of PolyP on the ingestion of bacteria, modified bacterial survival assays were conducted in which PolyP was only added after 4 hours (Fig. 3.9). The results of

these experiments matched that of previous experiments: the addition of PolyP increased long-term survival of bacteria at 48 hours despite being added after bacteria were ingested by *Dicty*.

Overall, the results above illustrate a growing complexity with extracellular PolyP signaling despite the clear role of PolyP as an inhibitor of bacterial killing since the effect of PolyP on ingestion and digestion changes over time. Interestingly, eukaryotes (including *Dicty*) also produce PolyP with shorter chain-lengths (42). Although previous literature has demonstrated the chain-length dependence of PolyP signaling, there are biological purposes for endogenous short-chain PolyP in human cells such as modulating signaling cascades for coagulation, the complement system, and bradykinin (43-47). Furthermore, short-chain PolyP has been shown to affect the differentiation, function, and motility of phagocytes in mammals (48-50). Endogenous PolyP produced by *Dicty* could therefore have an impact on various biological processes and the bacterial survival data collected. In order to account for the potential confounding variable of endogenous PolyP, recombinant PPX1 from *Saccharomyces cerevisiae* was purified by previously defined methods and added to experimental wells in the same fashion as PolyP both before and after 4 hours (4, 51). The addition of PPX1 was used to understand the effects of decreased endogenous PolyP on bacterial survival at 4 and 48 hours (Fig. 3.10). PPX1 appeared to upregulate particle uptake and 4-hour bacterial survival, and yet long-term bacterial survival at 48 hours in the presence of PPX1 was minimal. As shown in Figure 3.10A, the data suggested that PolyP potentiated a statitisically significant ($p<0.05$) decrease in particle uptake for Ax2 wild type, and PPX1 increased particle uptake per *Dicty* cell. However, despite the increased numbers of bacteria at 4 hours, *Dicty* cells exposed to PPX1 successfully cleared bacterial infections at 48 hours with numbers of bacteria in *Dicty* cells comparable to control conditions as depicted in Figure 3.10B. At both 24 and 48 hours, the addition of 15 µg/ml, 47

µg/ml, and 470 µg/ml PolyP after 4 hours induced a statistically significant ($p<0.05$) increase in CFUs as illustrated by Figures 3.10B and 3.10C.

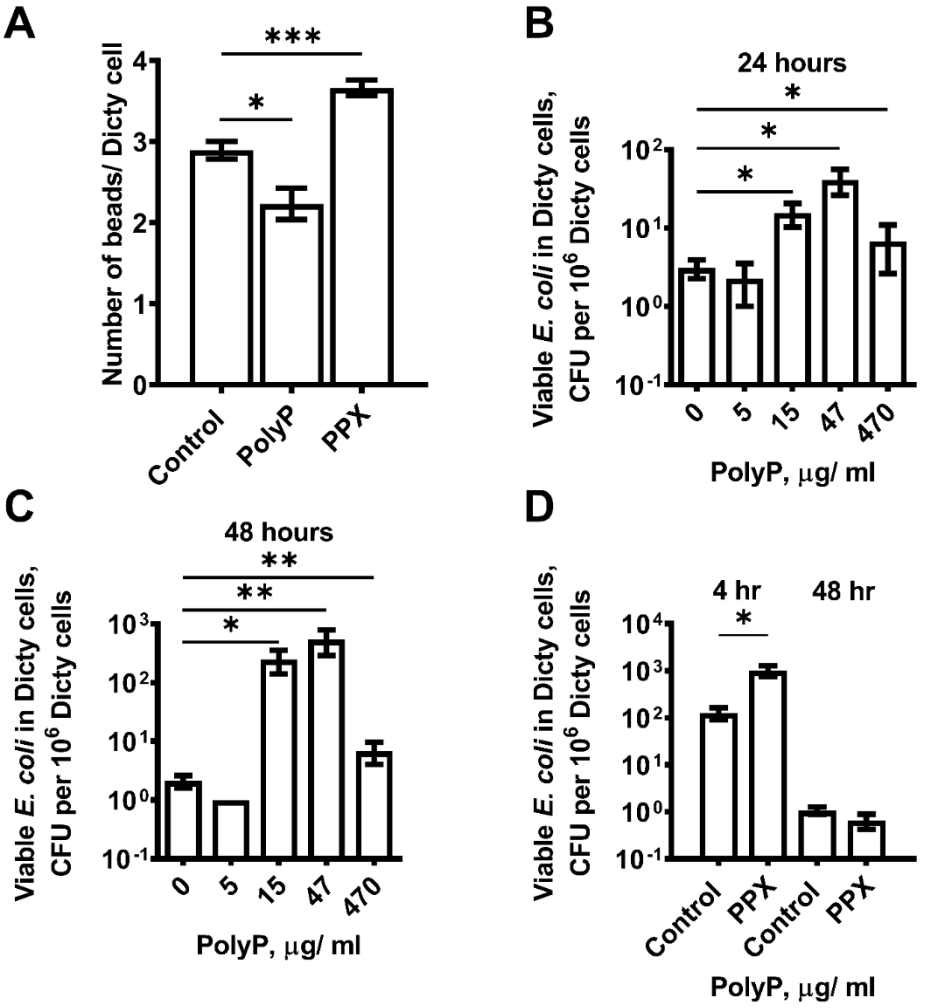

Figure 3.10: ***A)*** *Phagocytic index experiments were conducted with or without PolyP and recombinant PPX1. The addition of PolyP decreased the number of beads per cell while PPX1 increased the number of beads per cell.* ***B)*** *24-hour bacterial survival data was recorded for Ax2 wild type. The effect of PolyP on bacterial survival was tested by adding different concentrations of PolyP after 4 hours. 15, 47, and 470 µg/ml PolyP increased bacterial survival at 24 hours.* ***C)*** *48-hour bacterial survival data was recorded. The effect of PolyP on bacterial survival was tested by adding varying concentrations of PolyP after 4 hours, illustrating that 15, 47, and 470 µg/ml PolyP increases long-term bacterial survival.* ***D)*** *4-hour and 48-hour data for control and PPX conditions were graphed to illustrate that PPX increases the number of bacteria at 4 hours but has similar numbers of bacteria to Ax2 wild type in control conditions at 48 hours. Values are mean ± SEM from 3 independent experiments. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ by Mann-Whitney test.*

According to the results above, exogenous PPX that is added to *Dicty* cells in the presence of bacteria or particles stimulates both phagocytic ingestion and digestion. Since PPX

cleaves PolyP and the only PolyP in the experiment was that produced by the *Dicty*, these data imply that the PolyP produced endogenously by *Dicty* may have a role in host-pathogen dynamics. As a result, measurements of endogenous PolyP were conducted for Ax2 and 6 mutants. Since the production of PolyP depends on cell density, growth curves were generated for Ax2 and the mutants tested (Fig. 3.11). No clear significant difference was observed in total PolyP production between Ax2 and 6 mutants (Fig. 3.12). Mutant 1, however, had a statistically significant ($p < 0.05$) increase in the rate constant for PolyP production by nonlinear regression (analyzed in GraphPad Prism as a plateau followed by one phase association) of the PolyP data compared to Ax2 data over time (Fig. 3.13). On the other hand, Mutant 3 had a significantly lower rate constant ($p < 0.05$) for nonlinear regression (analyzed in GraphPad Prism as a plateau followed by one phase association) of the PolyP data compared to Ax2 data over time. The findings of the nonlinear regression of the overall PolyP production by the mutants did not provide a clear correlation between bacterial survival and endogenous PolyP because one would expect that Mutants 1 and 3 would have similar endogenous PolyP production if *Dicty* PolyP is a factor in bacterial survival.

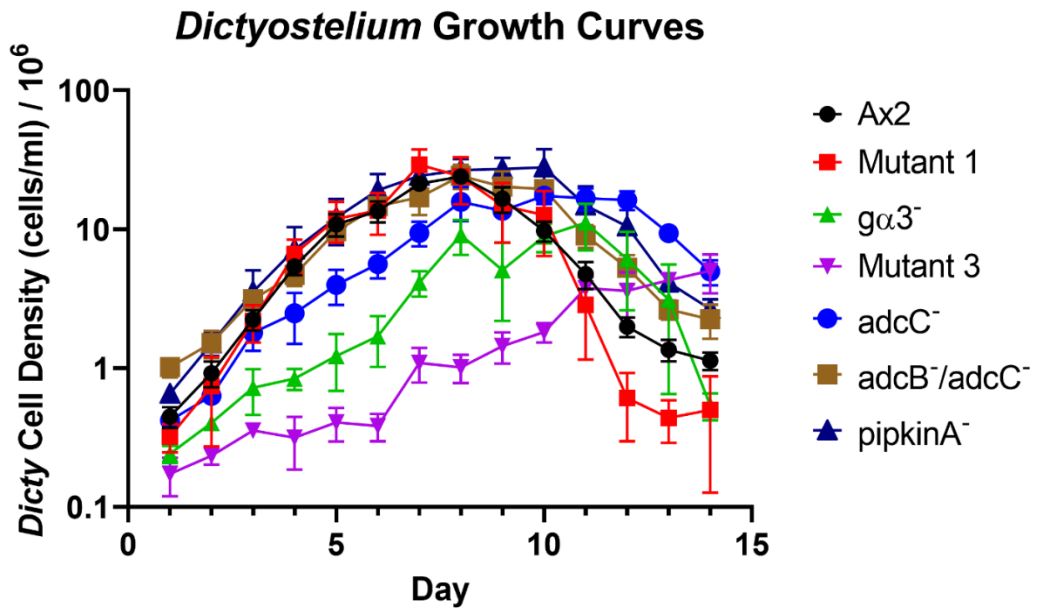

Figure 3.11: The growth of Dicty cells in shaking culture was measured over 14 days. The figure illustrates differences in proliferation by different mutants. Values are mean ± SEM from 3 independent experiments.

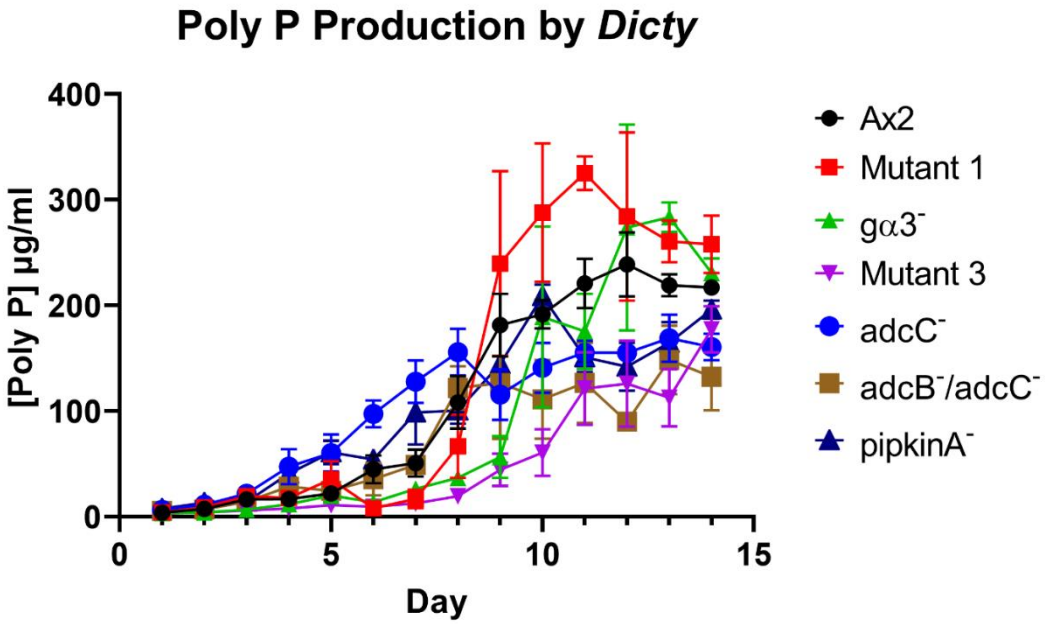

Figure 3.12: The production of endogenous PolyP by Dicty was measured over a period of 14 days. Values are mean ± SEM from 3 independent experiments.

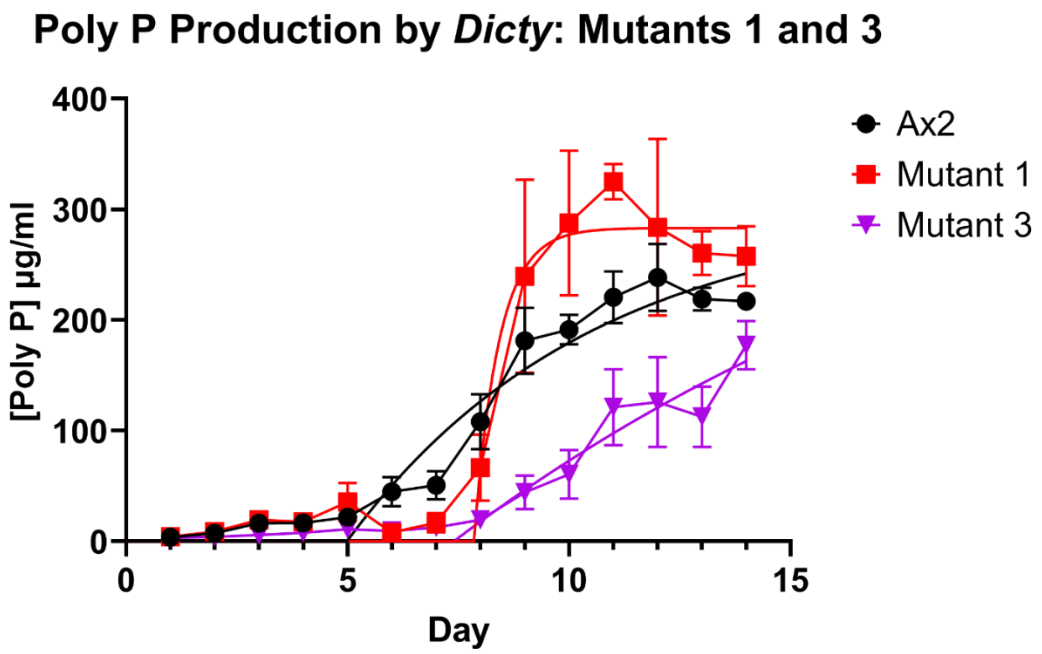

Figure 3.13: The production of endogenous PolyP by Dicty was measured over a period of 14 days. Values are mean ± SEM from 3 independent experiments. Nonlinear regression was performed and Ax2, Mutant 1, and Mutant 3 all have statistically different (p = 0.0002) rate constants by the Extra sum-of-squares F test. The values of the rate constants for Ax2, Mutant 1, and Mutant 3 were calculated as 0.1809, 1.785, and 0.06677 days^{-1}, respectively.

Based on the stunted growth of some mutants, as illustrated by Figure 3.11, the PolyP data shown above did not fully reflect endogenous PolyP production during the log phase of development, which was the cell density at which the bacterial survival experiments were conducted to ensure phagocytic morphology and behavior. In other words, Mutant 3 may have had a lower rate constant because, as depicted in Figure 3.11, it never completed its developmental cycle and proliferated slowly. To account for this, PolyP production was plotted against *Dicty* cell culture density at day three or log phase cell density (between 1 x 10^6 and 4 x 10^6 cells/ml) for Ax2 and the 6 mutants (Fig. 3.14). The comparative analysis illustrated that Mutant 3, Mutant 1, and *adcC*$^-$ cells appeared to produce more PolyP than Ax2 wild type in the log phase of development. To correlate this trend to previously described bacterial survival and phagocytic index data, the *Dicty* PolyP data was also plotted against the number of bioparticles

per *Dicty* cell (Fig. 3.15) and the number of viable *E. coli* at 4 hours and 48 hours (Fig. 3.16, 3.17). Based on these analyses, there was no clear relationship between the amount of endogenous PolyP produced by the *Dicty* cells and bacterial survival. The only correlation found was that Mutant 1 and Mutant 3 both produced more PolyP and ingested fewer bioparticles per *Dicty* cell than Ax2 wild type, thus providing evidence that endogenous PolyP affects the ingestion of particles but not bacterial survival.

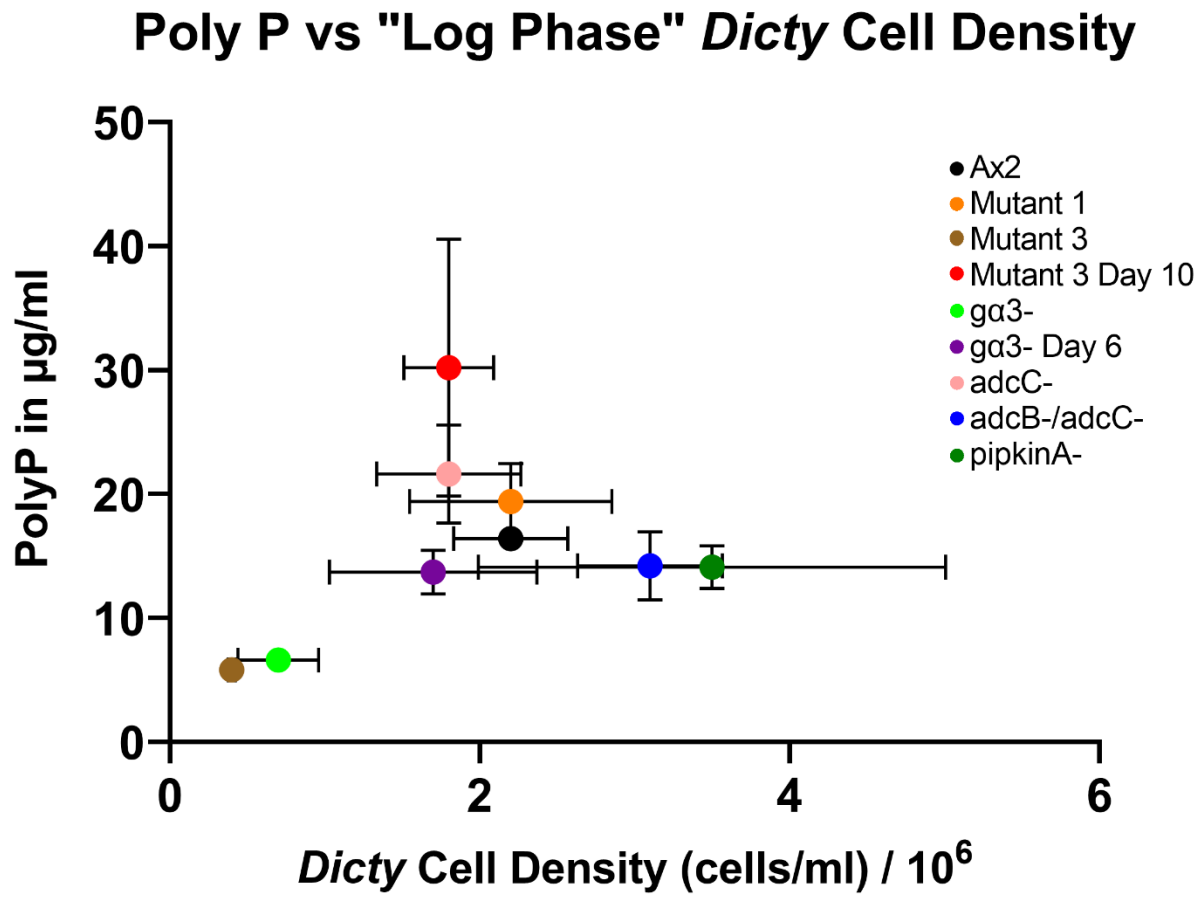

Figure 3.14: The endogenous PolyP measurement on day 3 was plotted against the measured cell density. Day 10 and day 6 PolyP values were also plotted for Mutant 3 and gα3^{-} because the cell density at these days was most similar to the log phase. Values are mean ± SEM from 3 independent experiments and the data are from figures 3.11 and 3.12. The figure illustrates comparisons of endogenous PolyP production between Ax2 and 6 mutants at different cell densities.

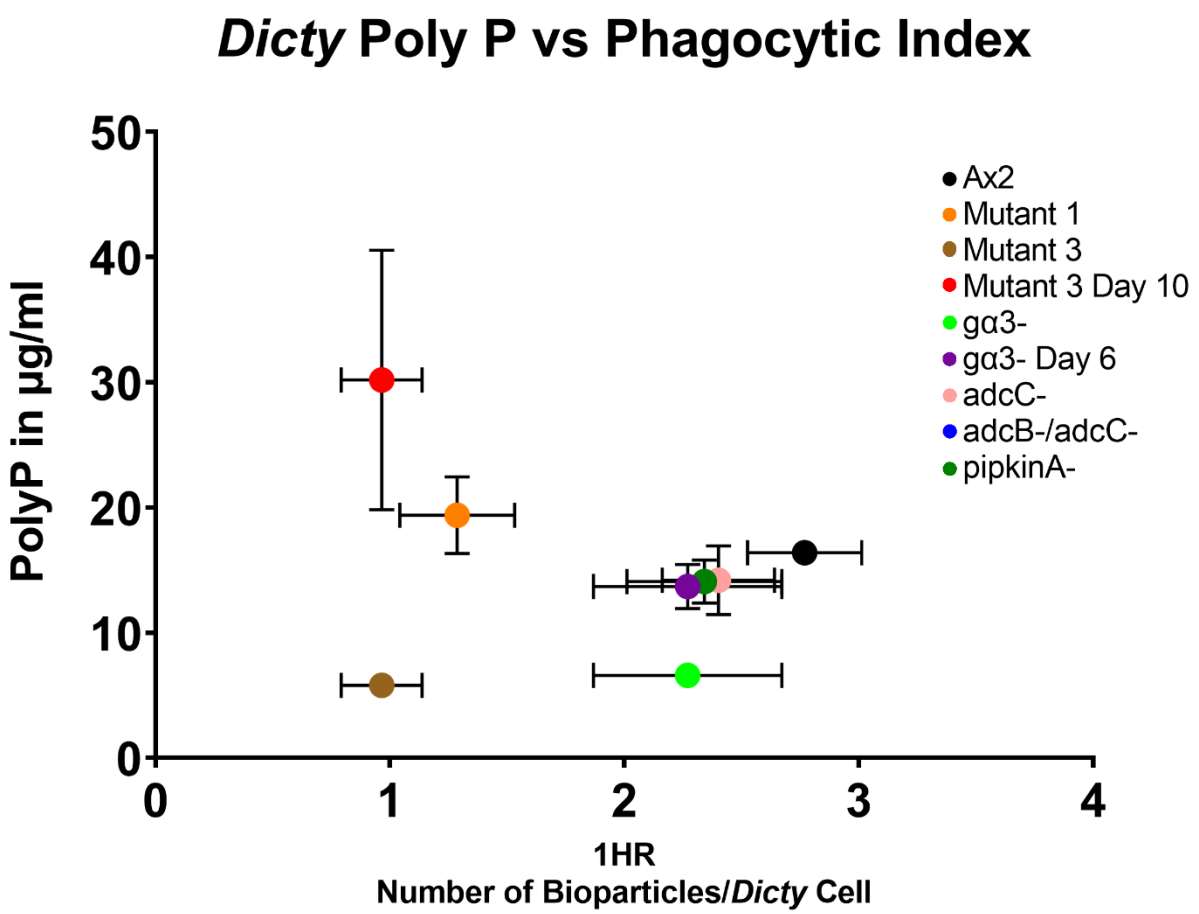

Figure 3.15: The endogenous PolyP measurement on day 3 was plotted against the number of ingested bioparticles per Dicty cell. Day 10 and day 6 PolyP values were also plotted for Mutant 3 and gα3^{-} because the cell density at these days was most similar to the log phase. Values are mean ± SEM from 3 independent experiments and the data are from figures 3.6 and 3.12. The figure illustrates comparisons of endogenous PolyP production to particle uptake for Ax2 and 5 mutants. Mutant 1 and Mutant 3 ingest fewer bioparticles per cell and produce more PolyP than Ax2 wild type.

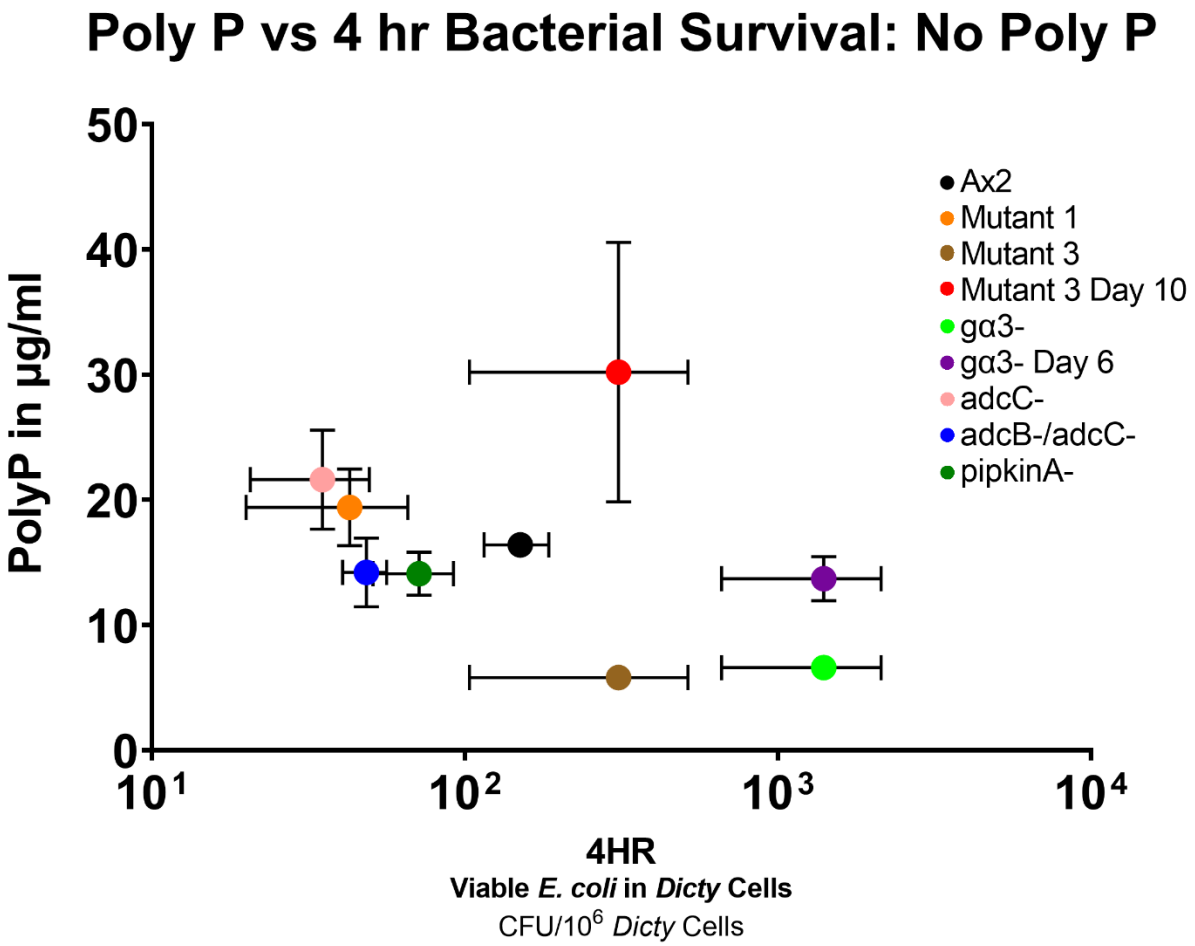

Figure 3.16: The endogenous PolyP measurement on day 3 was plotted against the number of viable E. coli per 10^6 Dicty cells at 4 hours. Day 10 and day 6 PolyP values were also plotted for Mutant 3 and gα3$^-$ because the cell density at these days was most similar to the log phase. Data are from figures 3.3 and 3.12. The figure compares endogenous PolyP production to the survival of bacteria at 4 hours for Ax2 and 6 mutants.

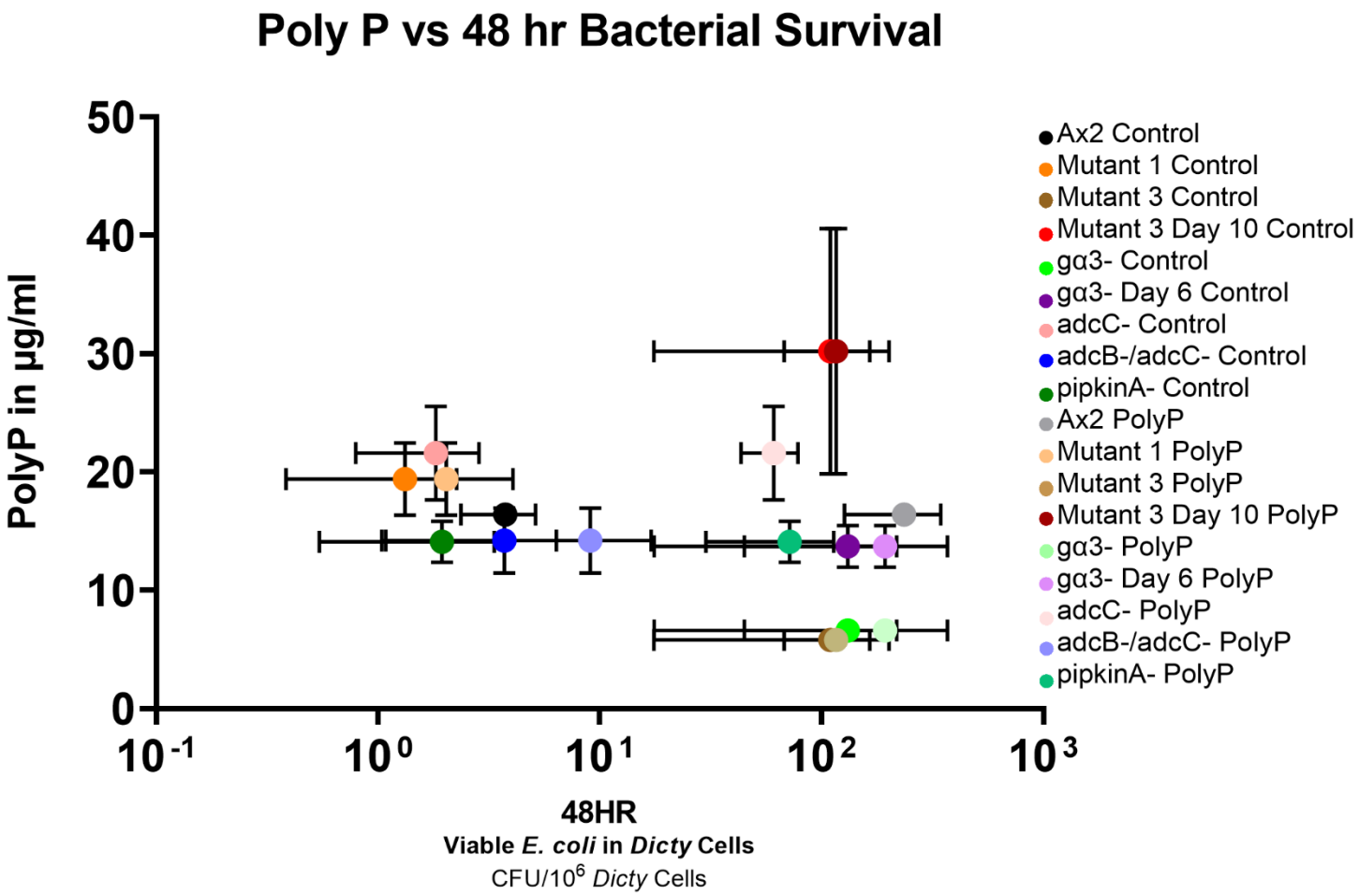

Figure 3.17: The endogenous PolyP measurement on day 3 was plotted against the number of viable E. coli per 10^6 Dicty cells at 48 hours. Day 10 and day 6 PolyP values were also plotted for Mutant 3 and gα3¯ because the cell density at these days was most similar to the log phase. Data are from figures 3.4 and 3.12. The figure compares endogenous PolyP production to the survival of bacteria at 48 hours for Ax2 and 6 mutants.

3.2 Discussion

Three proteins were identified as essential for PolyP signaling and bacterial survival in phagosomes. Mutants 1, 2, and 3 were lacking these key signal transduction proteins. Without these proteins, the mutant *Dicty* ignored the PolyP "don't kill me" signal and killed bacteria. The human homologs of the proteins that were absent from mutants 1, 2, and 3 could be inhibited in macrophages with novel drug therapies that could induce macrophages to kill intracellular pathogens. Importantly, the results further demonstrate that polyphosphate acts as an extracellular signal to protect bacteria from degradation. The majority of *Dicty* showed no response to polyphosphate at the earlier timepoint of 4 hours, which implies that polyphosphate is only a signal for long-term survival of bacteria. In fact, evidence from the phagocytic index data collected suggests that PolyP could even reduce the number of bacteria ingested rather than increasing this number at 4 hours, which indicates that PolyP likely does not induce particle uptake. If polyphosphate only potentiates long-term survival, then this matches the mechanism of pathogens like *Mtb*, which disrupt normal immune function after being ingested by macrophages (6). Biologically, it appears that polyphosphate plays a role as a conserved extracellular signal for pathogenicity because it has now been shown to induce long-term bacterial survival of *E. coli* in *Dicty* as well as *Mtb* in human macrophages (4). From these results, it can be hypothesized that polyphosphate signaling might allow for the intracellular survival of other pathogens like *Salmonella typhimurium* or *Legionella pneumophila,* for which there is already preliminary evidence (52-54).

Although the primary focus of this work was to characterize the proteins involved in PolyP and phagocytosis signaling, patterns in the data emerged that prompted further investigation. The phagocytic indices of many mutants were quantified, which shed further light

on the behavior of mutants in the bacterial survival assay. For example, the 4-hour data suggested that mutants *rasC*$^{-}$/ *rasG*$^{-}$ and *gα3*$^{-}$ both appeared to ingest more bacteria than wild-type cells (with or without polyphosphate) and yet failed to kill the bacteria effectively by 48 hours, even in control conditions. More bacteria survived in polyphosphate conditions than control conditions at 48 hours, but the fact that mutants *rasC*$^{-}$/ *rasG*$^{-}$ and *gα3*$^{-}$ retained a large number of intracellular bacteria at 48 hours even without polyphosphate suggests an inherent deficiency in these mutants' ability to kill bacteria. At 4 hours, *gα3*$^{-}$ cells contained more bacteria than Ax2 wild type, which implies that *gα3*$^{-}$ cells ingest more bacteria. However, the phagocytic index of *gα3*$^{-}$ was not significantly different from that of Ax2 wild type. In other words, *gα3*$^{-}$ cells were ingesting the same number of bacteria as Ax2, but the *gα3*$^{-}$ cells began retaining bacteria in both polyphosphate and control conditions even at 4 hours. At 48 hours, *gα3*$^{-}$ cells still have a significant number of bacteria in polyphosphate and control conditions. The retention of bacteria in *gα3*$^{-}$ cells at 4 hours, coupled with the continued intracellular survival of bacteria at 48 hours, in control conditions is consistent with a phenotype that lacks a key protein for bacterial killing. The protein Gα3 is associated with a G-protein coupled receptor, and has been shown to play a key role in cyclic adenosine monophosphate (cAMP) signaling for the early development of *Dicty* cells (19). cAMP is often cited as an intracellular secondary messenger that regulates gene expression by several well-studied phosphorylation cascades (55). However, in *Dicty* a receptor termed cAMP receptor 1 (cAR1) is utilized to detect cAMP as an extracellular signal for chemotactic aggregation and *Dicty* development (56). In humans, there is also evidence that cAMP can serve as a "third messenger" similar to the situation in *Dicty*, where it is exported from cells as a signal for other neighboring cells (57). Binding of cAMP to cAR1 serves as a positive feedback loop for cAMP synthesis and export in *Dicty*, thus *gα3*$^{-}$ cells lack

the ability to aggregate effectively and have reduced adenylyl cyclase activity (the enzyme which synthesizes cAMP) (19). The abolished activity of adenylyl cyclase results in a decrease in activity of cAMP's well-established downstream effector, protein kinase A (PKA), which is activated by cAMP and phosphorylates many other important kinases and influences transcription (58). Intriguingly, the exchange protein directly activated by cAMP (ECAP), an alternative target of cAMP, sometimes acts to either suppress or recover phagocytosis and has been shown to counter regulate PKA in certain cell types (59-61). Although cAMP has been previously posited as a master regulator of innate immune cell function which typically suppresses phagocytosis and pro-inflammatory responses, it is possible that the use of cAMP as a third messenger could act on a different pathway to increase phagocytic ability (62). In fact, in the pathogenic amoeba *Entamoeba histolytica*, which has a shared lineage with *Dicty*, the use of the pharmaceutical forskolin (FK), which acts to increase intracellular cAMP by activating adenylyl cyclase and modulates actin polymerization, increases phagocytosis (63-67). Furthermore, this matches the biology of neutrophils, which use EPAC and PKA signaling to upregulate phagocytosis and F-actin polymerization (59, 68, 69). The impact of Gα3 on these different downstream effectors has yet to be characterized, but my results indicate that Gα3 is involved in a cAMP-phagocytosis pathway similar to that of *E. histolytica* and human neutrophils.

On the other hand, *rasC*$^{-}$/ *rasG*$^{-}$ has a higher phagocytic index than Ax2 wild type and the addition of polyphosphate increased the uptake of particles even further. Due to an increased uptake of particles, the high numbers of bacteria surviving in *rasC*$^{-}$/ *rasG*$^{-}$ cells could be attributed to an overwhelming number of ingested bacteria, not an inherent deficiency in bacterial killing. It is unclear if *rasC*$^{-}$/ *rasG*$^{-}$ are truly sensitive to PolyP as a long-term inhibitor

of bacterial killing, or if the *rasC*$^{-}$/ *rasG*$^{-}$ cells simply consume more bacteria in the presence of PolyP because the addition of PolyP increased the number of beads per *rasC*$^{-}$/ *rasG*$^{-}$ cell. The RasC and RasG proteins are monomeric small GTPases that function as molecular switches, cycling between active GTP-bound and inactive GDP-bound states (70). Guanine-nucleotide-exchange factors (GEFs) activate Ras proteins, and inactivation of Ras proteins is catalyzed by GTPase-activating proteins (GAPs) that stimulate the hydrolysis of the bound GTP to GDP. The RasC protein in *Dicty* has also been connected to cAMP signaling by GPCR activation of adenylyl cyclase and downstream regulation of G-protein-coupled serpentine receptor activation of the phosphatidylinositide 3-kinase (PI3K) pathway, but not the mitogen-activated kinase (MAPK) pathway (23, 24). Studies of *rasG*$^{-}$ cells have found only partial inhibition of cAMP signaling and delayed aggregation, implying that RasC is more important than RasG for the cAMP pathway (24). No evidence of Ras participation in phagocytosis signaling has been found in *Dicty*, despite recent discoveries that *rasG*$^{-}$ cells have decreased macropinocytosis and *rasC*$^{-}$ cells have an increased rate of endocytosis (71, 72). Perhaps this is because RasG and RasC more actively regulate pseudopod formation and cytoskeletal rearrangements that are necessary for cell motility rather than the F-actin polymerization to form phagocytic cups (73, 74). The data from the bacterial survival experiments and previous literature therefore point towards no direct involvement of RasG or RasC in phagocytosis or polyphosphate signaling, and the increased rate of endocytosis by *rasC*$^{-}$ cells in previous work coincides with the increased phagocytic index found by the particle uptake assays for the double knockout (although *rasC*$^{-}$ cells themselves were found to have a decreased phagocytic index relative to control) (72).

Both *gβ*$^{-}$ and *pipkinA*$^{-}$ mutants showed a decrease in the percent of cells that ingest bioparticles in the presence of PolyP. This decrease in ingestion, however, did not appear to have

an impact on the long-term survival of bacteria in the *gβ*$^{-}$ and *pipkinA*$^{-}$ cells as shown in Figure 3.4, because PolyP remained a potent inhibitor of bacterial killing at 48 hours for both *gβ*$^{-}$ and *pipkinA*$^{-}$. As such, neither Gβ nor PipkinA proteins were found to be involved in PolyP signaling.

Dicty lacking phospholipase C (PLC) showed an increase in bacteria in the presence of PolyP at 4 hours. However, this did not coincide with the phagocytic index data, which indicated that there was no impact of PolyP on number of bioparticles ingested per cell, and in fact showed that PolyP decreased the percent of *plC*$^{-}$ cells with ingested bioparticles. Nevertheless, *plC*$^{-}$ cells are sensitive to the PolyP signal even at 4 hours and continue to retain bacteria at 48 hours in PolyP conditions. The response of *Dicty* lacking PLC indicates that the protein is not required to sense PolyP. The general lack of correlation between the 4-hour bacterial survival data and that of the 1-hour phagocytic index results is shown in Figure 3.8, indicating that the 4-hour data is not reflective of particle uptake but rather represents an early stage of bacterial digestion.

PolyP added after 4 hours (Fig. 3.9) continued to act as a “don’t kill me” signal for bacteria in *Dicty*. Importantly, these data illustrate the specificity of the PolyP signal as an inhibitor of long-term bacterial degradation because all extracellular bacteria have been eliminated prior to 4 hours due to the antibiotic gentamicin. The addition of PPX1 to Ax2 cells increased the ingestion of particles at 1 hour and the number of bacteria at 4 hours, and yet did not increase the number of bacteria that survived at 48 hours. Since the recombinant exopolyphosphatase from *S. cerevisiae* was added without any exogenous PolyP, and the *E. coli* selected for the bacterial survival assays do not produce any PolyP, the PPX1 was likely degrading endogenous PolyP produced by the Ax2 cells (75). PPX1 increased the number of ingested bacteria at 4 hours but did not affect long-term bacterial survival. In other words, the

data suggests that endogenous PolyP does not play a significant role in the bacterial digestion of non-pathogenic *E. coli* but affects the engulfment of *E. coli* into phagosomes. Furthermore, when measuring the endogenous production of PolyP by *Dicty*, the inhibitory effect of short-chain PolyP on particle uptake was supported since mutants with low phagocytic indices produced more PolyP. These findings point to a unique discovery: bacterial polyphosphate was shown to inhibit phagocytic killing without impacting ingestion whereas host polyphosphate appears to oppose this by inhibiting particle uptake but not bacterial digestion. From these results, it is likely that *Dicty* PolyP affects other cellular processes such as cytoskeleton polymerization or membrane dynamics, which impact the ingestion of particles but do not influence the downstream signaling of phagocytosis.

4. CONCLUSION

Polyphosphate and phagocytosis signaling, while interconnected, comprise two distinct and complex transduction pathways. The development and use of a novel *Dictyostelium* genetic screen as an avenue for understanding host-pathogen interactions led to the discovery of a G-protein α subunit vital to the phagocytosis pathway in *Dicty* and three potential targets for drug therapies against intracellular pathogens like *Mycobacterium tuberculosis*. *Dicty* mutants that produced the most short-chain polyphosphate were deficient in particle uptake, illustrating that host polyphosphate production inhibits particle ingestion, which can be alleviated by exopolyphosphatase. Furthermore, testing the time dependence of polyphosphate as an extracellular signal validated the inhibitory effect of long-chain polyphosphate on bacterial killing by phagocytes. The conserved biology of polyphosphate across species alludes to a shared and ancient history which is slowly being uncovered. As such, the polyphosphate signaling pathways have yet to be completely defined. However, future work in human macrophages utilizing gene deletions of identified *Dictyostelium* homologs is likely to provide new insights into the regulation of innate immunity by polyphosphate that can inform the creation of novel therapeutics and a deeper understanding of the universal molecular biology of polyphosphate.

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APPENDIX: CONSENT FOR USE OF DATA

I, *Te-An Chen*, hereby acknowledge that I have given my permission and consent for *Ryan Rahman* to use my data from Dr. Gomer's Lab in his Undergraduate Research Scholar Thesis.

Te-An Chen
Ph.D. Student | Zhao Laboratory
Department of Biomedical Engineering
Texas A&M University

Figure A.1: The figure depicts the expressed consent for the use of phagocytic index data collected by Te-An Chen.

Consent to Share Data

I, Shiyu Jing, do give permission for Ryan Rahman to use my data obtained as a part of the Gomer lab in his Undergraduate Research Scholar Thesis.

X Shiyu Jing
March 26, 2021

Figure A.2: The figure depicts the expressed consent for the use of PolyP data collected by Shiyu Jing.