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Long-term Survival of *Escherichia coli* in the Environment and Growth Advantage in Stationary Phase

by

Elizabeth Mengis

Undergraduate honors thesis under the direction of

Dr. Eric Achberger

Department of Biological Sciences

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Introduction

Clean, fresh water is vitally important to human health, industry, and the economy. Since the Earth's supply of fresh water is limited, the proper treatment of wastewater from sewage, farms, and industries is key. The presence of the fecal coliform bacteria *Escherichia coli* in water is the most common microbiological indicator of poor water quality, since its presence indicates probable contamination with other harmful fecal coliforms (1). *E. coli*'s lifespan in the natural environment is typically limited to less than fourteen days, after which it - and other harmful bacteria - tend to decrease and die off (2). The research presented in this paper examines a case found in a lagoon wastewater treatment system on an LSU research dairy farm, in which the *E. coli* present in wastewater did not die off as expected. This longer lifespan has been postulated to involve a phenomenon known as Growth Advantage in Stationary Phase, which has been demonstrated under laboratory conditions but is not well documented in the environment. The potential persistence of *E. coli* under normally unsurvivable conditions may have significant consequences for water sanitation and the determination of water safety.

Normal bacterial growth is characterized by four phases: lag, exponential, stationary, and death. Each phase involves specific biochemical reactions, expression of genes, and interactions of proteins unique to that phase. Lag phase is characterized by slow growth due to unbalanced growth in preparation for reproduction, followed by rapid growth during exponential phase. As essential nutrients are used up, the rate of bacterial growth slows and becomes equal to the

death rate in stationary phase. Certain survival genes become activated, and cellular compounds are synthesized at a different rate than exponential phase (3,4). To exhibit growth, bacterial cells must be able to scavenge essential amino acids released from dead cells. Any cellular changes that increase this ability to take up nutrients would confer a selective advantage (4). Finally, in death phase, starvation, changes in pH, and depletion of energy force the bacterial cells to break down (3,4). Bacteria cultures in standard liquid nutrient media follow these phases; it is expected that they follow a similar pattern in the environment. Harsh environmental conditions may cause exponential phase to shorten and quickly transition to death phase. However, about 0.1-1% of bacteria may gain mutations allowing prolonged survival in a long-term stationary phase during harsh environmental conditions (3).

Normal stress responses allow *E. coli* to survive changes in pH, temperature, or nutrient availability for a limited time in stationary phase before entering death phase (4). However, stressful environmental changes place a highly selective burden on the bacterial population, leading to a phenomenon known as the Growth Advantage in Stationary Phase (GASP) phenotype. Bacteria such as *E. coli* that display the GASP phenotype are usually found in older cultures and have genetic mutations allowing increased fitness in nutrient-depleted environments (5). These gene mutations are the result of a large, highly diverse bacterial population, which may undergo random mutations during each cell division. The GASP phenotype has been demonstrated numerous times in a laboratory environment when *E. coli* is incubated for several days in a Luria-Bertani (LB) broth batch culture. *E. coli* that

has been incubated for ten or more days displays an increased fitness advantage when grown in competition with the original day-old *E. coli* cells (5). The cells selected at ten days will eventually take over the mixed culture, as the original cells die off. GASP phenotypes may involve single or multiple genetic mutations, and they have been observed in a variety of bacterial species (3).

GASP phenotypes are relatively easy to observe, but it is much more difficult to determine which genetic mutations are allowing the cells to outcompete other cells and grow in stationary phase. Several common mutations associated with the GASP phenotype have been identified, many of which are involved with increased uptake of nutrients such as amino acids (3). Scavenging nutrients from the debris of dead bacteria cells requires large amounts of energy, since entire proteins, sugars, or strands of nucleic acids must be broken down into their constituent parts. Multiple genetic mutations are possible, and may be more advantageous than single mutations. The first GASP gene mutation identified, *rpoS819*, was in the *rpoS* gene, which codes for a sigma subunit of the RNA polymerase protein (RpoS) (5). Under normal conditions, RpoS has a key role in the bacterial stress response, and is involved in the transition into stationary phase. Studies have found that GASP mutations resulting in attenuated RpoS activity favored the increased uptake of amino acids and glucose into the cell, allowing a greater chance for survival (7). Attenuation of RpoS also leads to induction of the *bgl* operon, which is involved in the uptake and use of the glycosides salicin and arbutin (3).

Since the discovery of RpoS mutants, many other genetic mutations in *E. coli* have been linked with the GASP phenotype. Almost all of these mutations enable certain bacteria in stationary phase to increase their uptake of amino acids or sugars that are being released from other dying cells. In one mutation, the transposition and inversion of two IS5 mobile insertion sequences activates an operon for asparagine and glutamine uptake (3,6). Another mutation involving the *lrp* gene causes the loss of function of the leucine-responsive global regulator. Cells with this mutation have a heightened ability to scavenge the amino acids serine, threonine, and alanine, similar to *rpoS* mutants (3,7).

Work with *E. coli* Background

The *E. coli* isolated for our experiments with the GASP phenotype was taken from the LSU Dairy Farm's lagoon system for wastewater treatment. The lagoon system is a bifurcated three-stage treatment consisted of anaerobic lagoons, aerobic lagoons, and constructed wetlands (Figure 1). Sample Site A is water directly from the barn, which typically has a very high concentration of *E. coli* – about 10^6 to 10^7 bacteria per 100 mL of water – which is determined using the QuantiTray 2000 to enumerate *E. coli* (8). During a 120-day period, bacteria and other particles pass from Site A to Collection Site B in the anaerobic lagoon, and the concentration of viable *E. coli* drops about ten fold. Sample Site C is in the aerobic lagoon environment, where *E. coli* decreases 100 to 1000 fold during the approximately 120 days it takes to reach the constructed wetlands. There are three constructed wetlands for each branch of the system, and each was sampled independently (9).

Interestingly, whereas the typical lifespan of *E. coli* in the environment is less than two weeks, viable *E. coli* persist in the wastewater treatment for the better part of a year. Samples taken from Site D in the wetlands typically have an *E. coli* concentration of about 10^2 to 10^3 per mL (9). Water is considered to be impaired for primary contact, such as swimming, at a concentration of 153 *E. coli* per mL, meaning that even after such a large amount of time, wastewater from the dairy farm was still contaminated beyond a safe range (1). Since the lagoon system is a warm, open environment, wildlife frequent the water and can be responsible for up to 80% of the *E. coli* found (9). However, the *E. coli* from the dairy cows were still present in the water after so many months, which raises the question "Do all strains of *E. coli* survive in the wastewater equally, or is there a genetically distinct subset of *E. coli* that survives?"

Over an eight-month period, water samples were collected monthly from Sites A through D and *E. coli* were isolated and grouped using DNA Fingerprinting analysis. To isolate individual *E. coli* strains and quantify their number in a sample, water was analyzed using the QuantiTray 2000 system on Colilert medium (Idexx Laboratories). The Quanti-Tray is a long tray with individual wells used to estimate bacterial numbers using a Most Probable Number (MPN) approach. To make the test specific for *E. coli*, Colilert medium was used. Using Colilert, the well will turn yellow if the bacterium present can cleave ONPG (O-nitro-phenyl-β, D-galactoside), indicating that it is a coliform. Further, if the wells fluoresce blue under UV light, they can cleave MUG (4-methylumbelliferyl-β, D-glucuronide). Wells that exhibit

yellow color and blue fluorescence contain *E. coli* (8). The *E. coli* was isolated for analysis by DNA fingerprinting.

Bacterial cultures were analyzed using DNA fingerprinting through Random Amplification of Polymorphic DNA (RAPD) PCR, which uses arbitrary primers to generate DNA fingerprint patterns of DNA bands (10). DNA fingerprinting was used to identify individual *E. coli* strains from dairy cows found in the lagoon system, and eliminate bacteria that entered the water from wildlife or the environment (non-dairy). Based on the DNA fingerprinting analysis of the samples used in the study, about 40% were definitely dairy-related *E. coli*; they had 95% similarity. About 60% were probably dairy-related, at 90% similarity. Only strains with DNA fingerprints specific to dairy cows were used in the study. From the 1253 total fingerprints, 272 dairy-related strains were isolated from the barn at Site A and 244 were isolated from the wetlands at Site D.

These *E. coli* strains were analyzed with two DNA Fingerprinting reactions using RAPD PCR and different arbitrary primers. *E. coli* strains with the same DNA fingerprints were grouped, and only groups that contained DNA fingerprints from Site A and Site D were analyzed. We counted the number of times that each fingerprint appeared at the beginning and end of the lagoon system. All strains of *E. coli* generally died off as they passed through the lagoon system, but based on a ratio of the number of times a fingerprint appeared in the wetlands (Site D) relative to the barn (Site A), some *E. coli* strains persisted much longer than others. If there had been no genetic differences, the proportions of every bacterial strain in each water

sample would have been the same, indicating that they all died off at equal rates. Table I lists DNA fingerprint groups and their wetland/barn DNA fingerprint ratios. A group with a ratio significantly greater than 1.0 was classified as a survivor, and groups with a ratio significantly less than 1.0 were termed non-survivors. About half of the groups had a ratio between 0.75 and 2, and were not used because this data was inconclusive.

Materials and Methods

Survival Under Starvation Conditions

The strains designated as "survivors" and "nonsurvivors" were tested in the laboratory under carbon starvation conditions. Each strain was taken from exponential phase or stationary phase growth in L Broth (20 g Bacto Tryptone, 10 g Bacto Yeast Extract, and 10 g NaCl per liter), washed with Minimal A Medium (11), suspended in minimal media without a carbon source at 10^7 cells per mL, and incubated at 28 °C. Viable cell counts were determined as a function of time, and linear regression was used to calculate the exponential decay rate.

GASP Assays

The same *E. coli* strains analyzed under starvation conditions were then tested for the GASP phenotype. Spontaneous mutants that exhibited streptomycin, rifampicin, or nalidixic acid resistance were selected from cultures of each strain by spread plating 0.1 ml of an overnight L broth culture onto L agar (L broth with 16 g

agar per liter) containing 20 μg/ml of the antibiotic. The plates were observed after 24 and 48 hours and the largest colonies were selected. In our GASP assay, survivor strains typically carrying nalidixic acid resistance were tested against non-survivor strains usually carrying streptomycin resistance. Survivor and a non-survivor stationary phase cultures were mixed at a ration of 1:1000 survivor:non-survivor, and were incubated at 37°C . Viable cell counts of each strain were determined as a function of time using the different antibiotic resistances. The survivors were enumerated on nalidixic acid-containing medium, and the non-survivors on streptomycin-containing medium.

RpoS Testing

All *E. coli* strains collected in this study, both survivors and non-survivors, were assayed for a functional RNA Polymerase sigma factor (RpoS). *E. coli* ATTC 11775, an RpoS-deficient strain, was also tested as a control. To test for the ability to grow on succinate, all strains were grown on minimal media with succinate as the only carbon source (11,12). To test for the induction of catalases, the strains were grown on L agar for 24 hours at 37°C and then flooded with 6% hydrogen peroxide. The formation of bubbles indicated the presence of functioning catalases (12). Finally, to test for the accumulation of glycogen, all strains were grown on L agar for 24 hours at 37°C, then removed and grown at 4°C for 24 hours before concentrated iodine was added. Colonies producing and accumulating glycogen turn dark brown in color, while non-producers become a lighter tan color (13).

Test for colicin production

To test for the production of colicins, a overnight, L broth culture of every strain was spotted onto agar plates in 0.5 cm patches and grown to stationary phase. A soft agar overlay was made by adding an indicator strain sensitive to colicins to L agar containing 8 g per liter agar. The overlay was poured directly on top of the plates carrying our *E. coli* strains, and the plates were incubated for 24 hours at 37°C. Laboratory strains of *E. coli* including strains ATCC 11775, JM107, and DH5αMCR were used as indicator strains. The presence of a zone of clearing in the indicator strain immediately above the tested strains would indicate colicin production. *E. coli* ATCC 23716 carrying the ColE1 plasmid was used as a positive control for colicin production.

Results and Discussion

Decay Curves and GASP Assay

In the first stage of the experiment, *E. coli* cells were taken from exponential phase growth and placed under starvation conditions in minimal media lacking a carbon source. However, when the decay rates of survivors and non-survivors were compared, no significant difference was found between their respective half-lives. While this observation did not aid in determining the biological significance of the "survivor" designation, the observed death of both strains eliminated the possibility that there were "super bugs" able to grow in dilute suspensions under starvation conditions.

Upon further consideration, it was determined that it was unlikely that the *E. coli* cells entering the environment from the cow gut were still in exponential phase growth. The experiment was repeated with cells in stationary phase. When non-survivor cells from exponential phase (3 hours old) and from stationary phase (16 hours old) were starved, their decay rates were similar (Figure 2). The same experiment was done with survivor strains from exponential and stationary phase. The results showed that while survivors from exponential phase also died off at a rate consistent to non-survivors, but the survivor stationary phase cells remained viable for long periods (Figure 2) . Decay rates of multiple survivor and non-survivor strains taken from stationary phase were compared. The data indicates that the half-lives of survivor strains are at least twice as long as the non-survivors (Figure 3; Table II). These experiments revealed that the ability of the survivor *E. coli* strains to persist in the environment was related to physiological changes associated with stationary phase growth. It was hypothesized that the survivor strains possessed one or more naturally occurring GASP mutations that provided enhanced fitness in a harsh environment.

Isolated survivor and non-survivor strains were tested against each other in a series of assays designed to test whether or not the survival of *E. coli* in the environment was related to the GASP phenotype. Stationary phase cultures were mixed at a ratio of 1:1000 survivor:non-survivor, and viable cell counts were obtained over a period of ten to twelve days. The cell counts were used to create growth curves, allowing comparison of survivor and non-survivor strains. Three types of results were obtained, labeled as Class I, II, or IV based on the nomenclature

of Finkel *et al* (4). Class I is consistent with the ideal GASP phenotype, in which the minority strain outcompetes the majority strain for resources and is able to grow in larger numbers while the other is driven to extinction (Figure 4, Panel A). Class II is also consistent with the GASP phenotype as the minority strain multiplies to match the other strain (Figure 4, Panel B). Finally, Class IV results indicate that the minority strain was unable to outcompete the majority strain, and both die off (Figure 4, Panel C). We did not obtain Class III results, in which the survivor and non-survivor strain grow at similar rates, after which the survivor strain dies off quickly (4). In each case presented in this section, the survivor was the minority strain. After testing 42 combinations of seven survivor strains versus six non-survivor strains, the majority of the results were Class I or Class II, indicating the presence of the GASP phenotype (Table II; Table III). This is consistent with the survivor strains being naturally occurring GASP mutants.

Colicin Check

We performed a brief experiment to check that our results were not due to the ability of our strains to produce colicins, which are small inhibitory proteins made by one *E. coli* strain for the purpose of restricting the growth of closely related strains. Colicins may be RNAses, DNAses, or other enzymes that can pass from their cell of origin and penetrate the cell membrane of other *E. coli* nearby. They are encoded on a plasmid with two genes – one encoding the colicin itself, and the other encoding immunity to it. Non-immune, susceptible cells have specific membrane receptors for the colicin on their cell surface. Once inside the cell, they may stop

protein synthesis, break down DNA, or halt cellular respiration. Colicins undergo a variety of complex interactions with other proteins and receptors during transport to neighboring bacterial cells and within the cells themselves. It is still unknown why there are produced at high levels under certain conditions, such as DNA damage due to UV radiation (16). However, they have been found in a number of *E. coli* strains, and their presence could be responsible for one strain's ability to survive and "outcompete" another during the GASP assays.

To test for the ability of our strains to produce colicins, a liquid culture of every strain was spotted onto agar plates in 0.5 cm patches and grown to stationary phase. An overlay of soft agar was made, containing an indicator strain sensitive to colicins was mixed in. The overlay was poured directly on top of the plates carrying our *E. coli* strains, and the plates were incubated. If any of our strains were able to synthesize colicins, the indicator *E. coli* in the soft agar overlay would have been killed, producing a zone of clearing immediately around the colicin-producing strain. None of the *E. coli* strains used in our GASP experiments showed the ability to produce colicins. A laboratory *E. coli* strain known to produce colicin E1 did produce inhibition in our assay. This supports our results indicating that the GASP phenotype was being studied, rather than another common biological mechanism of inhibition.

Antibiotic Resistance and Strain Fitness

The antibiotic resistances employed in our experiments might have an effect on the fitness of a strain. In the data that we presented for our GASP assays, the survivors used were spontaneous mutants resistant to nalidixic acid, while the non-survivors had spontaneous mutations for streptomycin resistance. Previous studies on *E. coli* strains carrying antibiotic resistance have shown that in many cases, these bacteria are less fit. Antibiotic resistance develops spontaneously from genetic mutations, and in some cases the mutation may interfere with normal cell processes. However, it is also possible for these bacteria to develop a second, compensatory mutation that counteracts loss of function due to antibiotic resistance (14). The random nature of antibiotic resistance mutations led us to question whether or not it made an impact on our GASP assays.

To examine whether or not different antibiotic resistances had an effect on the fitness of an *E. coli* strain, a representative member of each DNA fingerprint group was tested against itself after selecting for spontaneous mutants resistant to rifampicin (*rpoB* mutant), nalidixic acid (*gyrA* mutant), or streptomycin (ribosomal protein mutations) (4). When tested in the GASP assay, some antibiotic resistant strains were able to outcompete the same strain with a different antibiotic resistance. The effects were most pronounced after ten days of incubation; because of this, all GASP assays were limited to ten days or less. Limiting the assays to ten days also minimized the possibility of selecting for new GASP mutations during the test.

In addition, we had experimental evidence showing that independently isolated, spontaneous antibiotic resistant mutants were not always equally fit in the GASP assay. Four independent rifampicin mutants were selected for one of the survivor strains and one of the non-survivor strains. Each rifampicin mutant was tested against the same strain that contained a spontaneous mutation conferring nalidixic acid resistance. In each case, the GASP assay results supported the existence of at least two different rifampicin resistance mutations that affected cell fitness to different extents. To minimize differences in cell fitness due to spontaneous antibiotic resistance mutations, we routinely selected the fastest growing spontaneous mutant for each antibiotic used. This reinforces the idea that because spontaneous resistance mutations can impose small changes on essential genes, fitness can vary between individual strains. In some cases, the mutation has no impact on the cell's normal metabolic processes. Studies have even shown that in a few instances the mutation may even provide slightly increased fitness, for reasons that are not well understood. However, in most cases where chromosomal mutations confer resistance (rather that acquisition of resistance through a plasmid), fitness of the strain typically decreases (15).

To test this, several GASP assays were repeated in which each non-survivor was tested as a nalidixic acid resistant strain and as a streptomycin resistant strain, with the survivor challenger bearing the other resistance. Representative plots are presented in Figure 5. In the first set of assays, the survivor strains representing groups C43 and C40 were able to outcompete the non-survivor representing group C27, giving Class I results. However, C27 was able to persist longer when it carried

nalidixic acid resistance. This suggests that when C27 carried streptomycin resistance, it was easier to drive to extinction (Figure 5, Panel A). In the second set, C43 and C40 were again able to outcompete the non-survivor representing group N2, giving Class I results for each. However in this case, changing which strain carries each antibiotic resistance did not have a significant effect (Figure 5, Panel B). In the third set of assays, Class I results were obtained when the group N8's representative carried streptomycin resistance. However, Class II results were obtained when the resistances were switched, and the non-survivor carried nalidixic acid resistance. Again, it seems that nalidixic acid resistance makes the non-survivor strain hardier relative to the streptomycin resistant survivor strains (Figure 5, Panel C). Finally, in the last set of assays, Class II results were again obtained when the non-survivor representing group C24 carried nalidixic acid resistance, but when the resistances were flipped Class I results were shown (Figure 5, Panel D).

These results indicate that streptomycin resistance may indeed make strains less fit; it may also be true that nalidixic acid resistance has a greater potential to improve a strain's fitness. However, the GASP hypothesis was not invalidated, since the survivor strains were still able to grow and outcompete the non-survivor strains.

Sources of the GASP Mutation

After determining that our survivor strains appeared to posses the GASP phenotype, the next step in our experiment was to characterize this phenotype in *E.*

coli. Common GASP genetic mutations have been identified in literature, especially the inactivation of the RNA Polymerase stationary phase sigma factor (*rpoS*$^{-}$ mutation); to determine whether or not this very common mutation was present in our strains, we used three separate techniques to test for the presence of a functioning RpoS protein. The cells with a wild-type RpoS protein will accumulate glycogen in stationary phase, induce catalase enzymes, and will have restricted ability to metabolize the dicarboxylic acid succinate. In our experiments, none of the *E. coli* strains collected from the lagoon grew on the succinate minimal media, while the laboratory control *E. coli* strain ATTC 11775 had luxurious growth. In the glycogen test, stationary phase colonies all turned brown rather than tan when exposed to iodine, indicating the ability to produce and accumulate glycogen. The control *E. coli* ATTC 11775 remained tan. Finally, stationary phase colonies rapidly produced bubbles upon addition of 6% hydrogen peroxide, meaning that the catalases were induced. Very weak catalase activity was observed for the control *E. coli* ATTC 11775. These results suggest that our strains – both survivors and non-survivors - do possess a normal, functional RpoS protein. Their GASP mutation(s) are not due to the common *rpoS*$^{-}$ mutation.

It is likely that a number of mutations are responsible for the GASP phenotype in different survivor groups that share a common DNA fingerprint. If this were true, a survivor with one GASP mutation would be able to outcompete another survivor with a different GASP mutation in the same GASP assay (4). To demonstrate this, a representative isolate from each of the survivor groups was tested in the GASP assay against the representative of the survivor group C15 (Table

II). The strains from C1, C40, and C43 outcompeted C15 and displayed the Class I GASP response. The strain from the N7 group displayed the Class II response and grew to equal numbers with C15. The N4 strain failed to compete with C15 and was classified as the Class IV response. Finally, the C14 strain displayed the Class III response, in which it started to increase in cell numbers relative to C15, but then died off at a rate greater than that of C15. We had not previously observed the Class III response in earlier GASP assays (4). This experiment supports the possibility of two, and possibly three or more mutations that confer the GASP phenotype in our survivor strains. Therefore, it may be difficult in the future to characterize the particular mutation or group of mutations leading to the GASP phenotype of *E. coli* in the environment. Further genetic studies are needed to determine if this is the case.

In addition, we have been working under the assumption that all strains sharing a common DNA fingerprint are genetically identical. However, we wondered if strains assigned to a DNA fingerprinting group may be slightly different from one another, or if they developed individual, unique mutations over time. This was explored using the GASP assay. Five strains with the DNA fingerprint pattern representing the N4 group of survivors were tested against one another. In the GASP assay, the challenger strain was diluted 1:1000 into a stationary phase culture of the majority strain. In the first set of tests, the challenger strain was resistant to nalidixic acid while the majority strains possessed rifampicin resistance. The test was repeated after switching the antibiotic resistances so the challenger strain was now rifampicin resistant. We would expect that none of the strains would be able to

outgrow the others in a GASP assay if they all possessed the same GASP mutation (*i.e.* they were genetically identical). Indeed, in almost every case, the two strains grew at the same rate and displayed Class II results. Three combinations did produce Class I results, indicating that the challenger strain was able to outcompete the majority (Table IV). This shows the possibility for mutations or other differences among members of the same DNA fingerprinting group. When the experiment was repeated with three strains of the C14 survivor group, no competition was observed (Table V). Each combination produced Class II results, showing that the strains were similar enough to grow together at the same rate. This suggests that any differences among strains with the same DNA fingerprint are minor. The few differences that were encountered among the N4 group could be explained by differences in cell fitness sometimes observed among spontaneous mutants selected for their antibiotic resistance.

Future Research

The GASP phenotype has been positively identified in our lagoon system *E. coli* strains designated as "survivors." On multiple occasions, this phenotype has been described by researchers in the laboratory setting (3,4). However, the natural occurrence of GASP mutants in the open environment –such as in our strains - is not well documented. Understanding the genetic changes that led to our strains' prolonged survival is the next step. Genome sequencing seems to be the most direct solution, but without one specific mutation to search for, it would be extremely

difficult to determine which of the many small changes to the DNA sequence contribute to the GASP phenotype. Multiple mutations may be responsible in one *E. coli* cell, and mutations may vary between strains regardless of their fingerprinting group.

We have evidence that the difference between survivors and non-survivors is related to their preparation for stationary phase of growth and starvation. Some steps have been taken to examine differential levels of protein expression between survivors and non-survivors during this transition. RNA samples were taken from strains in exponential and in stationary phase. The isolated RNA was reverse-transcribed to cDNA, which was fingerprinted and run on a polyacrylamide gel. We looked for differences in cDNA bands on the gel between survivors and non-survivors, as this indicated differences in the genes being transcribed when transitioning between exponential and stationary phase. Bands that appeared promising were cloned and sequenced. Real-time PCR is currently being used to quantify the levels of gene expression between survivors and non-survivors, since varying levels of gene expression reflect differential expression of proteins. Knowing how the proteins and cellular mechanisms vary between survivors and non-survivors is key in exploring the relationship between the GASP phenotype and survival in the environment. However, this process is lengthy and not always accurate. With unlimited funds, the best way to study the genetic changes in our strains would be through using a DNA microarray to isolate each strain's RNA and hybridize it to every known *E. coli* sequence. This would enable us to "see" which genes are turned on and off in survivors and non-survivors during the transition

from exponential to stationary phase (18). Using a DNA microarray, the cloning and sequencing steps would be eliminated to allow faster and more streamlined results.

Conclusions

In order to survive under starvation conditions, bacteria must adapt and become proficient at scavenging nutrients, or else they will quickly die off. One of the ways that bacteria can prolong survival is through Growth Advantage in Stationary Phase, which has been observed in a variety of bacterial species under laboratory conditions on numerous occasions. However, until this point there have been very few documented incidences of this phenomena occurring spontaneously in the natural environment. In the research presented in this paper, the results obtained from GASP assays on isolated *E. coli* strains from a dairy farm lagoon system indicate that these bacteria are naturally-occurring GASP mutants. Not only did our basic GASP assays give positive results in almost every case, but other experiments such as the colicin check allowed us to eliminate other possible sources of prolonged survival. We also checked to ensure that spontaneous antibiotic resistance in our strains did not have a significant negative impact on bacterial fitness. While working with environmental *E. coli* GASP mutants, it was imperative that we did not alter their genetic material or allow them to grow for long periods, possibly picking up new GASP mutations under laboratory conditions. This restricted the manner in which we were able to isolate and work with bacteria carrying antibiotic resistance, but the results of our antibiotic resistance assays demonstrate that there was no significant impact on bacterial fitness. This ensured

that our results from previous experiments are valid. After confirming the GASP mutation, we then tested all of the *E. coli* strains for the most common GASP mutation in literature, the *rpoS*⁻ mutation, and determined that it is not present in any of our strains. After performing assays in which survivors were competed against each other, rather than against non-survivors, we concluded that more than one mutation is likely responsible for our observed GASP phenotype. Future experiments designed to isolate specific cellular or genetic changes in our GASP mutants will allow us to understand their exact mechanisms of survival. *E. coli* is one of the most widely used bacterial indicators of water contamination. Therefore, understanding *E. coli*'s behavior in the environment is key, especially in cases where it can survive for long periods. This knowledge is necessary for preserving human health and sanitation.

Figures

Figure 1. **Model of the lagoon system.** Site A is the influent from the barn. Sites B are anaerobic lagoons, Sites C are aerobic lagoons, and Sites D are constructed wetlands (9,17).

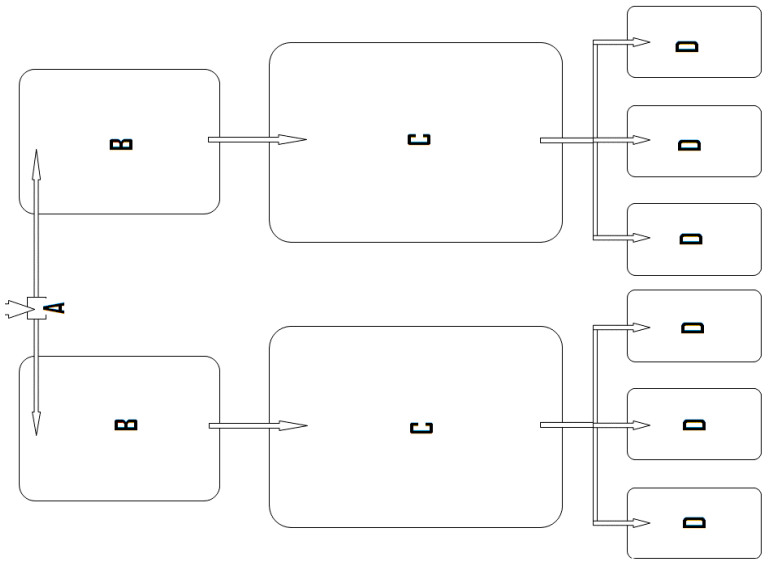


Figure 2. **Growth rates of survivors and non-survivors from exponential and stationary phase.** The right graph displays the growth rate of survivors (yellow) and non-survivors (purple) taken from exponential phase. The left graph shows results from same experiment repeated with cells taken from stationary phase. Here, survivors are able to maintain cell numbers at a steady rate, while non-survivors again die off.

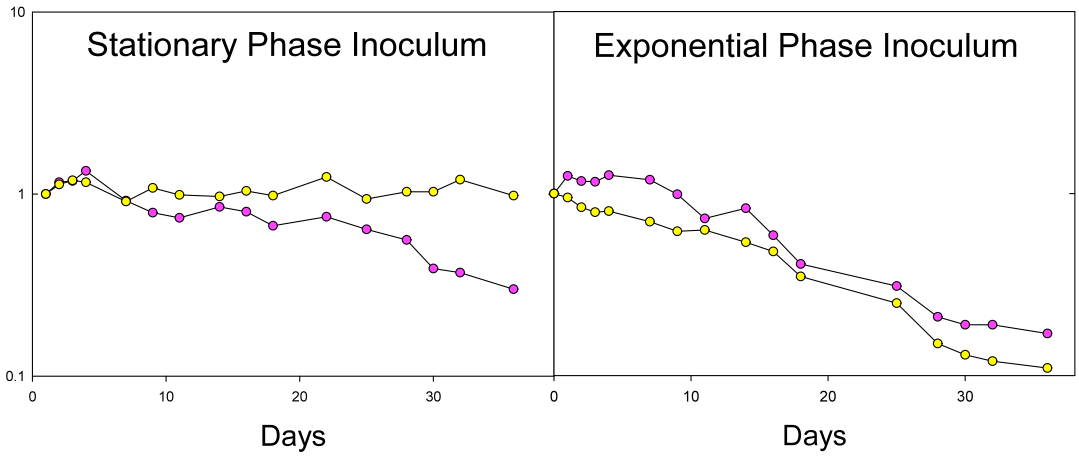


Figure 3. **Half-lives of survivors and non-survivors in stationary phase.** Cells were starved over time, and their half-lives were determined. Survivors are shown in yellow, with non-survivors in purple. DNA fingerprint groups are referenced in Table II.

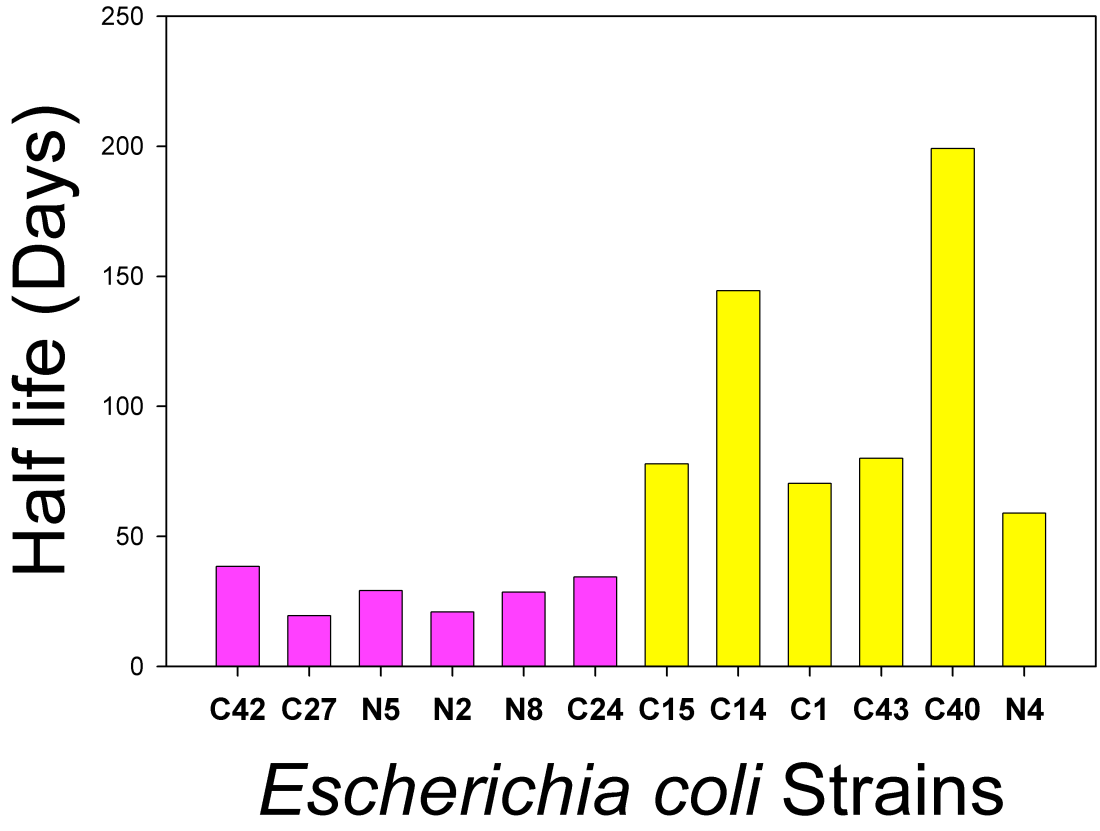
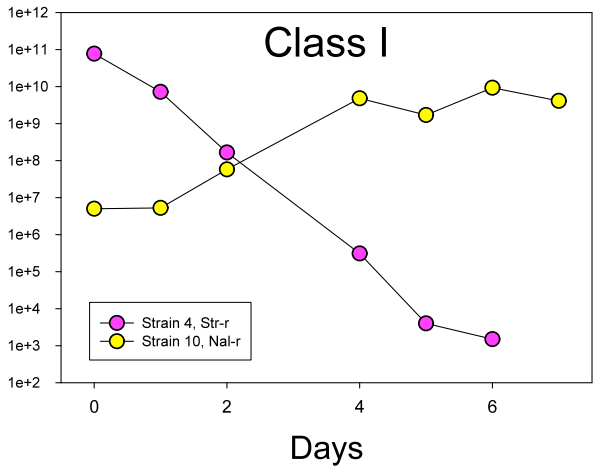
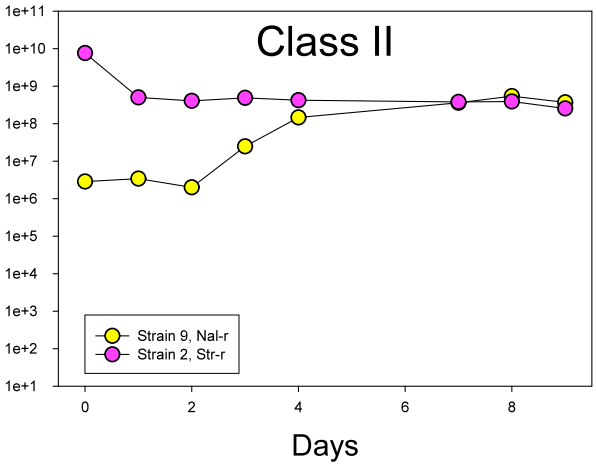


Figure 4. **Types of GASP assay results: Class I, II, and IV.** Panel A shows Class I results, in which the survivor strain drives the non-survivor to extinction. This is the most common response associated with the GASP phenotype. Panel B shows Class II results, also consistent with the GASP phenotype, where the survivor strain uses available nutrients to grow and match the cell numbers of the majority non-survivor strain. Finally, Panel C shows Class IV results, in which both strains die off quickly. This is not an example of the GASP phenotype.

Panel A



Panel B



Panel C

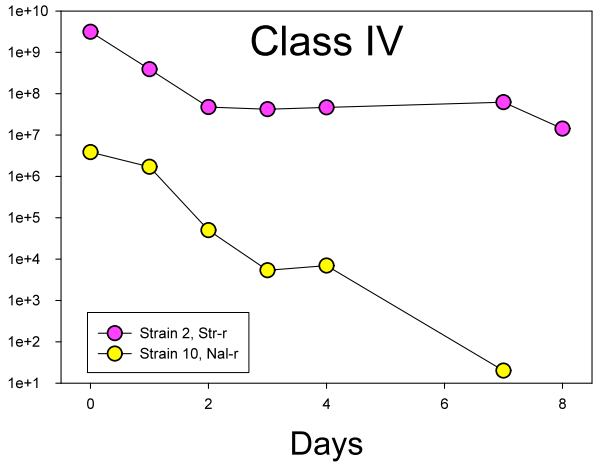
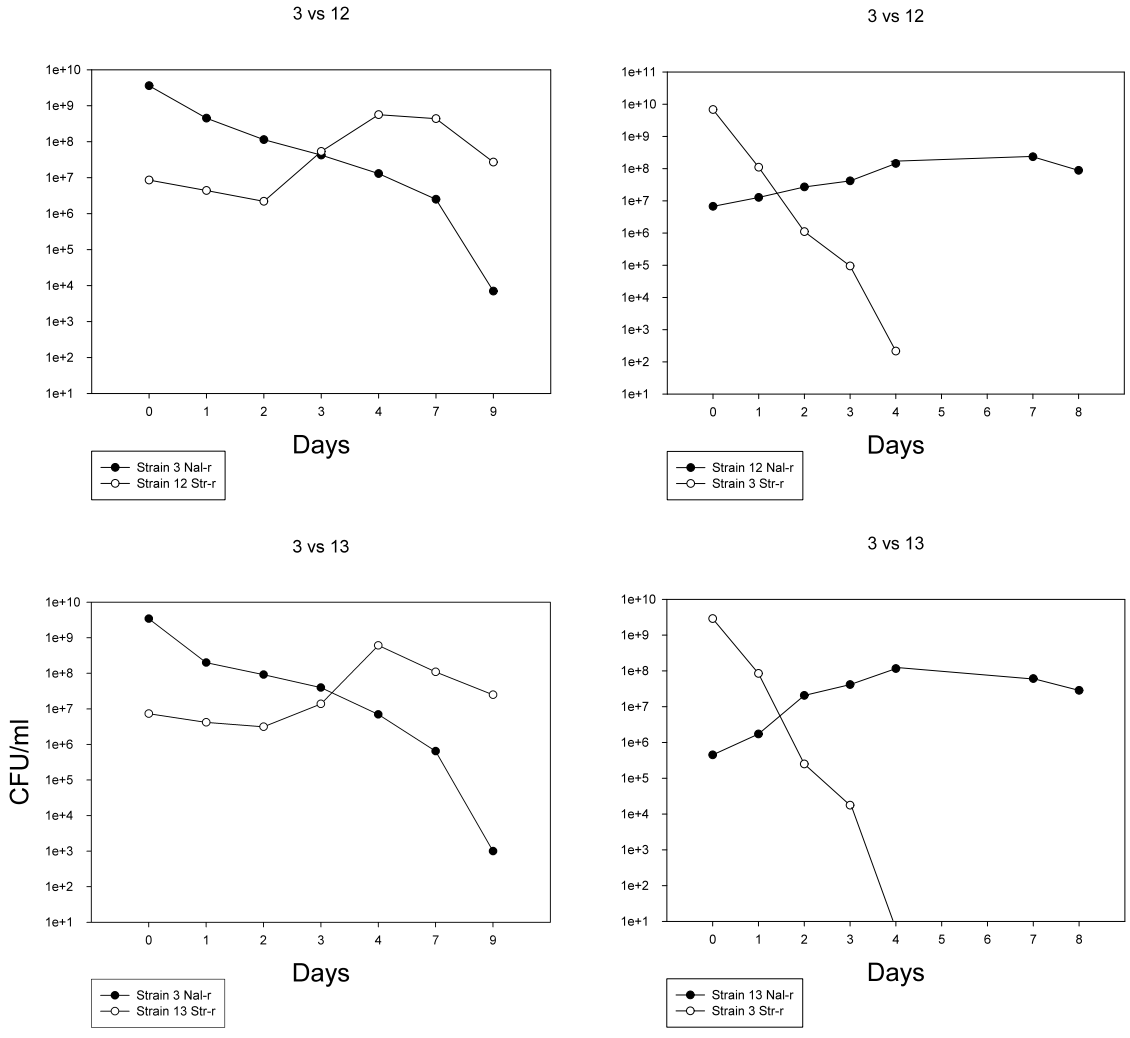
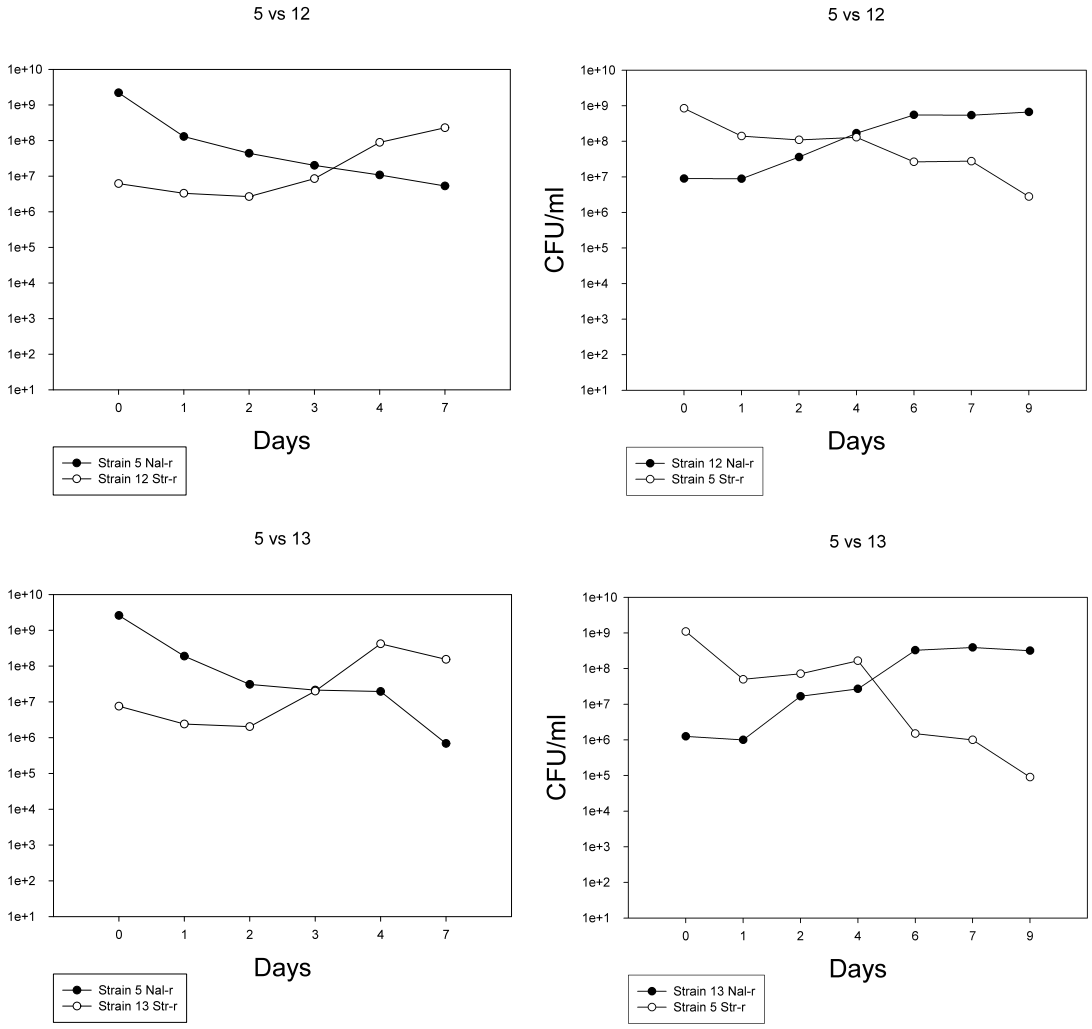

Class IV
1e+10
1e+9
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
1e+2
1e+1
0
2
4
6
8
Days
Strain 2, Str-r
Strain 10, Nal-r

Figure 5. **Effect of spontaneous antibiotic resistance mutations on fitness of strains in the GASP assay.** The GASP assay was used to compare representative survivor and non-survivor strains as a function of the antibiotic resistance used for each. Each non-survivor was tested as a nalidixic acid resistant (Nal-r) strain and as a streptomycin resistant (Str-r) strain with the survivor challenger bearing the other resistance. In each pair of graphs, data points for strains carrying nalidixic acid resistance are shown as closed circles, while strains carrying streptomycin resistance are depicted with open circles.

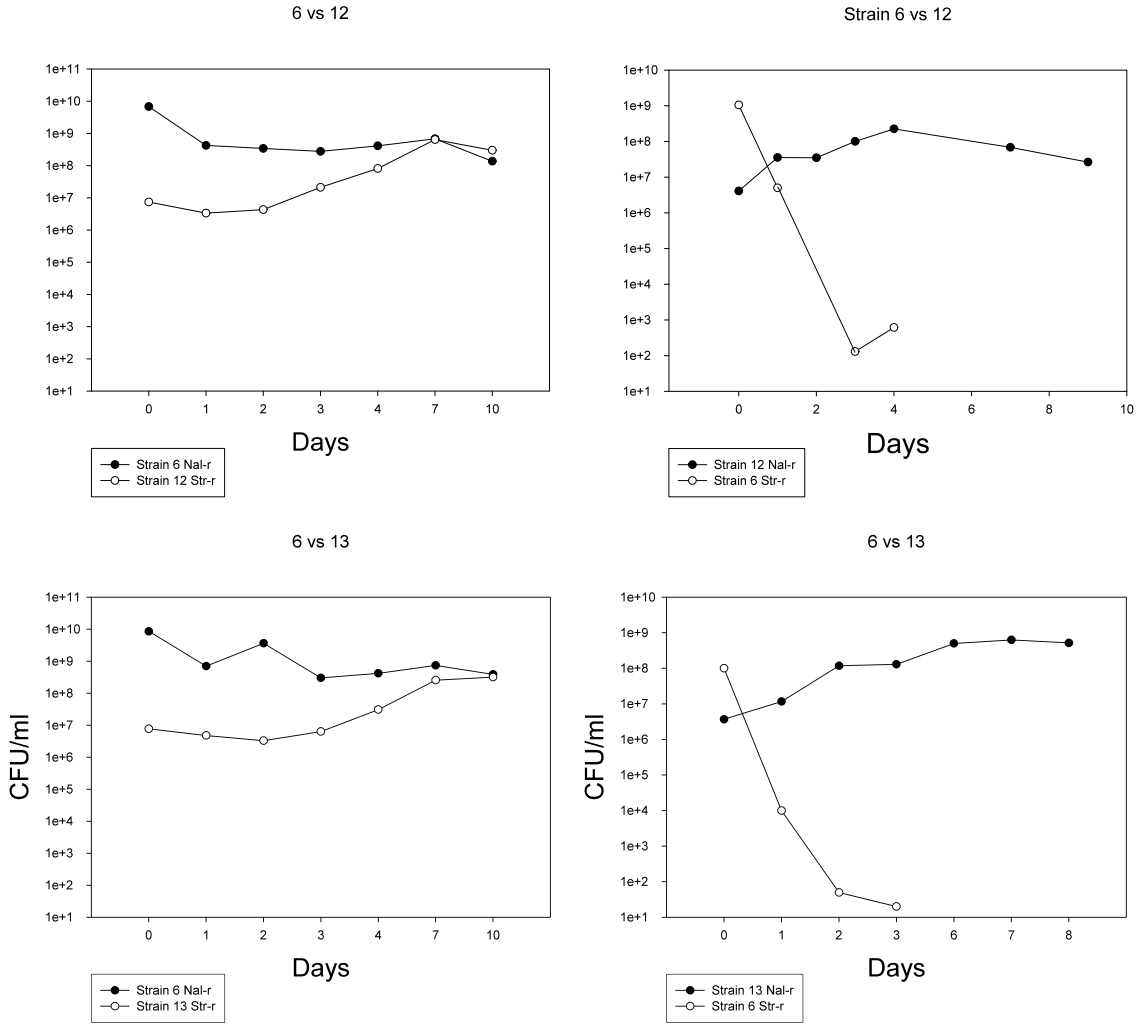
Panel A. The non-survivor strain from group C27, listed in the figure as strain 3, was competed against survivor strains from group C43 (strain 12) and group C40 (strain 13).



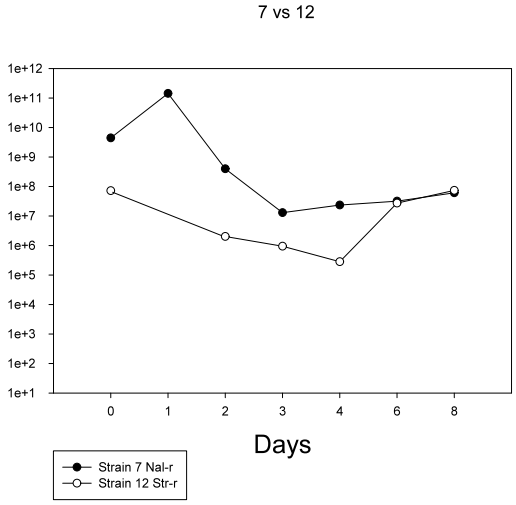
Panel B. The non-survivor strain 5 was a representative from group N2. The survivor strains representing group C43 (strain 12) and group C40 (strain 13) were used in each panel of the figure.

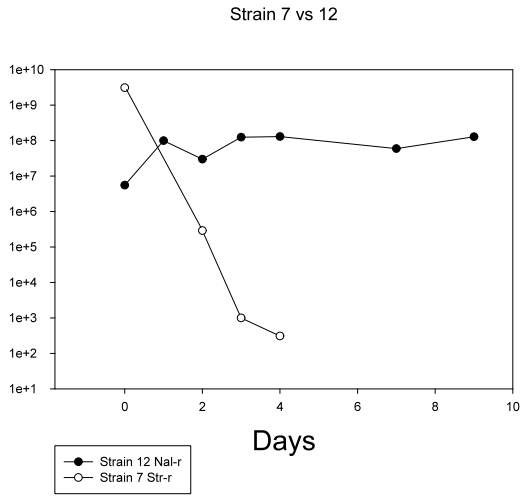


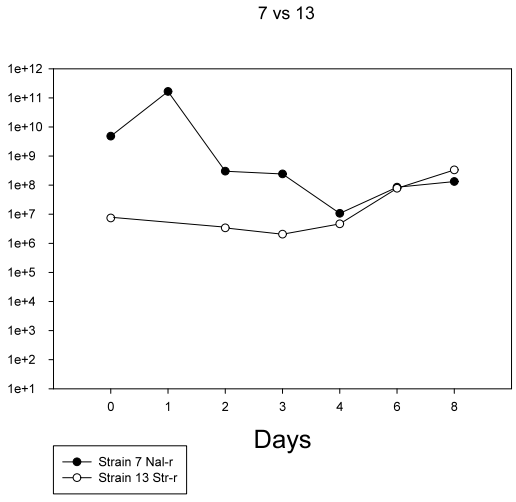
Panel C. The non-survivor strain representing group N8 (strain 6) was tested against the representatives of group C43 (strain 12) and group C40 (strain 13).



Panel D. The non-survivor strain representing group C24 (strain 7) was tested against survivors representing group C43 (strain 12) and group C40 (strain 13).







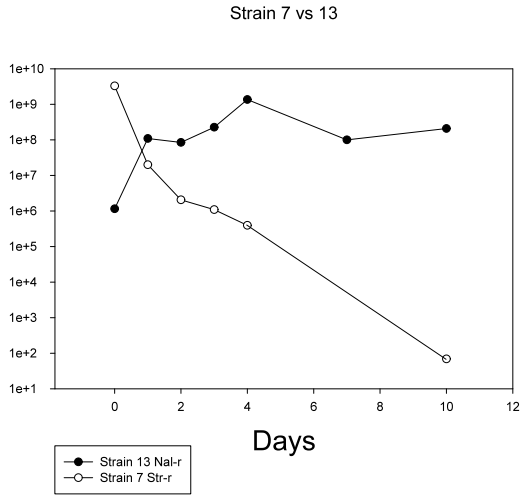


Table I. **Wetland to barn ratios of DNA fingerprinting groups.** Groups were identified as survivors or non-survivors based on the number of times a DNA fingerprint pattern appeared in the wetlands versus the barn. DNA fingerprint groups with wetland to bard (W/B) ratios significantly greater than 1.0, highlighted in yellow, were classified as survivors. Those with W/B ratios significantly less than 1.0 were classified as non-survivors, highlighted in purple. DNA fingerprint groups with ratios between 0.5 and 2 were deemed inconclusive and were not used in further experiments.

Signifigant Nodes and Clusters				
		Barn	*Wetland*	*(W/B)*
Nodes	N1	8	11	1.38
	N2	6	1	0.17
	N3	4	5	1.25
	N4	2	8	4.00
	N5	8	5	0.63
	N6	4	6	1.50
	N7	2	6	3.00
	N8	8	2	0.25
	N9	4	5	1.25
Clusters	C1	1	3	3.00
	C8	2	2	1.00
	C10	3	2	0.67
	C11	2	3	1.50
	C14	1	3	3.00
	C15	2	7	3.50
	C17	1	2	2.00
	C19	2	4	2.00
	C23	4	3	0.75
	C24	6	1	0.17
	C26	2	2	1.00
	C27	3	2	0.67
	C28	1	2	2.00
	C40	1	4	4.00
	C42	2	1	0.50
	C43	1	4	4.00
	C44	1	1	1.00

Table II. **Reference of strain designations based on strain number and fingerprint group.**

Strain Number	Fingerprint Group	Classification
2	C42	Non-Survivor
3	C27	Non-Survivor
4	N5	Non-Survivor
5	N2	Non-Survivor
6	N8	Non-Survivor
7	C24	Non-Survivor
8	N7	Survivor
9	C15	Survivor
10	C14	Survivor
11	C1	Survivor
12	C43	Survivor
13	C40	Survivor
14	N4	Survivor

Table III. **Class results of the GASP assays.** This table shows all of the combinations of survivor (yellow) and non-survivor (purple) strains that were competed against each other in individual GASP assays. Out of the 42 combinations, almost all gave either Class I or II results, indicating that the survivors carry the GASP phenotype and drove the non-survivors to extinction (17).

	2	3	4	5	6	7
8	II	I	I	I	I	I
9	II	I	I	I	I	I
10	IV	I	I	I	I	I
11	II	I	I	I	I	I
12	I	I	I	I	I	I
13	I	I	I	I	I	I
14	IV	I	IV	I	I	I

Table IV. **GASP Assay results for independently isolated strains with the DNA fingerprint pattern representing group N4.** All *E. coli* were identified as having the same DNA fingerprint pattern. The columns identify the majority strains, 19A13 and 38B15, in the GASP assay tested as either nalidixic acid resistant (Nal-r) or rifampicin resistant (Rif-r) strains. The rows are identified by the challenger strain, which exhibit the antibiotic resistance not found in the majority strain. In each case, the assay was initiated using a 1000:1 mixture of the majority strain:challenger strain. The plots of viable cell numbers over time were scored as Class I, Class II, or Class IV as described earlier.

	19A13	19A13	38B15	38B15
	Nal-r	Rif-r	Nal-r	Rif-r
19A13			Class II	Class II
23A5	Class I	Class II	Class II	Class I
26C13	Class I	Class II	Class II	Class II
2D5	Class II	Class II	Class II	Class II
38B15	Class IV	Class IV		

Table V. **GASP Assay results for independently isolated strains with the Group C14 DNA fingerprint pattern.** The columns identify the majority strains, 22E3 and 37B17, in the GASP assay tested as either nalidixic acid resistant (Nal-r) or rifampicin resistant (Rif-r) strains. The rows are identified by the challenger strain, which exhibit the antibiotic resistance not found in the majority strain. In each case, the assay was initiated using a 1000:1 mixture of the majority strain:challenger strain. The plots of viable cell numbers over time were scored as Class I, Class II, or Class IV as described earlier.

	22E3	22E3	37B17	37B17
	Nal-r	Rif-r	Nal-r	Rif-r
22E3			Class II	Class II
22E6	Class II	Class II	Class II	Class II
37B17	Class II	Class II		

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