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Yiming Zhang April 15, 2015

Factors Determining the Death Rate of Starved *E.coli* Cells

by

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract
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In nature, most bacteria survive in non-proliferating state due to the limitation of their access to nutrients. In such state, it has long been recognized that bacteria will progressively lose their viability. The factors determining the death rate of bacteria under starvation condition have been of great interest to scientists. Many of these factors have been very well studied on the molecular level, including the oxidative stress and the anaerobic respiration. In this research, we propose that the ongoing DNA replication in stationary phase is another factor that leads to bacteria death. We used two different methods in this study to compare the viability of cells with ongoing DNA replication and that of cells without such process. We took advantage of a temperature sensitive strain which is not able to initiate DNA replication at 43 ℃ but can finish ongoing replication at this temperature. Also we used the antibiotic rifampicin to achieve the same purpose. Our result shows that cells without open DNA forks do have extended lifespan under starvation condition. Moreover, we also compared the viability of rifampicin treated wild type cells with RpoS deleted cells. RpoS, a master regulator of stress response, is proved to be necessary in our research for cells to maintain the viability. We expect that the findings provide a new insight into underlying mechanisms of senescence and longevity for bacteria and other organisms.

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Title

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Abstract

In nature, most bacteria survive in non-proliferating state due to the limitation of their access to nutrients. In such state, it has long been recognized that bacteria will progressively lose their viability. The factors determining the death rate of bacteria under starvation condition have been of great interest to scientists. Many of these factors have been very well studied on the molecular level, including the oxidative stress and the anaerobic respiration. In this research, we propose that the ongoing DNA replication in stationary phase is another factor that leads to bacteria death. We used two different methods in this study to compare the viability of cells with ongoing DNA replication and that of cells without such process. We took advantage of a temperature sensitive strain which is not able to initiate DNA replication at 43 ℃ but can finish ongoing replication at this temperature. Also we used the antibiotic rifampicin to achieve the same purpose. Our result shows that cells without open DNA forks do have extended lifespan under starvation condition. Moreover, we also compared the viability of rifampicin treated wild type cells with RpoS deleted cells. RpoS, a master regulator of stress response, is proved to be necessary in our research for cells to maintain the viability. We expect that the findings provide a new insight into underlying mechanisms of senescence and longevity for bacteria and other organisms.

Keywords

Non-proliferating bacteria/ *E.coli*/ viability/ DNA replication/ RpoS

Introduction

In science researches, rich medium LB is used for cells to proliferate. However, in natural environment, most living organisms do not have access to enough nutrients to support continuous cell growth and replication. Therefore, studying the mechanisms through which cells sustain their viability and identifying the factors leading to cells death are of great importance. Regardless of the extensive researches on bacteria on the molecular level, not much about the death rate of starved cells has been explored so far.

The growth and death of bacteria population have been very well characterized in previous researches. In laboratory, bacteria cells are stored at -80 ℃. To initiate their proliferation, cells from -80 ℃ storage are transferred to LB rich medium and cells will recover their growth in such nutrient-rich environment.

In lab we describe bacteria population change with their cell density in liquid medium. The population density is measured through optical density, which is measured by an odometer. A tube of liquid medium containing cells is injected into the odometer which shines light onto the tube from one side and collects it on the other side. By calculating the difference in amount of light emitted and collected, the optical density (OD) can be measured and can be directly translated into population density. For bacteria, which have the size of about 600 nm, we use a wavelength of 600 nm on the odometer.

At the onset of cell growth, bacteria will first go through a lag phase. During this

lag phase, bacteria adjust themselves to the new environment. They take this time to manufacture proteins and other essential chemicals for their later growth. Meanwhile, cells only increase in size but not in number. Therefore during lag phase there is no increase in cell population density.

As cells are fully prepared for their growth, they enter the log or exponential phase, during which they rapidly proliferate and the cell density increases exponentially.

As the number of cells increases, nutrients in the liquid medium become depleted due to the increase in demand to support cell growth and survival. When cells sense the decrease in nutrients in the environment, their proliferation is checked and the cell density stops increasing and we define this as the beginning of stationary phase.

Furthermore, when one or multiple kinds of nutrients run out, cells begin to die. The death rate of cells is also population-dependent, which means that their viability also decreases exponentially.

Cell death under starvation condition has been the topic for many researches. On the molecular level, many factors determining cell death have been discovered, including oxidative stress and anaerobic respiration. Oxidative stress refers to the disturbance of the balance of redox reactions in cells. This imbalance can result in the production of many toxic chemicals including free radicals. These toxic species can then go on to react and damage other structures within the cell and hence leading to cell death. Anaerobic respiration is the mechanism for cells to draw energy when they do not have access to enough oxygen to undergo aerobic respiration. During

anaerobic respiration, cells can use other electron acceptors, for example, sulfate ion and sulfur.

While cells have ongoing DNA replication processes, if nutrients are depleted, cells will not be able to finish DNA replication and the DNA double helix will remain open, which is known as a DNA fork. Although the DNA double helix itself is a stable structure as the storage of genetic information, when it stays as a DNA fork, the stability of its molecular structure is undermined.

Therefore, in this research, we suggest that under starvation condition, the instability of the DNA fork will result in the increase in the death rate of bacteria.
In this study we chose *Escherichia coli* (*E.coli)* to be our model as this type of bacteria has been extensively studied in the past and many cell mechanisms are already well characterized.

During stationary phase when nutrients are depleted, we can measure the rate of cell death through the viability test: certain amount of culture is taken and diluted and plated on solid nutrient-rich medium. Each single alive cell will form a colony after an overnight incubation. By counting the number of colony forming units (CFU) per milliliter of culture used and plotting it over time, we will be able to evaluate the rate of cell death [Figure 1]. Again, since cell death is population-dependent, by using a log scale y-axis, we will be able to observe a linear relationship between CFU/mL and time [Figure 2].

To study the behavior of bacteria under starvation condition, in this research we induced the starvation by depleting carbon because carbon is one of the most

important elements supporting cell growth and survival. We think that by starving cells with carbon, we will be able to study the fundamental mechanisms underlying cell's behavior during stationary phase.

In order to test our hypothesis that in stationary phase, the instability of unfinished DNA replication fork leads to the loss of cell viability, we need to compare the viability tests of cells entering stationary phase with unfinished DNA replication with that of cells entering stationary phase without such DNA replication forks. There are two methods available to achieve this purpose. We first took advantage of a temperature sensitive strain (E508) whose optimal growing temperature is 30 ℃. This strain will stop growing or initiating new DNA replication at 43 ℃ but will still be able to finish ongoing replications. Our second method is to use the antibiotic rifampicin which inhibits the initiation of bacteria DNA replication, but still allows cells to finish ongoing replication processes.

Using the two methods we were able to observe a distinct extended lifespan in cells that do not possess open DNA replication forks.

Result

Cells entering a non-proliferating state after completing ongoing chromosome replication have significantly longer chronological lifespan.

Details of our experimental procedure can be found in methods section. In order to compare the viability of stationary phase cells with ongoing DNA replication and

that of cells without such process, we selected a temperature sensitive strain (E508). This temperature sensitive strain was first incubated at 30 ℃, at which temperature they would grow normally. Once they reached an optical density (OD) of 0.05 (at 600 nm), the temperature was switched immediately to 43 ℃. After10 hours, OD would completely stop and we reasoned that this was due to bacteria having finished their DNA replication and without initiating new ones. Then the cells were washed and suspended in carbon free medium at 30 ℃. For our control group, cells were incubated at 30 ℃ to an OD of 0.5. Then cells were directly washed and suspended in carbon free medium. The viability test shows an extended lifespan for cells that went through 43 ℃ [Figure 3].

We noted that this previous experiment involved a temperature shift, which might have changed the physiological states that led to the observed extension of lifespan. Therefore, to eliminate this possibility, we performed the next experiment using rifampicin as another method to prevent cells from initiating DNA replication.

Next, we grew wild type cells in a minimal medium supplemented with casamino acids, glucose and ammonium at 37 ℃. When they reached an optical density around 0.5 at 600 nm, we washed the cells and suspended them in carbon free medium to induce the stationary phase. Viability test was performed on these cells and the NFU/mL was plotted as a function of time on log scale. Another group of wild type cells are incubated with the same medium, but rifampicin was added at an optical density (OD) of ~0.1. The OD will stop increasing at around 0.5 and we then washed the cells and suspended them in carbon free medium. By measuring the viability, we

were able to observe that the cells which entered non-proliferating phase without DNA replication forks (the rifampicin treated group) had an obvious longer lifespan and a slower death rate, thus confirming our hypothesis [Figure 4].

The maintenance of viability is not due to dynamical equilibrium of growth and death of subpopulations

It occurred to us that the maintenance of viability could be due to a dynamical equilibrium of growth and death of subpopulations. In order to confirm that this dynamical equilibrium was not the cause for the maintenance of viability, we took advantage of the antibiotic, ampicillin. Ampicillin is known to attack only the cell wall of growing cells. If the bacteria are not growing with ongoing DNA replication, treating them with ampicillin will not kill them. With this idea, we first performed the experiment with temperature sensitive strain, and added ampicillin to the cells that had spent 10 hours in 43 ℃. The viability of cells from 43 ℃ was compared with that of cells without ampicillin treatment. The result shows that ampicillin did not affect the viability of cells [Figure 5]. Therefore we were able to conclude that for the cells whose viability was maintained, they did not have ongoing DNA replication.

We also performed the rifampicin experiment with ampicillin treatment. We first treated cells with rifampicin and then added ampicillin and measured viability. This viability is compared with rifampicin treated cells without ampicillin. The result was similar to that of the temperature sensitive train with ampicillin treatment, indicating that there were no growing cells in the experimental group [Figure 6].

RpoS is required for the long-term maintenance of viability

RpoS, a master regulator of stationary phase and general stress response, contributes to cell survival during various stresses, including starvation. To test that RpoS plays a role in the previous observed maintenance of viability, we used an RpoS deleted strain to study the effect of this gene. We performed the rifampicin experiment with both wild type strain and RpoS deleted strain. We discovered that while we were able to observe an extended lifespan for wild type cells after rifampicin treatment, the same trend did not emerge with RpoS deleted strain. In fact, after rifampicin treatment, RpoS cells rapidly lost their viability [Figure 7].

Discussion

In nature, most living organisms survive in non-proliferating states. As described in the introduction, we proposed that the instability of DNA replication fork is one of the major factors contributing to cell death under starvation condition.

From our results, it can be concluded that cells entering stationary phase without DNA replication forks have extended lifespan comparing to cells with unfinished DNA replication processes. Also, this extended lifespan is not due to a dynamical equilibrium between growing and dying cells, as proved by the ampicillin experiment. In addition, to attain this extended lifespan, RpoS gene is required.

Although the experiments lead to the conclusion that without DNA replication forks, bacteria viability during stationary phase can maintain for extended time, the relationship between DNA replication and lifespan can be indirect. We cannot rule out the possibility that by treating cells with antibiotics and changing temperatures, we in fact altered the physiological states of cells, which then provided them an extended lifespan.

In this study we used antibiotics to treat cells. In clinic, these antibiotics are used against microorganism infection. The fact that in this research treating bacteria with antibiotics actually leads to their extended lifespan can give us some important information to understand results from future clinic trials.

Lastly, a previous research has shown that starved yeast cells without ongoing DNA replication processes also have longer lifespan comparing to yeast cells with DNA replication forks. This similarity between eukaryotic cells (yeast) and prokaryotic cells (bacterium) may reveal some regularity governing the death of cells among all species.

Methods

Temperature sensitive strain and the rifampicin treatment experiments

E.coli K-12 wild type strain NCM3722, and a mutant temperature-sensitive for chromosome replication initiation E508, were used in our study. Our base medium is the N-C- minimal medium (which lacks carbon and nitrogen sources).

NCM3722 wild type cells were grown in N-C- minimal medium supplemented with 0.2% casamino acid, 20 mM NH_4Cl, and 20 mM glucose. When the OD at 600 nm of the culture reached ~ 0.5, we washed and suspended them in the N-C- medium (without other supplements). This marked the onset of starvation, the time zero on our graphs. Afterwards, the number of colony forming unit, CFU, was determined by platting cells on LB agar plates. The cultures and agar plates (after platting) were kept at 37 ℃ throughout the experiments.

For rifampicin treated group, cells were grown in N-C- minimal medium supplemented with 0.2% casamino acid, 20 mM NH_4Cl, and 20 mM glucose. When the OD at 600 nm reached ~0.5, we added 125 μg/mL of rifampicin, and waited for 6 hours. Then, the cells were washed and suspended in the N-C- medium containing no supplements (no rifampicin). Afterwards, CFU was determined by platting cells on LB agar plates. The cultures and agar plates (after platting) were kept at 37 ℃. To determine whether there existed a growing subpopulation in the culture prepared as above, we added 100 μg/mL of ampicillin to the culture. Note that right after the onset of starvation, CFU was very low, but was recovered to its maximum after a day. Thus we added the ampicillin a day after the onset of starvation, when CFU was fully recovered.

Our temperature sensitive strain was thymine-auxotrophic and 100 μg/mL of thymine was added to the medium throughout the experiments. The cells were grown in the N-C- minimal medium supplemented with 0.2% casamino acid, 20 mM NH_4Cl, 20 mM glucose and 100 μg/mL of thymine at 30 ℃. When the OD at 600 nm of the

culture reached ~0.5, we washed and suspended them in a N-C- medium with 100 μg/mL thymine (we performed a controlled experiment in which cells were suspended in a N-C- medium without thymine, but we did not see any difference in CFU). Afterwards, CFU was determined by platting cells on LB agar plates containing 100 μg/mL of thymine. The starved culture and agar plates (after platting) were kept at 30 ℃. For the temperature shift, cells were grown in the N-C- minimal medium supplemented with 0.2% casamino acid, 20 mM NH4Cl, 20 mM glucose and 100 μg/mL of thymine at 30 ℃. When the OD at 600 nm of the culture reached ~0.05, we shifted the incubation temperature to 43 ℃. The growth continued for some time (~8 hours) and completely stopped at OD at 600 nm of ~ 0.5. Then, we washed and suspended cells in the N-C- medium with 100 μg/mL thymine. Then we incubated the culture at 30 C. Afterwards, CFU was determined on LB agar plates containing 100 μg/mL thymine. The agar plates (after plating) were incubated at 30 C.

Author Contributions:

M.K. designed research.

Y.Z. and B.P. performed the experiments.

Y.Z. wrote the paper

Conflict of Interest:

The authors declare no competing financial interests.

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Figure Legends

Figure 1: viability test sample on regular y axis

This figure shows the death curve of wild type bacteria cells under carbon starvation condition. The number of colony forming unit per milliliter of liquid medium is plotted against time in hours.

Figure 2: viability test sample on log scale y axis

This figure shows the death curve of wild type bacteria cells under carbon starvation condition. The number of colony forming unit per milliliter of liquid medium is plotted against time in hours. The y axis is in log scale. Because cells die exponentially with respect to time, using log scale renders a linear relationship between CFU/mL and time (hour)

Figure 3: Temperature sensitive strain viability

Viability test result of temperature sensitive strain E508 is shown in this figure. Experimental group cells were grown in 30 ℃ to an OD (600 nm) of 0.05 and were then kept at 43 ℃ for 10 hours. We then washed the cells and suspended them in carbon free medium at 30 ℃ to measure viability. Control group cells were grown in 30 ℃ to an OD (600 nm) of 0.05. Then they were washed and suspended in carbon free medium. The viability test clearly shows that cells which had lived at 43 ℃ were able to maintain their viability for extended period.

Figure 4: Rifampicin experiment

Rifampicin group was grown in rich medium to an OD (600 nm) of 0.5 and was

treated with rifampicin for 7 hours and was then washed and suspended in carbon free medium. Control group was grown to and OD of 0.5 and was directly washed and suspended in carbon free medium. A clear difference in death rate of the two groups can be observed.

Figure 5: Ampicillin treatment of temperature sensitive cells

Temperature sensitive strain was grown at 30 ℃ and was transferred to 43 ℃. After sitting at 43 ℃ for 10 hours, one group of cells were washed and suspended in carbon free medium at 30 ℃ with ampicillin treatment and another group of cells were washed and suspended without ampicillin. It can be observed that there is no obvious difference between the death rates of two groups.

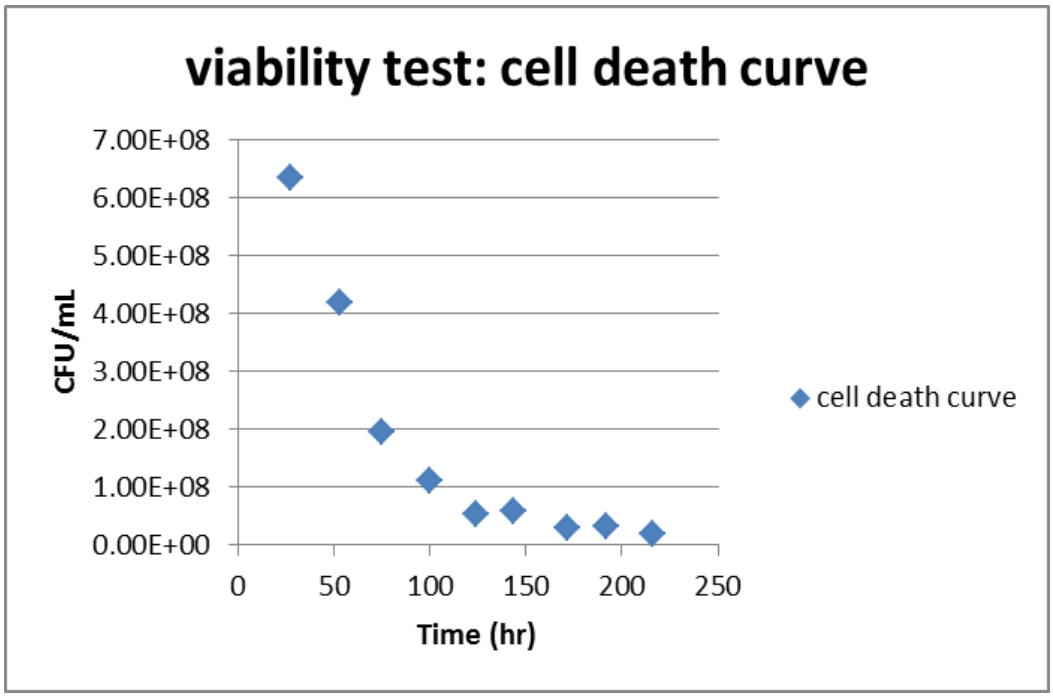
Figure 6: Ampicillin treatment of rifampicin treated wild type cells

Wild type cells were grown in rich medium to an OD (600 nm) of 0.5. Rifampicin was then added to the culture and cells were treated with it for 7 hours. Then the cells were washed and suspended in carbon free medium. One group of cells was then treated with ampicillin. The death rates of the two groups are compared in the graph. It is shown that there is no obvious difference in their death rates.

Figure 7: Rifampicin treatment with wild type and RpoS deleted cells

Wild type cells and RpoS deleted cells were grown in rich medium to an OD of 0.5. Then the cells were treated with rifampicin for 7 hours and were then washed and suspended in carbon free medium. The result for viability test is displayed in the graph. It is clear that cells without RpoS gene did not have extended lifespan, even after being treated with rifampicin.

Figure 1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6.00E+08
5.00E+08
4.00E+08
3.00E+08
2.00E+08
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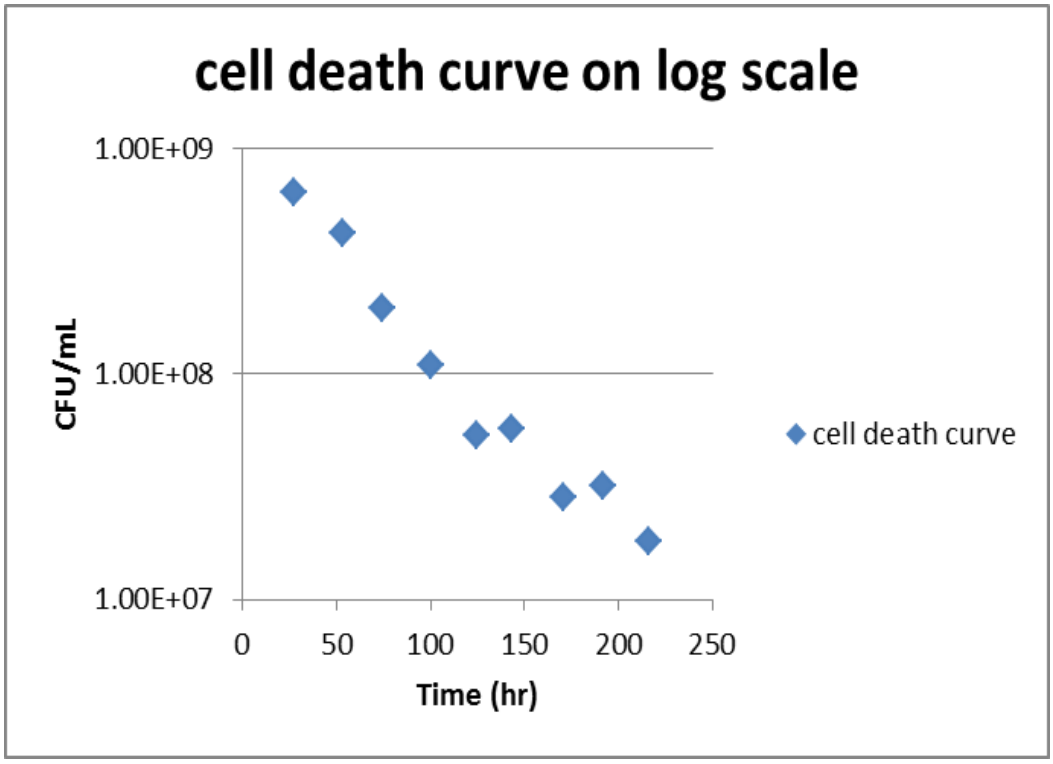
0.00E+00
CFU/mL
0
50
100
150
200
250
Time (hr)
cell death curve

Figure 2


cell death curve on log scale
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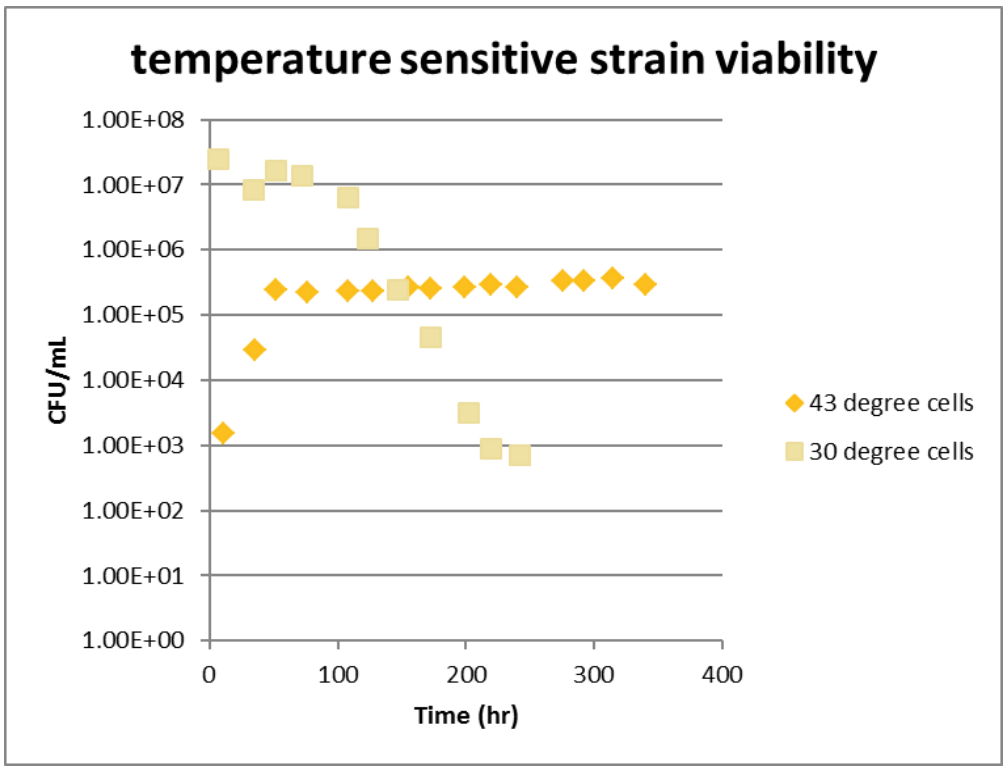
1.00E+07
CFU/mL
0
50
100
150
200
250
Time (hr)
cell death curve

Figure 3


temperature sensitive strain viability
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1.00E+03
1.00E+02
1.00E+01
1.00E+00
CFU/mL
0
100
200
300
400
Time (hr)
43 degree cells
30 degree cells

Figure 4

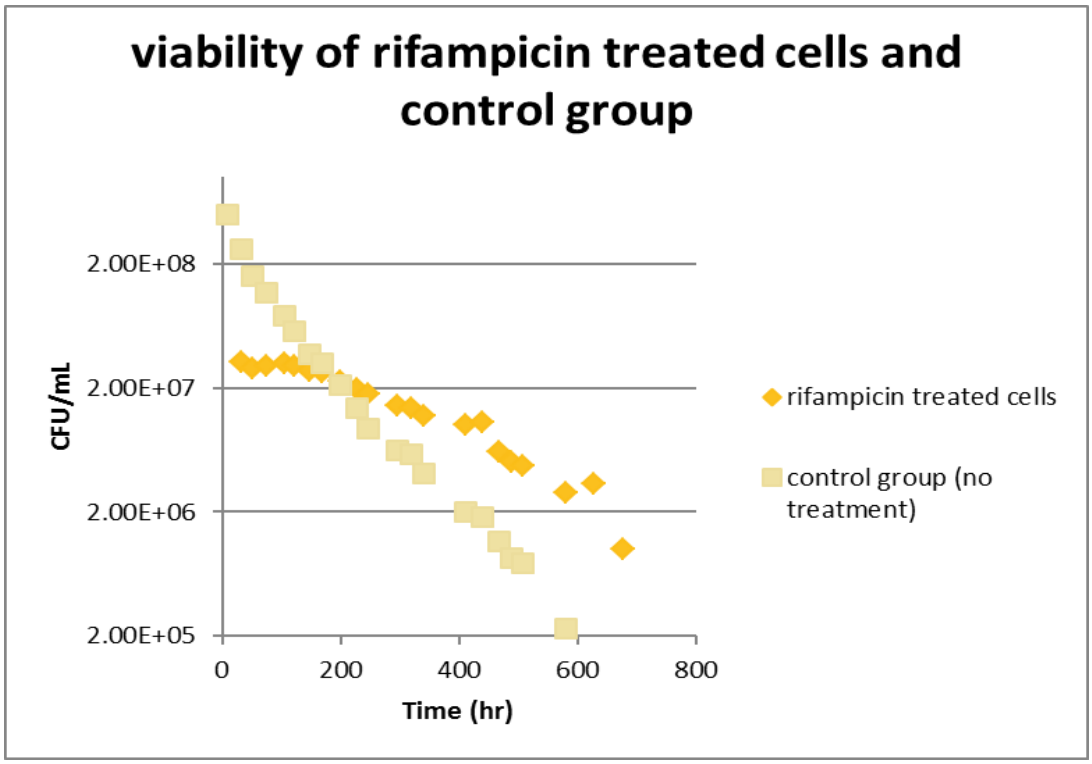

viability of rifampicin treated cells and control group
CFU/mL
2.00E+08
2.00E+07
2.00E+06
2.00E+05
0
200
400
600
800
Time (hr)
rifampicin treated cells
control group (no treatment)

Figure 5

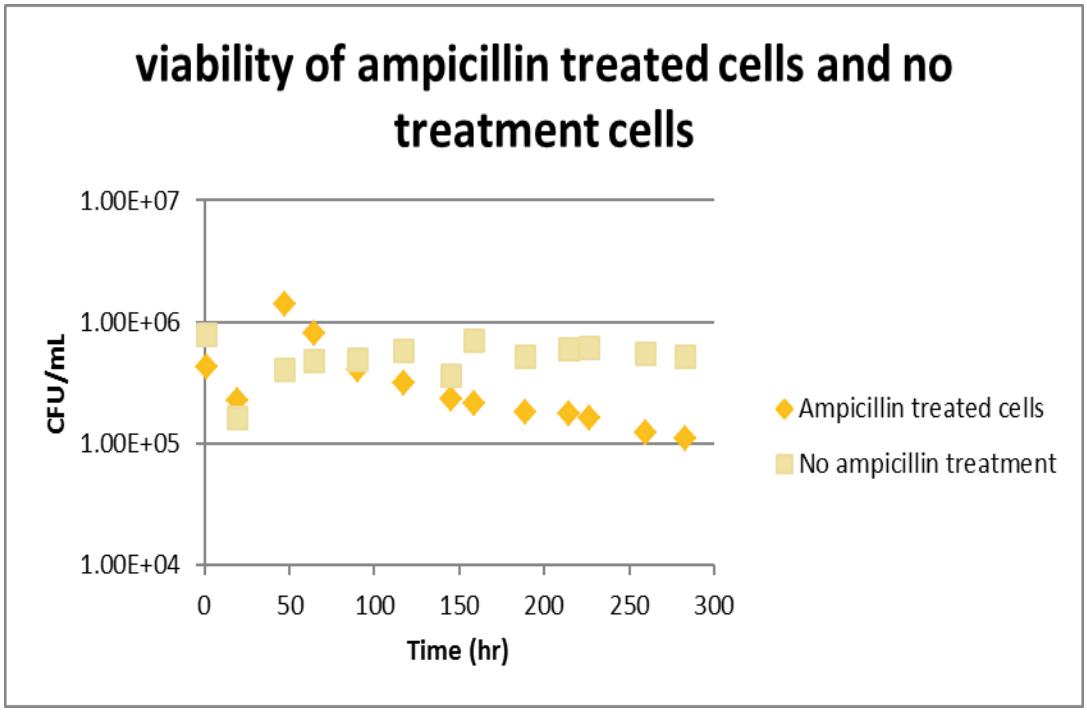

viability of ampicillin treated cells and no treatment cells
1.00E+07
1.00E+06
1.00E+05
1.00E+04
CFU/mL
0
50
100
150
200
250
300
Time (hr)
Ampicillin treated cells
No ampicillin treatment

Figure 6

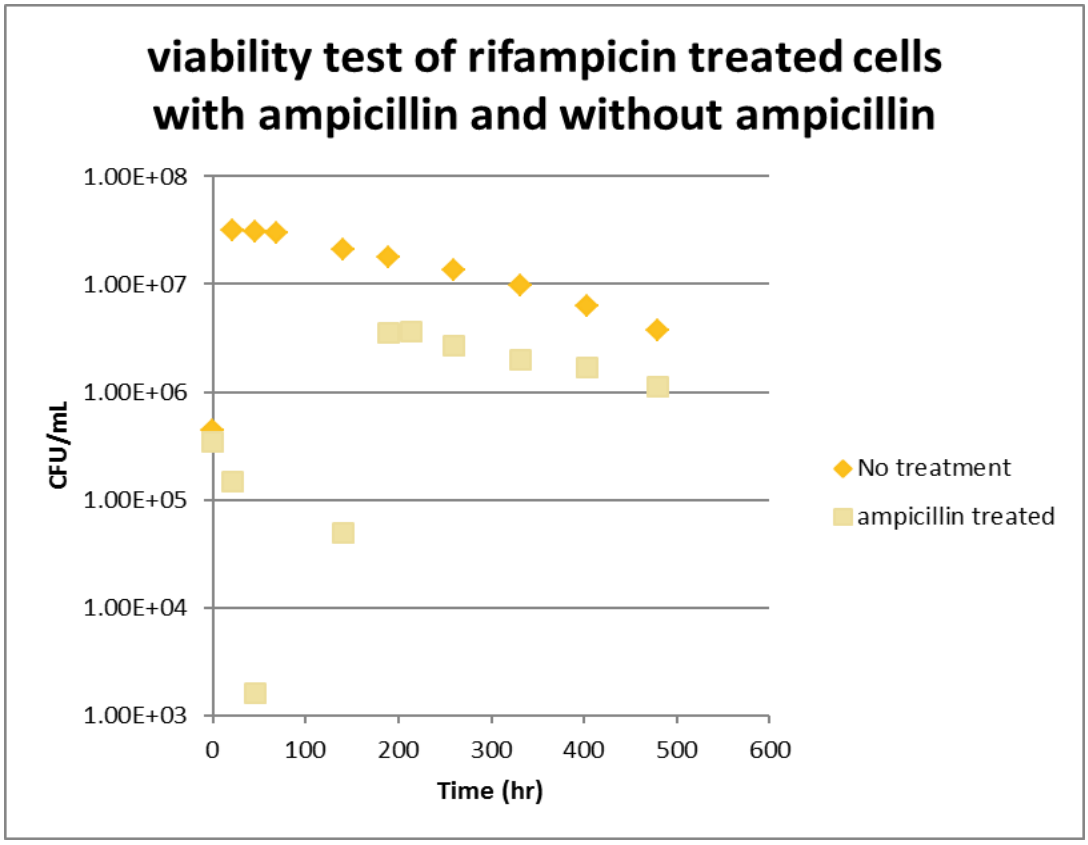
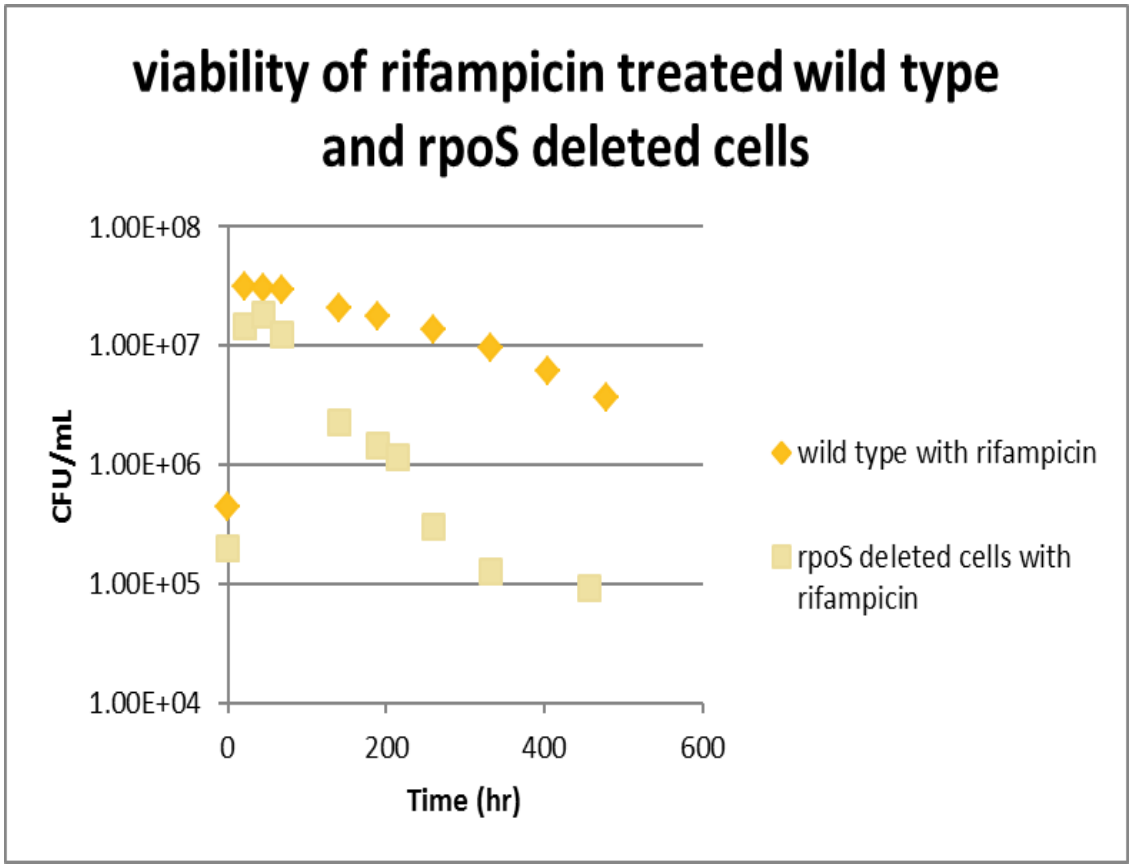

viability test of rifampicin treated cells with ampicillin and without ampicillin
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
CFU/mL
0
100
200
300
400
500
600
Time (hr)
No treatment
ampicillin treated

Figure 7


viability of rifampicin treated wild type and rpoS deleted cells
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
CFU/mL
0
200
400
600
Time (hr)
wild type with rifampicin
rpoS deleted cells with rifampicin