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**LIFESPAN OF PROKARYOTE MODEL ORGANISM
ESCHERICHIA COLI K-12**

by

Hyrum Gillespie

**Thesis submitted in partial fulfillment
of the requirements for the degree**

of

DEPARTMENTAL HONORS

in

**Crop Science (Research and Biotechnology Emphasis)
in the Department of Plants, Soils and Climate**

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Introduction

Bacteria are amazingly resilient organisms, in that they have evolved and adapted to the many extreme environments of Earth (from salt, to pH, to temperature extremes etc.). The bacterial lifespan and death rates are as important as its growth rates in these extreme environments. Bacteria would be useful to determine the effects of age on single cells, but because bacteria reproduce asexually by binary cell fission (clonal replication), calculating the lifespan has proven elusive. Without the determination of a lifespan, age studies using bacteria have limited application. Further, it has been proposed that organisms whose somatic cell line is not distinct from its germ line such as single celled organisms like *Escherichia coli* K-12 are immortal (Williams *et al.*, 1957; Johnson & Mangel, 2006).

Several theories exist with regards to aging. One theory, the “reliability theory of aging and longevity,” states that over time even systems with non-aging parts will deteriorate and die with age due to the failure of irreplaceable parts (Gavrilov & Gavrilova, 2001). This theory correlates to a recent study of *E. coli* wherein researchers attempted to characterize the effects of “age” in the bacterium by correlating it to the number of cell divisions in which a portion of the cell has participated. They used evidence suggesting

that division by binary fission is, in reality, asymmetric and over time some cells after division may decrease in their ability to divide (Steward *et al.*, 2005). Another interesting theory is the disposable soma theory of aging, which correlates aging and death to the allocation of resources by an organism to either self-maintenance or to reproduction (Drenos & Kirkwood, 2005; Kirkwood, 1981). Thus, an organism without the ability to divide would then devote its resources to self-maintenance, achieving a relative immortality.

Recent reports of bacteria isolated from extreme environments suggest some of these organisms are tens if not hundreds of thousands of years old. The chief criticism of these reports is that these are actually descendents of bacteria originally trapped long ago. This debate is based on a lack of information with respect to the lifespan of bacteria. I hypothesize that a definite lifespan of bacteria, like model organism *E. coli*, can be determined by inhibiting DNA replication and cell division.

A number of chemicals have been shown to cause the inhibition of DNA replication, including nalidixic acid, an antibiotic in the quinolone family (Goss *et al.*, 1965) and hydroxyurea (Koç *et al.*, 2004). Extensive research performed using *E. coli* and nalidixic acid (NAL) (Goss *et al.*, 1964; Pohlhaus *et al.*, 2008) suggests that NAL selectively targets and inhibits DNA replication in sublethal concentrations (Winshell & Rosenkranz, 1970; Deitz *et al.*, 1966), unlike antibiotics like streptomycin which target ribosomes and kill the cell. Although growth curves can be used to determine the relative concentration of NAL necessary to inhibit DNA replication and division, further sensitivity is required

in order to show that the concentration of NAL used is inhibiting but not proving lethal to the organism.

I will correlate the SOS response, the growth curves, and the expression of the *ftsZ* gene. FtsZ has been shown to be central to cell division through the formation of the “Z ring” (Shen & Lutkenhaus, 2010). Mutations of the *ftsZ* gene have been shown to prevent the formation of the septum in cell division. Initially in binary fission, FtsZ is found throughout the cytoplasm. However, as cell division progresses through elongation of the parent cell, FtsZ becomes localized in a ring in the center of the cell known as the Z ring (Lewin, 2000). Thus, the quantification of *ftsZ* expression will indicate whether at a given level of NAL cell division is occurring.

NAL has also been shown to trigger the SOS response in *E. coli* by inducing double stranded breaks through an unknown mechanism involving the stabilization of the cleavage complex in the gyrase reaction cycle (Pohlhaus *et al.*, 2008; Chen *et al.*, 1996). The SOS response is a stress response in bacteria induced upon severe DNA damage (Snyder & Champness, 1997).

Genes *recA*, *recC*, and *recN* have been shown to be directly involved in the SOS response (Pohlhaus *et al.*, 2008), and the detection of their substantially increased expression after SOS induction will show at what concentration NAL is cytotoxic. Gene *recA*, for example, senses single-stranded DNA resulting from damage and triggers the autocleavage of LexA (Snyder & Champness, 1997; Janion, 2001). When an insufficient

level of DNA damage exists, or when the SOS response has rescued the cell sufficiently from damage, *recA* is quickly repressed and the SOS response inhibited (Lewin, 2000). Thus, when the SOS response is induced in *E. coli* due to NAL, the concentration of antibiotic has passed from replicatory inhibition to cytotoxicity.

I will use these associations to determine a concentration of NAL that will inhibit cell division but not cause cell death. The determined concentration of NAL acid will then be used to create temperature a dependent death rate or “lifespan” for K-12 *Escherichia coli* as shown in **Figure 8a and b**.

Materials and Methods

A culture of *E. coli* K12 (accession # NC000913 of the National Center of Biotechnology) was started in 5 ml of LB in a 15 ml conical tube, and incubated overnight at 37 °C, shaken at approximately 120-140 rpm. Aliquots of 100 µl were inoculated into 15 ml glass test tubes containing ~5 ml of LB and incubated for approximately 2 h at 37 °C, and shaken at 120-140 rpm until reaching an optical density (absorbance 600 nm) of 0.2.

Nalidixic acid (NAL) was added from a 1 L liquid stock solution of NAL in LB (500 µg/ml or a 500X solution) to each test tube in order to obtain a final concentration of 0, .5, .75, 1, 2, 3, 5, 6, 7, 8, 9, 10, 20, 30 µg/ml NAL in 5 ml LB . Cultures were incubated for 1 h at 37 °C and shaken at 120-140 rpm in triplicate. The above concentrations were

separated into trials of 4 concentrations, repeated as many times as possible, with the control (*E. coli* in 0 ng/ml NAL) grown during every trial.

Absorbance at 600 nm were measured for each sample over a maximum of 7.5 hours. Results were averaged between replicates, and plotted as shown in **Figure 1**, with time in hours plotted against absorbance at 600 nm as measured by a spectrophotometer.

The maximum growth of *E. coli* under each concentration was correlated with the maximum slope of each curve depicted in **Figure 1**, as the maximum change in absorbance over time. Maximum growth was then plotted against concentration as shown in **Figure 2**.

E. Coli K12 was then grown using the above protocol, except that the quantity was increased to 10 ml. Cultures were grown in 50 ml tubes and incubated in NAL with concentrations of 0, 3, 4, 5, 7, 10, 15 µg/ml each grown in triplicate. Cells were pelleted at 4500 rpm, and RNA Protect® Bacteria Reagent (Qiagen) was used to stabilize the RNA. Cells were frozen and stored at -20°C. Total RNA was extracted using RNeasy® Mini Kit (Qiagen), quantified, and quality assessed using a bioanalyzer (Experion) (**Figure 3**). RNA was converted to cDNA using Quantitect® Reverse Transcription Kit (Qiagen).

Primers were designed for *recA*, *recC*, *recN*, *ftsZ*, and *gyrB* of *E. Coli* K12 and are shown in **Table 1**.

Table 1: Primers sets for *recA*, *recC*, *recN*, *ftsZ*, and *gyrB* of *E. Coli* K12.

<i>recA</i>	F: GGGTAACCTGAAGCAGTCCA
	R: GGGTTACCGAACATCACACC
<i>recC</i>	F: TTCTTGATCCAGACGCTGTG
	R:CGATTATCGCCGAAGGTTTA
<i>recN</i>	R: TACAAACGTCTGGCGAACAG
	F: ACTTTGCAGGTTTGCCTCTT
<i>ftsZ</i>	F: CTGTGCAAGGTATCGCTGAA
	R: ATCATTGCGTAGCCCATCTC
<i>gyrB</i>	F: ACGCTGCTGTTGACCTTCTT
	R: TGTTCCCTGCTTGCCTTTCTT

The Optimization of primers will occur through PCR, gel electrophoresis, and sequencing to ensure the amplification of the correct gene of interest and eliminate primer dimers

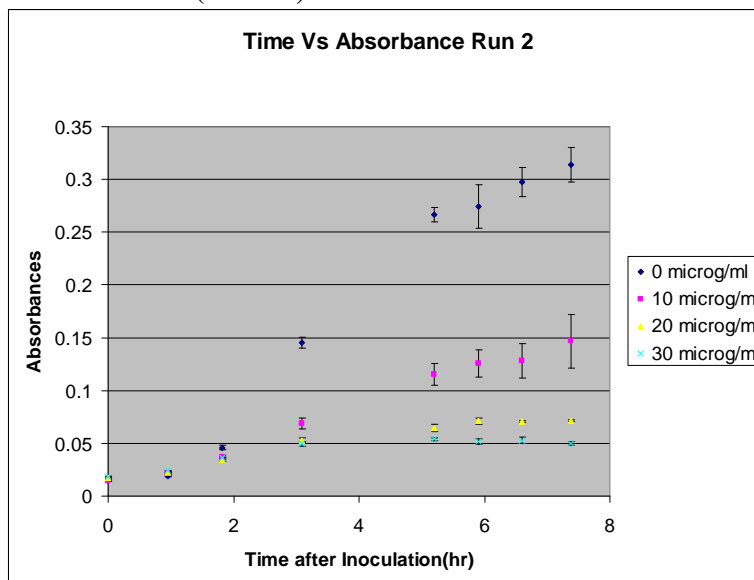
(Figure 4 and Figure 5).

Summary of Results and Project Outlook

Nalidixic acid was shown to decrease the growth of *Escherichia coli* K-12 over time

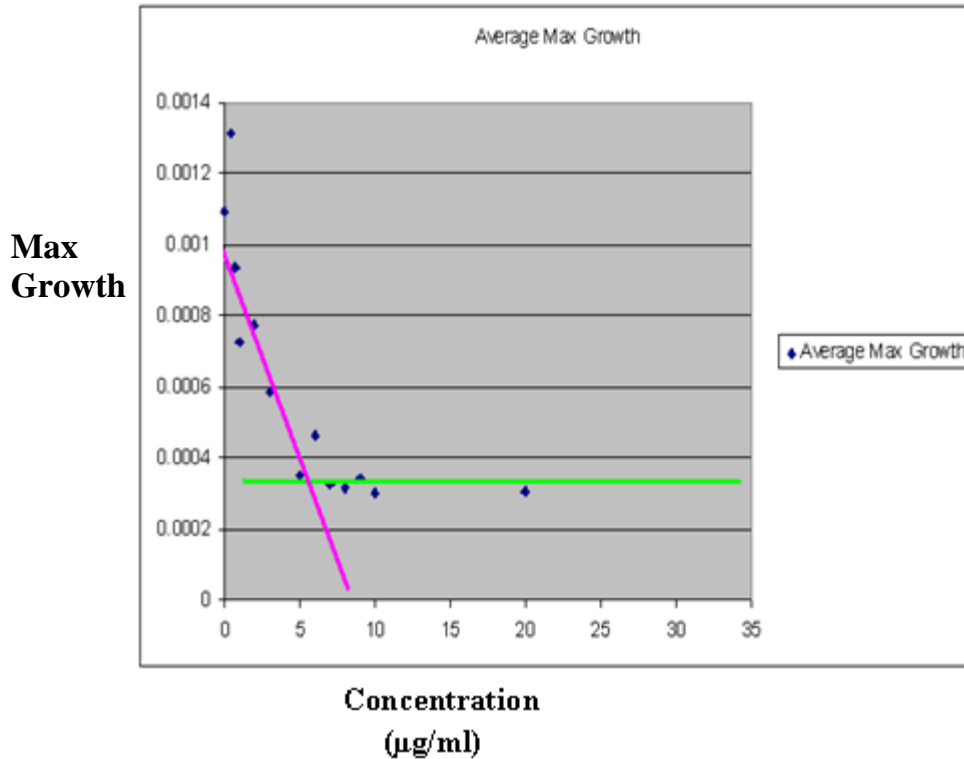
(Figure 1)

Figure 1: Growth curves of *E. coli* at concentrations of 0, 10, 20, 30 $\mu\text{g}/\text{ml}$ NAL. Time (hr) vs Absorbance (600 nm).



Nalidixic acid was found to inhibit but not kill *E. coli* at a concentration between 4-7 $\mu\text{g/ml}$ (**Figure 2**).

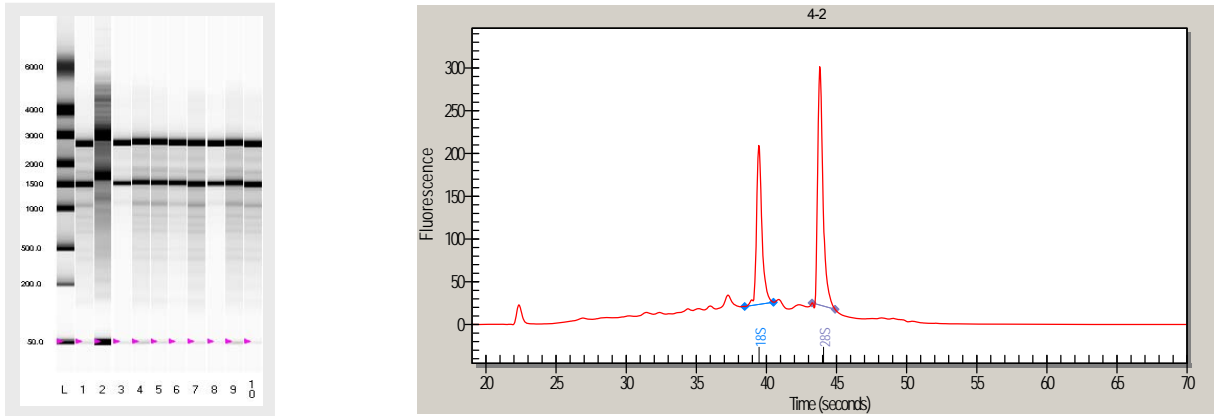
Figure 2: Concentration NAL vs Maximum Growth of *E. Coli*



As *E. coli* was grown under concentrations of NAL greater than this concentration, the maximum growth of *E. coli* did not show any significant decreases. This is expected, as concentrations higher than this range should kill the bacteria and hence there should not be any growth. However, the “zero” line of maximum growth is shown above zero due to several factors including: 1. Detection limits of the instrument (notice that the “zero” line or the maximum “change in absorbance/time in **Figure 2** is at approximately .0002). 2. A certain amount of increase in absorbance that may occur as a result of the time required for NAL to inhibit cell division.

RNA was quantified and a bioanalyzer analysis performed to ensure high quality of RNA (Figure 3).

Figure 3: Bioanalyzer results of nine extracted RNA samples to test for RNA quality and integrity (Left) and example of quality test performed for L3 (right).



All primers amplified DNA corresponding to the targeted sequence length as shown in

Figure 4.

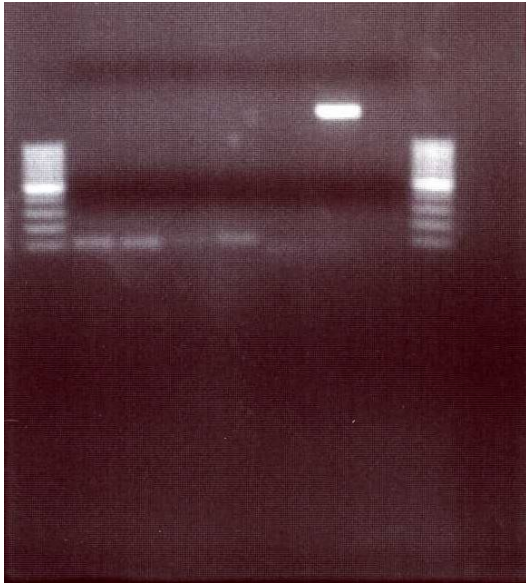
Figure 4: PCR products run on 1% agarose gel.

From Left to Right: L1-recN, L2-recA, L3-gyrB, L4-recC, L5-ftsZ, L6-Pos Control of cDNA L7-Neg Control of Primers(Water), L8-Pos Control of Primers, L9: Neg. control, Primers and no DNA (Water).



Initial concentrations of Primers for *recN*, *recA*, *recC* and *ftsZ* required further optimization to remove primer dimers as shown in **Figure 5**.

Figure 5: Primers run using E. coli Genomic DNA for optimization. From left to right L1-100 bp ladder, L2-*recN* Primers, L3-*recA* Primers, L4-*recC* Primers, L5-*ftsZ* Primers, L6-Negative Control (Water in place of DNA) & Primer Dimers, L7-Positive Control (16 S Primers) & Primer Dimers, L8-Nothing, L9-100 bp Ladder.



I have optimized the primers for *gyrB*, and the product of PCR on the cDNA shows that I have indeed reverse transcribed the *GyrB* RNA product into cDNA and replication of the desired sequence occurs using these primers (**Figure 6**). However, the primers are only transcribing a small quantity of DNA during the PCR, making sequencing difficult, and leading me to question whether I should redesign my *gyrB* primers because of inefficiency. As shown in **Figure 5**, further optimization of the *recC* primers needs to occur.

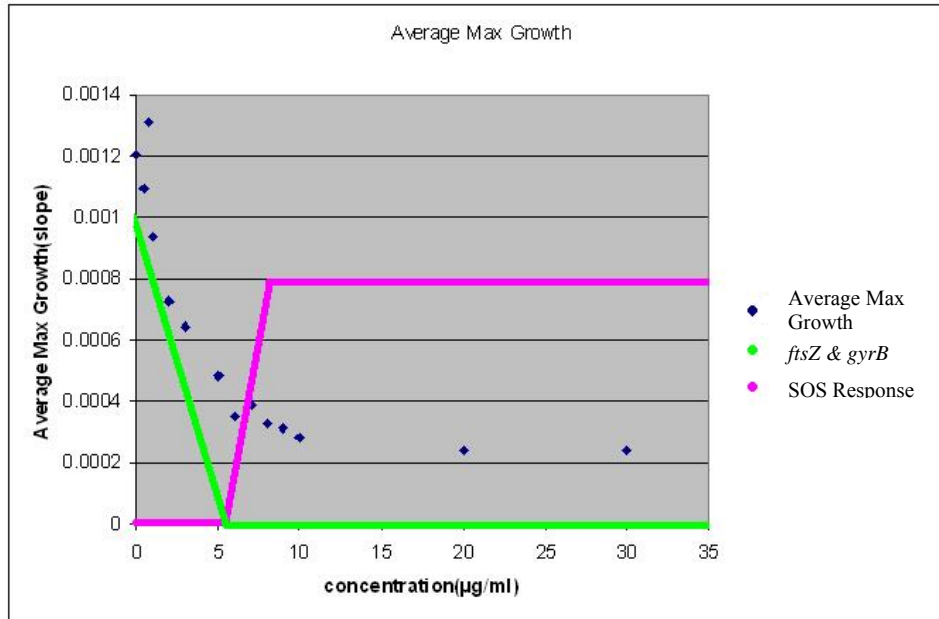
Figure 6: Sequenced portion of *gyrB* after PCR using *gyrB* primer set. Highlighted-The pairing of the Reverse *gyrB* Primer sequence.

Sequence:TCGTTGACGCGGTCACGTCTATATCGCTCAGCCGCCGCTGTACAA
AGTGAAGAAAGGCAAGCAGGAA

Gyrase Reverse Primer: TGTTCCTGCTTGCCTTTCTT

Upon optimization of all primers, PCR will be performed on cDNA samples to ensure that the intended sequence for each primer set is being replicated. After sequencing, each primer set will be used in semi-quantitative Reverse Transcription PCR (SYBR Green) to quantify the relative amount of RNA transcribed by the genes *recA*, *recC*, *recN*, *ftsZ*, and *gyrB* under NAL concentrations of 0, 3, 4, 5, 7, 10, 15 µg/ml. The correlation of the upregulation of *recA*, *recC*, *recN* and *gyrB* involved in the initiation of the SOS response. the downregulation of *ftsZ* involved in cell replication, and the maximum growth curve (**Figure 2**) will give added specificity to the concentration that completely obstructs replication in *E. coli*, but does not cause cell cytotoxicity. Furthermore, the correlation of these responses will show that one can indeed inhibit replication but not kill the cell (**Figure 7**).

Figure 7: Caricature of the expected correlation between Average Maximum growth vs Concentration (Blue), Expression of *ftsZ* (Green), Expression of SOS genes (Purple).



Last of all, after the desired concentration of NAL has been found, *E. coli* K-12 will be grown under varying temperatures, and its temperature dependent lifespan will be determined. Using a flow cytometer, the number of dead and live cells over time will be measured and graphed.

According to the hypothesis that bacteria have the capacity to live forever (Williams *et al.*, 1957), I expect to see one of two results as shown in **Figure 8a and b**. As temperature decreases, the lifespan of *E. coli* should approach infinity (**Figure 8a**) or reach a finite (**Figure 8b**) maximum lifespan. This is because when I measure how many cells are alive over time using a flow cytometer when cells are grown at 37 °C, I expect to find a finite lifespan (ex. a point where half of the cells have died). However, with this finite point, one can say nothing about *E. coli*'s ability to survive forever under cold

dormancy conditions. By growing bacteria over a range of temperatures, I should be able to determine if *E. coli* theoretically has the ability to enter an indefinite dormancy.

Figure 8a: Caricature of theoretical infinite lifespan of *E. coli* dependent on temperature

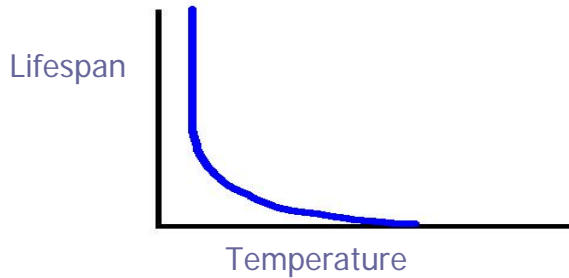
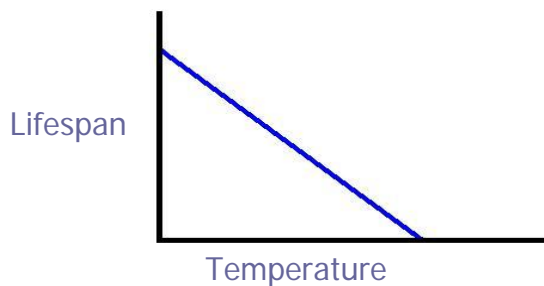


Figure 8b: Caricature of theoretical finite lifespan of *E. coli* dependent on temperature



The results of this study have significant application in the study of aging effects. This study will give evidence in support of a limited and specific lifespan for *E. coli* (**Figure 8b**) or evidence in support of the alternate hypothesis--immortality for organisms whose soma line is not distinct from its germ line (**Figure 8a**). If a lifespan for *Escherichia coli* and related organisms can be ascertained, they will then be ideally positioned for use in the study of the effects of aging in individual cells.

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