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PRELIMINARY DESIGN OF A LIVE VACCINE USING DIAMINOPIMELIC ACID-DEPENDENT *Y. pestis*

A Major Qualifying Project Report

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ABSTRACT

Diaminopimelic acid (DAP) is a modified lysine amino acid required for cell wall synthesis in most bacteria. This project's goal was to design a live growth-controllable vaccine using a strain of *Y. pestis*, the causative agent of plague, lacking the DAP gene to make its growth dependent on exogenous DAP while allowing host immune responses. *Y. pestis* starvation assays showed that DAP-starved cells cannot revive after 24 hours of starvation, and growth curves showed the minimum exogenous DAP concentration required per cell. Mock skin diffusion experiments showed that DAP diffuses through "skin", sustaining and limiting growth to areas of application. Results suggest that DAP-dependent *Y. pestis* can be a live vaccine controlled by DAP application.

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ACKNOWLEDGEMENTS

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BACKGROUND

General Plague Information

Yersinia pestis is a rod-shaped Gram-negative bacterium that causes three forms of plague: bubonic, septicemic, and pneumonic (Sebbane et al., 2009). The plague originated in rodents from China and spread through infected fleas (Morelli et al., 2010). The disease can be transmitted to humans through contact with infected animals, such as rodents and pets, or from a bite from their infected fleas. **Figure 1** depicts the various methods of *Yersinia* transmission.

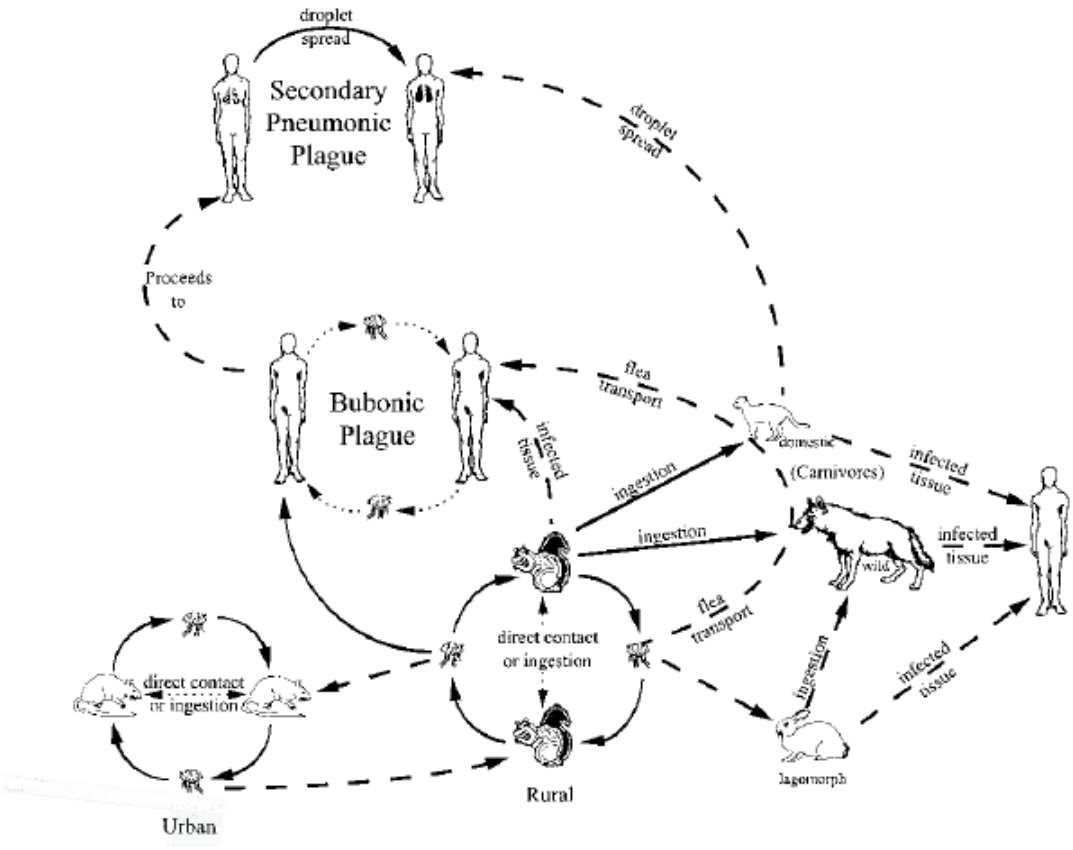


Figure 1: Plague Transmission Paths. The solid lines denote typical transmission paths, the dashed lines show occasional paths, and the dotted lines show uncommon paths (Perry and Fetherston, 1997).

Many different strains of *Y. pestis* have been traced back to the original strain from China. Over fifteen strains have been identified in three different branches of a phylogenic tree (Morelli et al., 2010). The original strain of *Y. pestis* actually morphed from its close relative *Yersinia pseudotuberculosis*, which is a gastrointestinal pathogen (Parkhill et al., 2001).

The incubation period for *Y. pestis* is about three to seven days in humans. Depending on how the patient became infected determines which type of plague (bubonic, septicemic, or pneumonic) is diagnosed, and each type affects humans differently. A bite from an infected flea causes the bubonic plague. It is characterized by swollen lymph nodes where the bacteria are doubling quickly. The infected lymph nodes can become so large that they become open wounds. The name, bubonic plague, comes from the term "bubo" which refers to the enlarged lymph nodes (Fact Sheet: Plague, 2005). In addition to swollen lymph nodes, other symptoms include headache, fever, chills, vomiting, nausea, and diarrhea (Perry and Fetherston, 1997).

Septicemic plague is similar to bubonic plague in that *Y. pestis* is introduced through the bloodstream. However, bacteremia in septicemic plague does not occur in the lymph nodes to form buboes. The cause of this type of infection could be from a flea bite or from contact with an infected animal if one has open cuts in the skin (Fact Sheet: Plague, 2005). The symptoms of septicemic plague are also similar to those of the bubonic plague: fever, chills, headache, and abdominal pains (Perry and Fetherston, 1997).

Pneumonic plague can be caused by inhalation of the bacteria from a patient with the bubonic plague (from sneezing or coughing) or by aerosolized droplets. This type is

characterized by infection of the lungs, which starts out like the flu but develops into pneumonia (Perry and Fetherston, 1997). Its incubation period can be shorter than other plagues (about one to three days) and it is extremely virulent, so patients must be kept in isolation. This is the only form of the plague which can be transmitted without the assistance of animals or fleas (Fact Sheet: Plague, 2005). Pneumonic plague is the most easily spread of the three types, and has a mortality rate of almost 100% (Titball and Williamson, 2001).

Treatment and Prevention

Because *Y. pestis* is a bacterium, bubonic plague can be treated fairly well with antibiotics if caught early, however it is very difficult to catch septicemic and pneumonic forms early because of how rapidly they develop (Titball and Williamson, 2001). The typical antibiotic used for treatment is streptomycin, but tetracycline and chloramphenicol can be used as well. Patients are contagious until they have been treated for at least two days, and should be quarantined until then. Even with treatment, about 50% of septicemic patients die (Perry and Fetherston, 1997).

Antibiotics have also been used in precautionary measures to prevent infection. This is atypical and only practiced when people are in close contact with infected patients or materials. A live vaccine for the plague has been in use since 1908, EV76. It is an attenuated strain of *Y. pestis* that is pigmentation negative, whereas all virulent strains are pigmentation positive (Russell et al., 1995). EV76, however, is not completely safe as a 1% fatality rate was found in mice studies (Titball and Williamson, 2001). A second type of vaccine was a killed formalin-fixed virulent whole cell vaccine, but this type is no longer distributed. When in use, the killed vaccine was only given to military personnel

and those working with virulent strains. It required three shots over the course of nine months and additional booster shots were needed every two years (Perry and Fetherston, 1997).

Treatment with antibiotics is mostly effective, only if it is diagnosed at a very early stage. Because plague can resemble other gram negative bacterial infections and be difficult to diagnose early, it is important to find a more effective vaccine (Perry and Fetherston, 1997). Moreover, bioterrorism is an imminent threat because *Y. pestis* is readily available in many laboratories around the world, and it is easily aerosolized to cause mass epidemics of the pneumonic form of plague. It has also been found that there is a high frequency of antibiotic resistance gene transfer to *Y. pestis* in the midgut of fleas, which poses a huge threat to human health (Hinnebusch et al., 2002).

Live Vaccines

Vaccination is important for developing immunity to harmful diseases and establishing healthy communities. There are different types of vaccines distributed: killed, attenuated, sub-unit, and DNA. They all have advantages and disadvantages, and some are better for certain diseases.

Killed vaccines contain a dead version of the normal infectious agent while sub-unit vaccines contain components of the infectious agent, such as surface proteins. Both of these vaccines elicit a sufficient immune response, but booster shots are required for complete immunity. Because they are inactivated they can be used for immune-deficient patients. On the other hand, sometimes they can be too weak and not provide an effective immunity (Virology, 2010).

A new development on DNA vaccines has possible advantages for stability and flexibility. DNA vaccines are plasmids that contain a gene encoding an antigen of the infectious agent. The DNA sequence can be engineered easily and produced in large quantities but there are also severe disadvantages, such as the plasmid integrating into the host genome, or the immune system creating anti-DNA antigens (Virology, 2010). Because this research is so novel and the exact repercussions are unknown, it is not yet a safe method of vaccination.

Attenuated vaccines are live agents but are mutated to be non-pathogenic. Because the vaccine is the actual bacteria or virus, it elicits all parts of the immune system and provides the best immunity long term. The immunity is quickly established after inoculation, and there is no need for booster shots. Most attenuated vaccines are inexpensive to make and transport easily (Emergency Preparedness and Response, 2007).

Measles, mumps, rubella, and chicken pox are all successful stories of live attenuated vaccines. Once vaccination is complete, patients are completely immune to these diseases and do not need booster shots later in life. The major disadvantage to live vaccines is that they could mutate themselves and become dangerous. There is also concern that the live vaccine could spread to others in contact with the patient and accidentally inoculate them as well (Emergency Preparedness and Response, 2007). The worst case scenario would be that the vaccine mutated, is now pathogenic, and then spreads to others.

A live vaccine for the plague would provide greater protection against all strains of *Y. pestis* than a sub-unit vaccine which would have limited immunogenic responses. One study created an attenuated plague vaccine by mutating the bacterial tyrosine

phosphatase, YopH, which is a strong factor in virulence. *Y. pestis* appears to be avirulent when YopH is deleted, therefore it should be a practical live vaccine, but its growth is not controllable (Bubeck and Dube, 2007).

The EV76 attenuated vaccine that is pigmentation negative is not avirulent, and therefore it is not permitted for use in humans (Titball and Williamson, 2001). It is important to find a bacterial mutation that makes the vaccine avirulent without killing the bacterium. *Y. pestis* can be attenuated by mutating virulence factors or by mutating its physical structure. If a virulence factor is removed, then the vaccine will not give the patient all the symptoms of plague but the bugs can still infiltrate the body. Mutating the structure of *Y. pestis* causing it to depend on an outside nutrient would make the bacterium avirulent, but its replication would be controllable and it would still elicit a full immune response.

DAP and Live Vaccines

Diaminopimelic acid (DAP) is an optically inactive amino acid in the aspartate family, and is an epsilon-carboxy derivative of lysine. Its main function is as a critical component in the cell wall of many bacteria. In gram negative bacteria such as *E. coli* and *Y. pestis*, the peptidoglycan layer is composed of linear chains of alternating N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), linked by β-(1,4)-glycosidic bonds (Meadow et al., 1957). Each NAM attaches to a 4-5 residue chain of D-alanine, D-glutamic acid, and meso-diaminopimelic acid. This composition is thought to protect the bacteria from attacks by peptidases. In bacteria, the decarboxylation of the meso isomer αε-diaminopimelic acid forms lysine, a process that is fueled by L-aminoadipic acid in yeasts and fungi (Bukhari and Taylor, 1970).

The *asd* gene encodes a key enzyme in the biosynthetic synthesis of DAP, β-semialdehyde dehydrogenase (Galan et al., 1990). Bacteria lacking this enzyme are identified by Δasd. Lack of the *asd* gene renders the bacteria unable to synthesize the cell wall independently, making the bacteria dependent on an outside source of DAP for cell wall replication. If no outside source of DAP is provided, the cells will lyse due to the lack of the cell wall.

A Δasd mutant of *S. flexneri* was used in a study by Shata et al. (2000) who created an attenuated strain designed to efficiently escape the host endosome, allowing for direct access to the cytoplasm of the host cells. This strain allows for direct targeting of the lymphoid tissue in colonic mucosa to elicit muscosal and systemic immune responses. Using this method, a LacZ reporter plasmid was successfully delivered to cultured human cells, and expression of the LacZ gene was detected along with an observable rise of β-gal-specific antibodies and T-cell proliferation. The uptake of the LacZ gene can be attributed to the lack of DAP to the bacteria – without DAP they could not synthesize the cell wall and subsequently lysed, releasing the gene into the lymphoid tissue (Shata et al., 2000). Vaccination approaches using Δasd mutants of *Y. pestis* have not yet been tested.

PROJECT PURPOSE

Y. pestis is a highly infectious bacterium that causes three forms of the plague. While it is possible to block bubonic plague with a course of antibiotics if caught at the earliest stages, this method does not work for pneumonic plague, and would not be sufficient if *Y. pestis* is ever used in an act of bioterrorism where the agent would spread human to human via aerosols. The previous killed vaccine for bubonic plague requires three initial inoculations, and boosters every few years thereafter. A topical live vaccine would not only be less painful and easier to administer, but would provide longer lasting, stronger immune responses. Linking the administration of a cell wall-deficient strain to the topical application of the missing cell wall component would also contain the bacterial replication to the area of application, reducing the risk of peripheral illness sometimes experienced with vaccine injections. Diaminopimelic acid (DAP) is a modified lysine amino acid required for cell wall synthesis in most bacteria including *Y. pestis*, so the goal of this project is to test a DAP-deficient strain previously created by the Goguen laboratory at UMASS Medical Center to prove it requires exogenous DAP for survival, and to determine the minimum DAP concentration required for survival. In addition, the project will test the replication of Δasd mutant *Y. pestis* on artificial skin (20% polyacrylamide gel) to verify the replication is restricted to sites of DAP application.

METHODS

Bacterial Strains

Y. pestis JG150Δasd and JG150Δasd pML 001 were obtained from Jon Goguen at UMASS Medical Center.

Hourly Growth Curves

In order to determine the amount of DAP required per cell, growth curve experiments were performed. JG150Δasd cells were grown overnight at 37°C in TB^{+}DAP25 media, and the optical density (OD) was measured. In order to test the effect of different DAP concentrations, these cells were distributed into 25 mL of media containing different concentrations of DAP. In order to quantitatively compare the results, each fresh culture was started at an OD of 0.05. This was calculated from the starting OD of 2.06 using the formula cv = cv. The DAP concentrations used were 2 μM, 2 nM, 20 pM, 0.2 pM, 2 fM, and 0 DAP as a negative control. The positive control was 2 μM, as previous experiments performed in our lab had shown this to be a more than a sufficient amount of DAP to sustain the mutant strain. These cultures were incubated at 37°C, and optical density at λ600 was measured every hour.

A repeat of this experiment was performed using the same methods described above with DAP concentrations of 2 μM, 1 μM, 200 nM, 100 nM, 20 nM, 10 nM, 2 nM, and 0 DAP.

Overnight Growth Assays

After establishing a testable range of DAP concentrations, an overnight growth assay was run. Again, JG150Δasd cells were grown overnight at 37°C in TB^{+}DAP25 medium. The cells were washed, and resuspended in fresh media of varying concentrations of DAP; including 2 µM, 700 nM, 600 nM, 500 nM, 400 nM, and 0 DAP as a negative control. After an overnight incubation at 37°C, the ODs of these cultures were measured and compared. The OD measurements from this experiment were used to calculate the amount of DAP each cell requires.

Luminescence Assay

In preparation for future experiments, growth curve experiments of the mutant strain containing an additional luminescence plasmid were performed. A culture of JG150Δasd pML001 cells was grown overnight in TB^{+}DAP25, AMP100 media, washed and resuspended into fresh media containing 2 µM DAP, using the same methods and concentrations described in the previous section. A 30 second luminesce measurement was taken with the Packard Pico-Lite Luminometer Analyzer for Bio- and Chemi-Luminescence every hour to track the vitality of the cells in culture over time. Concurrently, the optical density of the culture was measured for comparison purposes.

DAP Diffusion Through Soft Agar Containing JG150Δasd

JG150Δasd pML001 cells were grown in TB+ DAP25 overnight, and 10^{7} cells from the culture were washed with TB+ medium. Soft TB+ agar was melted, aliquoted into six 3.5 mL tubes and set in a 42˚C water bath to cool. Five different DAP

concentrations (131 μM, 65.5 μM, 2 μM, 700 nM, 600 nM) were spread onto five TB+ AMP 100 plates, respectively. No DAP was spread onto a sixth plate as a negative control. After the DAP was dried, 100 μL of the washed cells were added to each aliquot of soft agar and spread evenly over each plate. Once dry, the plates were incubated at 37˚C for 7 hours before recording data to allow the cells to reach log phase growth. Starting at hour 7, each plate was photographed using the SBIG ST-402 cooled CCD camera every hour for 8 hours. The camera set up is shown in **Figure 2**.

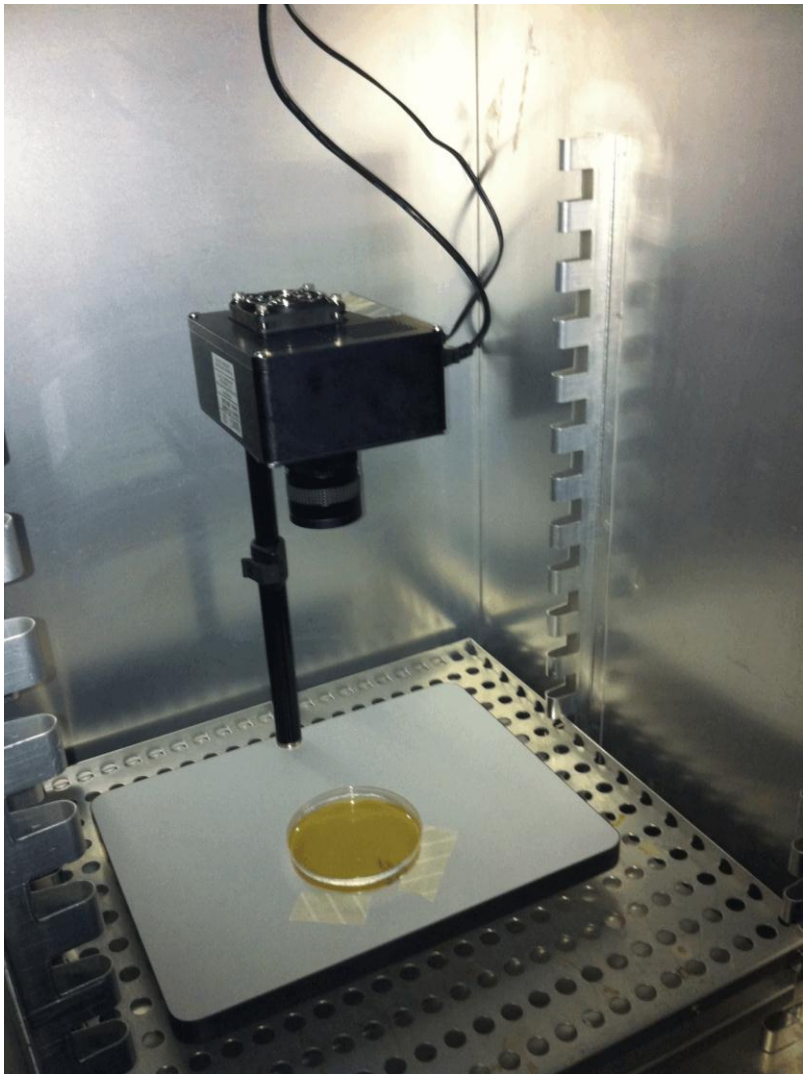


Figure 2: Set Up of the Light Sensitive SBIG ST-402 Cooled CCD Camera. Camera was placed in an incubator in a dark room to ensure light detected was from luminescent cells.

Mock-Skin Experiment

JG150Δasd pML001 cells were grown overnight in 25 mL of TB+ DAP AMP100 medium, then 6 sets of 10^7 cells were washed in TB+ medium. The cells were added to 0.5 mL of TB+ top layer soft agar and poured into a small plastic washer. The washer was placed on top of a 20% polyacrylamide gel. The gel was made with 30% Acrylamide/bis, 1.5 M Tris-HCl (pH7.4), distilled deionized water, 0.05% TEMED (final concentration), and 10% APS (0.05% final concentration). On the opposite side of the gel, another washer was placed. This washer held various concentrations of DAP (131 µM, 2 µM, and 0 µM) in 0.5 mL of TB+ top layer soft agar. Each DAP concentration was tested on its own gel. The gels were placed in empty plates with damp sterile gauze (the complete setup is shown in **Figure 3**). The plates were grown at 37˚C for 7 hours in a desiccator. At the 7th hour the gels were photographed every hour for 8 hours using the SBIG ST-402 cooled CCD camera.

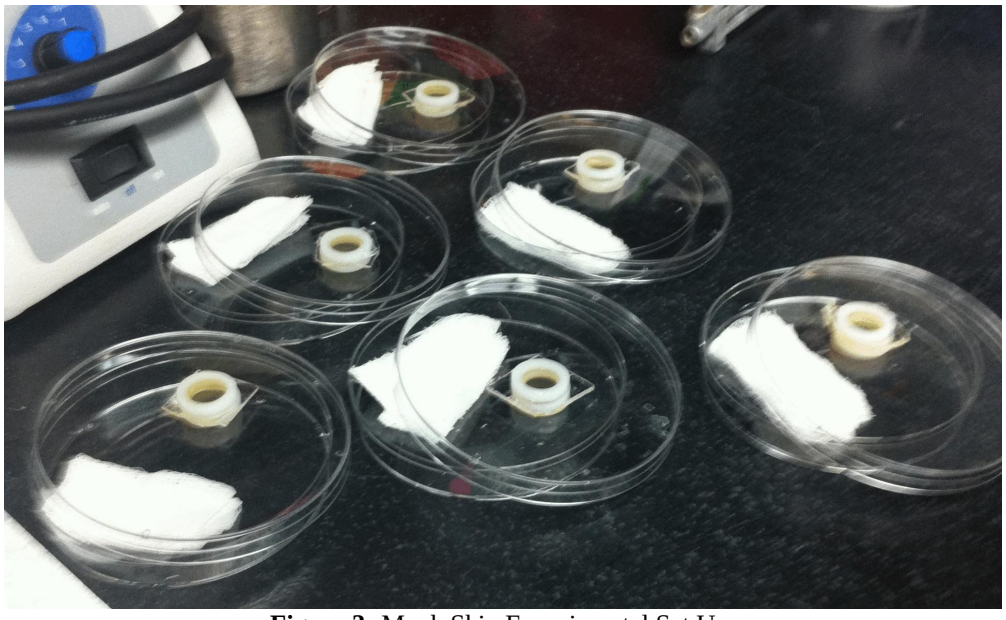


Figure 3: Mock Skin Experimental Set Up.

Troubleshooting Mock-Skin Experiment

Since the JG150Δasd pML001 cells did not grow in the mock-skin experiments, steps were taken to figure out the reason. The same 20% polyacrylamide gel was prepared and the same set up was used, except only 0 μM and 32750 μM DAP concentrations were tested. To determine if the cells were not receiving enough oxygen for growth, a washer was placed on top of a 20% polyacrylamide gel and 175 μL of TB+ soft agar containing 10^7 cells and 131 μM DAP was added to the washer. In another setup to test oxygen deficiency a 20% polyacrylamide gel was placed between two washers. One washer contained 400 μL of TB+ soft agar with 131 μM of DAP. The other washer contained 175 μL TB+ soft agar with 10^7 cells. A final test was performed to determine whether the acrylamide was killing the cells. A washer containing TB+ soft agar with 10^7 cells and 131 μM DAP was placed on top of a 20% acrylamide gel. For all of the troubleshooting experiments mentioned, the washer-gel setups were placed in empty plates with damp sterile gauze. The plates were grown in a desiccator at 37˚C for 7 hours. Then the gels were photographed using the SBIG ST-402 cooled CCD camera to observe growth by luminescence.

Starvation Assay

JG150Δasd cells were grown in 25 mL of TB+ DAP25 medium overnight. The OD of the culture was measured, the volume containing 300 cells was calculated, and 100 cells were spread onto three TB+ DAP25 plates. The plates grew over two nights at 37˚C, then the colonies were counted. The rest of the culture was split in half and washed separately with TB+ medium. One half of the culture was resuspended and grown in 5

mL of TB+ DAP25, while the other half was resuspended and grown in 5 mL of TB+ medium. Both cultures were grown at 37˚C over two nights. Again, the OD's of both cultures were measured, calculations for 300 cells from each culture were determined, and 100 cells were spread onto three TB+ DAP25 plates. The plates were left on the bench for three days to grow, and were then counted for colonies.

RESULTS

The goal of this project was to test a DAP-deficient strain Δasd of *Y. pestis* previously created by Jon Goguen to prove it requires exogenous DAP for survival, to determine the minimum DAP concentration required for survival, and to test its replication on artificial skin (20% acrylamide gel) to verify the replication is restricted to sites of DAP application.

Hourly Growth Curves

To verify that the *Y. pestis* attenuated strain JG150Δasd requires DAP for survival, and if so, to determine the minimal concentration of DAP required for survival, optical density readings were taken at hour intervals for JG150Δasd cells grown in TB^{+} medium containing various concentrations of DAP: 2 μM, 2 nM, 20 pM, 0.2 pM, 2 fM, and 0. An overnight reading was also taken to evaluate the final OD of the cultures. From this curve (**Figure-4**), it is apparent that growth increases for a period of time, then begins to plateau due to the exhaustion of the DAP supply. Because of the large gap in final OD readings, the DAP concentrations were altered for subsequent experiments.

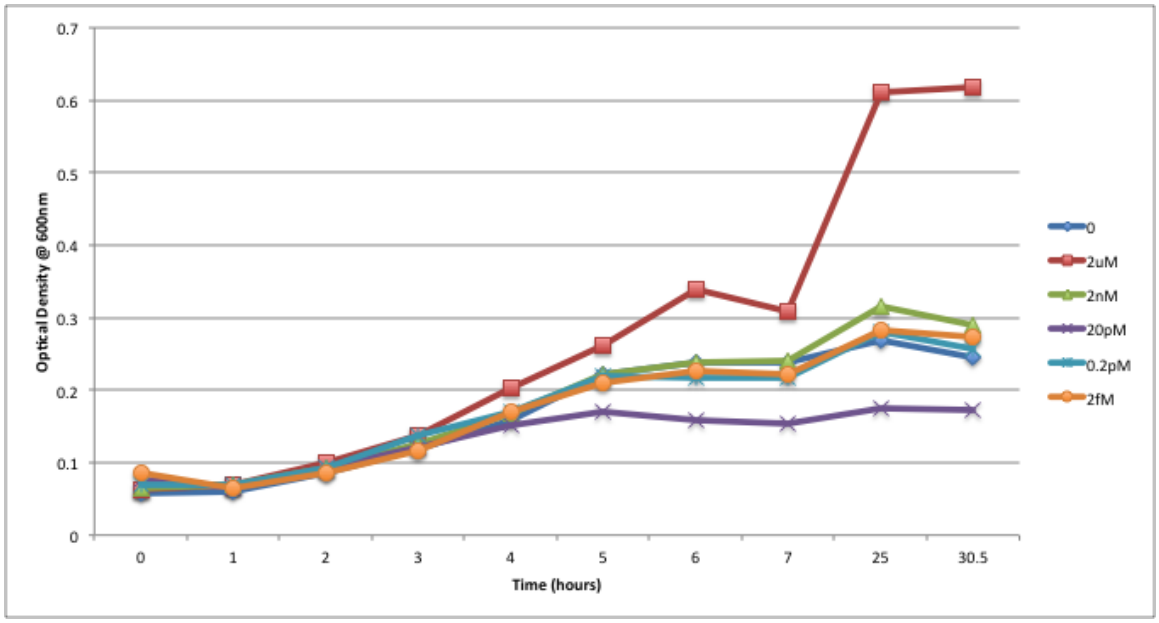


Figure 4: Hourly growth of JG150Δasd cells in varying concentrations of DAP measured by optical density readings at 600 nm, Trial 1.

A repeat growth curve was performed in the same manner as previously mentioned, using higher concentrations of DAP (**Figure-5**). Concentrations used in this trial included 2 µM, 1 µM, 200 nM, 100 nM, 20 nM, 2 nM, and 0. This growth curve further displays the effect of varying concentrations of DAP on JG150Δasd survival. Then based on these results, the DAP concentrations were further altered to cover the gap observed between 2 µM and the lower concentrations.

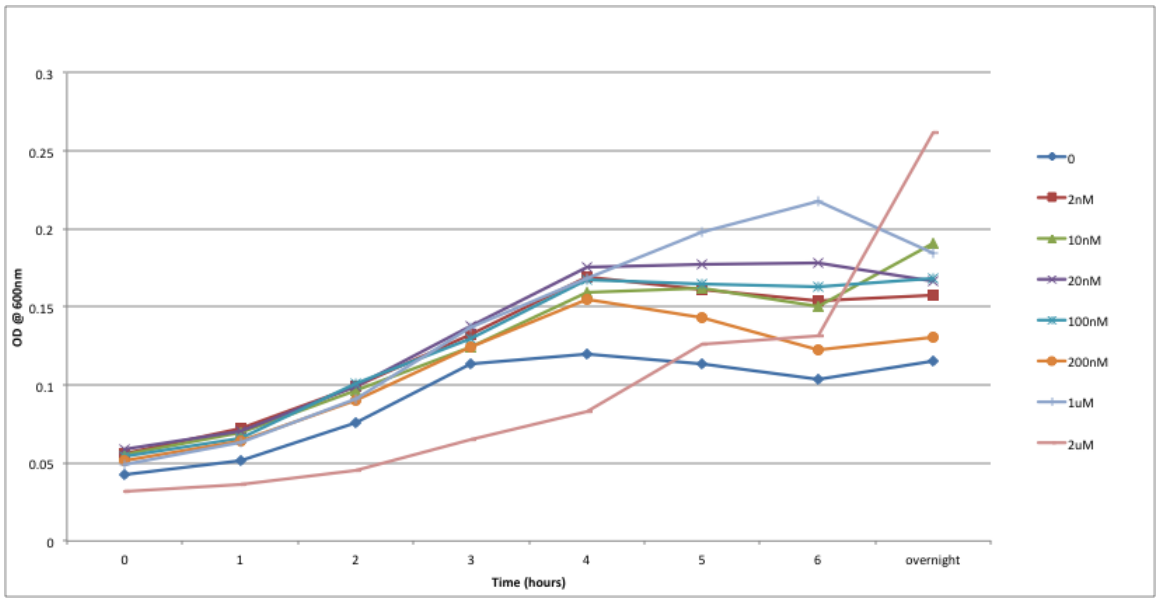


Figure 5: Hourly growth of JG150Δasd cells in varying concentrations of DAP measured by optical density readings at 600 nm, Trial 2.

Overnight Growth Assays

Due to the very small range of final cell OD readings in the previous set of data, the concentration of DAP in the media was greatly increased to a maximum of 2,000 nM (2 μM). JG150Δasd cells were grown in TB^{+}DAP medium containing the following concentrations of DAP: 2 μM, 700 nM, 600 nM, 500 nM, 400 nM, and 0 nM. These cultures were incubated overnight, and their final OD readings were measured (**Figure 6**). In this case, there is a very clear increase of growth of the bacteria when the DAP concentration is higher. From this data, we were able to calculate the amount of DAP required per cell to sustain growth. The average DAP required per cell was calculated to be 2.29×10^{-12} grams.

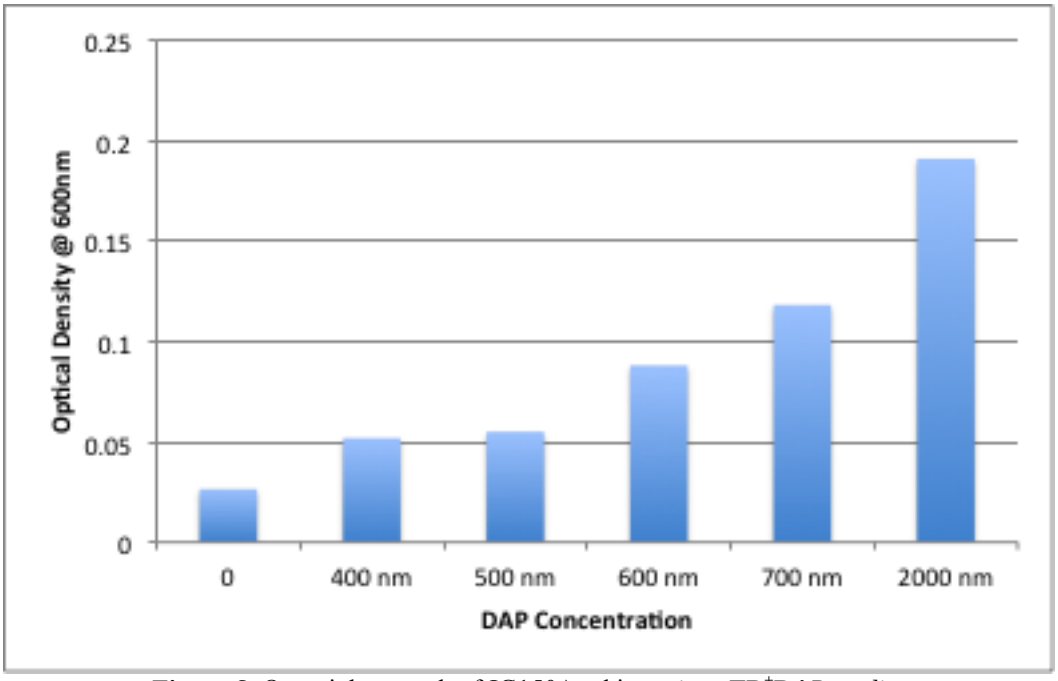


Figure 6: Overnight growth of JG150Δasd in various TB^{+}DAP media.

Luminescence Assay

In order to examine the behavior of JG150Δasd cells containing the luminescence plasmid pML001, an hourly luminescence assay was run on a culture of cells grown in medium containing 2 μM DAP. Luminescence readings (in photons) were taken every hour and are shown in **Figure 7, Panel A**. Optical density was measured concurrently, and the growth curve is shown in **Figure 7, Panel B**. As the cells ran out of available DAP they subsequently died and ceased to glow, causing the luminescence reading to decrease over time.

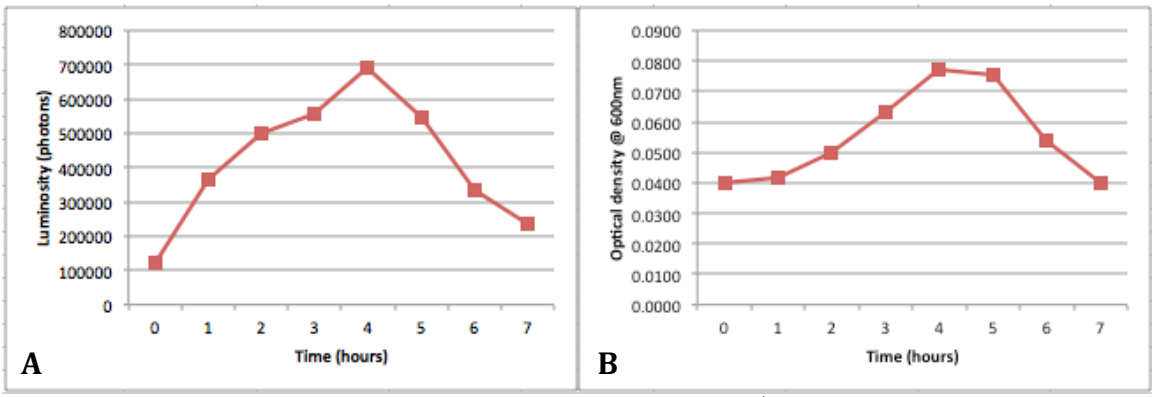


Figure 7: A) Luminosity of JG150Δasd pML001 cells in 2 µM TB^{+}DAP AMP100 media over time; B) Optical density of JG150Δasd pML001 cells in 2 µM TB^{+}DAP AMP100 media over time

Ability of DAP-Starved Cells to Revive

JG150Δasd cells were grown overnight in TB+DAP25 (DAP25 has a concentration of 131 µM; high concentration). One hundred cells were plated onto TB+DAP25 media at time zero. The remaining culture was divided in half, one allowed to grow again overnight in the presence of DAP, and the other not containing DAP. From these cultures, 100 cells were plated onto TB+DAP25 plates to determine if the starved cells would be able to grow. As **Table 1** shows, the culture initially starved of DAP was not able to revive on media containing DAP (no colonies). The cells plated at time zero and the cells grown in DAP 25 are controls as they both were able to grow.

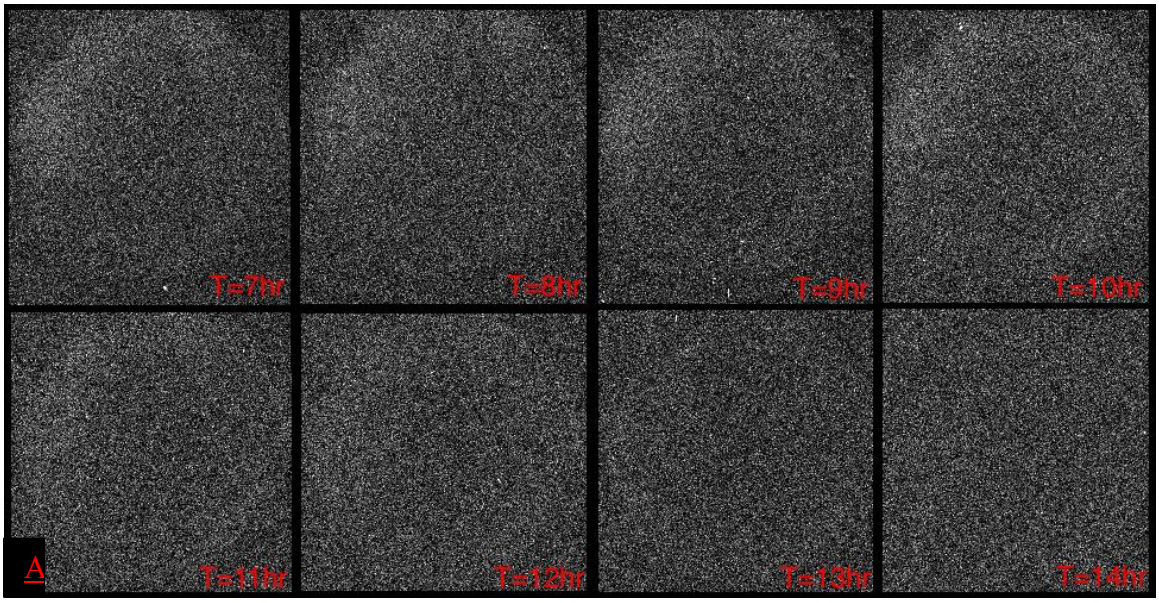
Plate	131 µM DAP (Time =0)	131 µM DAP (over two nights)	No DAP (over two nights)
1	74	67	0
2	63	88	0
3	57	83	0
Average:	64.7	79.3	0

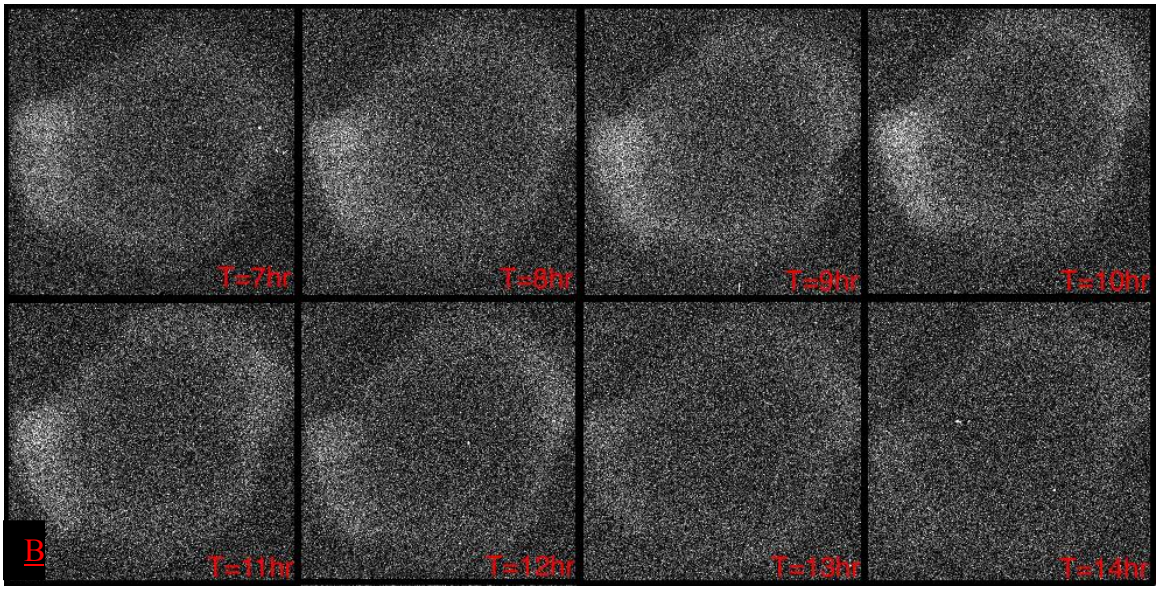
Table 1: Number of colonies observed at time 0 and after two days growth

DAP's Diffusion Ability

In order to measure the ability of DAP to diffuse through soft agar, JG150Δasd pmL001 cells were added to TB+ top layer soft agar and poured onto TB+AMP100

plates with varying DAP concentrations (131 µM, 65.5 µM, 2 µM, 700 nM, 600 nM, and 0). After 7 hours of growth, pictures of the plates were taken hourly for 8 hours to observe growth by luminescence glowing. **Figure 8** shows the different concentrations of DAP (each has its own panel A-E) and how they affected *Y. pestis* growth indicated by luminescence. All plates with DAP glowed, thus DAP at various concentrations was able to diffuse through TB+ top layer soft agar to reach the cells. As earlier experiments showed, the cells begin to plateau and die once the available DAP has been used. The time of highest growth or brightest glowing is around hour 10.

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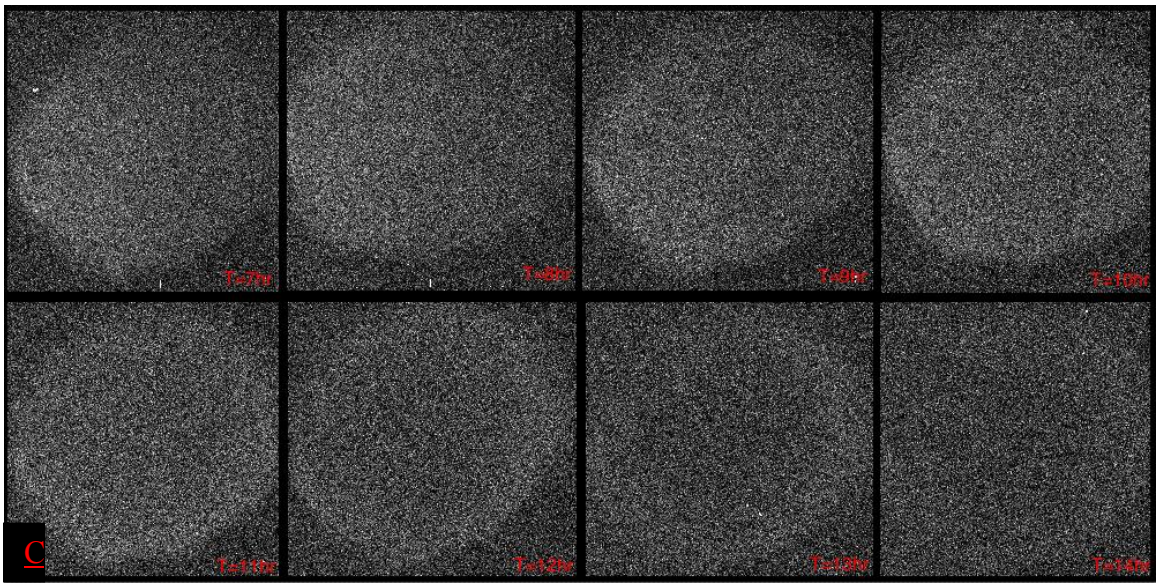
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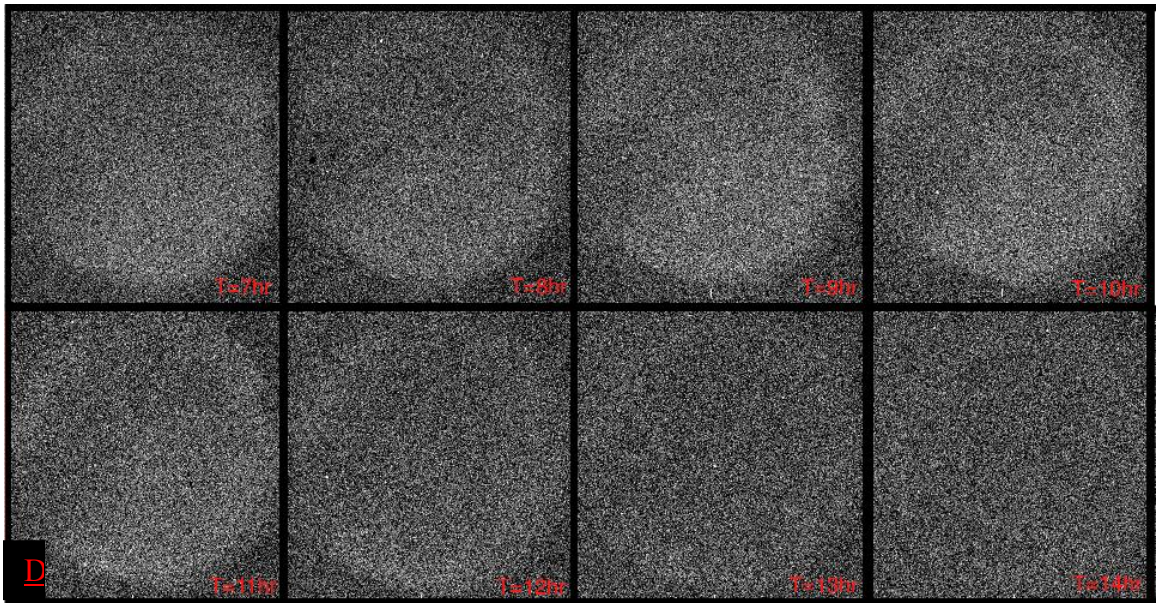
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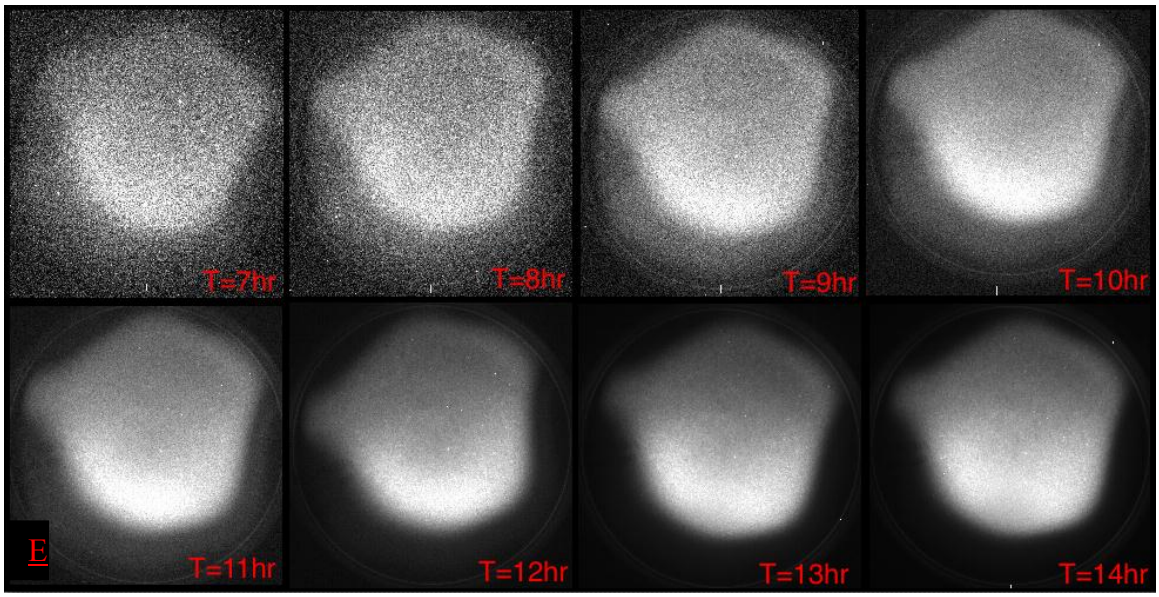
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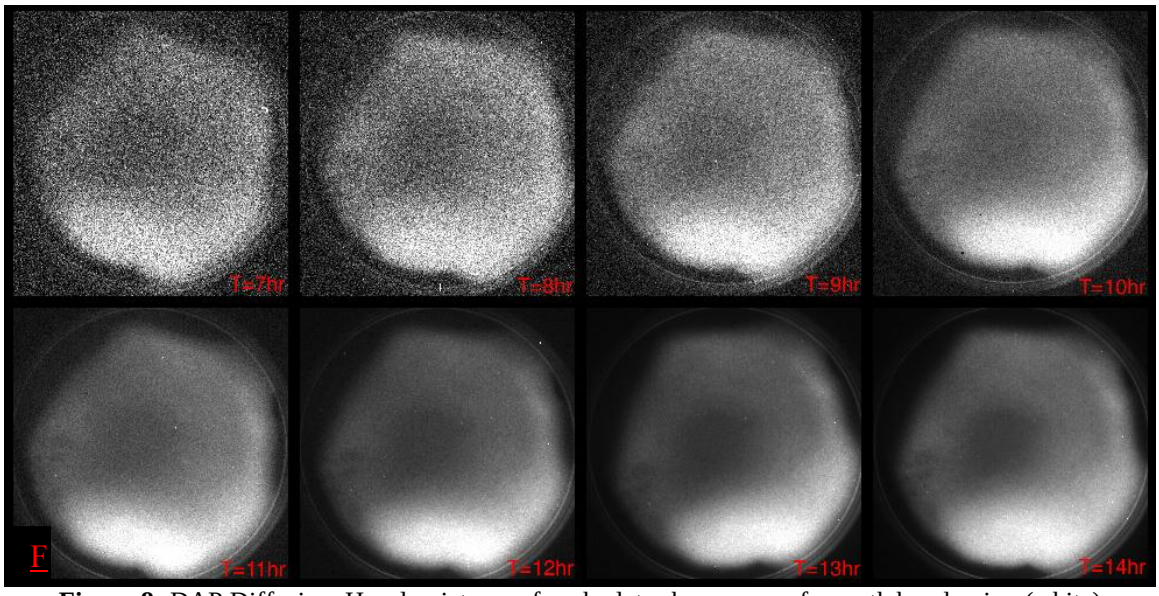

Figure 8: DAP Diffusion. Hourly pictures of each plate show areas of growth by glowing (white) *Y. pestis* cells. Each panel in the figure is a series of pictures from a specific DAP concentration. Panel A is 0 µM, B is 600 nM, C is 700 nM, D is 2 µM, E is 65.5 µM, and F is 131 µM.

Mock-Skin Experiments

No luminescence was detected in the mock-skin experiment setup detailed in the methods section. Subsequent experiments were set up to test different aspects of the procedure to determine which factor was hindering growth. Results of these experiments indicated that a component of the polyacrylamide gel was toxic to the cells, rendering them unable to luminesce.

DISCUSSION

This project explored different aspects of designing a controllable live vaccine for plague using a diaminopimelic acid (DAP)-dependent strain of *Y. pestis*. Being a novel approach to vaccine development, experiments performed in this project established the foundation of the potential vaccine. The first step was to verify that growth of the mutant strain was indeed dependent upon DAP, and if so, to determine the minimum amount of DAP required by each cell for survival. This was achieved by performing both hourly and overnight growth assays using different concentrations of DAP. Next, a strain of DAP-dependent *Y. pestis* containing the luminescence plasmid pML001 was tested by diffusing different DAP concentrations through soft agar to observe growth using a light sensitive camera. This assay showed that higher concentrations of DAP were needed to diffuse through the soft agar to sustain cell growth compared to the liquid media growth curves. An attempt at modeling the diffusion of DAP through skin was performed using polyacrylamide gel as a skin substitute. However, growth was not observed in this assay, and diagnostic experiments indicated that the polyacrylamide gel was toxic to the cells.

Because the polyacrylamide gel is toxic to the cells, an alternative material to model skin needs to be used in subsequent experiments. This would likely be chicken skin initially, then progressing to mouse skin. After establishing the amount of DAP needed for application to the skin, a lotion will be developed containing an appropriate amount of DAP. Mice will be infected with JG150Δasd pML001 then the DAP lotion will be applied to the inoculation site. Growth of the cells on the skin will be monitored using a light sensitive camera. Ideally, the growth will be contained to the area that

received the lotion, preventing the mice from experiencing adverse symptoms previously observed in the *Y. pestis* EV76 live vaccine (Titball & Williamson, 2001).

A topical live vaccine for the plague will have many advantages over its predecessors. The killed vaccine mentioned in Perry and Fetherston (1997) required three injections over nine months, and booster shots were necessary every two years to sustain immunity. A live vaccine would offer a simpler administration as well as a stronger and longer lasting immunity. This would be advantageous if *Y. pestis* was ever used in an act of bioterrorism, as it is highly contagious and easily spread via aerosols.

BIBLIOGRAPHY

Bubeck SS, Dube PH (2007) *Yersinia pestis* CO92_*yopH* Is a Potent Live, Attenuated Plague Vaccine. *Clinical and Vaccine Immunology* **14**: 1235-1238.

Bukhari HI, Taylor AL (1970) Genetic Analysis of Diaminopimelic Acid- and Lysine-Requiring Mutants of *Escherichia coli. Journal of Bacteriology.* **105**: 844-854.

Emergency Preparedness and Response: The Live Virus Smallpox Vaccine (2007) Web. <http://www.bt.cdc.gov/agent/smallpox/vaccination/live-virus.asp>.

Fact Sheet: Plague (2005) Web. <http://www.who.int/mediacentre/factsheets/fs267/en/>.

Galán JE, Nakayama K, Curtiss R III (1990) Cloning and Characterization of the asd Gene of *Salmonella typhimurium*: Use in Stable Maintenance of Recombinant Plasmids in *Salmonella* Vaccine Strains. *Gene* **94**: 29-35.

Hinnebusch BJ, Rosso M-L, Schwan TG, Carniel E (2002) High-Frequency Conjugative Transfer of Antibiotic Resistance Genes to *Yersinia Pestis* in the Flea Midgut. *Molecular Microbiology* **2**: 349-354.

Meadow P, Hoare DS, Work E (1957) Interrelationships between Lysine and αε-Diaminopimelic Acid and their Derivatives and Analogues in Mutants of *Escherichia coli*. *The Biochemical Journal* **66**: 270-282.

Morelli G, Song Y, Mazzoni CJ, Eppinger M, Roumagnac P, Wagner DM, Feldkamp M, Kusecek B, Vogler AJ, Li Y, Cui Y, Thomson NR, Jombart T, Leblois R, Lichtner P, Rahalison L, Petersen JM, Balloux F, Keim P, Wirth T, Ravel J, Yang R, Carniel E, Achtman M (2010) *Yersinia pestis* Genome Sequencing Identifies Patterns of Global Phylogenetic Diversity. *Nature Genetics* **42**: 1140-1143.

Parkhill J, Wren BW, Thomson NR, Titball RW, Holden MTG, Prentice MB, Sebaihia M, James KD, Churcher C, Mungall KL, Baker S, Basham D, Bentley SD, Brooks K, Cerdeno-Tarraga AM, Chillingworth T, Cronin A, Davies RM, Davis P, Dougank G, Feltwell T, Hamlin N, Holroyd S, Jagels K, Karlyshev AV, Leather S, Moule S, Oyston PCF, Quail M, Rutherford K, Simmonds M, Skelton J, Stevens K, Whitehead S, Barrell BG (2001) Genome Sequence of *Yersinia pestis*, the Causative Agent of Plague. *Nature* **413**: 523-527.

Perry RD, Fetherston JD (1997) *Yersinia pestis*—Etiologic Agent of Plague. *Clinical Microbiology Reviews* **10**: 35-66.

Russell P, Eley SM, Hibbs SE, Manchee RJ, Stagg AJ, Titball RW (1995) A Comparison of Plague Vaccine USP and EV76 Vaccine-Induced Protection Against *Yersinia pestis* in a Murine Model. *Vaccine* **13**: 1551-1556.

Sebbane F, Jarrett C, Gardner D, Long D, Hinnebusch BJ (2009) The *Yersinia pestis* caf1M1A1 Fimbrial Capsule Operon Promotes Transmission by Flea Bite in a Mouse Model of Bubonic Plague. *Infection and Immunity* **77**: 1222-1229.

Shata MT, Stevceva L, Agwale S, Lewis GK, Hone DM (2000) Recent Advances With Recombinant Bacterial Vaccine Vectors. *Molecular Medicine Today* **6:** 66-71.

Titball RW, Williamson ED (2001) Vaccination Against Bubonic and Pneumonic Plague. *Vaccine* **19**: 4175-4184.

Virology-Chapter Eight, Vaccines: Past Successes and Future Prospects (2010) Web. <http://pathmicro.med.sc.edu/lecture/vaccines.htm>.