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Testing artificial genes designed to inhibit the growth of E. coli as an alternative to traditional antibiotics

Abstract:

The CDC states "antibiotic resistance is one of the world's most pressing public health threats". Through mutation, bacteria too easily defeat traditional antibiotics by rendering their narrow attacks ineffective. This research explores a novel alternative to traditional antibiotics by specially designing artificial genes to broadly disrupt bacterial systems. Unnaturally high quantities of hydrophobic residues were used within the genes to cause uncontrolled aggregation, exhaustion of intercellular resources, and to overwhelm the chaperone systems. One sequence was highly hydrophobic (H) while the other was highly hydrophobic and highly acidic (HH). These genes were delivered to bacteria in pET11a plasmid vectors through artificial transformation. A liquid growth media experiment was conducted. Nine groups, based on the type of bacteria (H, HH, or untransformed) and the amount of IPTG used (1 mM, 0.1 mM, or none), were cultured and data was collected on their growth via spectrophotometry. Partial growth inhibition was observed in all the groups with IPTG. However, with the H gene at 1 mM IPTG, nearly total growth inhibition occurred. This novel antibiotic has great potential for combating bacterial resistance. Weakening the bacteria by this technique would likely allow the natural immune system to eradicate the remaining bacteria. Though artificial transformation is not a usable delivery system in the human body, promiscuous bacterial conjugation shows distinct possibilities for significantly enhancing the human bacterial flora's effect on the immune system. Through further development of this technique, the presently grim future of bacterial infection and resistance could be significantly and positively altered.

Introduction:

The Centers for Disease Control and Prevention state on their website that "Antibiotic resistance is one of the world's most pressing public health threats".[1] Unfortunately, too many of the present antibiotics are being rendered ineffective by antibiotic resistance developing in the bacteria they are meant to fight.[2] The costs to the U.S. healthcare system from antibiotic resistant bacteria exceeds $20 billion per year.[2] Societal costs add another $35 billion to the financial expenditures stemming from this crisis.[2] The problem with antibiotic resistant bacteria is a growing danger. Presently, around 70% of bacteria that are responsible for infections in hospitals

are resistant to at least one of the antibiotics used to treat them.[3] The facts of biology tell us this number will only increase. Investigative reporting from USA Today, has found that just one particular bacterium, *Clostridium difficile*, caused 30,000 deaths in the U.S. in 2012.[4] When considering the accelerating, life-threatening, and costly situation antibiotic resistant bacteria are causing in the U.S. and world's societies, it is clear something *must* be done.

The widespread use of traditional antibiotics has promoted the growth of resistant strains of bacteria. (Neu, 1192) Bacteria have not experienced similar natural environmental pressures, such as those created by antibiotics, in the past. As a result, novel antibiotic processes are needed to create functional treatments. This investigation is the second phase in the development of a novel treatment which may possess capabilities which will make it a more effective option to combat bacterial pathogens than traditional antimicrobials.

This study examines the hypothesis that artificially competent bacteria may produce protein aggregates and experience cell death after they take in DNA coding for aggregate forming polypeptides. Protein aggregates can be toxic to bacteria. (González-Montalbán et al, 2005) The mechanism of this may entail disturbing the homeostasis of the cytoplasm by blocking cellular processes and/or absorbing essential proteins and molecules. In eukaryotes, amorphous aggregates have been shown to be toxic because their development results in impairment of cellular processes by causation of oxidative stress and problematic interaction with cell membranes. (Stefani and Dobson, 2003) Similar events are likely taking place in bacteria experiencing overabundant inclusion body formation. Indeed, the results of this study indicated that the source of the toxicity may have been a product of the bacteria consuming cellular resources while synthesizing and degrading the aggregates.

The artificial gene sequences used in this investigation were specially designed to yield large insoluble aggregates and cause general chaos in the bacterial cytoplasm. In part, this was accomplished through the utilization of the strong T7 promoter in the pET11a plasmid. Polypeptides that are not normally part of bacterial systems are likely to cause inclusion bodies to form if they are expressed by a sufficiently strong promoter. (Baneyx and Mujacic, 2004) Such a large volume of protein would also be more likely to cause a disturbance in a cell. In addition, the presence of hydrophobic residues increases the likelihood for aggregates to occur because hydrophobic residues attempt to bury themselves within other proteins and result in many polypeptides "sticking together" to form aggregates. (Idicula-Thomas et al, 2005) Bacteria

have developed protein systems to combat excessive inclusion body formation. (O'Donnell and Lis, 2006) Because of this, the two artificial DNA sequences I designed were equipped with a variety of features to increase their effectiveness. One of the polypeptides was composed almost entirely of hydrophobic amino acids with the exception of some scattered glycine, a few aspartic acids, and several polar amino acids on the n- and c-terminal sequences to avoid degradation by proteases which recognize bulky hydrophobic or basic residues at those locations. (Wickner et al, 1999) The other gene sequence was similar but had the addition of acidic residues every 5 amino acids in an effort to prevent DnaK from binding to the proteins. (González-Montalbán et al, 2005) By necessity, the polar amino acids were also included at the ends of both sequences. It should be noted that no aromatic residues were included in either polypeptide because they tend to be targeted by the disaggregase ClpB and the Lon protease when exposed to the intracellular milieu. (Baneyx and Mujacic, 2004) (Gur and Sauer, 2008) By these mechanisms, the gene sequences were specifically designed to overcome the defenses of the bacteria in an attempt to inhibit or, ultimately stop, their growth.

The sequence which was composed almost entirely of hydrophobic amino acids may have exhibited toxicity despite being recognizable by many chaperone systems because it was so vastly hydrophobic that any other proteins might have experienced difficulty in interacting with it at all. Such a highly hydrophobic protein is not present in nature, and therefore, has not had selective pressure in the past. In addition, it may have been hydrophobic enough that it began to aggregate before translation was complete. This would be a beneficial process for achieving bacterial death because chaperones likely would begin to work upon proteins while they were still translating (O'Donnell and Lis, 2006) and the aggregates would, in this case, be able to compete with the chaperones and thus still exhibit toxicity. Finally, as mentioned before, it may have been a potent drain on the resources of the bacteria.

The other sequence was similar to the first one but with aspartic acids placed at intervals between every five residues. The reason for this was to prevent or inhibit binding by various chaperones and proteases, especially DnaK. DnaK has been shown to be necessary for cell viability in cultures overexpressing inclusion body prone polypeptides. (González-Montalbán et al, 2005) In addition, the many extra aspartic acids resulted in this sequence being highly acidic which may have interfered with the functions of proteins in the bacterial cells in other ways. One possibility is that some proteins might have experienced difficulties in folding optimally

near clusters of the aspartic acid rich polypeptides because of the lowered PH. This may have allowed the polypeptides to be protected against chaperones and proteases alike thus increasing their power.

There are a variety of reasons these gene sequences could prove to be more difficult to gain resistance against than traditional antibiotics. Since aggregates cause general chaos in the cell and do not bind to a target site, the bacteria should not be able to defend themselves by their usual mechanism against antibiotics of altering a site or sites. Bacteria have been specifically known to alter such target sites in resistance to antibiotics such as rifamycins and quinolones. (Lambert, 2005) Because these gene sequences affect many processes within bacteria beyond a given target site, the cell altering one, or even more, sites would not protect them from this newly designed attack. The bacteria would also have to deal with the fact that aggregates are large and insoluble, and therefore, cannot be removed through efflux adaptations. A new exocytosis pathway would have to develop by the bacteria and this would be very difficult for organisms possessing cell walls. Decreasing membrane permeability to the DNA would not be an option for the bacteria either because calcium-induced competence does not involve alterable membrane transport proteins. This already shows that they have no defense for this. Bacteria would likely be restricted to improving their chaperone systems or metabolic efficiency but this would also prove difficult for them. Because of all these factors, the introduction of these specific gene sequences would be much more difficult for bacteria to develop resistance to than the present antibiotic system we have today.

In the event that resistance did develop, the gene sequence antibiotic would be easy to modify making it newly effective again. The DNA sequence could simply be altered and re-cloned. One could even induce a variety of random mutations and test the many new versions on many separate cultures. Then, the most successful versions could be selected and used to combat the newly adapted pathogens. This would be a faster and less costly tool for in the race against antibiotic resistance. Perhaps with future incarnations of this research, we might truly find an answer to the mounting costs and real threat of antibiotic resistance bacteria.

Methods:

The artificial DNA sequences cloned into the pET11a plasmid described in the introduction were ordered from GenScript.com. Below, the ORF sequences are displayed in DNA bases and amino acid single letter code.

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Highly Hydrophobic (H)

5-CATATGATGTCTAACACCTCTGTTATCATGTGCATGATCG
GTGTTATCGGTATGATCGGTGACTGCATCATCGTTATCGTTATGG
GTCCGCCGGGTGTTGACATCGTTATCTGCGGTGGTTGCATCGCGA
TCGGTATGCCGCCGGGTATCTGCATCGTTATCGACGGTATCGTTC
CGCCGGGTATGTGCGGTATCATCATGATGGTTATCGGTATCGTTT
GCATCGGTGTTGTTATCTGCGGTGGTGTTGTTATCATCGTTATCA
TCATCATGTGCGGTGTTGGTATCGTTATCTGCGTTGGTGTTGGTG
TTATCGGTGACGTTATCATCCCGCCGGCGATCGCGATCGTTTGCG
TTATCATCGTTATGATGATCGTTCCGCCGGACTGCATCATGATCG
CGATCATGATCGTTGTTGGTATGATGTGCGTTATCCCGCCGATCG
TTGGTGTTATCATCGGTGACGTTATCATCGTTATCGGTGTTGTTA
TCTGCATCCCGCCGGGTGACGTTATCATCTGCGGTGGTATCATCG
TTAACACCTCTAACACCTCTTCTTAAGGATCC-3
M M S N T S V I M C M I G V I G M I G D C I I V I V M G P P G V D I V I C G G C I A I G
M P P G I C I V I D G I V P P G M C G I I M M V I G I V C I G V V I C G G V V I I V I I I M
C G V G I V I C V G V G V I G D V I I P P A I A I V C V I I V M M I V P P D C I M I A I M I
V V G M M C V I P P I V G V I I G D V I I V I G V V I C I P P G D V I I C G G I I V N T S N
T S S Stop

Highly Hydrophobic and Highly Acidic (HH)

5-ATGATGTCTAACACCTCTGACGTTATCATGGGTATGG
ACGCGTGCATCGTTATCGACATCGGTATCATGATCGACCCGCCGA
TCTGCGGTGACGCGCCGCCGGTTCCGGACCCGATCGTTTGCATCG
ACCCGTGCGTTATCGCGGACCCGCCGGTTATCGGTGACTGCATCG
CGATCGGTGACATGTGCCCGCCGATCGACGCGGTTATCGCGGGTG
ACATCGTTTGCATGGGTGACCCGCCGATCATCATGGACATGATCG
TTGCGTGCGACATCGTTGTTATCTGCGACGGTATCGCGCCGCCGG
ACGCGGTTATCGCGATCGACGTTGCGGTTATCTGCGACGTTGCGC
CGCCGATGGACGCGGACATCATGTGCGGTGCGGACGGTGTTGTTC
CGCCGGACGCGGTTGCGATCGCGGACGCGATGGGTGTTATCGACG
CGGTTCCGCCGGCGGACATCATCGGTGTTTGCGACCCGCCGGTTA
TCGCGGACATCGGTGTTATCGTTGACGTTATCGCGGTTATGGACA
ACACCTCTAACACCTCTTCTTAA-3
M M S N T S D V I M G M D A C I V I D I G I M I D P P I C G D A P P V P D P I V C I D P
C V I A D P P V I G D C I A I G D M C P P I D A V I A G D I V C M G D P P I I M D M I V
A C D I V V I C D G I A P P D A V I A I D V A V I C D V A P P M D A D I M C G A D G V
V P P D A V A I A D A M G V I D A V P P A D I I G V C D P P V I A D I G V I V D V I A V
M D N T S N T S S Stop

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Experiment 1: Solid Growth Media Plates

The bacteria were made artificially competent in order to ready them for the intake of the gene sequences, and then, the gene sequences were introduced in order to transform the bacteria. *E. coli* BL21(DE3) were used in order to express the artificial genes via the T7 promoter. The plasmids arrived in the form of 4 µL of lyophilized DNA. They were refrigerated at proper temperatures for several days before use. Then, 40 µL of buffer was added to each of the two samples to make a 1:10 dilution. Competent *E. coli* BL21(DE3) were put on ice. The buffer/plasmid solutions were centrifuged for three seconds, then were vortexed for a few seconds. This was repeated twice more, but only with one second increments for each process. Next, 1 µL of buffer/plasmid solution was added to each of the two samples of bacteria. That is, one sample received the H plasmids and one sample received the HH plasmids. The solutions were returned to ice for another 15 minutes. Finally, the bacteria were heat shocked by being placed in a hot water bath at 42°C for 30 seconds. Afterwards, they were returned to ice. This procedure transformed one sample of bacteria with H plasmids and one sample of bacteria with HH plasmids.

Plates of bacteria were prepared by two methods on four plates to ensure good growth and usable samples. The transformed bacteria (stored in 1.5 mL tubes) were dropped into a flask. The flask was then placed in an environmental shaker incubator and the bacteria were left to grow for one hour. Four AMP plates were selected. 40 µL of SOC broth was added to each of these plates before spreading. After this, 10 µL of bacteria were spread across two plates with a plastic spreader; one of the plates received 10 µL of H bacteria and the other received 10 µL of HH bacteria. 70 µL of bacteria of each type were pipetted onto two more plates. The use of these two methods was to ensure growth in case of aberrations in the culturing process. These plates were incubated overnight in a normal incubator at 37.5°C. Colonies grew on all the plates, although there were fewer colonies on those which had been given 10 µL of bacteria originally. The two plates with less growth were disposed of in a biohazard bag while the two plates with better bacterial growth were used as the working samples.

Continuing with the experiment, the transformed bacteria containing the gene sequences were plated in order to observe for bacterial growth or inhibition of growth. Four new AMP plates were selected. Two of them were treated with a mixture of 5 µL of 1 mM IPTG and 40 µL of

distilled water by spreading the solution onto the plates with a plastic spreading stick. (IPTG is a chemical commonly used to induce genetic expression.) Two plates were left without IPTG as controls. All four plates were streaked, using disposable plastic inoculating loops, with the transformed bacteria. The streaking was performed so that with each rotation of the plate, the density of the bacteria was lowered. With each new plate, a single sample from the original growth plates was taken and spread. Two of the plates received the bacteria containing gene sequence H and two of them received the bacteria containing gene sequence HH. The plates were then incubated overnight at 37.5°C. Photographs of the plates were then taken. This procedure was then repeated once more giving a total of eight plates tested and observed for results.

Experiment 2: Liquid Growth Media with Varioskan

Since the plate experiments showed indications of inhibition of bacterial growth, a more refined technique was selected to acquire more accurate data of the results. A liquid growth experiment on a 96 well plate was conducted. An aliquot of LB broth (growth medium) was prepared in a flask; 5 mL were distributed to three different tubes. Then, 5 µL of Ampicillin (AMP) 100 was delivered to two of the tubes; the ones that would soon contain the transformed bacteria. The third tube did not receive AMP because untransformed bacteria were used in it as a control. For each of the two experimental tubes, a pipette tip was used to transfer a colony from the transformed cultures. For the control, 5 µL of *E. coli* BL21(DE3) from a 1.5 mL storage tube were transferred to the one tube. The three tubes were incubated at 30°C for a few hours before they were moved to a 37.5°C environmental shaker incubator to grow overnight. After the bacteria grew overnight, three more tubes were prepared in a similar manner to those mentioned previously but, this time, 50 µL of each of the grown cultures were transferred to their appropriate tube. This created a 1:100 dilution. The new tubes were placed in the environmental shaker (37.5°C) to grow for the next few hours. Finally, the absorbance of each tube was measured in a spectrophotometer set to measure with 600 nm light. Zeroing was performed with tubes containing LB broth but no bacteria. The absorbencies read as follows; untransformed bacteria: 0.8545, H bacteria: 0.345, HH bacteria: 0.5675. From these findings, the amount of sample to use in order to start growth with approximately the same number of bacteria was calculated. ($CV = C^1V^1$.) The calculations revealed that 5.85 µL of untransformed bacteria, 14.49 µL of H bacteria, and 8.81 µL for HH bacteria were needed to give equal numbers of bacteria for the three samples.

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For the next step of the experiment, the tubes needed to be prepared for these equaled bacterial samples to prepare for their final growth. To start, 5 mL of LB broth was added to each of nine tubes. Six of these tubes were also treated with 5 µL of AMP in anticipation of later addition of transformed bacteria. Three tubes were left without AMP for the later addition of untransformed bacteria controls. The tubes were separated into sets of three, two AMP-prepared and one *not* AMP-prepared tubes. Each set was given 50 µL of the appropriate dilution of different *E. coli* (untransformed, H, and HH bacteria) as described earlier. The untransformed bacteria were delivered to the tubes without AMP. In addition, across the sets, each different *E. coli* was given a different amount of IPTG; one of every three tubes was given 5 µL of 0.1 mM IPTG, one of every three was given 5 µL of 1.0 mM IPTG and one of every three was not given any IPTG. This variation in amounts of IPTG (0.0, 0.1, and 1.0 mM) allowed for a control for the antibacterial effect that has sometimes been seen with IPTG while also allowing for its intended use within the experiment to induce gene expression across all three bacterium, untransformed, H, and HH bacteria. This allowed for important control comparisons within the results.

In the final step of the liquid growth experiment on a 96 well plate, the nine previously prepared tubes of solution were distributed onto the plate and put into the Varioskan to gather data. A 96 well plate was labeled, appropriately identifying for each of the 9 different solutions. (Table 1) Since 59 wells would remain empty of solution, a multichannel pipette was used to deliver 100 µL of distilled water to each. This would maintain the desired humidity of the plate. Next, 100 µL of previously prepared solution from each of the nine tubes was pipetted into a well on the 96 well plate. This was repeated three times from each tube giving 27 solution-filled wells. These twenty seven wells were filled adjacent to each other in three groups of nine on one side of the plate to avoid discrepancies in the reading occurring as a result of those on opposite sides of the plate being read slightly differently by the plate reader Varioskan. (This is a common aberration that can happen when using the device if prepared incorrectly.) After the labeled lid was exchanged for a blank lid to prevent the labels from interfering with the readings and with each of the 96 wells full, the plate was inserted into the Varioskan. The Varioskan was set to measure the absorbance of the wells containing liquid growth media every twenty minutes for fifty hours. The final step provided the opportunity to compare data from the nine different solutions under well-controlled conditions.

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Results:

In both the trials with the solid growth media plates and those conducted using liquid growth media on a 96-well plate, the bacteria infected by the plasmids containing artificial genes exhibited a reduction in growth.

Results from Experiment 1: Solid Growth Media Plates

The solid growth media trials exhibited approximately the same degree of growth reduction for both the H (Highly Hydrophobic) and the HH (Highly Hydrophobic and Highly Acidic) gene sequences. For these tests, bacterial growth was often nearly as thick in the control as in the manipulated for the first streaks, but reduced in the second streaks, and sharply reduced in the third. In fact, the only manipulated group plate which showed any visible growth in the third streak was the HH plate for the first replicate. Positive, though difficult to quantify, results were found in the solid growth media trials.

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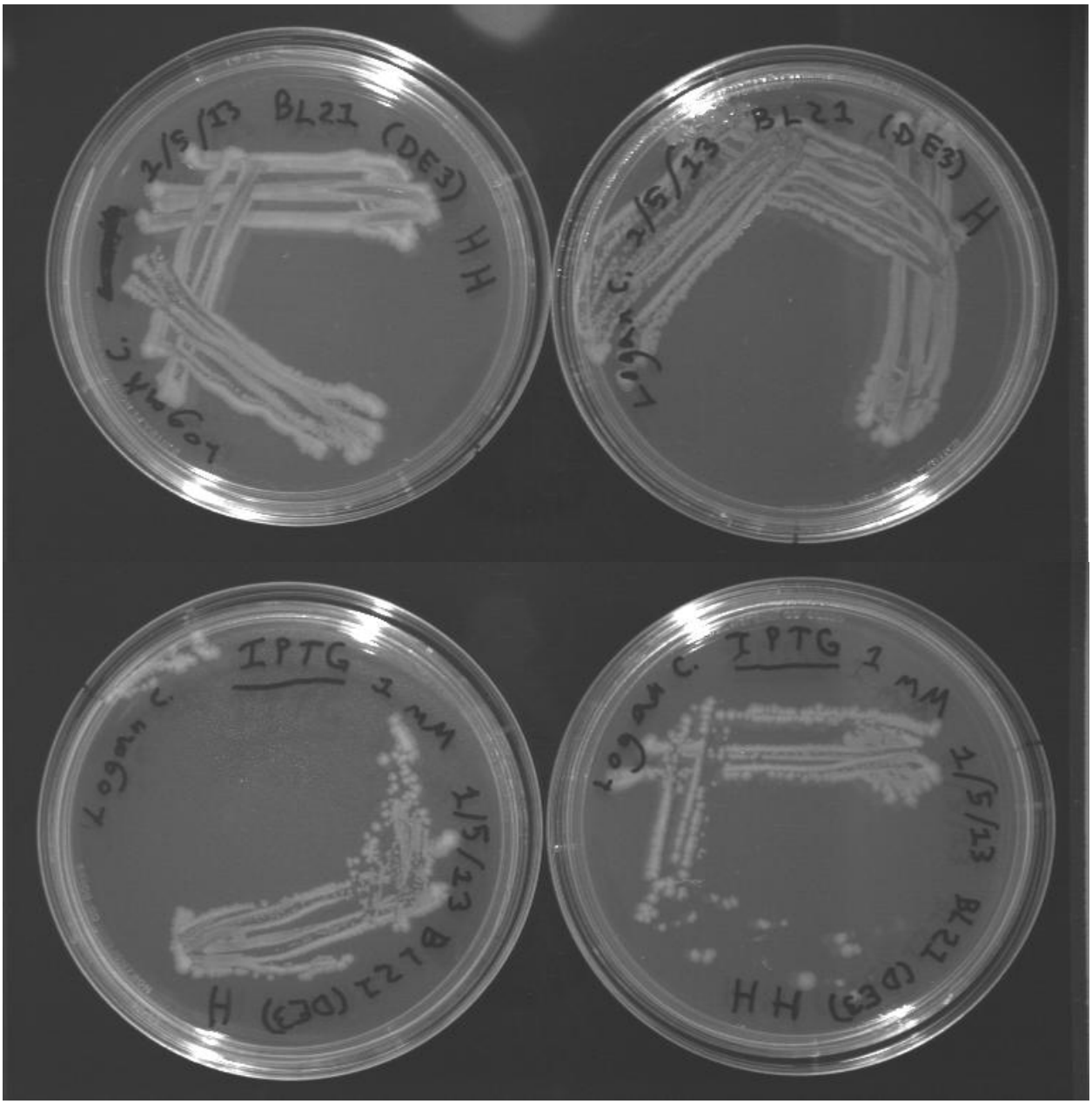

Fig.1 A comparison of the bacterial growth on the control and manipulated plates for the first solid growth media trial. (The manipulated plates are labeled with an underlined "IPTG.")

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Fig. 2 A comparison of the bacterial growth on the control and manipulated plates for the second solid growth media trial. (The manipulated plates are labeled with an underlined “IPTG.”)

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Results from Experiment 2: Liquid Growth Media with Varioskan

Similar results were seen in the liquid growth experiment. However, there were several marked differences observed due to the more precise data collected by the Varioskan. The wells containing bacteria transformed with H in the 1 mM IPTG group, exhibited almost no growth, nearly total bacterial death.

Since this finding was so significant, it needed to be critically assessed. There was some possibly that this effect was due to a pipetting error. However after thoroughly analyzing the graphs, the corresponding growth patterns of the other groups and the growth pattern of the H transformed bacteria itself. This was especially seen in the graphing of the bacteria transformed with HH in the 1nM IPTG since it also showed almost no growth of the bacteria for the first four hours. Also, the H transformed bacteria did show a minimal amount of growth over the course of the full fifty hours. If it had been a pipetting error where no bacteria were added, this well should have shown no growth. In addition, the fact that growth of the third streaks on both of the solid growth media results for the H gene sequence showed bacteria that were visibly less concentrated, further confirms the finding of the same sequence in the liquid media. In further efforts to account for all variables that could account for the bacterial death, it is important to remember that the IPTG and its potential for causing bacterial death was controlled for within th experiment. Based on the results of the liquid growth, the IPTG alone caused minimal toxicity to the bacteria. However, the concentration of 1 mM IPTG induced greater toxicity from the artificial genes than the 0.1 mM concentration of IPTG. Since the role of the IPTG is to induce gene expression, it appears that the higher concentration allowed for better expression of both of the gene sequences resulting in greater bacterial growth inhibition. Considering all these elements, it is likely that the near total bacterial death resulting from the bacteria transformed with H in the 1 mM IPTG, was a valid finding.

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Tables and Graphs

Well	Materials Used
B02	*E. coli* BL21(DE3)
B03	*E. coli* BL21(DE3)
B04	*E. coli* BL21(DE3)
C02	*E. coli* BL21(DE3) transformed with H gene.
C03	*E. coli* BL21(DE3) transformed with H gene.
C04	*E. coli* BL21(DE3) transformed with H gene.
D02	*E. coli* BL21(DE3) transformed with HH gene.
D03	*E. coli* BL21(DE3) transformed with HH gene.
D04	*E. coli* BL21(DE3) transformed with HH gene.
E02	*E. coli* BL21(DE3), 1 mM IPTG
E03	*E. coli* BL21(DE3), 1 mM IPTG
E04	*E. coli* BL21(DE3), 1 mM IPTG
F02	*E. coli* BL21(DE3) transformed with H gene, 1 mM IPTG
F03	*E. coli* BL21(DE3) transformed with H gene, 1 mM IPTG
F04	*E. coli* BL21(DE3) transformed with H gene, 1 mM IPTG
G02	*E. coli* BL21(DE3) transformed with HH gene, 1 mM IPTG
G03	*E. coli* BL21(DE3) transformed with HH gene, 1 mM IPTG
G04	*E. coli* BL21(DE3) transformed with HH gene, 1 mM IPTG
B05	*E. coli* BL21(DE3), 0.1 mM IPTG
B06	*E. coli* BL21(DE3), 0.1 mM IPTG
B07	*E. coli* BL21(DE3), 0.1 mM IPTG
C05	*E. coli* BL21(DE3) transformed with H gene, 0.1 mM IPTG
C06	*E. coli* BL21(DE3) transformed with H gene, 0.1 mM IPTG
C07	*E. coli* BL21(DE3) transformed with H gene, 0.1 mM IPTG
D05	*E. coli* BL21(DE3) transformed with HH gene, 0.1 mM IPTG
D06	*E. coli* BL21(DE3) transformed with HH gene, 0.1 mM IPTG
D07	*E. coli* BL21(DE3) transformed with HH gene, 0.1 mM IPTG

Table 1. Materials used in wells on the 96 well plate.

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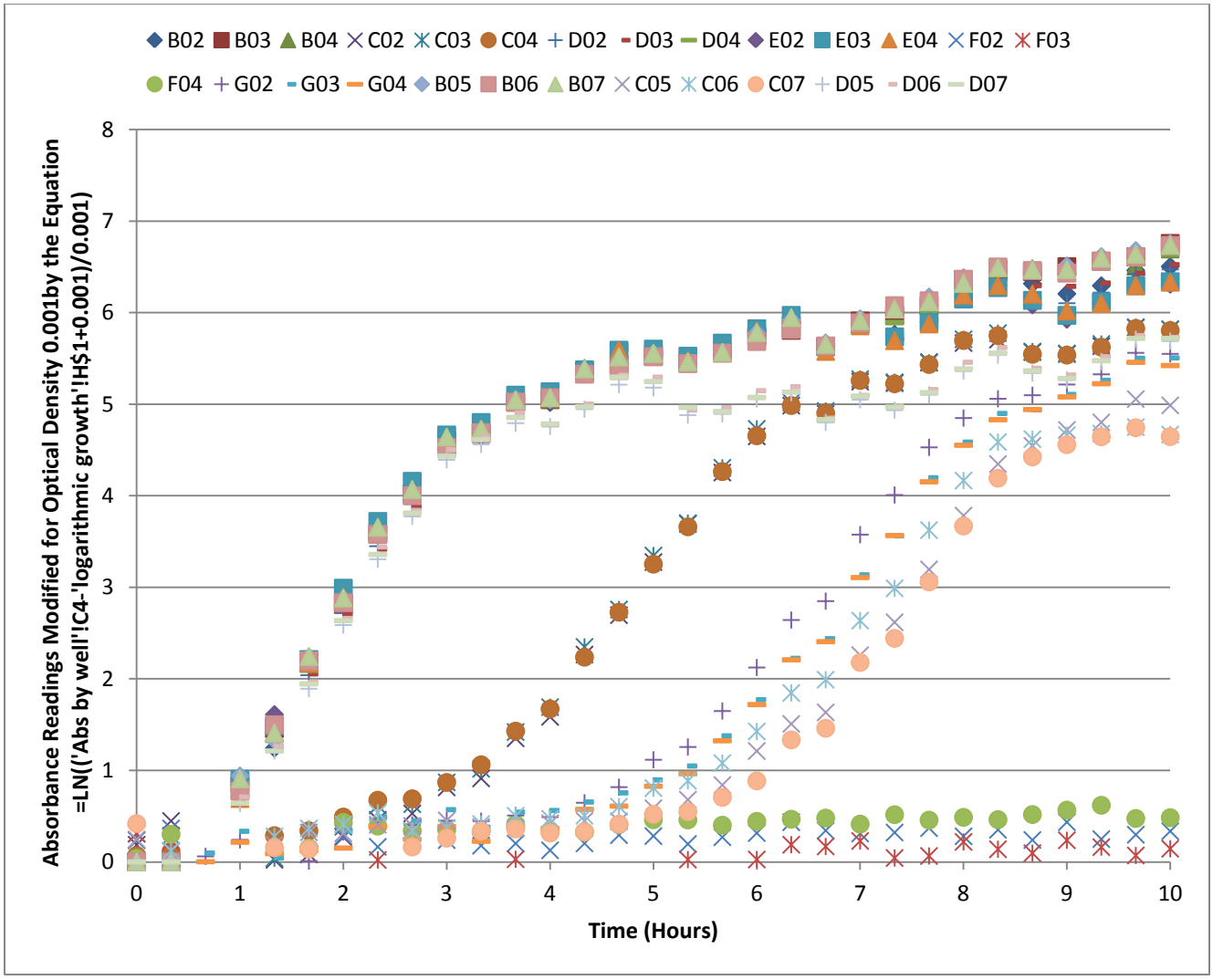

Fig. 3 Growth curve for all wells for the first ten hours.

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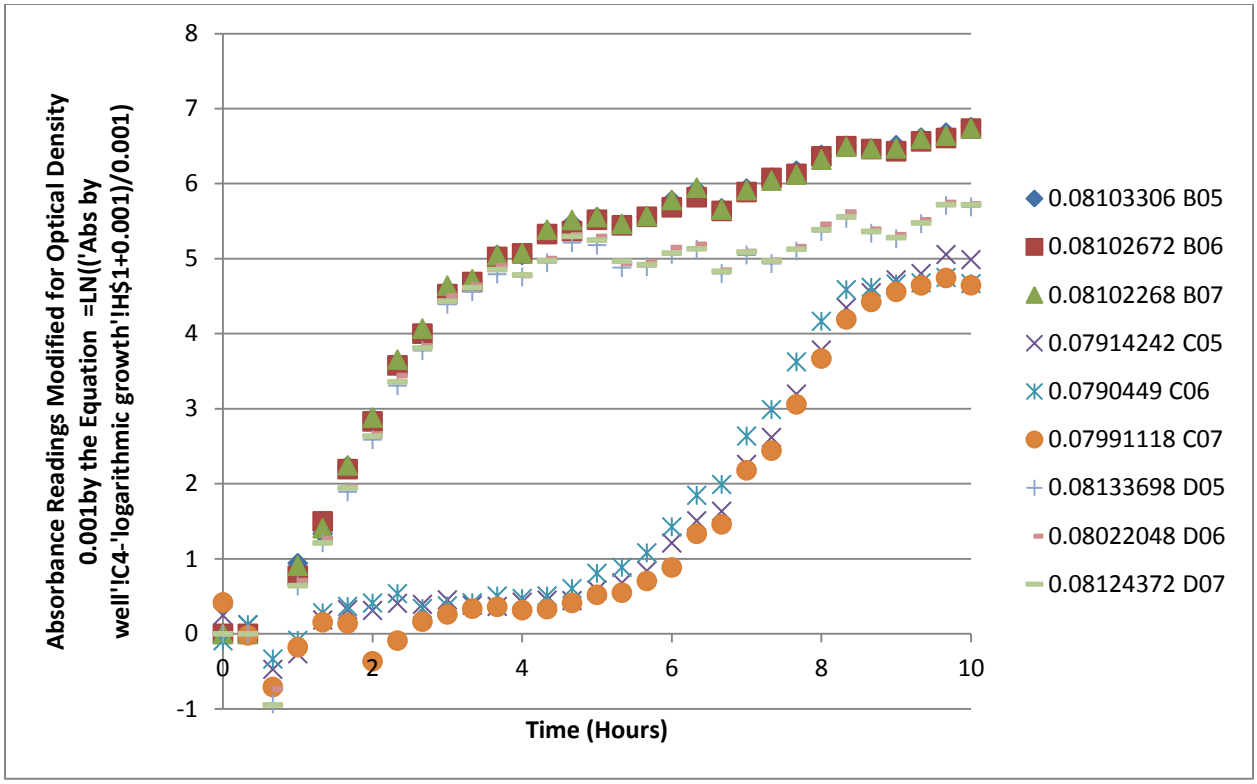

Fig. 5 Growth Curve for all wells with 0.1 mM IPTG for the first ten hours.

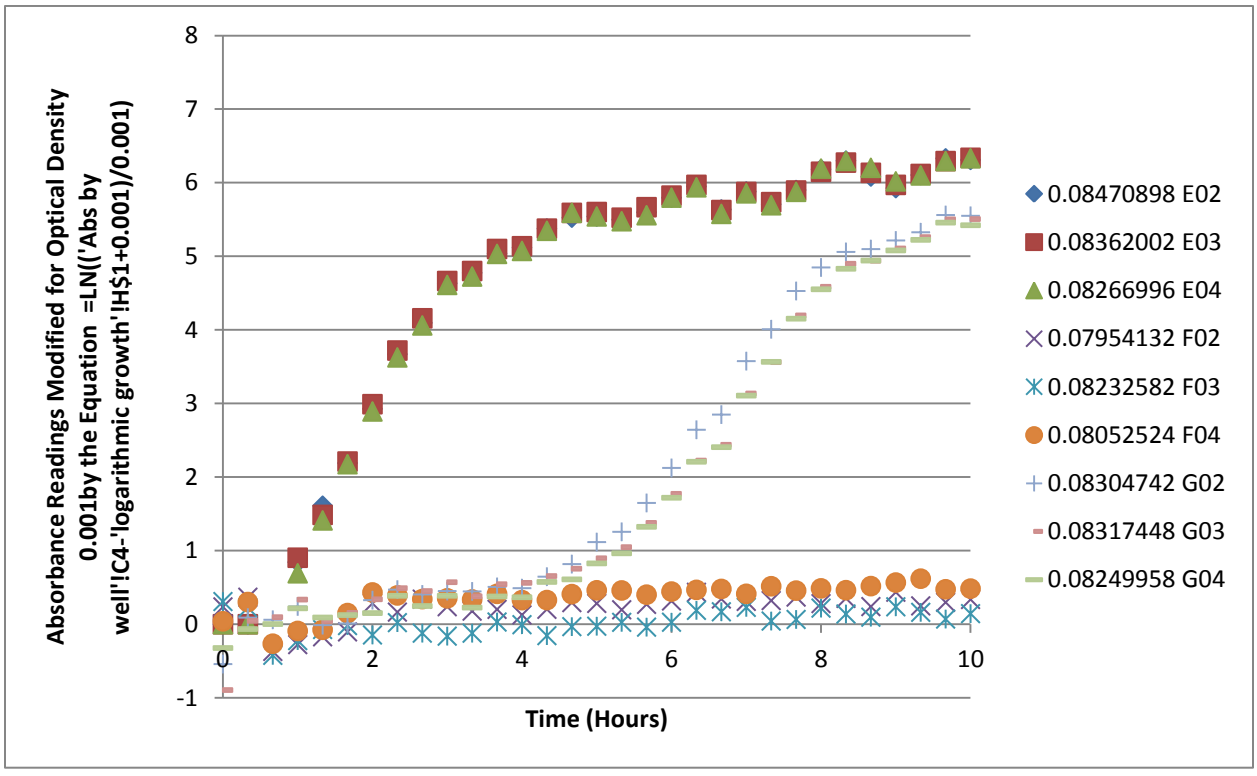

Fig. 6 Growth Curve for all wells with 1 mM IPTG for the first ten hours. Note that the growth reduction increases from the degree it was at for the 0.1 mM IPTG results by similar amounts for both the H and the HH genes.

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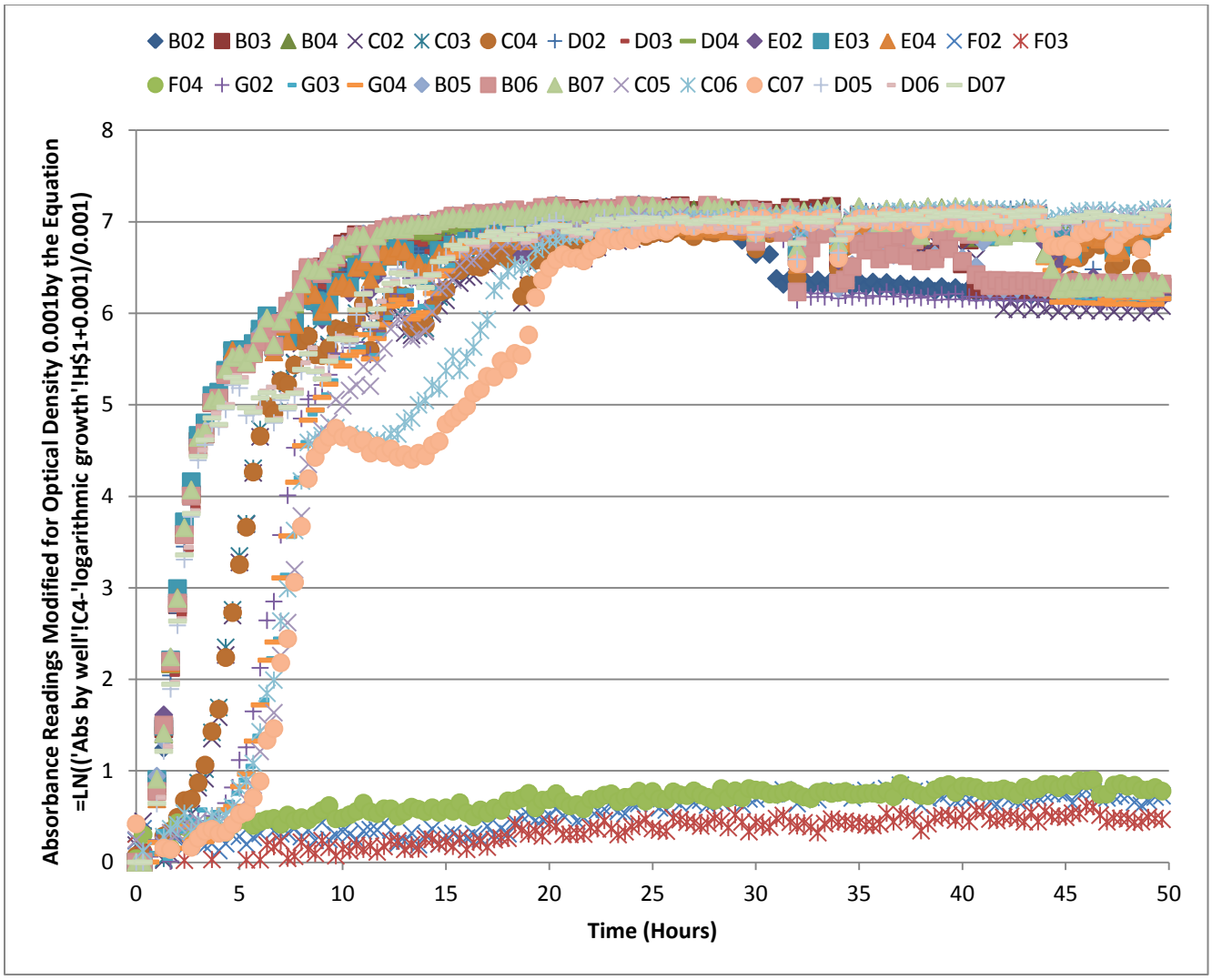

Fig. 4 Growth curve for all wells for the full fifty hours.

After the full fifty hours of data collection, all the groups except for the H group with 1 mM IPTG grew to approximately the same level. Nevertheless, the partial reduction in growth of the other groups still hold valuable possibilities for this technology while the highly expressed H group appears extremely promising.

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Discussion:

Both artificial genes caused partial growth inhibition in the *E. coli* under all the experiment conditions of this study. The one possible exception to this was in one of the two HH groups where it was difficult to determine if any growth inhibition had occurred in the third streak through simple observation. However, the H group with 1 mM IPTG for the third streaks on the plates (solid growth media) and in the growth curve on the graph (liquid media experiment), showed nearly total growth inhibition occurred. This indicates that in bacterial populations with few members, as in the third streaks and the diluted liquid cultures, total growth inhibition can be induced with this gene sequence.

Bacteria growing in suboptimal conditions, such as within the human body, are more likely than their experimental counterparts to be susceptible to intervention. If caught before becoming well-established, they could be considered to be in a similar "few members" population as described above. Also, because they are already subject to attack by the innate and adaptive immune system and must accomplish the difficult task of finding a place to establish colonies, they are likely to be extremely vulnerable to this technology. It must be noted, of course, that while every member of the bacterial populations tested in this experiment had transformed the plasmids, delivery to infectious pathogens in vivo would not be so all inclusive. Nevertheless, their vastly increased vulnerability due to the immune system and suboptimal physical environment would likely outweigh the lack of universal uptake, especially if/when further research to maximize the delivery and toxic effect of the genes was established. There is great promise in the use of such artificial genes as a treatment for bacterial infections.

The artificial genes designed for this experiment possess several benefits over naturally existing toxic genes. Both the H and HH genes use up large amounts of cellular resources. The overproduced polypeptides take up a massive number of amino acids, especially when considering that many of the same amino acids were incorporated into the sequences. In addition, the most frequently used molecular chaperones DnaK and GroEl are put under heavy strain. Due to these chaperones being forced into high ATPase activity, they consume a great deal of ATP draining the cells energy supply. GroEL even uses seven ATP molecules per cycle. Finally, although the polypeptides were designed to be protease resistant, some protease activity may still have occurred. Because of the low binding efficiencies of bacterial proteases to the custom polypeptides, upregulation to produce high levels of proteases may have been used by the *E. coli*

to adjust to the conditions. This may have been an additional factor in the strain on the resources of the bacteria. This type of metabolic drain may be capable of greatly increasing the vulnerability of pathogens towards starvation and in attacks from other sources. This metabolic drain would explain the fact, that in this investigation, the bacteria tested experienced difficulty in establishing colonies, but once they were able to accomplish this, grew somewhat successfully with the exception of the H transformed bacteria in 1 mM of IPTG. These many characteristics of the designed gene sequences resulted in greater bacterial growth inhibition than would have been possible with natural toxic genes.

There are a variety of other possible sources of toxicity contributing to the cell stress which inhibited the bacterial growth. In later studies, more techniques with which to control for such things will need to be utilized. The unnaturally high hydrophobicity of the H polypeptides (and to a lesser degree the HH polypeptides) may have resulted in the denaturing of native proteins in the cytoplasm and other problematic interactions with cytosolic and membrane components. The HH polypeptides possessed a low net charge of -30.5. This in combination with their somewhat small size means that they are quite acidic compounds. Even in the event of small inclusion bodies forming, this could create “acidic clumps” which might have detrimental effects on cellular processes. Despite these additional possibilities for reasons that the artificial polypeptides were likely toxic, the beneficial qualities described in the introduction are likely to still apply.

This technology has the potential to develop into a novel treatment for bacterial infections. Our society is in desperate need of an alternative to traditional antibiotics. This may provide that alternative. There are a variety of components and improvements still requiring development before this goal can be accomplished.

At this stage of the research, *E. coli* BL21 (DE3) were used to test the genes. *E. coli* BL21 (DE3) is a genetically engineered strain which possesses a special RNA polymerase that is able to bind to the T7 promoter. Infectious pathogens generally do not have this type of RNA polymerase. Because of this, for real pathogens targeted by this treatment, appropriate promoters must be selected. This may be accomplishable through selecting promoters from various lytic bacteriophages and incorporating them ahead of the open reading frames of the toxic genes within the plasmid vectors. If bacteriophage x infects bacterial pathogens w, y, and z then the promoter from that bacteriophage, which will be powerful because it is used to overproduce viral

capsid components in viral reproduction, can be used to overproduce the custom toxic polypeptides when fighting bacteria w, y, and z. Before targeting any human pathogens, new and powerful viral promoters from viruses capable of infecting those bacteria will need to be selected and tested.

Most naturally occurring bacteria possess a set of restriction enzymes which cut DNA at specific sequences. The genome and extragenomic elements of the bacteria themselves are protected at these sites by methylation. To prevent the digestion of plasmid vectors carrying toxic genes, the plasmids will need to be treated with bacterial methylases specific to the pathogen they are designed to infect. The online database, ReBase will be invaluable in managing and accomplishing this task. Alternatively, plasmids could be cloned in bacteria of the same strain they would be designed to attack but with their toxic genes turned off. These bacteria would have their genes for restriction enzymes knocked out but their genes for methylases still present. This technique may provide a way with which to prevent unknown restriction enzymes from digesting invasive plasmids.

Finally, a delivery mechanism capable of distributing the plasmids carrying the toxic genes to populations of pathogenic bacteria within the human body is essential. This mechanism will need to function in a way that does not allow the bacteria to easily devise ways to prevent the delivery through adaptation. There are several possibilities for such a mechanism. Bacteriophages could be used to deliver the genes along with their viral genomes. The co-evolution between the phages and bacteria would provide a way to circumvent resistance to the delivery. However, this extension of phage therapy would not be currently allowable in the United States as phage therapy itself is still a distance from being approved. The plasmids could also be conjugated to gold nanoparticles which sink through bacterial membranes. Because of this nonspecificity of this method of delivery, it would be difficult to develop adaptations capable of repelling the nanoparticles. (For example, in the case of some antibiotics a membrane transporter might allow them to enter the cell. This membrane transporter could easily be altered to block the antibiotic. The nanoparticles on the other hand would be more difficult to obstruct.) Unfortunately, it must be noted that in gram negative bacteria, their double membrane provides a more problematic barrier to nanoparticles. The most promising possibility for delivery is the use of promiscuous bacterial conjugation systems to move the plasmids among microbes via horizontal gene transfer. There would be additional benefit in this technique because the rate of bacterial conjugation

increases sharply when biofilm formation occurs. (Biofilms form in the majority of bacterial infections.) The plasmids could be spread among native human microbial flora and then, in the event of foreign invasion, by bacteria carrying one of the promoters used in the plasmids. Multiple gene copies with different promoters could be used to target more than one type of pathogen. In this way, the native flora would be able to deliver the toxic genes to the foreign invaders. This technique would require cloning onto an F+ plasmid which includes both an OriT sequence and Tra genes which code for proteins used to build the sex pilus and accomplish other components of conjugation. Because the F+ plasmid itself is nearly one hundred thousand base pairs in length, this may present difficulties in cloning. Less problematic, artificial versions of this plasmid (such as plasmids with the RP4 transfer system) may be useful for solving this issue. Because conjugative transfer frequencies tend to be too low to be significant for this purpose, it is necessary for plasmids to be able to transfer conjugative capabilities so that the conjugation will able to occur exponentially. (One bacterium infects two or three others, then they each infect a few more, ect.) For this delivery method in particluar, the use of artificial toxic genes rather than natural ones may possess an advantage because bacteria have been reported to be capable of preventing conjugative transfer of naturally occurring toxic genes in one study at the Weizmann institute. It may be that genes, which have never before appeared in the natural world, might not be recognizable by these bacteria's defense mechanisms. If this did become an issue, more research would be needed to circumvent the problem. Delivery of the artificially-designed, toxic gene sequences into the human body will be an interesting area for further expansion of this research and an area I feel confident has high potential for success.

As mentioned in the introduction, in the event of resistance developing against this treatment, the antibiotic would be easy to modify because the DNA sequence could be modified in a variety of ways. This might include random mutagenesis or genetic engineering of the plasmids to include new harmful genes which may replace or complement the original sequences. This would be a faster and less costly than traditional drug development. With such variety in the possibilities for modifying this treatment, this technology provides real hope for overcoming the life- threatening and costly dangers that come with the world-wide problem of ever-evolving antibiotic resistant bacteria. I look to the future of medicine with hope, that with the vast array of new possibilities and opportunities presented by these promising research findings, we may finally be able to defeat infectious bacterial disease.

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