Pseudomonas aeruginosa Displays a Dormancy Phenotype in Water.

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ABSTRACT

Background
Our laboratory is interested in the survival of the environmental bacterium *Pseudomonas aeruginosa* in water as this is a reservoir for infection. Our hypothesis is that *P. aeruginosa* is capable of long-term survival in water by existing in a dormant state due to the presence of particular genes.
Methods
P. aeruginosa wild type PAO1, as well as mini-Tn*5-lux* mutants were inoculated in sterile water and incubated at room temperature. At various time points, bacterial quantitation was performed and the samples were subjected to live-dead staining for visualization by fluorescence microscopy and analysis by flow cytometry. Stained samples were sorted by fluorescence-activated cell sorting (FACS) in order to separate live and dead populations and bacterial quantitation was performed. Further analysis by flow cytometry was carried out using the following fluorescent dyes: Pico Green, Redox Green, and Alexa Fluor 633 Hydrazide in order to confirm the ability of the organism to exist in a dormant state.
Results
PAO1 survives in water for > 6 months, but two physiologically different populations of cells were observed. Fluorescence microscopy of *P. aeruginosa* in water showed that almost 80% of bacterial cells in water stained red with propridium iodide, indicating they were dead. Flow cytometry analysis confirmed this result. However, the red fractions grew on nutrient agar following FACS, indicating these cells were viable. Further analysis with PicoGreen, Redox Sensor Green, and Alexa Fluor Hydrazide fluorescent dyes demonstrated that the organism maintains a constant population of DNA containing cells that do not die, that the cells are not metabolically active, and yet, the cells are capable of maintaining a membrane potential throughout time points of up to one month.
Conclusions
P. aeruginosa is heterogeneous in water and many cells stain dead, although they are viable. Other fluorescent dyes used in conjunction with flow cytometry analysis have demonstrated that the organism is viable, but not metabolically active. This indicates that *P. aeruginosa* may exist in a dormant state. Determining the mechanisms by which *P. aeruginosa* is able to survive in low nutrient environments will further the development of solutions to disease prevention. Dormant organisms are not susceptible to treatment with antibiotics and re-introduction of these organisms to a nutrient-rich environment if transmitted through a contaminated water supply will result in infections.

INTRODUCTION

Pseudomonas aeruginosa is an environmental bacterium capable of causing disease in humans and is one of the most common causes of hospital-acquired infections. This bacterium is capable of long-term survival in the environment and is often transferred into the hospital whereby it persists on surfaces and in the water supply (7). Studies have shown that *P. aeruginosa* can survive for long periods of time in water, however, the mechanism for this survival has never been understood.

The goal of this project was to investigate whether *P. aeruginosa* is capable of dormancy in a low nutrient environment. Dormancy refers to the ability of an organism to exist without replicating, utilizing nutrients, or synthesizing any proteins. Therefore, it must be determined whether or not there are physiological and/or metabolic changes between the vegetative growth form, and the dormant growth form (1). *P. aeruginosa* has been shown to survive in water for over 145 days, significantly longer compared to two other bacterial pathogens, *Escherichia coli* and *Staphylococcus aureus* (5). We previously utilized a *P. aeruginosa* mini-Tn*5-lux* mutant library to screen for genes involved in water survival (2, 3, 6, 8). In subsequent studies looking at the survival of *P. aeruginosa* and the mutant library we have found that a population of the cells in water appear to be dead when subjected to live/dead staining, but are viable when plated on nutrient agar. Persistence has been characterized, but dormancy has never been reported for *P. aeruginosa* in low nutrient environments (4).

MATERIALS AND METHODS

Survival of *P. aeruginosa* in water:
PA01 was grown overnight in LB, washed 3 times, and added to sterile distilled water at a concentration of 10^7 cfu/ml. Samples were loosely capped and incubated at room temperature. At each time point 100 µl of sample was serially diluted and plated for bacterial quantitation.
Live/dead staining, flow cytometry, and fluorescence-activated cell sorting (F.A.C.S.) to determine dormancy:
At each time point for eight weeks, 1 ml (10^7 cells) of sample was taken for flow cytometry and F.A.C.S. and was centrifuged at 13, 000 rpm for 3 minutes and resuspended in 1 ml of 0.9% saline. Syto9 (green) and propridium iodide (red) were added at a final concentration of 30 and 10 µM respectively. Samples were then subjected to flow cytometry for the purposes of quantitating the number of green (live) and red (dead) cells based on their emission wavelengths. Once the proportion of green and red cells was determined, 10^6 of these cells were sorted by fluorescence-activated cell sorting to separate the live and dead populations into separate vials. PAO1 incubated in water was also stained with PicoGreen for analysis of DNA content, Alexa Fluor Hydrazide for accumulation of carbonyl compounds as a measurement of metabolic activity, and Redox Green to determine electron transport activity. All of these were subjected to flow cytometry to analyze the amount of stain bound by the cells, as an indicator of cell viability (Pico Green) and metabolic activity (Alexa Fluor Hydrazide and Redox Green).
Assessment of ATP production of PAO1 live and dead populations:
PAO1 stained with PI and Syto9 was subjected to F.A.C.S. to separate the living and dead fractions meaning that the red (PI) and green (Syto 9) fractions were isolated. ATP production of each fraction was assessed using the BacTiter-Glo Microbial Cell Viability Assay, which measures the amount of ATP present in a sample as a function of luminescence.
Fluorescence microscopy of live/dead stained cells:
At each time point for 8 weeks, 1 ml of the water sample containing either PAO1 was centrifuged and resuspended in 0.9% saline. Syto9 and propridium iodide were added to 10 µl of the sample at a concentration of 30 and 10 µM respectively and 2 µl of the sample was added to an agarose well (0.1% agarose) on an agarose-coated glass slide and sealed with a glass coverslip. Slides were visualized on a Leica fluorescence widefield microscope.

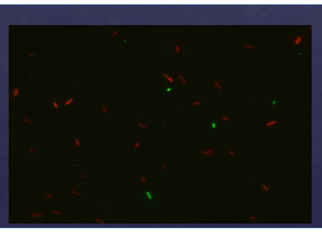


Figure 1. Live/dead staining results of *P. aeruginosa* strains PAO1 following incubation in water for one week. PAO1 was subjected to live/dead staining with Syto9 and propridium iodide and viewed on a fluorescence microscope for visualization of live and dead cells in the population.

Table 1. Counts per Second (CPS) of luminescence assay to measure ATP. Samples used equal volume of bacteria suspension and ATP reagent. ATP reagent component of BacTiter-Glo Microbial Cell Viability Assay from Promega.

Week	PAO1 (S9)	PAO1 (S9PI)
1	16 504	15 657
2	671	789
7	636	698
8	460	632

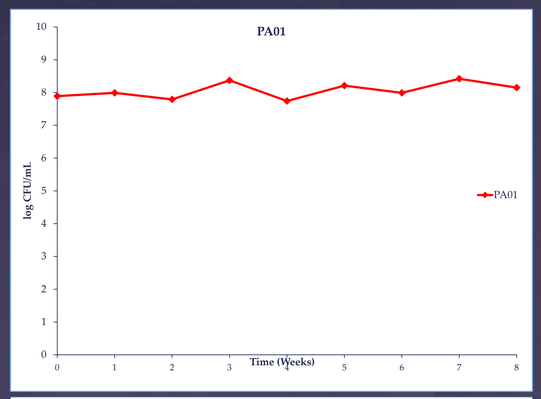


Figure 2. Survival of *P. aeruginosa* PAO in water. PAO1 was inoculated into sterile water at a concentration of 10^7 cfu/ml and incubated at room temperature. At each time point bacterial quantitation was performed by serial dilutions and plating.

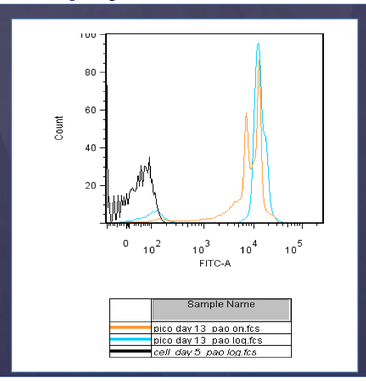


Figure 3. Pico Green staining and flow cytometry results for *P. aeruginosa* PAO in water. Both log and stationary phase cultures of PAO1 were obtained and inoculated into water for incubation. Each sample was stained with Pico Green and subjected to flow cytometry over a series of time points to determine cell viability as a function of double-stranded DNA content. This figure indicates the results obtained for samples following 13 days of incubation in water.

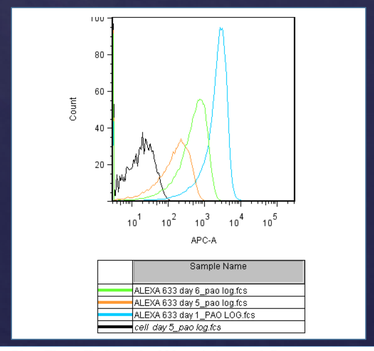
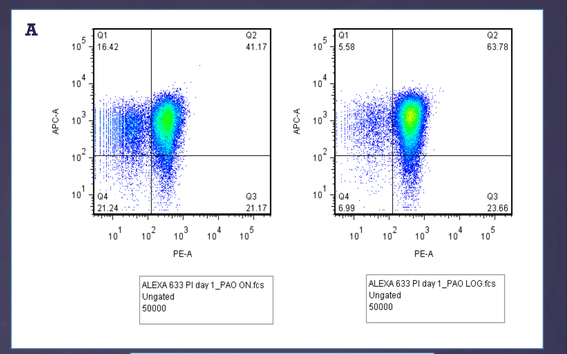
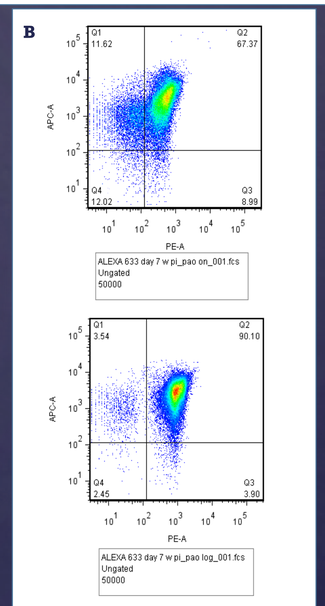


Figure 4. Alex Fluor Hydrazide 633 staining and flow cytometry results for *P. aeruginosa* PAO in water. Both log and stationary phase cultures of PAO1 were obtained and inoculated into water for incubation. Each sample was stained with Alex Fluor 633 and subjected to flow cytometry over a series of time points to determine cell viability as a function of double-stranded DNA content. This figure indicates the results obtained for log phase cells following 1, 5, and 6 days of incubation.





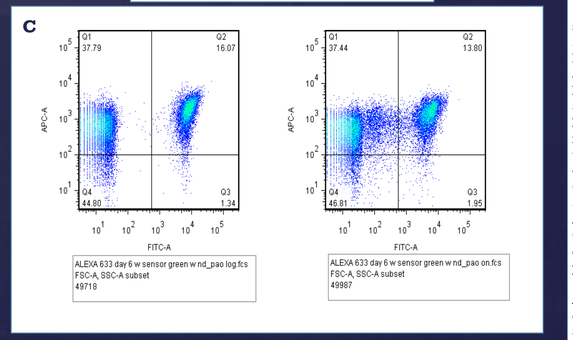


Figure 5. Alex Fluor Hydrazide 633 and Propridium Iodide (PI) or Alexa Fluor Hydrazide 633 and Redox Sensor Green staining and flow cytometry results for *P. aeruginosa* PAO in water. Both log and stationary phase cultures of PAO1 were obtained and inoculated into water for incubation. Each sample was stained and subjected to flow cytometry over a series of time points to determine cell viability and metabolic activity. Figure 5A, PAO1 stained with Alexa Fluor 633 and PI at Day 1. Figure 5B, PAO1 stained with Alexa Fluor 633 and PI at Day 7. Figure 5C, PAO1 stained with Alexa Fluor 633 and Redox Sensor Green on Day 6.

DISCUSSION

Pseudomonas aeruginosa is capable of long-term survival without nutrients, and this study demonstrates that this may be due to the ability of the organism to exist in a dormant state. The organism appears dead by live/dead staining (Figure 1), yet, when the dead cells are plated on a nutrient medium, they are viable. This explains why the number of cells does not diminish over prolonged incubation in water. A conclusion that can be made with regards to Syto9 and propidium iodide (PI) is that their ability to bind to nucleic acids does not necessarily reflect that a cell is either dead or alive. It is likely that *P. aeruginosa* is capable of altering its membrane in water and this may assist the organism in surviving in a low nutrient environment. This alteration would explain the ability of PI to penetrate the membrane and stain the DNA. Further studies are currently being conducted to investigate whether the membrane has increased permeability in water and whether strains harbouring mutations in membrane genes are less likely to survive in water.

P. aeruginosa can survive for months in water and maintain its initial inoculum size. The number of colony forming units remains the same over time (Figure 2). We have previously shown that PI-stained cells are viable by fluorescence-activated cell sorting and performing bacterial quantitation. In this study, the PAO1 was also shown to be viable as staining with Pico Green revealed that the same population of cells persists over time and contains double-stranded DNA, an indicator of viability (Figure 3). Although the cells are viable in water, the amount of metabolic activity diminishes. ATP production decreased over time in cells that stained with Syto9 and well as PI (Table 1). Flow cytometry analysis of PAO1 cells stained with Alexa Fluor Hydrazide 633 demonstrated that the amount of carbonyl compounds also decreases over time, which indicates that the metabolic activity of the cells is not increasing (Figure 4). Cells that stain with PI as well as Alexa Fluor 633 maintain a constant profile in flow cytometry, indicating that the cells that appear to be dead are not metabolically active and that it is the same population of cells, not cells that are dying and replicating due to the available nutrients of dead cells (Figure 5). Results of staining with Redox Sensor Green suggest that although the cells may not be metabolically active, they are capable of maintaining a membrane potential (Figure 5).

The ability of *P. aeruginosa* to survive in water makes it a reservoir for infectious disease. Since this organism is readily transferred into the hospital where it causes infection, it is important to understand why this organism is able to survive in environments such as water, that contain few nutrients for growth. This will contribute to a better understanding of the organism which can lead to better solutions for the prevention of infection. Finally, determining the mechanism for survival in a low nutrient environment will be beneficial for understanding how other microorganisms may persist in similar conditions.

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