

Investigating the Role of the Type VI Secretion System in the Rhizobacterium *Azospirillum* *brasiliense*

By

Sean Matthew Smith

Dr. Gladys Alexandre (Advisor)

Biochemistry Senior Honors Thesis

The University of Tennessee, Knoxville

Biochemistry, Cellular, and Molecular Biology Department

Chancellor's Honors Program

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1. ABSTRACT

Bacteria possess a diverse array of secretion systems that enhance their ability to survive in many environments through the production of effector molecules. One of the most recently discovered of these systems is the type VI secretion system (T6SS), which was identified through studies of *Vibrio cholerae* and *Pseudomonas aeruginosa* in 2006. Although best characterized in pathogenic relationships such as the interspecific competition of bacteria where killing via the T6SS is contact dependent, the T6SS is now known to mediate other biological relationships such as symbiosis between bacteria and eukaryotic hosts. *Azospirillum brasilense*, a well-studied soil diazotroph that colonizes the plant roots of important agricultural crops, is one example of a species in which the T6SS may serve a nonpathogenic function. The T6SS in *A. brasilense* is upregulated by the plant hormone auxin, which suggests its involvement in a bacteria-plant symbiotic relationship. However, no evidence currently exists to rule out the possibility that the *Azospirillum* T6SS is involved in bacterial competition, perhaps with other root colonizing bacteria. Here we attempt to describe the role of the T6SS in *A. brasilense* through mutation of ClpV, a AAA+ ATPase essential to T6SS function, and characterization of the mutant phenotype.

2. INTRODUCTION

Azospirillum brasilense is a free-living diazotroph that colonizes plant roots and lives in the rhizosphere in the soil. *Azospirillum* species are economically important due to their ability to promote the growth of many important agricultural crops and are found as components of biofertilizers. In addition to the fixation of nitrogen, *Azospirillum* is capable of the production of a number of plant signaling molecules, such as nitric oxide, auxin, and phytohormones, which are responsible for many of *Azospirillum*'s growth-promoting properties [2]. The role of auxin, a plant developmental hormone, is especially important in plant-microbe interactions and can act as a reciprocal signaling molecule, mediating plant growth and microbial symbiosis [7]. While the study of *Azospirillum* species has proven fruitful for agricultural industries, research on this organism has also helped to elucidate basic bacterial processes, for example cell signaling, chemotaxis, biofilm formation, and motility [8-11]. A recent study published in *Microbial Ecology* reported that the exogenous addition of indole-3-acetic acid to *A. brasilense* resulted in a broad transcriptional response of transport and cell surface proteins [12]. One of the most

interesting observations of this study was that IAA resulted in the upregulation of type VI secretions system (T6SS) genes in *A. brasilense*. Because the T6SS is best characterized in pathogenic relationships, this observation raised the question of this system's role in *Azospirillum* behavior and ecology.

Seven secretion systems have been identified in bacteria, with the T6SS being one of the most recent additions. These secretion systems are involved in a number of diverse biological processes, from signaling to intercellular and host interactions to virulence [13-17]. The T6SS is a multiprotein secretion apparatus assembled across the inner and outer membranes of gram-negative bacteria, perhaps most notable for its similarity in many respects to components of bacteriophage proteins which suggest a shared evolutionary origin [4, 18, 19]. The T6SS was first characterized in 2006 [20, 21] and has since come to represent an important pathogenic weapon in many species, some of which include *Escherichia coli*, *Acinetobacter baumannii*, *Pseudomonas fluorescens*, *Campylobacter jejuni*, *Agrobacterium tumefaciens*, and *Salmonella gallinarum*, among others [22-29]. Despite its obvious role in virulence, T6SS gene clusters are present in over a quarter of gram-negative bacteria [30], many of which are not known pathogens, and studies are appearing which begin to describe the T6SS in a diverse array of nonpathogenic roles [31]. Because *Azospirillum* is not recognized to be pathogenic its T6SS may be involved in other interesting interactions that could be important in plant root colonization or interspecies relationships with other bacteria. To explore these possibilities, we sought to describe the T6SS in *A. brasilense* through the characterization of a mutant strain deficient in type VI function.

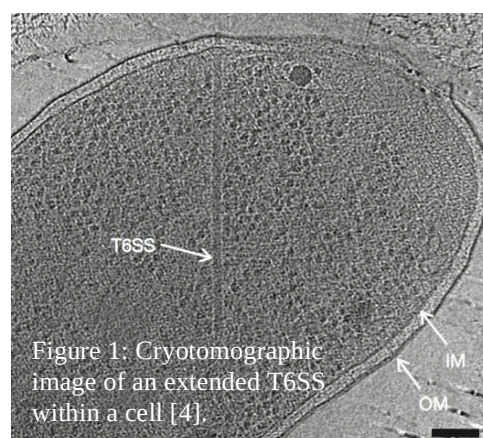
One of the essential components of a functional type VI apparatus is the ClpV ATPase, which remodels dimeric VipA/VipB tubular complexes, a required process in the type VI contraction and secretion cycle [32-34]. Through allelic exchange mutagenesis mutants were

constructed in which the *clpV* gene was deleted in order to produce a type VI-deficient *A. brasilense* strain. A number of experiments were then conducted to characterize the phenotype of these $\Delta clpV$ cells in comparison to wild type cells. The cells were observed in liquid and solid culture and differences in growth, motility, and colony morphology were noted. The plant hormone indole-3-acetic acid (IAA) was used additionally because IAA is known to upregulate the type VI genes in *Azospirillum*. Because *A. brasilense* is also known to produce auxin, the cells were tested for their auxin production abilities. Finally, competitive growth assays and root colonization assays were performed in order to describe the mutant cells' interactions with plants and other bacteria.

3. BACKGROUND

In 2006 John Mekalanos and colleagues at Harvard Medical School published two articles reporting the identification of virulence loci in two pathogenic bacteria, *Pseudomonas aeruginosa* and *Vibrio cholerae*. Although clues suggestive of a “type VI” secretion system had been present in the literature since the 1990s, these two papers were the first to explicitly characterize and tentatively recognize the type VI secretion system, as it is now understood. Mekalanos and colleagues reported the identification of a virulence locus in *P. aeruginosa* that likely functions in chronic infections, and detected hemolysin co-regulated protein 1 (Hcp1) in the blood sera of cystic fibrosis patients along with Hcp1-specific antibodies [21]. The second important paper published by the same group characterized a virulence locus in *V. cholerae* associated with contact dependent cytotoxicity against a *Dictyostelium* host model, and described VgrG as an effector protein of this “virulence-associated secretion” system [32]. Hcp1 and VgrG are now recognized to be required components of the T6SS.

A full T6SS is composed of several different protein components and is built spanning the inner and outer membranes of gram-negative bacteria. *In vivo*, the T6SS can be quite large. An electron cryotomography image of a T6SS in a live cell depicting its size is presented in Figure 1 [4]. Variation has been observed in the composition of type VI secretion systems between different species with the number of components ranging from 12 to 20 and some species harbor multiple type VI gene clusters [13, 35]. Despite this variation, it is thought that the minimal apparatus is composed of 13 core components [1]. These 13 core components have been determined by systematic mutagenesis studies in *Edwardsiella tarda* and *Vibrio cholerae* and may be grouped into three categories: membrane-associated proteins, bacteriophage homologs, and proteins of unknown structure/function [5]. Type VI gene loci are often accompanied by additional accessory genes that may encode regulatory proteins or proteins of other functions. As a whole, the T6SS can be viewed as a phage injection



system, repurposed and repositioned as an injection weapon anchored to bacterial cell envelope. Figure 2 depicts the similarities between bacteriophage and T6SS injection cycles.

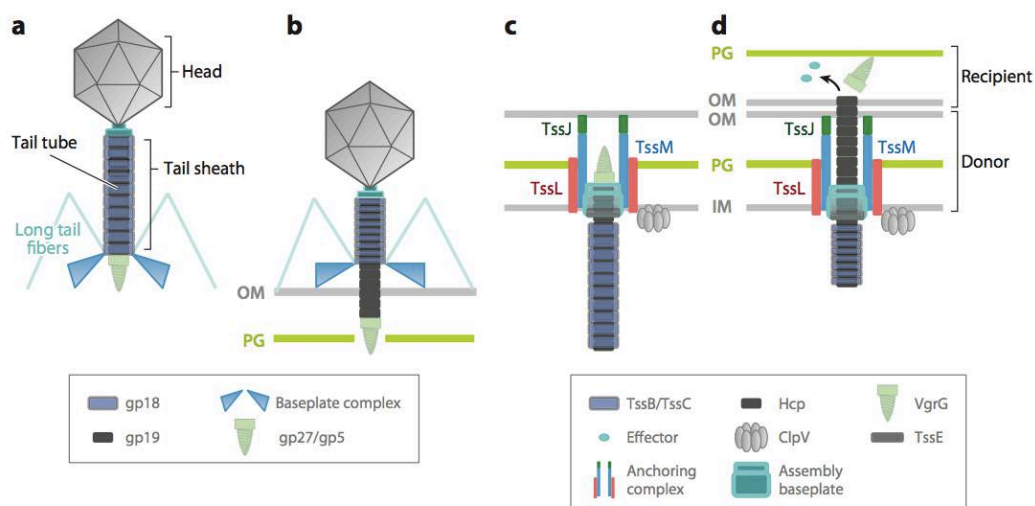
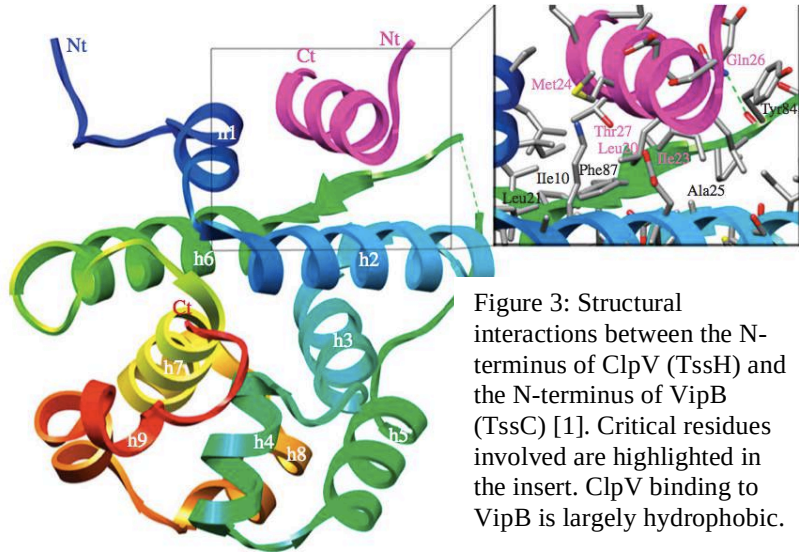


Figure 2: Diagram comparing T6SS components to their bacteriophage counterparts [5]. Homologous proteins are colored the same. Injection is driven by contraction of the sheath-like structure composed of TssB/TssC.

The aforementioned Hcp1 and VgrG proteins are essential components and most bacteria that possess a T6SS secrete these proteins into culture supernatants [13]. VgrG is homologous to the bacteriophage tail spike and as a trimer it functions as the puncturing device of the T6SS. Hcp proteins form large hexameric oligomers within an open pore space of sufficient diameter to allow passage of type VI substrates [1, 36]. The Hcp ring forms a tubular structure, which is surrounded by a sheath that is composed of VipA/VipB dimers. The VipA/VipB dimers form a contractile sheath around the Hcp tube and together these components are the equivalents of the bacteriophage tail sheath, tube, and spike [32, 37]. Other essential components are involved in forming the baseplate that anchors the contractile structure to the cell envelope. Two of the components of this system are related to the type VI secretion system, TssM and TssL (“type-six-secretion” M and L), while the other protein, TssJ, has no known homology [38]. Aside from the ClpV AAA+ ATPase, the remaining core components are either putative cytosolic proteins or soluble proteins, some of which share homology with bacteriophage proteins and are involved in type VI assembly and other functions. These include TssA, TssE, TssF, TssG, and TssK [1]. Despite much initial controversy, it is now clear that ClpV comes into play during the type IV contraction cycle [39]. ClpV is a chaperone ATPase of the heat shock protein family involved in protein quality control mechanisms and serves to disassemble the contracted form of the type VI secretion apparatus in order to prepare it for further rounds of firing. Post-contraction, ClpV interacts specifically through its N-terminal domain with VipB to oligomerize and disassemble the VipA/VipB dimer, a remodeling step that is required for tubule removal and recycling of the secretion apparatus [33, 34, 40]. This ATP-dependent recycling process provides the energy for type VI firing by allowing a cell to ‘reaccess’ the energy stored in a contracted type VI

apparatus; in this way ClpV mediates T6SS turnover [39]. The unique largely hydrophobic N-terminal interaction of ClpV with VipB is depicted in Figure 3 from Cascales and Cambillau [1].

Inferences have been drawn into the mechanism of T6SS injection based on the architecture of the multiprotein apparatus, the likely functional similarity of components that are homologous to phage proteins, and through experiments that directly



observe the T6SS in action. Experiments conducted by Mekalanos and colleagues in 2012 have proved important in verifying mechanistic models of type VI action proposed by others. Here

Mekalanos and colleagues examined the *V. cholerae* T6SS through time-lapse fluorescence light microscopy by labeling the VipA protein with superfolder green fluorescent protein [4]. Their experiments demonstrate that the type VI secretion cycle proceeds through stages of assembly, quick contraction, disassembly, and re-assembly, and thus validate a mechanistic model in which the

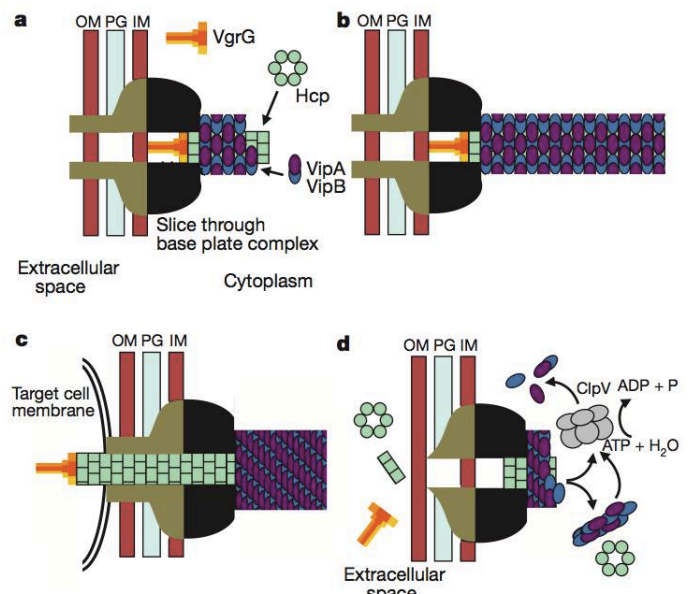


Figure 4: Proposed model of the type VI firing cycle [4]. Assembly to the extended conformation (a-b) is followed by contraction; “firing” (c). ClpV-dependent disassembly (d) prepares the system for a second firing round.

T6SS proceeds through firing cycle in a swift, dynamic manner. Their model is presented in Figure 4. More recent experiments using similar techniques have revealed other interesting features of the type VI secretion apparatus. In late 2012, for example, Mougous and colleagues reported that type VI activity in one species raised the susceptibility of this species to being killed by the T6SS of a heterologous species [41]. In 2013, Mekalanos and colleagues reported that type VI activity in either *V. cholerae* or *Acinetobacter baylyi* prey species directly stimulated a counterattack from an opposing *P. aeruginosa* T6SS [42, 43], a phenomenon which they have termed “T6SS duelling” [42]. These observations suggest that cells can sense type VI mediated activity and that T6SS activity is regulated by cell contact.

As these experiments suggest, T6SS activity to date has largely been implicated in pathogenicity and virulence, with type VI secretion systems serving to inject toxic effector proteins into other bacteria or eukaryotic hosts. The type VI toxic effector proteins of *P. aeruginosa* were characterized by Mougous and colleagues in 2011, for instance, and exert their effects through peptidoglycan degrading activity in target cells [22]. These type VI effector proteins (Tse1-3) have been well characterized and *P. aeruginosa* protects itself from these toxins by encoding cognate immunity proteins [22, 44, 45]. Eric Cascales and colleagues have documented through fluorescence time-lapse microscopy the contact-dependent killing of *E. coli* prey by pathogenic strains of *E. coli*, which is depicted in Figure 5 [46]. These time-lapse images

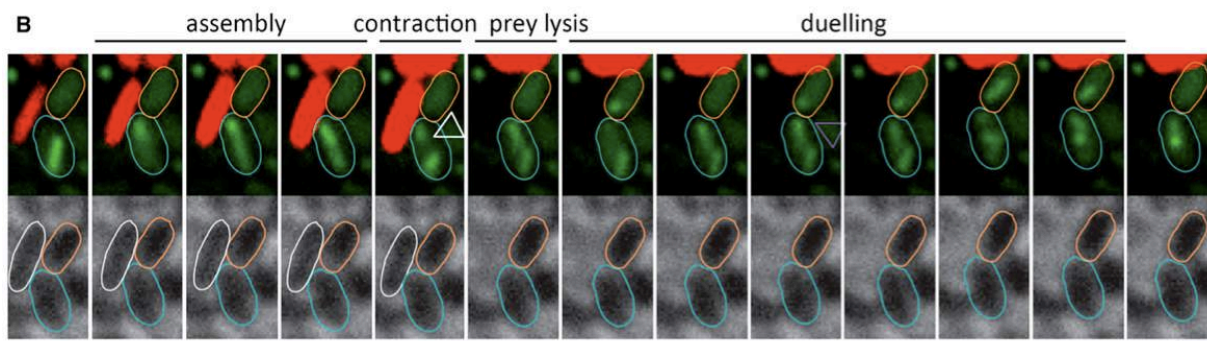


Figure 5: Time-lapse fluorescence microscopy of a T6SS-dependent killing event between enteroaggregative *E. coli* (EAEC) predator cells (green) and nonpathogenic *E. coli* prey cells (red) [3].

demonstrate the rapidity and efficiency of T6SS-dependent killing. Although the T6SS clearly is an important player in the virulence of many pathogens, it is also clear that it is involved in a number of non-pathogenic relationships. This is most apparent through its wide prevalence throughout gram-negative bacteria; indeed, type VI gene loci are present in over 100 sequenced genomes [13] and studies have demonstrated that the T6SS may be important in antipathogenesis, commensal relationships, and mutualisms [31, 47]. One study of *Helicobacter hepaticus*, for instance, demonstrated that the T6SS is involved in reducing inflammation during infection and thus serves to establish a balanced host relationship [48]. Nevertheless, studies are still desired to provide a more complete picture of the diverse roles the T6SS may be employed in.

Another interesting role for the T6SS was described unwittingly in 2003 by a research group working in the Netherlands that reported that the *imp* gene locus (for impaired in nodulation) in *Rhizobium leguminosarum* was responsible for secreting proteins that blocked *Rhizobium* infection of pea plants, thus limiting symbiosis and influencing host specificity [49]. One of the effector proteins was identified as a periplasmic ribose-binding-like protein, however its method of action has remained unclear [50]. Like *R. leguminosarum*, *Azospirillum brasilense* (Figure 6) is a plant growth promoting nitrogen-fixing bacterium of the alphaproteobacteria class and one example of an organism in which the T6SS serves potentially nonpathogenic functions [51, 52]. However, the only

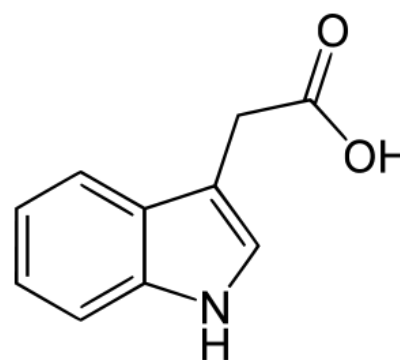
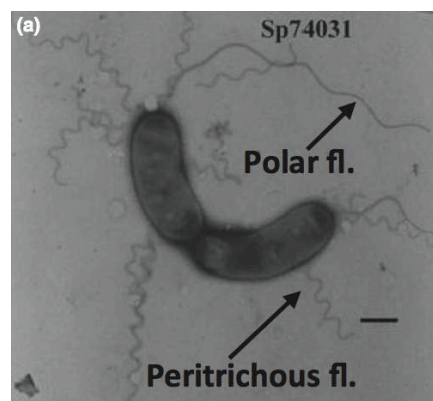


Figure 6 (above): *Azospirillum brasilense* [2].

Figure 7 (below): Chemical structure of the plant hormone indole-3-acetic acid (IAA), also known as auxin [6].

publication to date discussing the T6SS in *A. brasilense* reported results from a transcriptome analysis that the type VI gene locus was upregulated in the presence of exogenously added indole-3-acetic acid [12]. IAA (Figure 7) is an important plant hormone with diverse effects, such as stimulating cell division and enlargement, triggering vascular tissue differentiation and root initiation, delaying fruit ripening, and promoting flowering [53]. Here, based on their observations of the IAA-dependent upregulation of the T6SS and other gene expression changes the authors concluded, “IAA provides a means of communication in *Azospirillum*-plant interactions”.

Given these observations, we sought to characterize *A. brasilense* mutants deficient in T6SS activity to describe the role of this novel secretion system in *A. brasilense*. Drawing from current knowledge of T6SS function in *R. leguminosarum* and the observations that T6SS activity is contact dependent and involved in symbiotic relationships we hypothesized that the T6SS in *A. brasilense* could influence its symbiosis with plant roots. Another possibility is that the *Azospirillum* T6SS is involved in interbacterial interactions with other members of its species or other species and that these interactions are ecologically adaptive. Consequently, impairing the *Azospirillum* T6SS should impact these relationships or behaviors involved in interaction.

4. METHODS AND RESULTS

The first step taken in this project was the generation of a mutant strain of *Azospirillum brasilense* that was deficient in the type VI secretion system. Mutants were desired in order to elucidate the role of the T6SS in *A. brasilense* through an understanding of what functions were lost in the absence of this system. This was accomplished by allelic exchange mutagenesis targeting the gene for the ClpV ATPase, a protein whose function is required for type VI activity. Mutants were selected and two mutants were verified by sequencing to be used in further

experiments and designated as *clpV*₃ and *clpV*₉. The mutagenesis was carried out with help from the graduate student Priyanka Mishra.

Once the mutants were constructed a number of experiments were carried out to examine the phenotype and behavior of the mutant $\Delta clpV$ strains in comparison to the wild type Sp7 strain. These included growing the cells on different solid media and in different liquid cultures and observing different growth morphologies or patterns, growing swarm plates, and growing cells with and without exogenously added auxin, which is known to up-regulate T6SS genes in *Azospirillum*.

First, cells were grown in liquid media and examined under the microscope to observe if there were any gross observable differences between the wild type and mutant cells. These experiments were combined with cultures that had been induced with varying concentrations of the plant hormone indole-3-acetic acid, dissolved in water. The IAA stock solutions were prepared by dissolving different quantities of solid IAA crystals in water and the inductions were conducted by adding different volumes of IAA solution to cells in media. Because auxin is light and temperature sensitive, the IAA stock solutions were wrapped in foil and stored at -20°C. Solutions of 10mM (0.175g IAA in 10mL H₂O) and 1mM (0.0175g IAA in 10mL H₂O) were prepared and used to induce cell cultures. One of the first observations was of the appearance of clusters of cells of varying shapes and sizes. The clusters appear to be aggregates of cells that were stuck together to form clumps; often cells attached to the periphery of clusters could be seen continuing to swim.

The original presentation of this clumping phenotype was somewhat irregular so more systematic inductions were performed to investigate the nature of this phenotype. Because of the irregularity of results even these experiments had to be repeated many times. Experiments

conducted in April 2012 in which new inoculations were observed at 6, 25, and 44-hour time intervals and overnight cultures were induced with 1.25 uL of 100 mM IAA solution and observed at 6 hours. These tests indicated that the clumping phenotype appeared predominantly in Sp7-induced cultures only, with clusters being wholly absent from $\Delta clpV$ cultures (Figure 8; this and the remaining figures are appended after the References section). Some Sp7 cultures exhibited very large cell clusters. Repeating this experiment at a later date using a new IAA stock solution resulting in conflicting results, however, raising questions regarding problems with the IAA stock solutions such as contamination. The later experiment suggested that the clumping phenotype was not triggered by auxin but rather present only in the *clpV*-deficient cells and only at small levels because it was not observed in the Sp7 cultures. Another possibility is that the clumping phenotype and/or the role of auxin were influenced by cell aggregation which was reduced in these experiments because the cultures were less advanced in growth. These observations coupled with the fact that the clumping phenotype was inconsistently observed and somewhat challenging to elicit suggests caution in the conclusion that IAA stimulates cell-to-cell clustering in wild type, and not $\Delta clpV$, cell cultures, although this was supported by the initial observations depicted in Figure 9.

The clumping phenotype raised questions about the ability of the cells to flocculate. To probe the mutant cells for flocculation, the wild type and mutant cells were grown in flocculation media (which is a minimum medium replete in carbon but limited in nitrogen) and incubated with shaking at 28°C, conditions that induced cell to cell aggregation and subsequent formation of visible flocs (flocculation). This was repeated at a later date and both cultures flocculated to the same extent after approximately 24 hours of growth. Wild type and mutant cells were also grown on MMAB (minimal media) solid plates with either 20uL of Congo Red dye or 20uL

Trypan Blue dye, which can reveal differences in the polysaccharide composition of the cell surfaces, but no differences in the colonies were observed (Figure 10).

Because the clumping phenotype suggested that the T6SS function could be related to cell contact further experiments were designed to observe colony growth under conditions of contact. An experiment was performed to evaluate the growth of the mutant and wild type cells on solid media when they are in contact with one another (Figure 11). Three pairs of colonies were inoculated so that they would be in contact during their growth. The first pair was Sp7, the second *clpV*₃, and the third a combination of mutant and wild type. This experiment was repeated on TY and MMAB solid media but no substantial differences were observed. These experiments and observations made during the previous IAA-induction experiments had suggested that the mutant cells might be impaired in growth in some way. Based on these observations, further experiments were pursued to investigate if *clpV* deficiency resulted in growth impairment. Mutant and wild type cells were grown in serial dilutions on TY solid plates to demonstrate marked growth impairment for the $\Delta clpV$ strains (Figure 12) and this was confirmed by experimental repetitions.

To further test the phenotypic differences between the wild type and mutant cell strains were grown on TY and MMAB media and various observations were made such as colony size and morphology. Furthermore, some colonies were induced with IAA to test if auxin played a role in colony size or morphology. These experiments led to some interesting observations. In general, when allowed to grow for an extended period of time, $\Delta clpV$ colonies grew to a smaller ring diameter than wild type colonies, corroborating the observation that $\Delta clpV$ cells are impaired in growth. Furthermore, when observed at 7-10 days post inoculation, $\Delta clpV$ colonies were distinctly pink colored and presented different morphology patterns compared with wild

type colonies (Figure 13). No differences were observed between IAA-induced and non-induced cells.

To further investigate growth differences swarm plate assays were conducted to examine cellular motility. Wild type and mutant cells were grown on semi-solid agar media and different motility patterns were observed (Figure 14). To further elucidate the motility difference, cells were grown on semi-solid plates in the presence of malate with and without nitrogen. Plates with malate and then with and without nitrogen were used to determine if nitrogen and carbon source played a role in the altered motility phenotype. The results indicated that there was no difference in motility, however the wild type cells also grew to a larger ring size than the mutant cells (Figure 15).

To better understand the growth impairment of the $\Delta clpV$ cells a microplate reader was used to construct growth curves for the wild type and mutant strains. Interestingly, despite the previous observations that suggested impairment in growth, multiple experiments demonstrated no appreciable difference in the growth of wild type cells versus mutant cells over a 25-hour period in liquid media (Figure 16). Initially, this observation suggested that rather than growth impairment *per se* the lack of a type VI secretion system resulted in an elevated level of cell death. In order to test this possibility a live/dead assay was conducted, however the results did not clearly indicate that $\Delta clpV$ was subject to elevated levels of cellular death. Results of three independent experiments are presented in Figure 17. Repeating this experiment with a broader sample size and more exact counting methods could improve the results obtained.

It is known that *A. brasilense* is an auxin-producing bacterium and it was thought that the $clpV_3$ mutant strain might be deficient in the production of auxin. In order to test this hypothesis, Salkowski reagent was used to assay the auxin production in cells. The Salkowski reagent was

prepared by dissolving 0.811g anhydrous FeCl_3 in 10mL H_2O and then taking 1mL of this 0.5M solution and adding it to 50mL of 35% HClO_4 [54]. The Salkowski reagent reacts with indole-3-acetic acid to form a pink color that can be quantitatively visualized using a spectrophotometer at 535-530nm [55]. First, 100uL of the Salkowski reagent were reacted with known concentrations of IAA to establish a standard curve of IAA concentration (Figure 18), and the Salkowski reagent was used to assay auxin production in wild type and mutant cells. To do this, 150uL of standardized cells that had been grown overnight were added to 100uL of Salkowski reagent and allowed to react in the dark for at least 30 minutes in a 96 well microplate [55]. A microplate reader was then used to quantify the color intensity at 535-530nm [55], and a comparison with the standards curve was used to estimate the IAA concentration in the cells. This was done first over a 72 hour period for Sp7 and *clpV*₃ cultures grown in TY liquid (Figures 19 and 20), and then repeated with additional cultures that had been induced with 1uL IAA/5mL TY at the start of growth (Figure 21). The Salkowski assay was also conducted for cultures grown in flocculation media (Figure 22), and for this experiment a separate standard curve for auxin concentration was constructed using MMAB liquid media as the background. The assay in flocculation media was repeated a second time, obtaining similar results.

The results to date suggested further lines of inquiry into the adhesion and interaction of $\Delta clpV$ cells. Accordingly, a biofilm assay was conducted to probe the ability of $\Delta clpV$ cells to form biofilms. An initial experiment suggested that $\Delta clpV$ cells might be capable of forming a biofilm to a greater extent than Sp7 cells, however significant error was observed in the first experiment. The assay was repeated more carefully and demonstrated that $\Delta clpV$ cells were capable of forming biofilms to a slightly greater extent than Sp7 cells (Figure 23).

To further probe the behavior of the $\Delta clpV$ mutants in terms of cell interactions, competitive growth assays were conducted with other strains of *Azospirillum* to investigate if the absence of a functional T6SS impacted growth in the presence of heterologous strains. Cells were spread on plates and grown overnight and then inoculated with 50uL of the test strain, which was allowed to grow overnight. Each combination was repeated in reverse. These results are presented in Figure 24. Six strains were tested and differences were observed in combinations with three strains. Inoculating Sp7 or $\Delta clpV$ cells with 106-RC-4, 202-orchG-1, or 209-01G-B3 strains resulted in differences between the Sp7 and $\Delta clpV$, whereas inoculations with Alfa1B-104-1, 106-01G-3, or 209-orchG-21 strains resulted in no differences. Controls performed inoculating Sp7 against Sp7 and $\Delta clpV$ against $\Delta clpV$ demonstrated no observable difference.

The natural environment of *Azospirillum brasilense* is the soil, in which it is involved mutualistic interactions with plant roots. Accordingly, it is likely that the function of the T6SS in this rhizobacterium is involved in or mediated by plant root colonization and a possible hypothesis is that the T6SS could be involved in plant root colonization. In order to evaluate if $\Delta clpV$ is impaired in plant root colonization, an experiment was performed where both wild type and mutant cultures were inoculated with plant seeds in Fahraeus semi-solid media. The Fahraeus media was prepared with 0.4g agar, 0.1g $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 0.12g $\text{MgSO}_4 \cdot \text{H}_2\text{O}$, 0.1g KH_2PO_4 , Na_2HPO_4 , 0.005g ferric citrate, and trace amounts of sodium molybdate per 1 liter of H_2O [56]. This was boiled on a hot plate before autoclaving and boiled again before using. For the experiment, the Fahraeus semi-solid media was poured into glass test tubes. Plant seeds were sterilized through a series of washes in commercial bleach containing 6% hypochloride (Clorox), water, and ethanol. After sterilization the seeds were allowed to germinate by placing them

between two small squares of Whatmann paper over a bed of cotton in a Petri dish and storing them overnight in the dark. Germinated seeds were then planted in the Fahraeus media in the glass test tubes and 100uL of standardized cells were added to the center. Twelve test tubes were inoculated with Sp7 cells and twelve with *clpV*₃ cells. The plants were allowed to grow for 23 days in an indoor greenhouse. The plants were then analyzed for any difference in growth and morphology.

In the analysis of the plants any plants showing substantial levels of fungal growth, which are often observed with cereals even after surface disinfection, were excluded (7 of the 12 Sp7 plants and 5 of the 12 $\Delta clpV$ plants). For the remaining plants, pictures were taken and the total biomass of the plant and the root system were recorded. The length of the longest leaf and the number of total leaves were also recorded. The pictures of the root systems and plants are presented in Figure 25a-b and biomass measurements are presented in Figure 25c. The root systems were then homogenized in 3mL filter-sterilized phosphate buffer solution (PBS) in a tissue grinder and plated out onto MMAB plates (MMAB solid media + salts + ampicillin and fungicide) in serial dilutions of 10^{-2} , 10^{-4} , and 10^{-6} . The results of the serial dilutions are presented in Figure 25e. Generally the root systems of $\Delta clpV$ -inoculated plants appeared to be more elaborate than Sp7-inoculated plants (Figure 25a) however the root to total biomass ratio and longest leaf measurements were somewhat greater for Sp7 cells over $\Delta clpV$ cells (Figure 25d). On the other hand, the number of colonies recovered from the homogenized root systems was slightly greater for $\Delta clpV$ cells than Sp7 cells (Figure 25e).

5. DISCUSSION

The type VI secretion apparatus was originally identified in pathogenic species and has since come to be characterized in numerous pathogenic interactions. Intriguingly, however, the

genes required for this novel secretion system are present in a number of species that are not recognized pathogens and studies are beginning to document how the T6SS can be involved in non-pathogenic interactions. *Azospirillum brasilense* is an ideal specimen for these lines of inquiry: this non-pathogenic soil diazotroph is well studied and harbors a single type VI gene locus. Here, we sought to elucidate the function of the T6SS in *A. brasilense* through mutagenesis of one of its key components, the AAA+ ClpV ATPase. Our results indicate that the *A. brasilense* T6SS may mediate interactions depending on cell-to-cell contact.

The initial experiments presented here suggested that the *A. brasilense* T6SS influenced a cell clumping phenotype observed in liquid culture, however the results proved too inconsistent to determine conclusively any reproducible behavior (Figures 8 and 9). These and other experiments suggested that the mutant cells were impaired in growth and further experiments confirmed this (Figures 12-15). Growing Sp7 and $\Delta clpV$ cells on solid plates and in serial dilutions demonstrated that $\Delta clpV$ is impaired in growth in comparison to wild type cells. Furthermore, differences in swarm plate ring morphology were observed, suggested motility differences as well. In addition to these observations, $\Delta clpV$ cells grown for an extended period exhibited a distinct pink coloration due to carotenoid production, which is indicative of cellular stress. These observations collectively suggest that the absence of a T6SS produces varied effects that generally appear to be detrimental to cell growth and survival.

Interestingly, however, when the cells were grown in liquid culture no difference was observed in their growth to stationary phase (Figure 16). Similar results were obtained from the live/dead assay in which comparable levels of cells were observed to die for both wild type and mutant cell strains (Figure 17). These observations, when combined with the growth observations on solid media, suggest that the growth impairment and motility differences may

only manifest themselves when the cells are grown on solid media. Thus, the T6SS may be involved in growth or motility in such a way as to only impair the $\Delta clpV$ cell strain when it is grown on solid media, that is, when cell to cell contacts are more likely frequent and abundant. This observation would be consistent with the generally assumed function of bacterial T6SS and could also explain the impairment of growth seen only for the $\Delta clpV$ mutant. Indeed, Mougous and colleagues reported that type VI activity in one species raised the susceptibility of this species to being killed by the T6SS of a heterologous species [41]. This conclusion is supported by the biofilm assay and competitive assay results. The biofilm assay indicates that the $\Delta clpV$ cells possess a slightly improved ability to form biofilms over the wild type cells (Figure 23) and the competitive growth assay revealed that the $\Delta clpV$ cell strains responds differently to growing in combination with three heterologous strains, 106-RC-4, 202-orch-G-1, and 209-01G-B3 (Figure 24). Together, these results suggest that the T6SS in *A. brasilense* mediates intercellular relationships during growth between bacteria species.

Observing auxin production in the Sp7 and $\Delta clpV$ cells resulted in no appreciable difference in auxin levels (Figures 18-20). The same result was obtained when the analysis was performed in flocculation media or in cells induced with auxin prior to growth (Figures 21-22). These results suggest that the T6SS likely does not play a role in auxin production even though its activity is known to be stimulated by the action of auxin as a signaling molecule.

The inoculations of plants with wild type and mutant cells produced interesting results. Because *A. brasilense* is normally found colonizing plant roots and plants utilize auxin as a signaling hormone, plant root colonization is a potential process in which the *A. brasilense* T6SS may be involved. In general, plants inoculated with $\Delta clpV$ and allowed to grow for an extended period of time presented elaborate root systems, however the total root biomass was slightly

greater for Sp7-inoculated plants, as was the ratio of root system to total plant biomass (Figure 25). Of significance is the observation of a difference in the length of the longest leaf. This suggests that the different root morphologies have an effect on the above ground tissues and growth. Although Sp7-inoculated plants exhibited a slight advantage in growth based on the biomass and leaf measurements, serial dilutions of root system homogenate revealed that $\Delta clpV$ -inoculated plant roots harbored more cells than Sp7-inoculated plants. Although replicas were conducted for both cell strains, these experiments could be repeated or conducted with different plants to improve the results. Nevertheless, the results presented here tentatively provide a link between the T6SS and the ability of *A. brasilense* to colonize plant roots.

Collectively, the results reported here are consistent with other studies of the T6SS that demonstrate that its activity is contact dependent. In this case, the *A. brasilense* T6SS may be involved in contact dependent interactions with other bacteria or with eukaryotic host plants. Further experiments could examine in more detail the interactions between *A. brasilense* and other bacteria or plant root systems. Fluorescence microscopy of fluorescently tagged Sp7 and $\Delta clpV$ cells could elucidate more exactly what interaction is taking place. It would also be interesting to perform competitive growth assays with pathogenic bacteria, such as pathogenic *E. coli* strains. Another possibility is fluorescently labeling type VI components and observing T6SS activity *in vivo*, perhaps through the application of auxin. Finally, more competitive root colonization assays would be useful in determining if there is a substantial difference in plant growth and bacteria survival in the absence of a T6SS. Understanding the role of the T6SS in *A. brasilense* will not only enhance our knowledge of this important plant growth promoting bacteria but will also improve our knowledge of the diverse applications of the T6SS.

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Figure 8: Original experimental images in control and IAA-induced cultures.

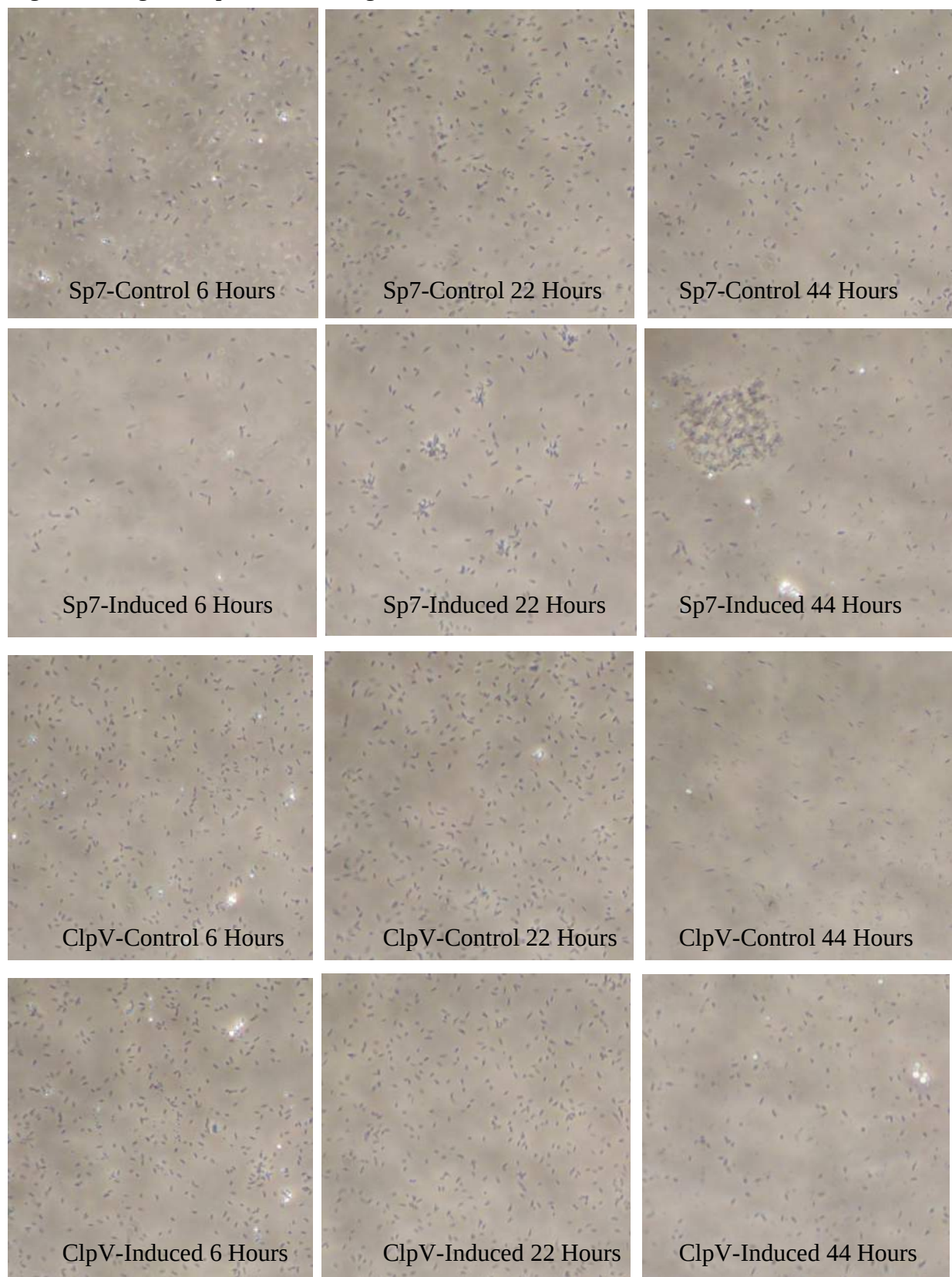


Figure 9: Control and IAA-Induced cultures from a second experiment in which the clumping phenotype was demonstrated only in Δ ClpV cultures, conflicting with the original data.

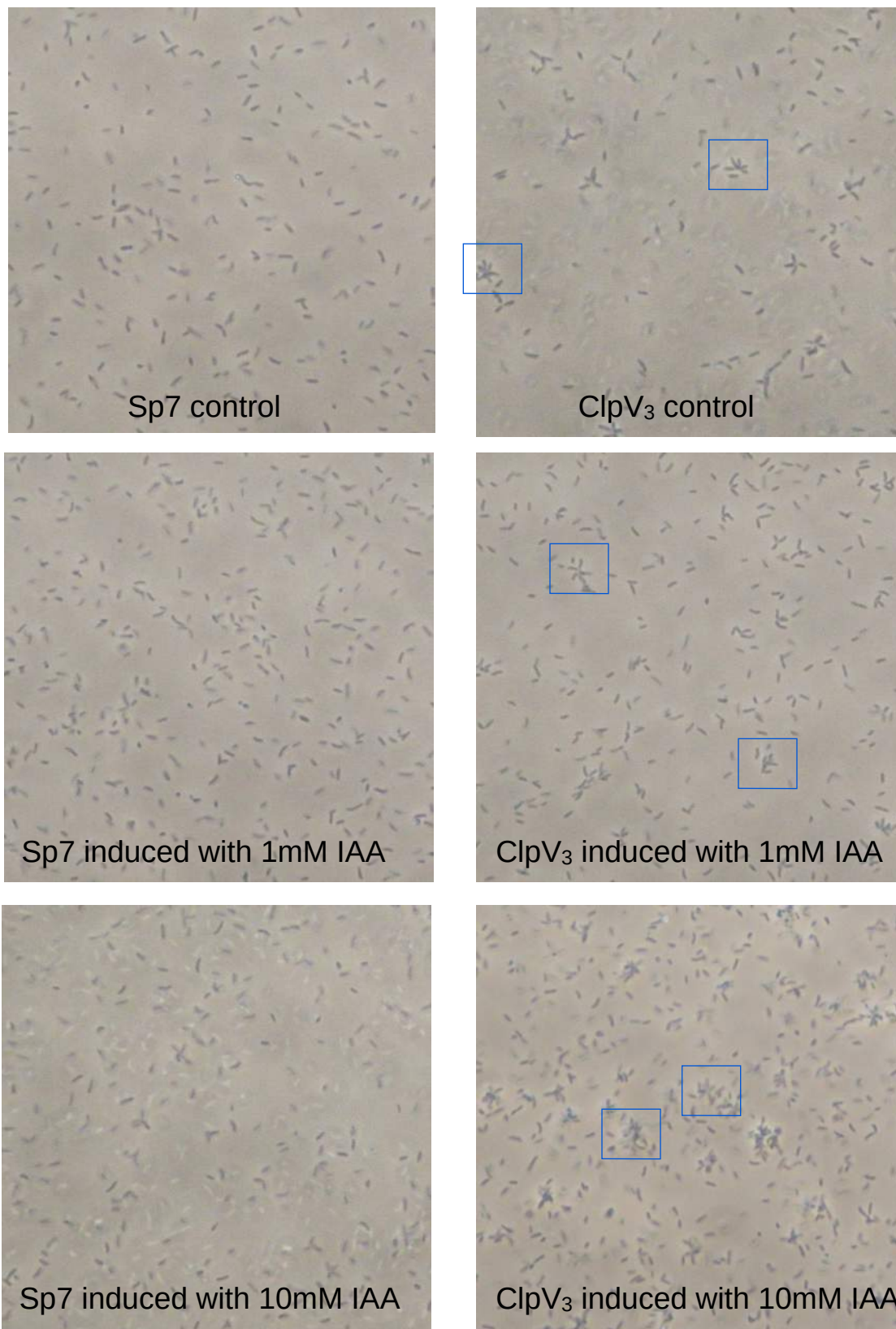


Figure 10: Congo Red (left) and Trypan Blue (right) plates with Sp7 and ClpV₃ cells. 20uL of dye were added to 20mL of MMAB solid.

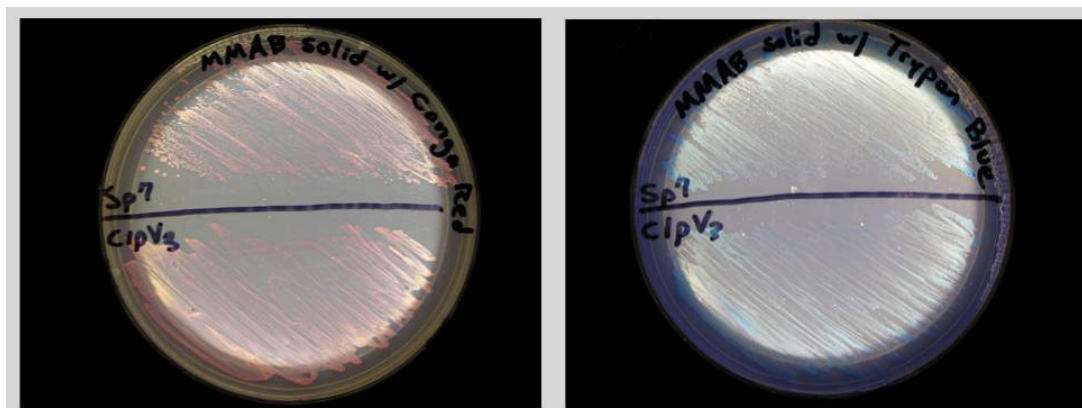


Figure 11: Figure 5: TY solid plate (left) and MMAB solid plate (right) with cells grown in contact with one another. The leftmost pair is Sp7, the middle ClpV₃, and the rightmost pair is a combination of Sp7 (top) and ClpV₃ (bottom).

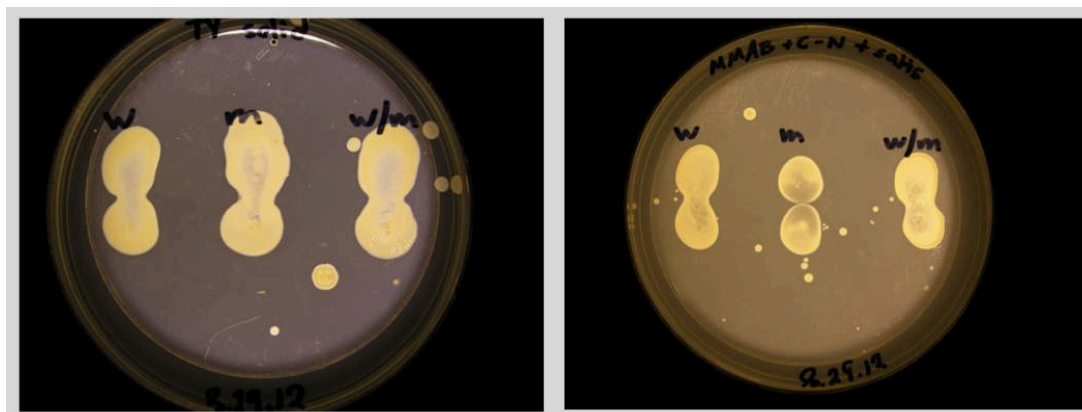


Figure 12: The Δ ClpV mutant strain is impaired in growth on TY solid media. Dilution plates are illustrated comparing the growth of wild type (Sp7, left plate) and Δ ClpV (right plate). Sp7 colonies grow at 10^{-4} and 10^{-5} dilutions while no Δ ClpV colonies grow for these dilutions at the same time interval.

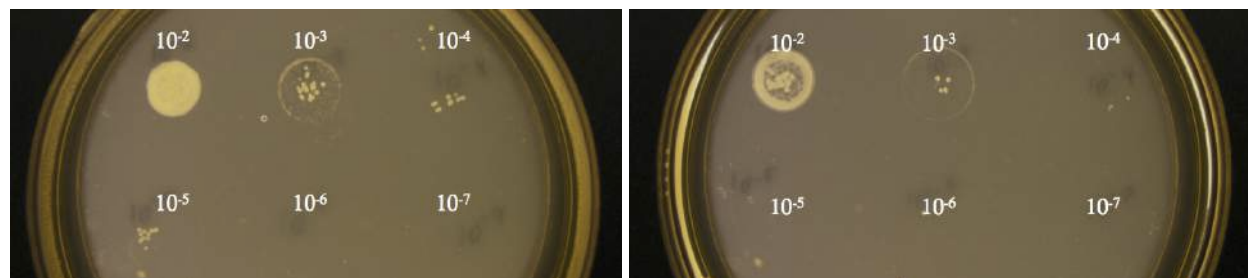


Figure 13: Cells grown on TY solid media for an extended period exhibit differences in colony color and morphology indicative of cellular stress.

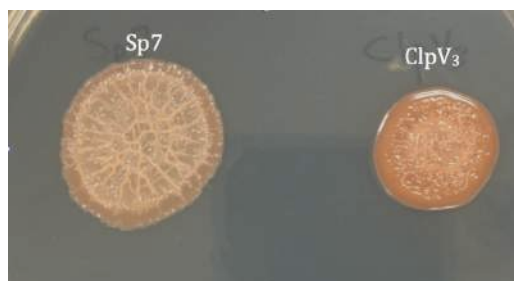


Figure 14: TY semi-solid swarm plates depicting differences in ring size and motility; pictures taken after 24 hours growth.

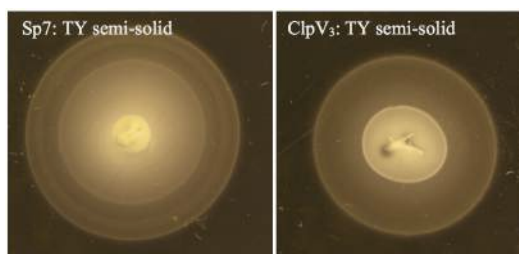


Figure 15: MMAB swarm plates with and without malate and nitrogen (pictures taken at 72 hours). While there was little noticeable difference in motility, a consistent difference in ring size was observed with Sp7 having larger rings.

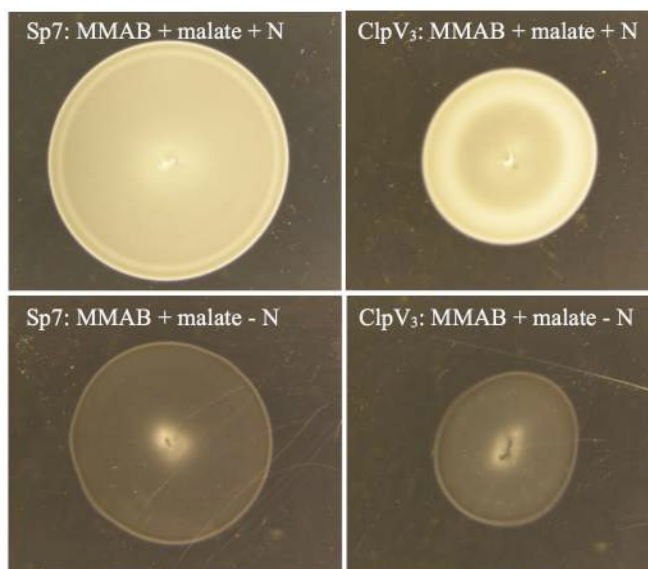


Figure 16: Growth curve data from three independent experiments.

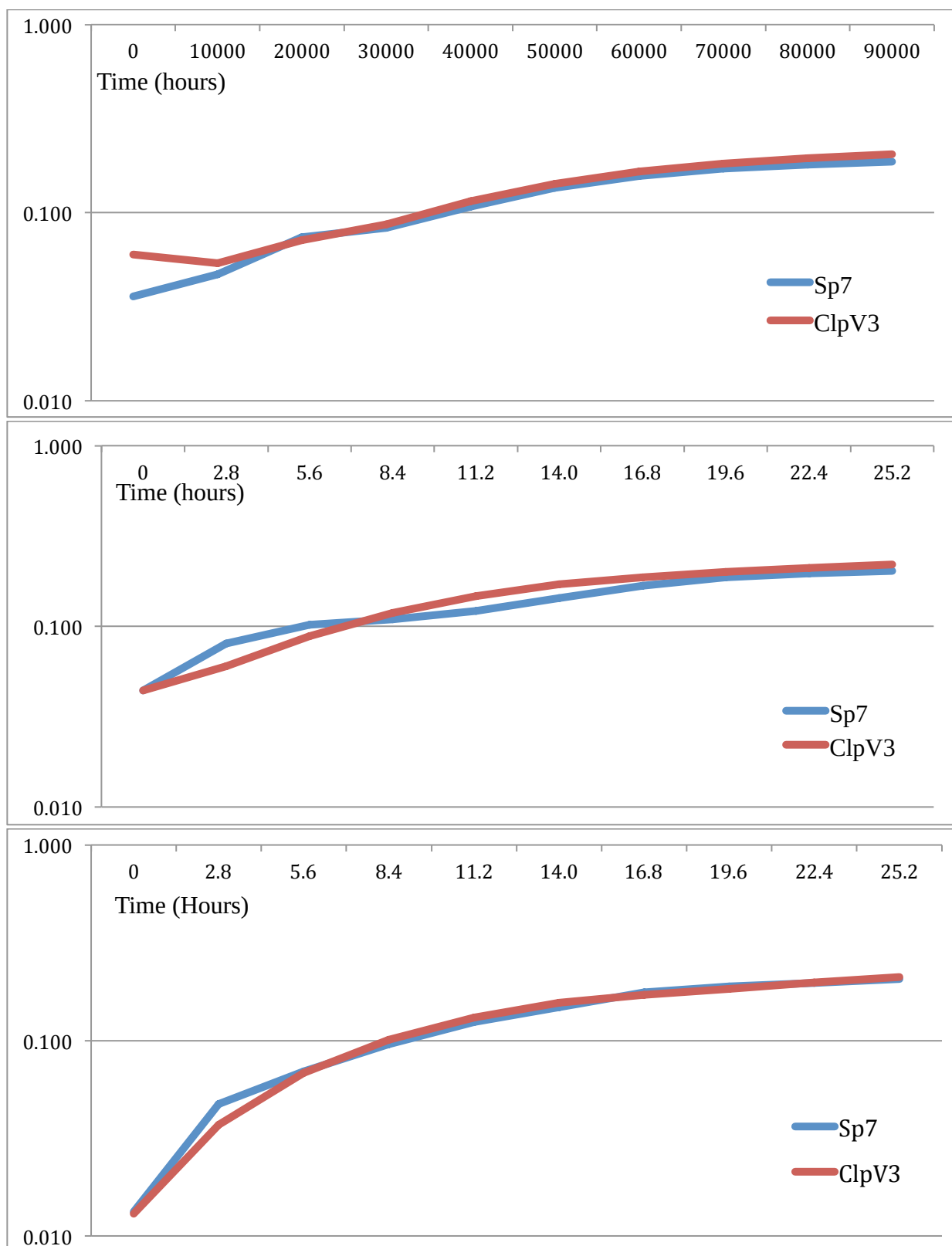


Figure 17: Representative Images from the Live/Dead Assay.

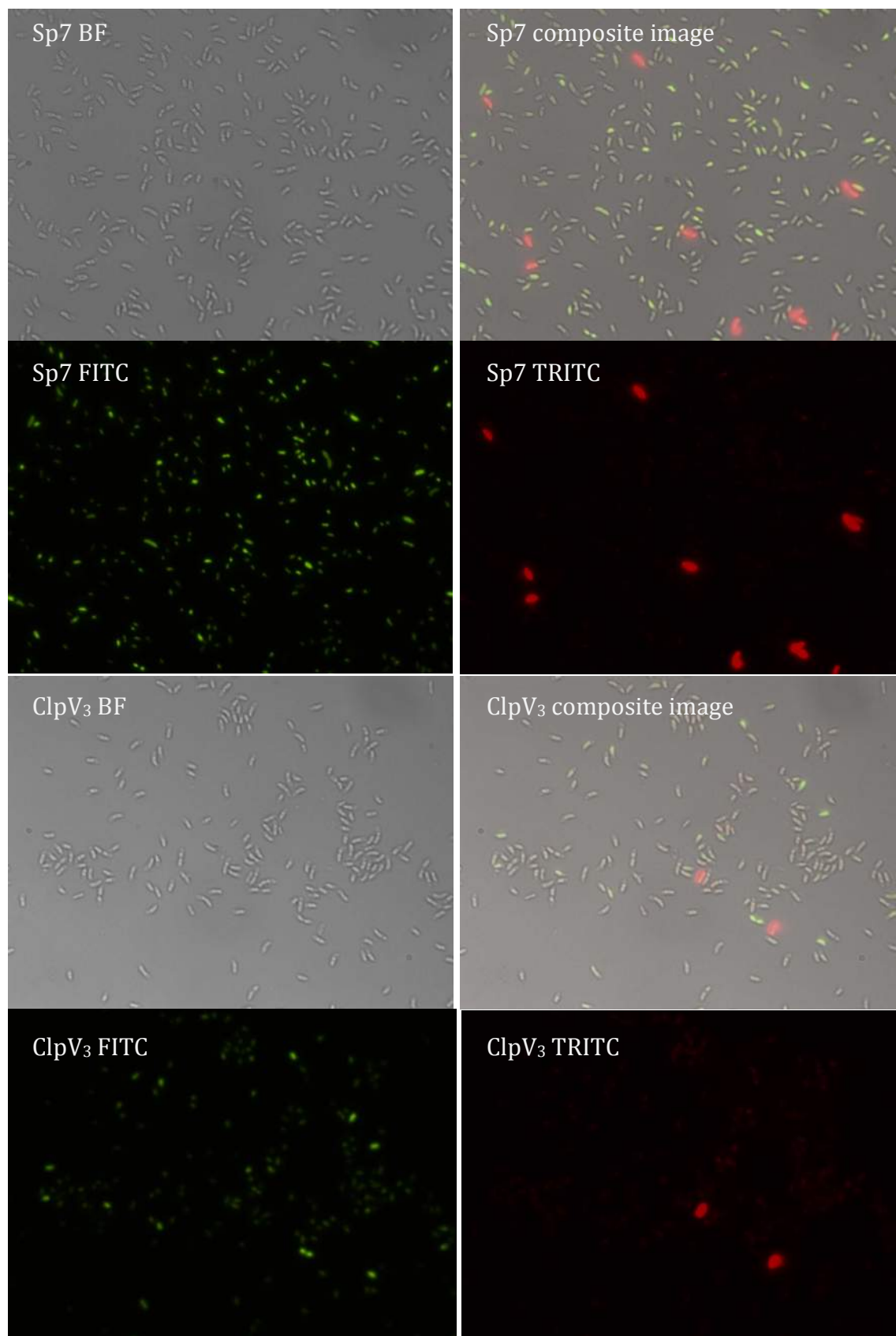
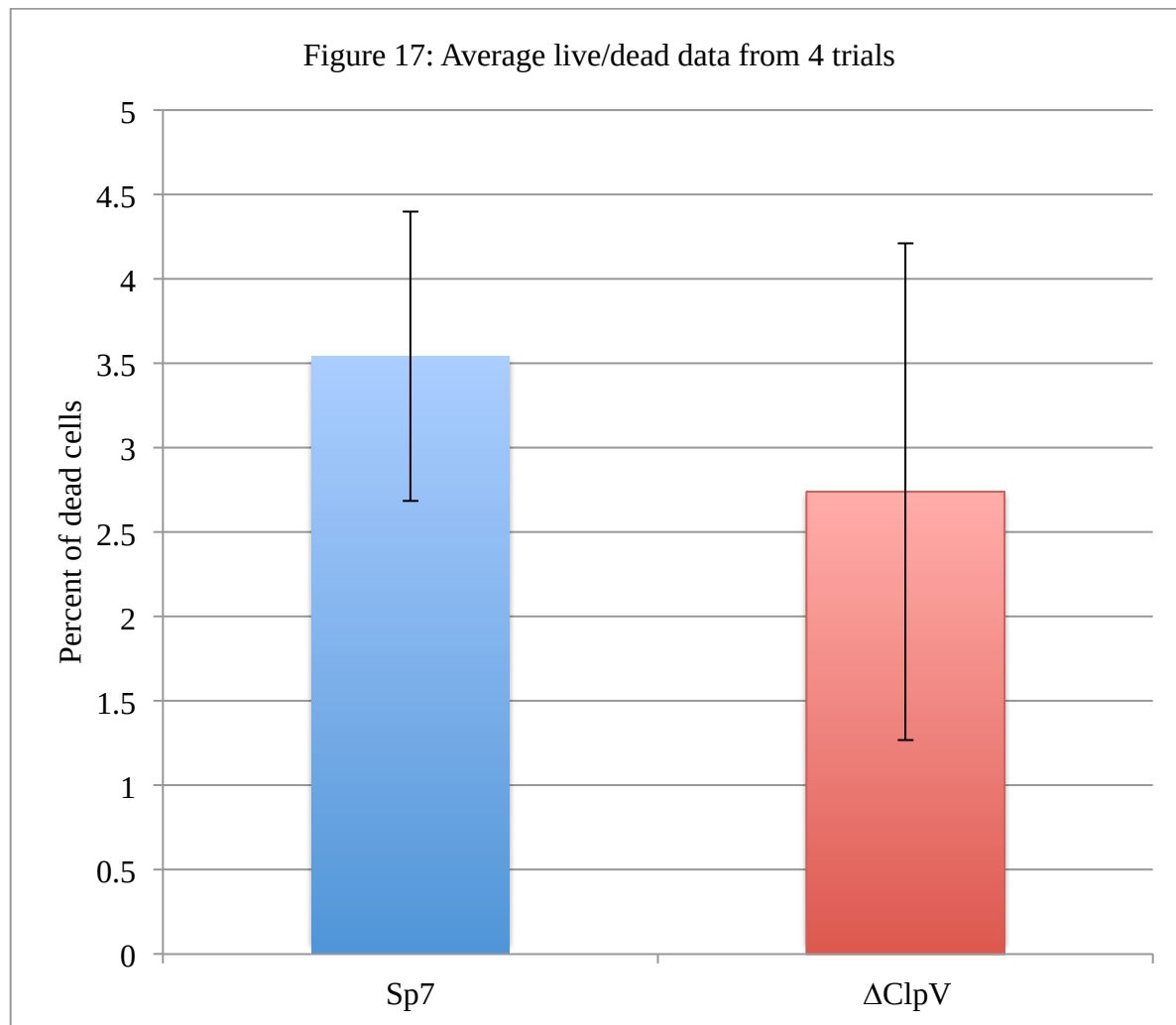
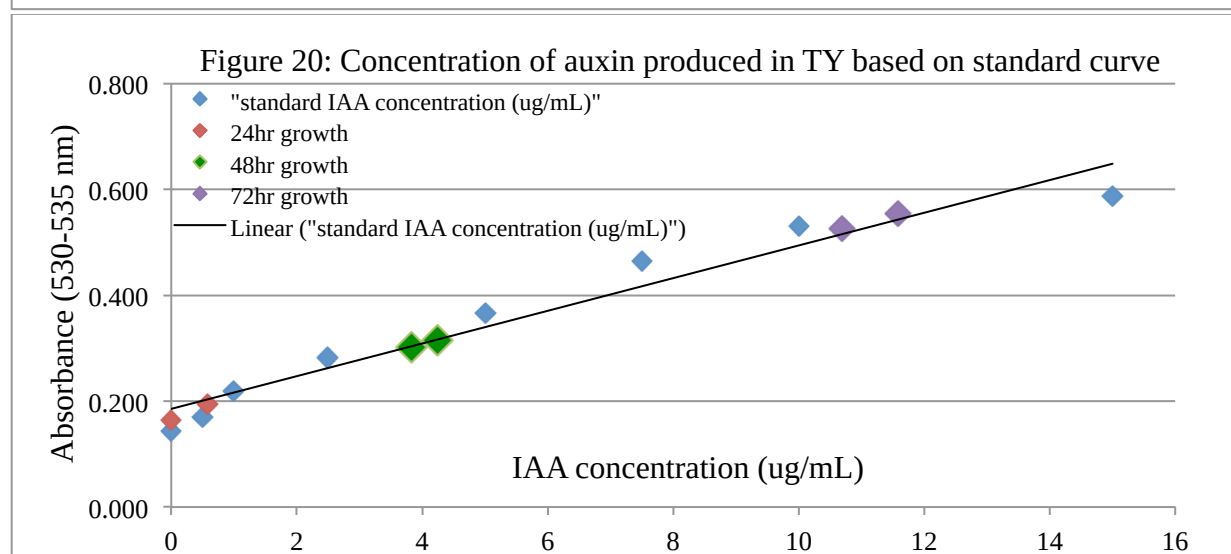
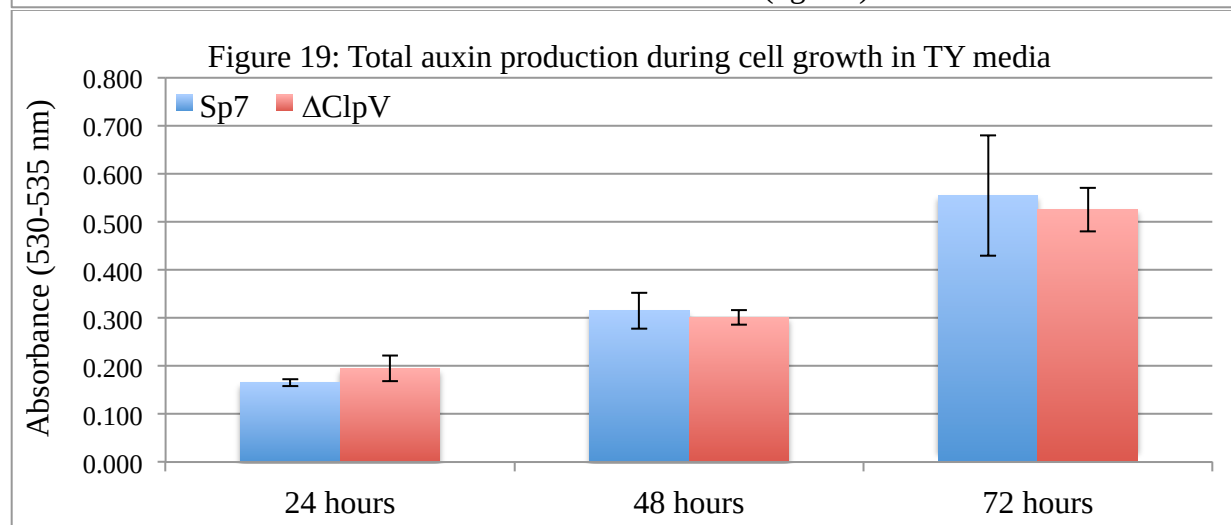
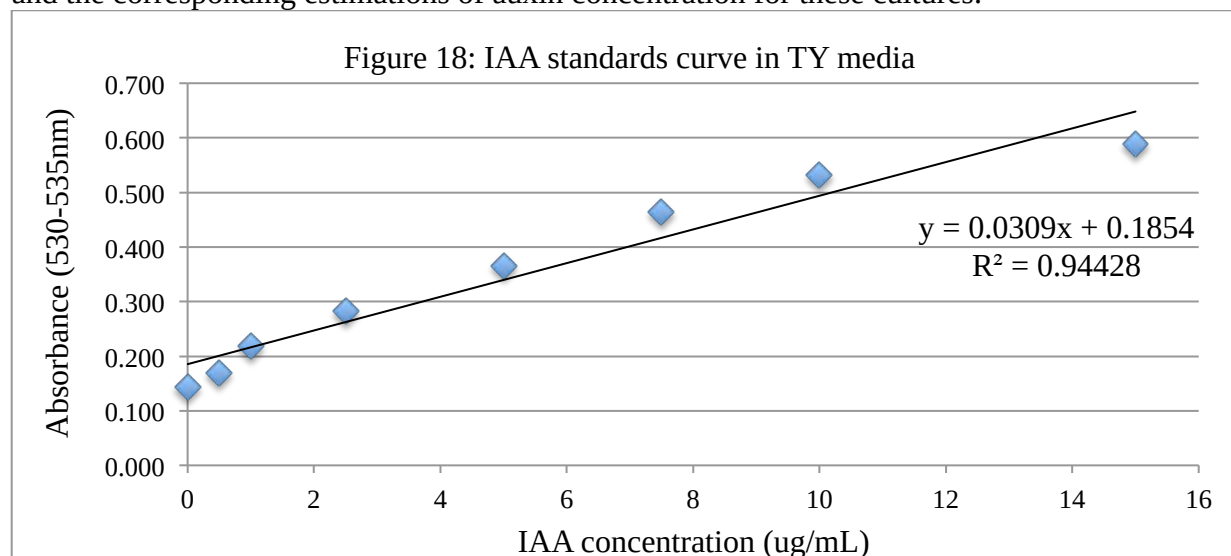


Figure 17 (continued): Quantified results from the live/dead assay.



Figures 18-20: IAA standards curve for Salkowski assay, auxin production during cell growth and the corresponding estimations of auxin concentration for these cultures.



Figures 21-22: Quantification of IAA in induced and non-induced cultures and auxin production in flocculation media.

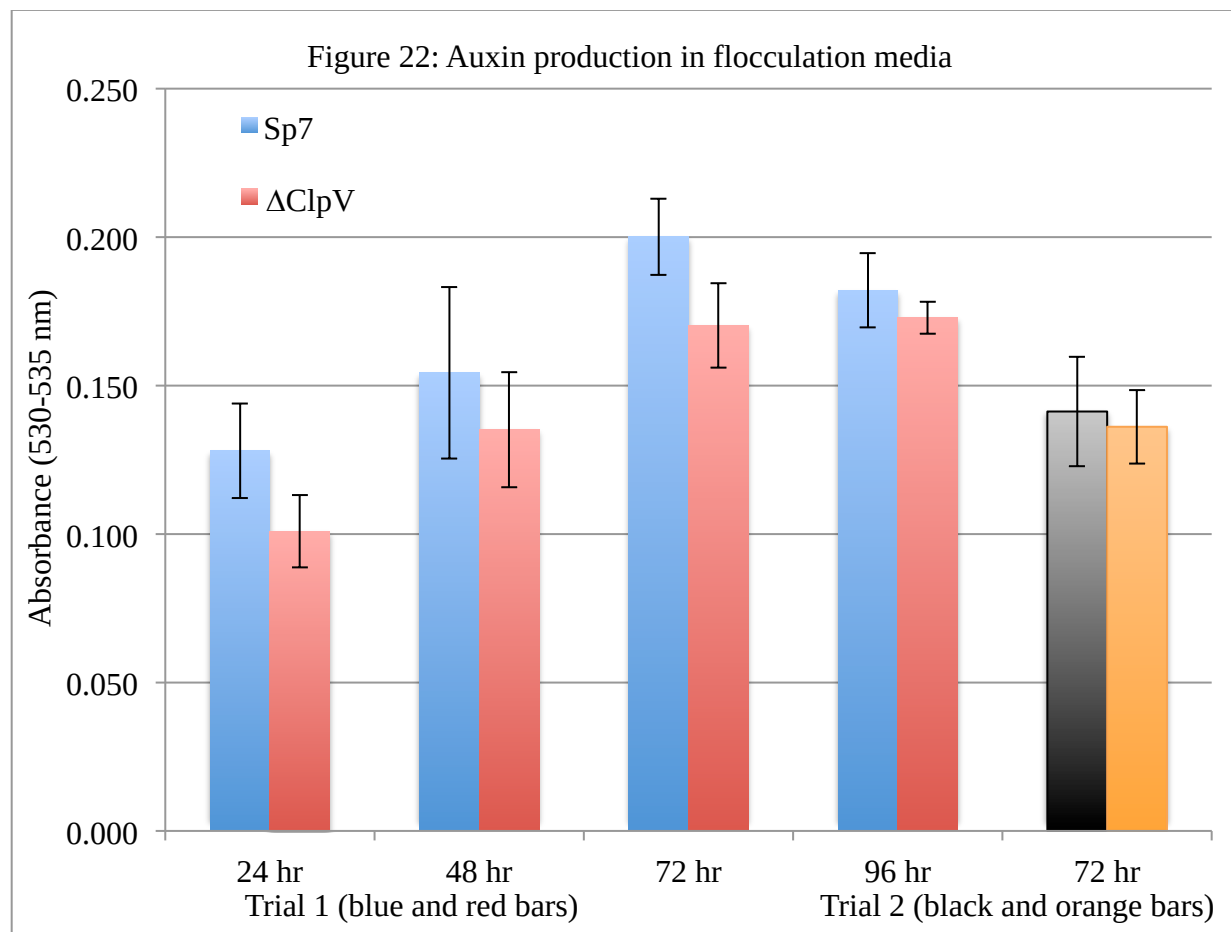
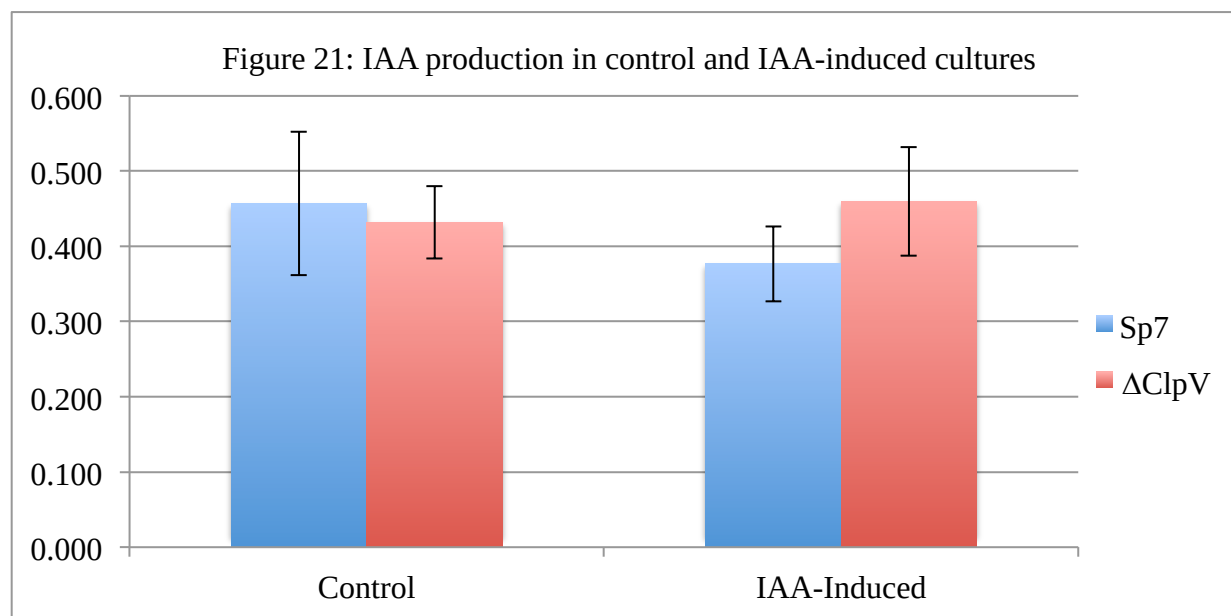
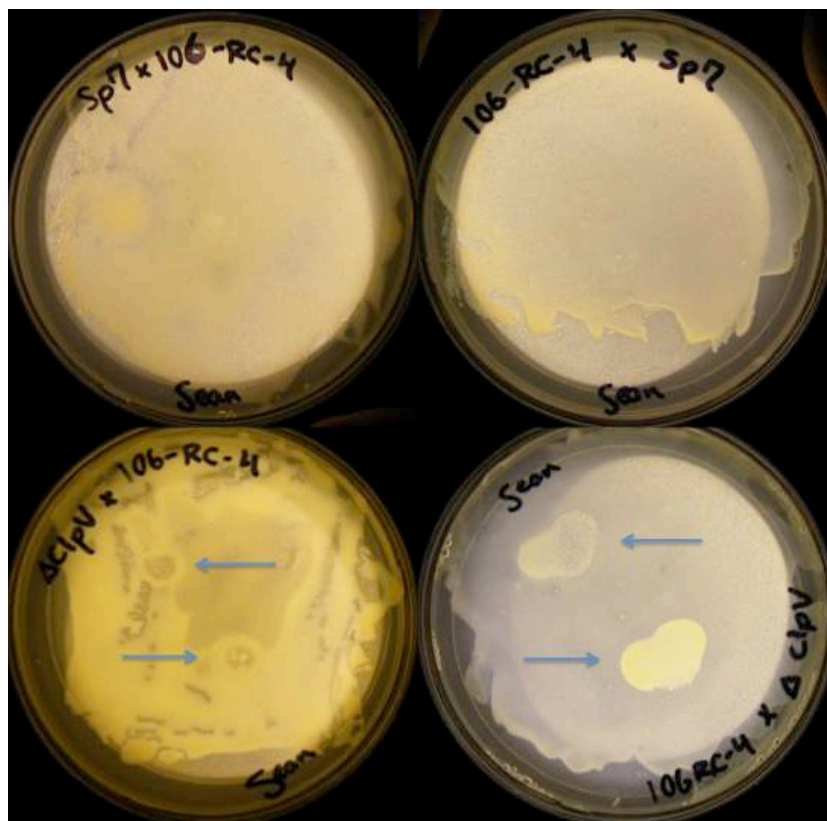
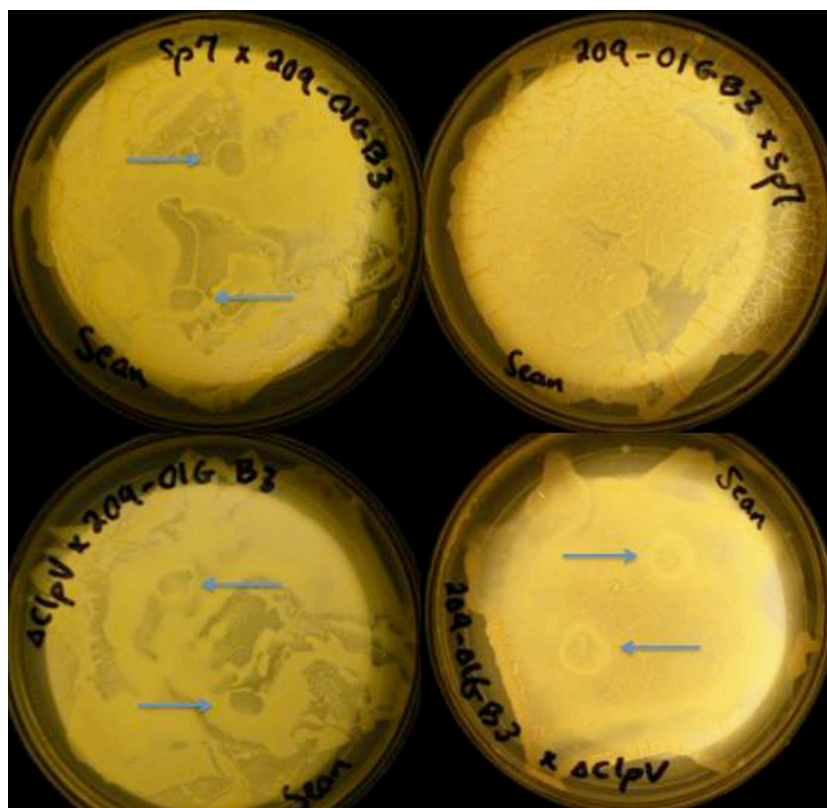


Figure 24b: Photograph examples of differences observed in the competitive growth assay between different strains.



Photographs of competitive assay results for Sp7 and Δ ClpV combinations with the 106-RC-4 strain. Growth of 106-RC-4 against Sp7 was clearly diminished (top two photos) compared to growth against Δ ClpV (bottom two photos, colonies marked by arrows).



Photographs of competitive assay results for Sp7 and Δ ClpV combinations with the 209-01G-B3 strain. Growth of 209-01G-B3 after Sp7 or Δ ClpV were similar (left two panels) but Sp7 was unable to grow after 209-01G-B3 whereas Δ ClpV was (right two panels). Colonies where visible are marked by arrows.

Figure 25a: Photographs of root systems from selected Sp7 and Δ ClpV-inoculated plants.

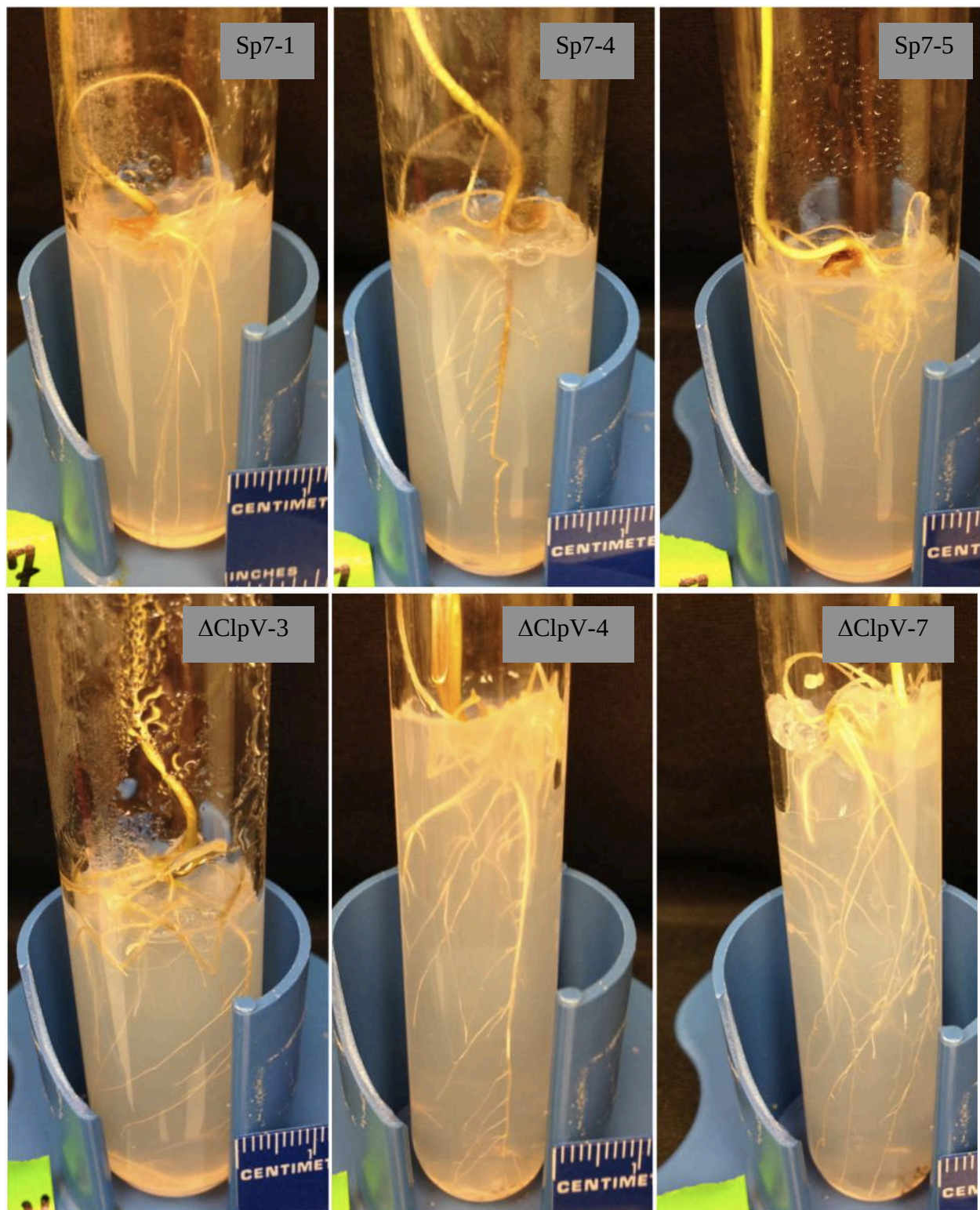
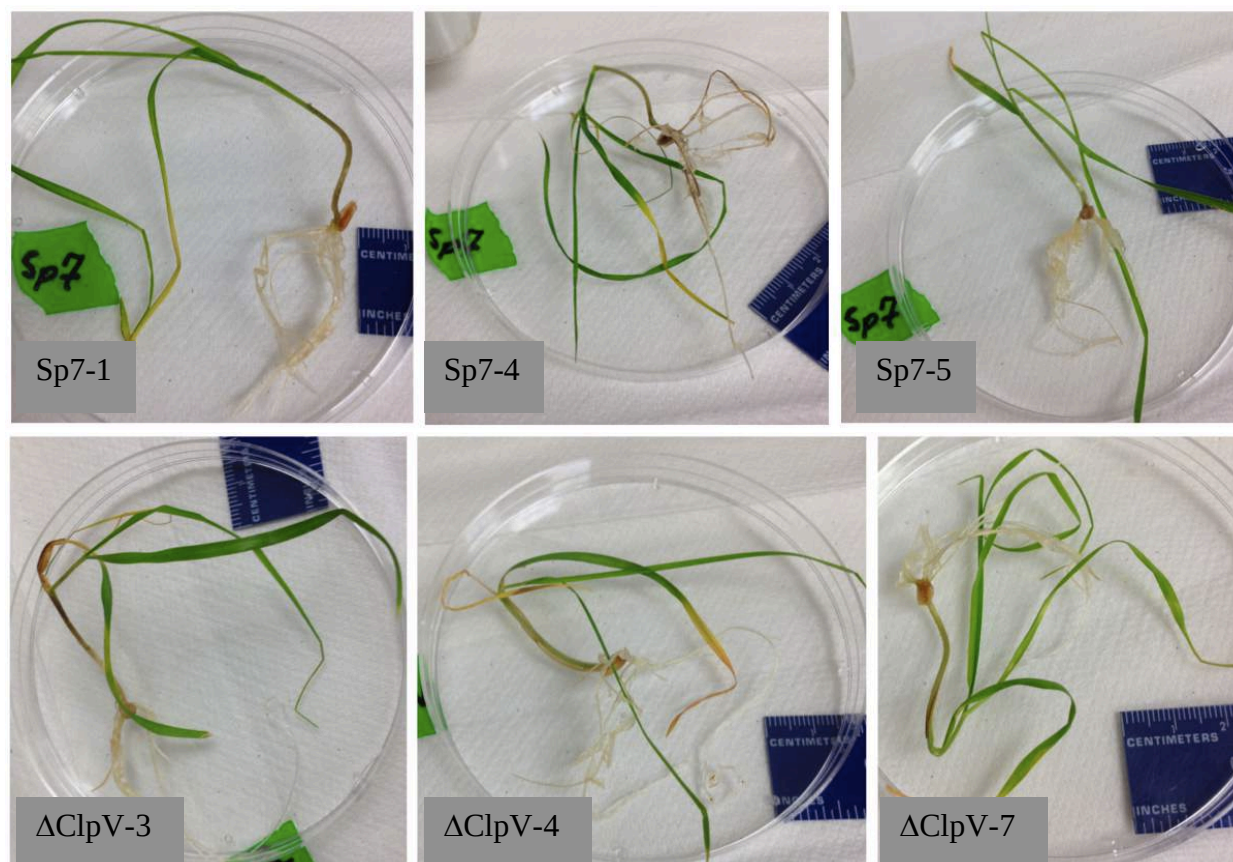


Figure 25b: Photographs of plants from selected Sp7 and Δ ClpV-inoculated plants.Figure 25c: Biomass measurements of root systems and full plants, measurement of longest leaf, number of colonies in 10^{-4} serial dilutions, and calculation of colonies/root biomass.

Cells	Roots (g)	Plant (g)	Roots/Total	Leaf (cm)	10^{-4} colonies	10^{-4} /root (g)
Sp7-1	0.24	0.40	0.60	19.50	12.00	49.42
Sp7-2	0.10	0.16	0.63	18.50	8.00	77.29
Sp7-3	0.12	0.23	0.51	23.00	10.00	84.96
Sp7-4	0.19	0.34	0.55	18.00	5.00	26.53
Sp7-5	0.26	0.39	0.67	22.00	9.00	34.86
Avg.	0.18	0.31	0.59	20.20	8.80	54.61
Cells	Roots (g)	Plant (g)	Roots/Total	Leaf (cm)	10^{-4} colonies	10^{-4} /root (g)
Δ ClpV-1	0.15	0.22	0.69	19.00	4.00	26.65
Δ ClpV-2	0.28	0.44	0.65	19.50	21.00	74.42
Δ ClpV-3	0.12	0.24	0.51	13.50	10.00	81.04
Δ ClpV-4	0.12	0.24	0.48	14.50	9.00	76.34
Δ ClpV-5	0.13	0.31	0.43	22.00	42.00	314.84
Δ ClpV-6	0.06	0.24	0.22	14.00	5.00	90.91
Δ ClpV-7	0.21	0.34	0.61	20.50	12.00	57.55
Avg.	0.15	0.29	0.51	17.57	14.71	103.11

Figure 25d: Graphs of biomass and leaf measurements.

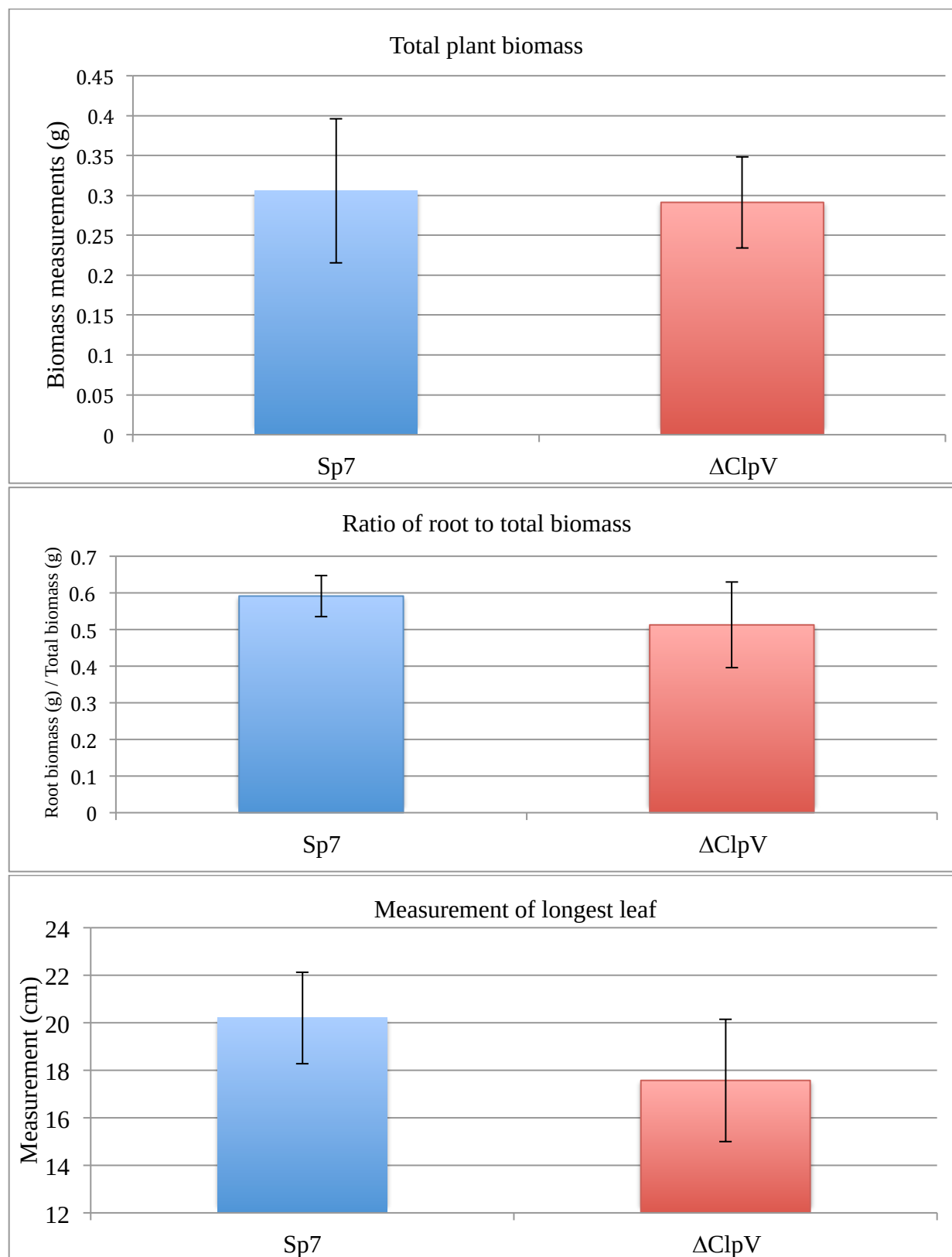


Figure 25e: Graphs of serial dilution colony counts.

