ANALYSIS OF FULL LENGTH GCD AND INTERNAL TRUNCATIONS OF TGCD

CLONING AND CHARACTERIZATION OF FULL LENGTH GCD AND INTERNAL TRUNCATIONS OF TGCD

By

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Abstract

The increase in antibiotic resistance has accelerated the search for novel antibacterial agents. As proteins with toxic properties appear to be less susceptible to common resistance mechanisms, they may serve as potential substitutes for current antibiotics. The present study focuses on a toxic peptide called tGcd derived from the *Escherichia coli* genome that can cause cell death when it is expressed. To better understand the mechanism of tGcd and to decipher the sequence determinants for its toxic phenotype, sequence analyses of this peptide were conducted. First, we conducted growth analysis for two full-length Gcd constructs to test whether the observed growth suppression was caused by the overexpression of tGcd in *E. coli* cells. We also generated a series of truncation mutants to determine the minimal sequence required for toxicity. Finally, to test whether tGcd's toxicity was due to differences in expression levels, Western blot analysis was conducted. This study reveals that full length Gcd does not have an effect on bacterial growth when overexpressed. We also observed that some internal amino acids of tGcd can be truncated without the loss of the toxicity to the *E. coli* cells. Finally, Western blot analysis showed that the toxicity of this peptide is independent on its expression levels. These findings offer additional insights into properties common to membrane targeting antimicrobial peptides in Gram negative microbes, and contribute to the discovery of small bacterial peptides that may be useful for combating multidrug-resistant bacteria.

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List of Abbreviations and symbols

AMP	Antimicrobial peptide
aTc	Anhydrotetracycline
bp	base pairs
C- terminus	Carboxy terminus
DNA	Deoxyribonucleic acid
Gcd	Glucose dehydrogenase
IgG	Immunoglobulin G
Kan	Kanamycin
kDa	KiloDalton
LB	Luria-Bertani broth
MC	Multiple cloning site
min	Minute
mL	Millilitre
N- terminus	Amino terminus
nm	Nanometer
nt	Nucleotides
OD_{600}	Optical density measured at 600 nanometers
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
pNYL9	Vector plasmid containing multiple cloning site downstream of a tetracycline inducible promoter
PQQ	Pyrollo-quinoline quinine
PVDF	Polyvinylidene fluoride
RBS	Ribosome binding site
RNA	Ribonucleic acid
rpm	Rotations per minute

SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sRNA	Small RNA
t	Truncation constructs
TBS	Tris-buffered saline
TE	Tris-EDTA Buffer
Tet	Tetracycline
TetR	Tetracycline inducible promoter
tGcd	Truncated glucose dehydrogenase
Tween 20	Polysorbate 20
µL	Microlitre
˚C	Degrees Celsius

Prelude

The genomic screen in search of novel antimicrobial agents, which led to the identification of L1R 4-4, was performed by Courtney Barker and Naveen Kumar. Subcloning of L1R4-4 and initial characterization of tGcd were done by Bobbi Sawchyn. All subsequent analysis was carried out by me as part of the requirements in fulfillment of the degree Masters of Science.

1. INTRODUCTION

Antibiotics are compounds that can effectively stop bacteria and fungi from growing or kill them outright. They can do so by being bacteriostatic – stopping bacteria from growing – or bactericidal – causing cell death. Chloramphenicols, for example, are bacteriostatic, and penicillins, are bactericidal. There are also antibiotics that can display bacteriostatic activity in some circumstances and bactericidal in others [1,2].

With the increase in antibiotic resistance in recent years, the search for novel antibacterial agents has become more and more important [3]. Evolutionarily conserved peptides of the innate immune response – antimicrobial peptides (AMPs) – are candidates for the development of novel antibiotics [4,5].

AMPs are a significant component of the innate immune system of a wide variety of animals and plants, forming the first line of defense against invading microbes. Some of these peptides are well characterized, like melittin of bee venom. There are also various newly identified peptides like peptide toxins of spiders and sea anemones. AMPs are broad-spectrum antimicrobials with activities against bacteria, viruses, fungi and parasites (some of them even exhibit activities against tumor cells and spermatozoa) [4]. These peptides are not only important antimicrobials, they are also multifunctional mediators of the immune defense. Numerous antimicrobial peptides are under study due to their potential as valuable new therapeutics.

My study focuses on a toxic peptide named tGcd. It is derived from the glucose dehydrogenase enzyme in *Escherichia coli* and has growth suppression properties when expressed. Even though the precise mechanism of action of tGcd remains elusive, analysis shows that it shares various characteristics with AMPs. Thus, analysis of this peptide could provide us with insight on what is needed to suppress bacteria growth and may aid in the development of new therapeutics. The three main focuses of my study is summarized in Figure 1.

Antimicrobial peptides are evolutionarily conserved and widespread. In mammals, native antimicrobial peptides and proteins represent bridges between innate and adaptive immunity. They are major defense mediators. In humans, antimicrobial peptides not only exhibit a direct antimicrobial activity, but also provide a range of non-antimicrobial bioactivities related to defense, inflammation and wound healing [6].

The diversity of antimicrobial peptides is great; thus, it is difficult to categorize them except on the basis of their secondary structures. There are four major classes of AMPs, including α-helical, β-sheet (which contain many disulfide bridges), looped (single disulfide bridge), and extended (Figure 2a). The fundamental structural principle for all four classes is their amphipathic characteristic which gives them the ability to adopt a shape in which clusters of hydrophobic and cationic amino acids are spatially organized in discrete sectors of the molecule. They are also small (12-50 amino acids), and have an overall net positive charge. Due to their amphipathicity, cationic charge and size, they can easily attach to and penetrate the membrane bilayer, then either disrupt the membrane integrity directly or pass through the membrane to block important cell

pathways. AMPs inhibit the growth of the target cell once they enter the cell membrane through various mechanisms [5]. These mechanisms include the barrel-stave model, carpet model, and toroidal-pore model, each distinct in the role which the peptide plays in the bacterial membrane. Alternatively, as opposed to disrupting the cell membrane, the antimicrobial peptide may have an intracellular target. Many AMPs are associated with the regulation of both proteins and nucleic acids. For example, buforin II, which is derived from the histone H2A, displays growth inhibition without lysing the cells, and is further able to bind nucleic acids with a much higher affinity when compared to the membrane targeting AMP magainin. The binding of buforin to the nucleic acid molecules subsequently inhibits further cellular processes such as transcription and translation, successfully halting microbial growth. Thus, they are further divided into membrane lytic or non-membrane lytic groups (Figure 2b) [7].

These unique properties of AMPs give them many advantages over traditional antibiotics. Unlike traditional antibiotics, the induction of resistance for antimicrobial peptide is difficult because it requires dramatic changes in phospholipid composition with pleiotropic effects on transport and enzymatic systems [6]. Indeed, bacteria have been exposed to AMPs for millions of years, and with the exception of a few species like *Burkhoderia* spp., very little resistance has been reported. Currently, more than 1000 natural cationic peptides with antimicrobial properties have been identified [7].

Even though very few antimicrobial peptides are currently in use in the pharmaceutical market, many are progressing through clinical trials for the treatment of various diseases. The effectiveness of AMPs is seen in how they combat bacterial

resistance in both Gram positive and Gram negative microbes. Bacteria can be divided into two classes based on their cell wall structure – Gram positive and Gram negative. The bacteria cell wall protects a cell from the effects of osmotic pressure, aids in cell division, and serves as a primer for its own biosynthesis. Thus, the cell wall biosynthetic processes of the bacteria historically have been the site of action of the largest number of antibiotics.

One of the significant differences between the membranes of Gram positive and Gram negative bacteria is the composition of a complex polysaccharide called peptidoglycan. There can be as many as 40 sheets of peptidoglycan, comprising of 50% of the cell wall in Gram positive bacteria, and only 1 or 2 sheets, comprising only 5-10% of the cell wall for Gram negative bacteria. This difference in structure affects the bacterial susceptibility to traditional antibiotics, since the traditional antibiotics (beta-lactams) target the peptidoglycan.

In contrast, the AMPs have broad spectrum of activity against both Gram positive and Gram negative microbes. Gram positive bacteria such as *Staphylococci, Streptococci*, and *Enterococci*, which are major culprits in hospital outbreaks, can be efficiently inhibited by AMPs. One good example is the effective treatment for multidrug resistant Gram negative *Pseudomonas aeruginosa*. Currently, *P. aeruginosa* has been increasingly recognized for its ability to cause significant hospital-associated outbreaks of infection; severe cases can result in patient death.

Even though beta-lactam has successfully treated *P. aeruginosa* related diseases in the past, there has been an increase in *P. aeruginosa* strains displaying resistance to these

antibiotics. Not only has *P. aeruginosa* a thin layer of peptidoglycan, but it also has an asymmetric bilayer surrounding the peptidoglycan layer, which is chemically distinct from all other biological membranes. The outer leaflet of this membrane is composed of lipopolysaccharides (LPS), while phospholipids and proteins make up the inner leaflet. The outer leaflet of the outer membrane protects the Gram negative bacteria by excluding both hydrophobic and hydrophilic molecules. This outer membrane causes large antibiotic molecules to penetrate it slowly, which allows for high antibiotic resistance. The LPS in the outer leaflet is made of sugar and a lipid known as lipid A. Lipid A is an endotoxin; it can trigger fever, vasodilation, inflammation, shock, and blood clotting in humans. Since a large amount of lipid A is released when the cell dies, treating gram-negative bacteria with antibiotics may potentially cause more harm than leaving the disease untreated. Unlike traditional antibiotics, LPSs are an excellent target for AMPs because they have the potential to both directly inhibit the growth of multidrug-resistant bacteria and neutralize the action of released LPS due to its stimulation of immune cells [8].

In contrast, AMPs' amphipathic nature allows them to effectively interact with LPS. Antimicrobial peptides generally bind to LPS through electrostatic interactions between their cationic amino acids (lysine and arginine) and head groups of LPS, and this complex is stabilized through hydrophobic interactions between the hydrophobic amino acids of the peptide and fatty acyl chains of LPS. For example, Sushi peptides, which are derived from Factor C, can disrupt LPS aggregates through detergent-like action and also have LPS-neutralizing activity. In terms of reducing inflammation, several AMPs prevent

LPS-induced cytokine induction in macrophages, resulting in interruption of the development of septic shock in animal models [9-12].

Through previous research, our lab identified truncated Gcd (tGcd) – a small peptide that is able to cause significant growth inhibition in *E. coli* when expressed at high levels. One of the research focuses of the Li lab is to identify existing materials that are lethal to the host organism upon its overexpression. A screen involving a tetracycline inducible promoter was employed in order to identify novel toxic molecules in *E. coli*. First, the bacterial genome was digested with the frequent cutter endonuclease Sau3A. This created fragments of an average size of 256 nucleotides in length. These fragments were subsequently inserted into the pNYL9-MCS11 vector - a plasmid derived from pZE21-MCS1 [13]. This plasmid forms a tetracycline-inducible system that can regulate the expression of the gene of interest. Only upon the addition of anhydrotetracycline (aTc) – a more potent yet less toxic analogue of tetracycline – overexpression of the genomic insert is induced. If the expression of the protein affects growth, toxic phenotype would be displayed for cells containing the inserted gene. Next the vector was introduced by electroporation into *E. coli* DH5α-Z1 cells, which had been engineered to endogenously express the TetR repressor. Approximately 4,500 such clones were screened and five constructs displayed a growth inhibition phenotype. One of the constructs, termed L1R4-4, showed great potency. L1R4-4 is derived from the N-terminal membrane anchor of the glucose dehydrogenase enzyme in *E. coli* and it is able to significantly disrupt the electrochemical gradient and hinder proper cell division. L1R4-4 is composed of four random gene fragments from the annotated genes of Gcd, ydiA, intG

and ydhU. Further sequence analysis of L1R4-4 showed an open reading frame (ORF), which include the Gcd fragment plus two codons of the ydiA fragment, and it is responsible for the toxic effect of L1R4-4. This fragment is termed tGcd (Figure 3).

Through previous research, several observations about tGcd were made by the Li lab. First of all, tGcd is a small peptide toxin that can cause cell death when expressed. The coding sequence for tGcd has 318 nucleotides, translating into 106 amino acids. tGcd corresponds to three and a half of five transmembrane domains within the N-terminal membrane anchor of the native Gcd enzyme in *E. coli* (Figure 3). Truncational analyses of the N and C termini were conducted to determine the sequence requirements for toxicity. Furthermore, internal transmembrane segments were successfully removed without compromising the toxic effect of tGcd [13]. It was demonstrated that the peptide can be reduced from 106 to 62 amino acids without losing toxicity. The precise mechanism of the toxin is still elusive, but through fractionation experiments, it is known that it does target the bacterial membrane.

Previous experiments indicated that tGcd significantly inhibited *E. coli* growth. However, it is entirely possible that the inhibitory effect is only caused by the overexpression of the tGcd protein, thus this result alone cannot prove that the tGcd peptide is toxic to *E. coli*. Therefore, my first goal is to determine whether the growth inhibition effect of tGcd is due to toxicity of the peptide itself. To achieve that, we need to examine the effect of full length Gcd on cell growth when overexpressed. If *E. coli* growth suppression is caused by the tGcd sequence rather than the effect of overexpression, the full length Gcd should be non-toxic to the host when overexpressed.

To this end, the full length Gcd was cloned and growth analysis was conducted. Our results showed that full length Gcd does not suppress growth when expressed in pNYL-MCS11 vector.

Additionally, I am trying to determine the shortest motif sequence required for the toxicity of tGcd. There are many unique attributes that tGcd has which could potentially make it a suitable candidate for new antimicrobial therapeutics and new biological tools. However, currently, the shortest tGcd peptide that retains the toxic phenotype is 62 amino acids long. The length of this peptide makes it rather impractical to use as a therapeutic agent. The main reason that synthetic AMPs, which tGcd is similar to, have not progressed more successfully through the clinic is their large size. These complications include the cost of synthesizing larger peptides, their labiality to proteolytic degradation, and their unknown toxicology profile when administered systemically [14-17].

Since 2000, several synthetic AMPs have entered clinical trials and at least 15 peptides or mimetics are in clinical trials as antimicrobial or immunomodulatory agents. In a Phase I/II trial, the AMP hLF1–11 (which is composed of amino-terminal amino acids 1–11 of human lactoferrin) was found to be safe and well tolerated when delivered intravenously. In phase III trials, Pexiganan, a derivative of magainin that showed equivalence to an oral fluoroquinolone for foot ulcer infections in patients with diabetes, has demonstrated efficacy, but it has not yet been approved due to complications associated with the large size. Currently, pharmaceutical companies are employing several strategies to overcome the issue of stability, toxicity, and cost [17,18]. This current trend shows that in order for tGcd to have practical therapeutic use in the

pharmaceutical market, further reduction in size would be an advantage. Thus, further successful truncations of tGcd leading to a smaller peptide can further advance its progress towards using tGcd for a wide range of clinical application including infective, inflammatory and immune system-based disease [19]. Furthermore, learning more about the sequence requirement for the toxicity of tGcd can also aid in our understanding of toxic peptides in general. Thus, gaining more insight into the makeup of toxic peptides can help us in designing new toxic molecules for therapeutic and biochemical uses. In order to find out the essential motif for the toxicity of tGcd, a number of truncation mutants were generated by crossover PCR (Figure 4). Result from growth curve analysis shows that the length of this peptide can be further minimized.

Even though tGcd shows ability to suppress cell growth through growth curve analysis, the growth curve does not provide solid proof that the truncation mutants were being expressed. Furthermore, the levels of expression for each constructs cannot be shown by the growth curve analysis. Previous studies have shown that the overexpression of membrane proteins have two effects on *E. coli* cells. First of all, overexpression of membrane proteins may affect the integrity of the bacterial membrane, which leads to reduced bacterial growth and hampered cell division. In addition, overexpression of membrane proteins may lead to saturation of the protein sorting and translocation machineries, possibly preventing biogenesis of endogenous proteins [20]. Typically membrane proteins are targeted via the SRP pathway to the SecYEG translocon where they are inserted co-translationally into the membrane. The unnaturally high abundance of co-translationally inserted SecYEG substrates may lead to an increased occupation of

SecYEG translocons by translating ribosomes, leaving fewer translocons for post-translational translocation of secretory proteins. This phenomenon is observed in a study conducted by Wager et al., illustrating that the abundantly overexpressed membrane proteins may titrate out targeting components, which may lead to diminished synthesis of endogenous substrates, such as subunits of respiratory chain complexes [21]. Thus, the toxic effect of tGcd truncation may be the result of overexpression.

To test whether the toxic properties of tGcd truncations are the direct effects of overexpression, I employed a Western blot analysis for 4 truncation constructs in which two of the constructs were able to suppress *E. coli* growth, and the other two constructs had lost their toxic effects. By comparing the expression levels of these constructs, I was able to determine whether higher tGcd expression levels directly lead to higher levels of toxicity [22].

For this analysis, the four tGcd truncation constructs were genetically fused with N-terminal FLAG-tags (Met-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys). A thrombin cleavage site was also introduced in between the FLAG-tag and the gene for each FLAG-tag fused construct, allowing for future removal of the tag from the protein if needed. These FLAG-tag constructs were transformed into *E. coli* DH5α-Z1 cells, and the growth curve analysis was conducted in a similar manner as that of the untagged full length or truncated Gcd. The results are compared with the ones obtained from the untagged version to show that the FLAG-tag does not affect the expression of level of the tGcd proteins. Then, western blot analysis was conducted to illustrate that the toxicity of tGcd

is not directly correlated to the expression level of the protein. More details are provided in the following sections of the report.

2. RESULTS

2.1 Testing the Toxicity of Full Length Gcd

In order to investigate whether full length Gcd is toxic when over expressed, clones with two different ribosome binding sites were tested: clones containing pNYL plasmid with the native ribosome binding site within the original gene for Gcd and clones that contain the consensus ribosome binding site. The clones expressing full length Gcd using the native ribosome binding site was made using forward primers containing the sequence TAATGAATTGAAATGGTGTCTCTTT; on the other hand, the clones expressing full length Gcd using the consensus ribosome binding site were made using forward primers containing the sequence TAAGGAGG. The reason for using both ribosome binding sites to clone the full length Gcd is due to the fact that tGcd was cloned using the modified ribosome binding site prior to my arrival.

Growth analysis was conducted either in presence or absence of aTc for both constructs, and the results obtained for each construct was compared and analyzed. The extent of growth inhibition was measured by observing the cell density at OD_{600} and comparing it to the negative control, pNYL9, and the positive control, tGcd and LIR 4-4. The result can be verified by plating the cells on agar plates in presence or absence of aTc. The effect of the expression of full length Gcd was determined by the appearance of the colonies on the agar plate.

The full length Gcd was expressed under the same condition as LIR4-4, pNYL9, and tGcd for both constructs. Agar plate growth analysis was conducted using pNYL9 as

the negative control and L1R 4-4 and tGcd as positive controls. The plate was supplemented with kanamycin and incubated overnight at 37˚C. The result obtained demonstrates that when full length Gcd is expressed, cells can grow to similar intensity as that of the negative control for both constructs. Thus, full length Gcd does not have a toxic effect on the cell when it is overexpressed (Figure 5).

This observation was also confirmed by the growth curve analysis. Selecting single colonies grown on the kanamycin supplemented plates after re-transformation, the effect of the expression of the insert was assayed for suppression of bacterial growth. *E. coli* cells were grown in LB media with selective antibiotics kanamycin in the presence and absence of aTc for the induced and uninduced condition, respectively. The optical densities were measured using a plate reader from a 96 well plate at a wavelength of 600 nm. Readings were taken every hour for 8 hours after induction in aliquots of 200 µL at a time. All constructs were tested in triplicates.

The result for both constructs shows that the full length Gcd was not able to suppress cell growth like that of L1R 4-4 or tGcd (Figure 6); the growth of bacteria in the induced sample grown in presence of aTc was similar to that of the negative control – the cells containing only empty pNYL. Taken both results together, one can infer that the reduction in cellular growth observed when tGcd is overexpressed is not a mere artifact of Gcd overexpression; rather it is the peptide that is responsible for the growth reduction. This growth reduction is due to the presence of overexpression of a toxic peptide.

2.2 Truncation Analysis of tGcd

tGcd is derived from the N-terminal membrane anchor of the glucose dehydrogenase enzyme in *E. coli*. Glucose dehydrogenase contains five N-terminal membrane-spanning segments, with the N terminus located in the cytoplasm and the C terminus located in the periplasm. The C-terminal periplasmic domain binds PQQ, contains the site of catalytic activity and is able to interact with ubiquinone in the cytoplasmic membrane [13]. Compared to glucose dehydrogenase, tGcd is composed of three and a half transmembrane domains within the N-terminal membrane anchor of the larger glucose dehydrogenase protein. Though previous research, the tGcd peptide was reduced to 62 amino acids. This construct has the two internal membrane spanning domains removed, thus, leaving only the N- and C- terminal transmembrane segments while retaining their topology (Figure 2).

One of my goals is to identify the sequence elements required to maintain tGcd's ability to affect cell growth by conducting internal truncations. I have generated a number of truncation mutants using crossover PCR (please refer to appendix for a detailed outline of the method) [23], which minimized the tGcd amino acid sequence by five amino acid residues from the middle of the shortest tGcd sequence that retains its toxicity created before my arrival. 12 truncations were created by three rounds of cloning (Figure 8). Please refer to the appendix for a list of truncations and the rationale behind each construct.

The first set is composed of truncations 1 to 5 (t1-t5), the locations of these five truncations are close to middle of the tGcd gene based on the observation that toxicity is abolished if tGcd is truncated from either ends. The locations of 1-5 are also randomly chosen because little is known about the sequence requirement for toxicity. Growth curve analysis was conducted in liquid media using the same procedure as described for full length Gcd. The data obtained 8 hours after induction with aTc supports that t1-5 were all able to significantly suppress *E. coli* growth, however, to a less extent than the positive controls (tGcd and L1R4-4).

The second set, which contains truncations 11- 14, are designed based on the observation that t1-5 suppresses growth. The growth curve analysis in presence of aTc shows that tGcd and t11-t13 were able to significantly suppress *E. coli* growth; however, t14 has lost some toxicity.

We are then interested in testing whether truncating five amino acids before and after t14 would also abolish some toxicity. Thus, t15 and t16 were designed, truncating five amino acids before and after t14. The analysis of truncation 17 (t17) was conducted simultaneously with t15 and 16. Truncation 17 was designed based on the observation that truncation 13 in the second set of truncations showed toxic phenotype. Growth analysis shows that truncation 15 was able to suppress growth, though with reduced activity. Truncations 16 and 17 have lost almost all of their toxicity.

2.3 Western Blotting Analysis for tGcd Truncation Constructs

In order to detect the presence of truncated proteins inside *E. coli* to monitor expression, the western blotting technique was employed. In this case, the following 4 truncation constructs were chosen: two positive constructs which showed growth suppression - pNYL9 +t2, pNYL9+t13, two negative constructs which had limited growth suppression - pNYL9 +t16, pNYL9+t17. pNYL9 and pNYL9 + tGcd were used as controls.

Before I examined the expression level of the protein constructs by the Western blot technique, it was necessary to show that the addition of the N-terminal FLAG-tag did not affect the toxicity (or function) of the tGcd constructs. Therefore, the 4 truncation constructs of tGcd were genetically fused with the FLAG-tag (Figure 9). Then, these FLAG-tagged constructs were transformed into *E. coli* DH5α-Z1 cells and a growth curve analysis was conducted in the same manner as the constructs without the FLAG-tags. Results obtained from both the growth curve analysis and the plate analysis for the tagged constructs are the same as those of the untagged constructs (Figure 10, 11).

Since *E. coli* growth was not altered by the addition of the FLAG-tag, the Western blot technique was used to analyze bacterially expressed proteins in *E. coli* following the growth curve analysis for the constructs above. Anti-FLAG antibodies were employed to monitor the expression of these constructs by the Western blot analysis. Cells were grown to mid-log phase at which point tGcd expression was induced for 3 hours. No protein expression could be detected with our negative controls, L1R4-4 and tGcd lacking a

FLAG-tag, or in any of the un-induced samples. However, a strong signal was detected at approximately 8 kDa, which corresponds to the 6.7 kDa protein fragment with the addition of a 1.0 kDa FLAG-tag (Figure 12).

3. DISCUSSION AND FUTURE DIRECTIONS

tGcd - a novel protein fragment derived from glucose dehydrogenase - was analyzed in this study. To address the question whether or not the toxic phenotype we observed is a consequence of tGcd overexpression, growth analysis was conducted for two full length tGcd constructs: clones containing pNYL plasmid with the native ribosome binding site and clones containing the consensus ribosome binding site. Studies revealed that full-length Gcd does not have an effect on bacterial growth when expressed for both clones. This observation is supported by both the growth curve analysis in liquid media and on solid agar plate. This observation supports the hypothesis that the *E. coli* growth suppression observed is caused by the tGcd sequence rather than the effect of overexpression. This unique property makes further studies on tGcd rather intriguing. Further investigation into the characteristics of this peptide will help us gain more insight into the mechanisms of truncated peptides.

Through previous studies, the tGcd peptide was reduced to only 61 amino acids while maintaining its toxicity. However, truncation experiments indicate that the length of this peptide can be further minimized. Growth analysis of the 12 truncations revealed that all truncations lost toxicity to some extent. Truncations 1 to 13 showed a significant ability to suppress growth, similar to that of the positive control (tGcd). Truncations 14 and 15 were able to suppress growth, however, to a much less extent than tGcd. Truncation 16 and 17 showed very limited toxicity.

This observation is consistent with previous studies. Truncation experiments prior to my arrival revealed that the N- and C- termini of tGcd play a crucial role in its toxic activity. Deletion of more than two amino acids from either end of the protein almost completely abolished its toxicity. However, the two internal, hydrophobic transmembrane segments were successfully removed without compromising the toxic effect of tGcd [13]. Truncations 1 to 13, which were able to suppress growth significantly, are located in close proximity within the first hydrophobic segment, toward the middle of the tGcd peptide. On the other hand, truncations 14 through 17 are located more towards the N and C termini of the peptide; these peptides all span either completely or partially into the hydrophilic region of the tGcd. Thus, this observation supports previous studies in terms of both the location of truncation and the hydrophobic/hydrophilic nature of the peptide. Both my observations and observations made from previous studies show that the amino acids towards the middle of the peptide can be truncated without significantly affecting its toxicity. In terms of hydrophobicity, previous studies demonstrated that the two segments that could be removed without abolishing tGcd's toxicity are hydrophobic in nature, which is in support of the observation that truncations 14 through 17 consist of segments of five amino acids that are either completely or partially located in the hydrophilic region of tGcd. Thus, the data obtained through a growth analysis of the 12 truncations further support the idea that the N- and C- termini of tGcd play a crucial role in its toxic activity, whereas the internal transmembrane segments, which are hydrophobic in nature, can be truncated.

Western blot technique was used to analyze bacterially expressed proteins in *E. coli*. Four truncation constructs of tGcd were FLAG-tagged at the N-termini and cloned into the pNYL plasmid expression vector. Truncations 2 and 13 were chosen because they were able to suppress *E. coli* growth when induced by aTc. On the other hand, truncations 16 and 17 were chosen because they lost their toxic properties. These constructs were FLAG-tagged and transformed into DH5α-Z1 cells.

Growth analysis was conducted for the FLAG-tagged constructs, using pNYL and FLAG-tagged tGcd as controls. The results obtained were as expected. Growth curve illustrates that the constructs, truncation 2 and truncation 13, were able to suppress *E. coli* growth significantly; truncation 16 and truncation 17 were not able to suppress *E. coli* growth. This result indicates that recombinant peptides remained active despite the addition of the FLAG-tag.

Once we confirmed that the FLAG-tag does not alter growth, we induced protein expression by adding aTc. The protein produced could then be detected by Western blotting. Since no protein expression was detected with our negative controls, L1R4-4 and tGcd lacking a FLAG-tag, or in any of the un-induced samples, the assay did work as expected. In addition, an intense band was detected at approximately 8.0 kDa on the electrophoresis gel, which directly corresponds approximately to the 6.7 kDa tGcd truncation protein fragment with the addition of a 1.0 kDa FLAG-tag. This confirms that a peptide was being expressed, ultimately leading to the growth defects observed.

In order to test whether these inactive mutants were due to differences in expression levels, the intensity of the bands on the Western blot were compared. Bands were observed for truncations 2 and 13 three hours following induction with aTc. Truncation 2 and 13 were able to cause growth suppression, whereas truncation 16 was not able to cause growth suppression. Truncation 16 shows the most intense band, and then followed by truncations 13 and 2, respectively. No band was observed for truncation 17. Since band intensity corresponds to the expression level of the protein, this result indicates that the protein coded by truncation 16 is not only being expressed, but is also present at a higher level compared to the other constructs. This observation is rather interesting because constructs coded by truncations 16 and 17 both are not able to suppress growth significantly when expressed; however, the levels of expression for these proteins are very different: there are no protein being expressed for truncation 17, but truncation 16 is being expressed at a high level, even higher than that of truncations 2 and 13, which were able to suppress growth. This observation demonstrates that the lack of activities of truncation 16 and 17 is not a result of low expression levels.

Due to the importance of cellular toxins and AMPs and because tGcd appears to have many properties similar to AMPs, further investigation on tGcd will aid in our understanding of what is needed to inhibit microbial growth and could potentially contribute to the challenge in finding novel antimicrobial agents.

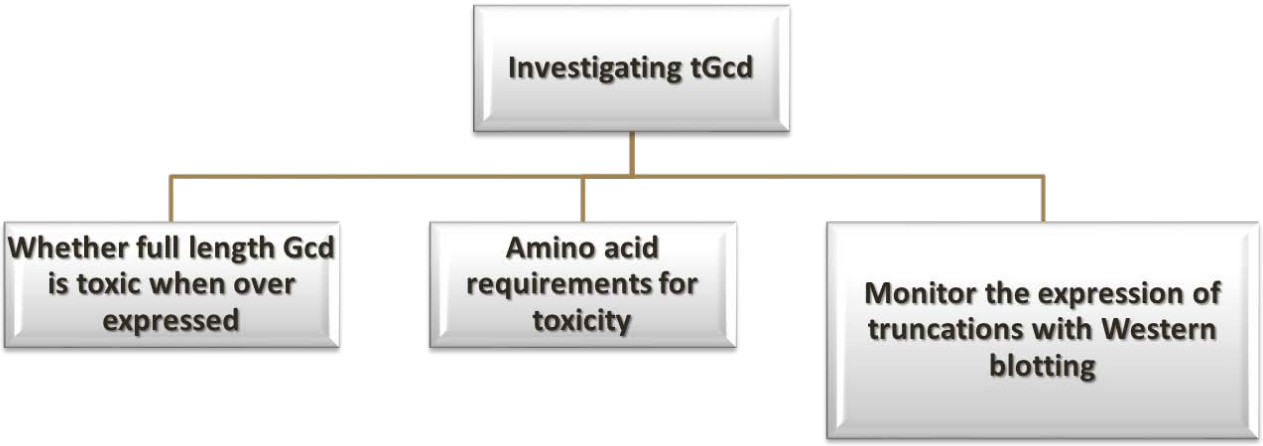


Figure 1. The three main focuses for my research. Aim 1: To test the hypothesis that *E. coli* growth suppression is caused by the tGcd sequence rather than the effect of overexpression. Aim 2: Investigating the sequence requirement by truncation to learn more about sequence requirement for toxicity and to develop a smaller peptide. Aim 3: Western blot analysis to detect the expression of truncation constructs.

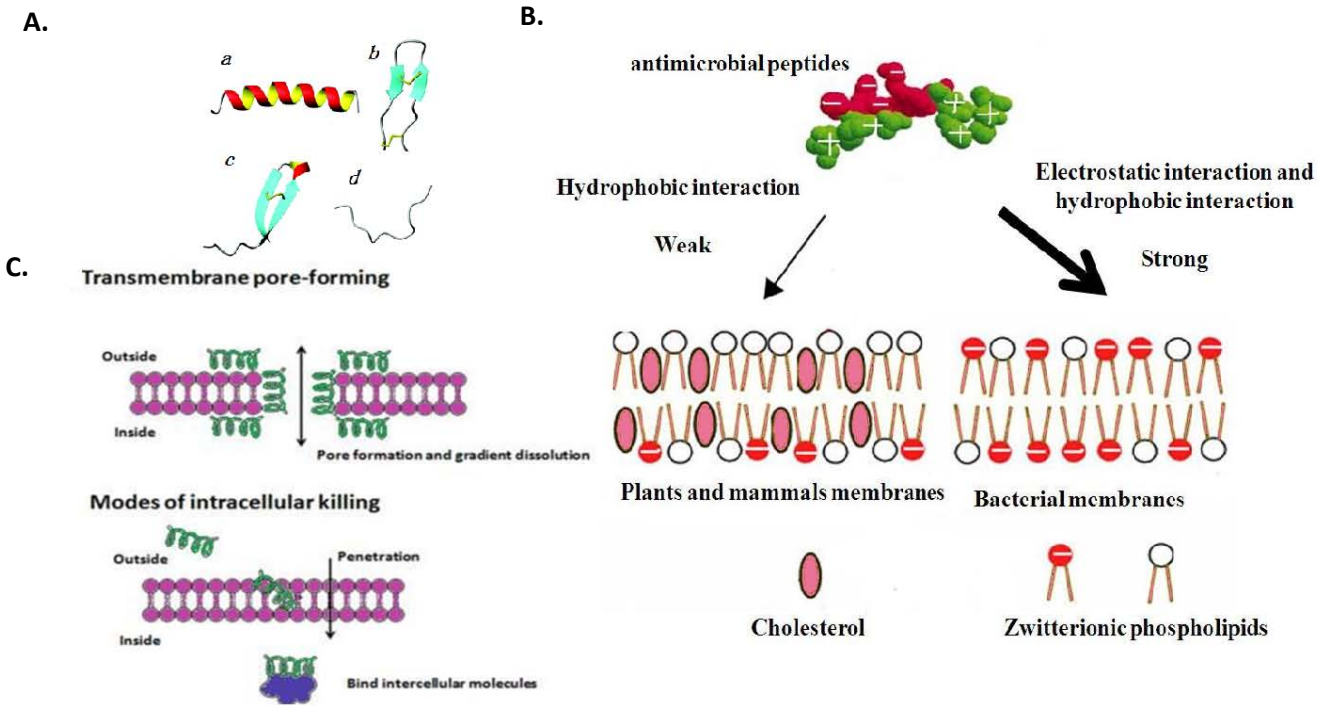


Figure 2. Structure and mechanisms of antimicrobial peptides. (A). Structural classes of antimicrobial peptides. (a) Alpha helical (helical magainin-2) (b) ß-sheet (ß -sheeted polyphemusin) (c) looped (looped thanatin) (d) extended (extended indolicidin). The disulfide bonds are indicated in yellow [16]. (B). The initial contact between the peptide and the target organism is electrostatic because most bacterial surfaces are anionic. (C) The modes of action by which antimicrobial peptides kill bacteria. Mode of action includes disrupting membranes (top panel) and targeting cytoplasmic components (bottom panel). Their amino acid composition, amphipathicity, cationic charge and size allow them to attach to and insert into membrane bilayers or allow them to penetrate into the cell to bind intracellular molecules which are crucial to cell living.

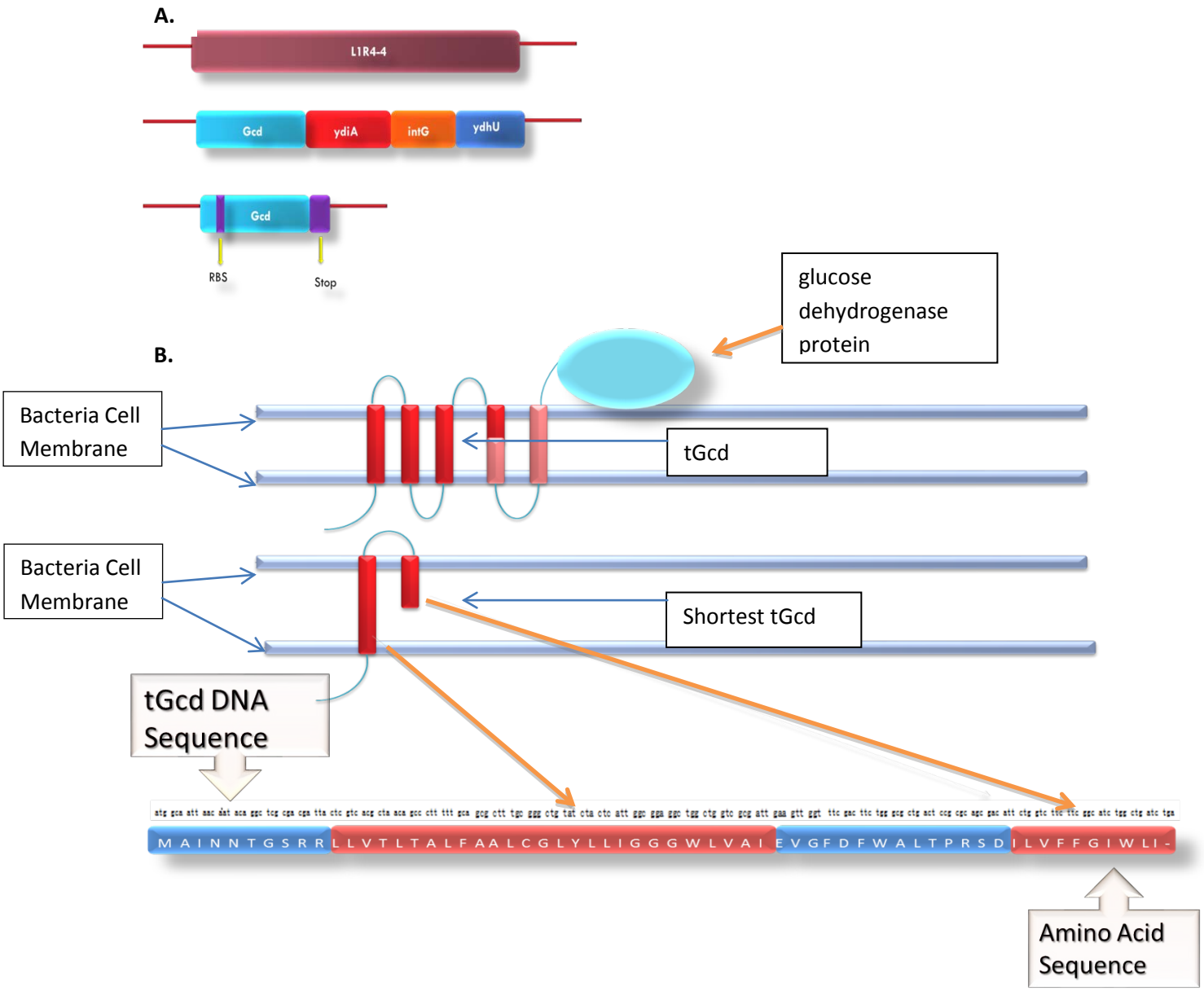


Figure 3. Structural context of tGcd. L1R 4-4 contains the tGcd peptide which cause growth suppression. (A) The Gcd and ydiA sequences contain an open reading frame named tGcd. (B) tGcd corresponds to three and a half of the five transmembrane domains within the N-terminal membrane anchor of the larger glucose dehydrogenase protein. The hydrophobic regions are displayed in blue; the hydrophilic regions are displayed in red.

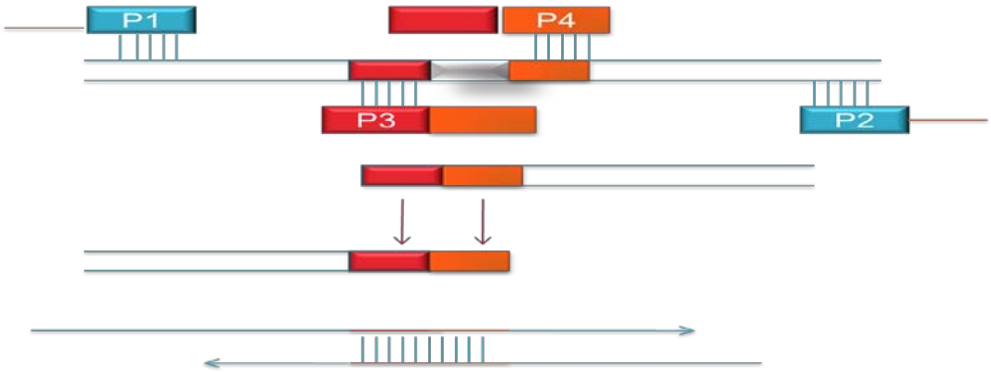
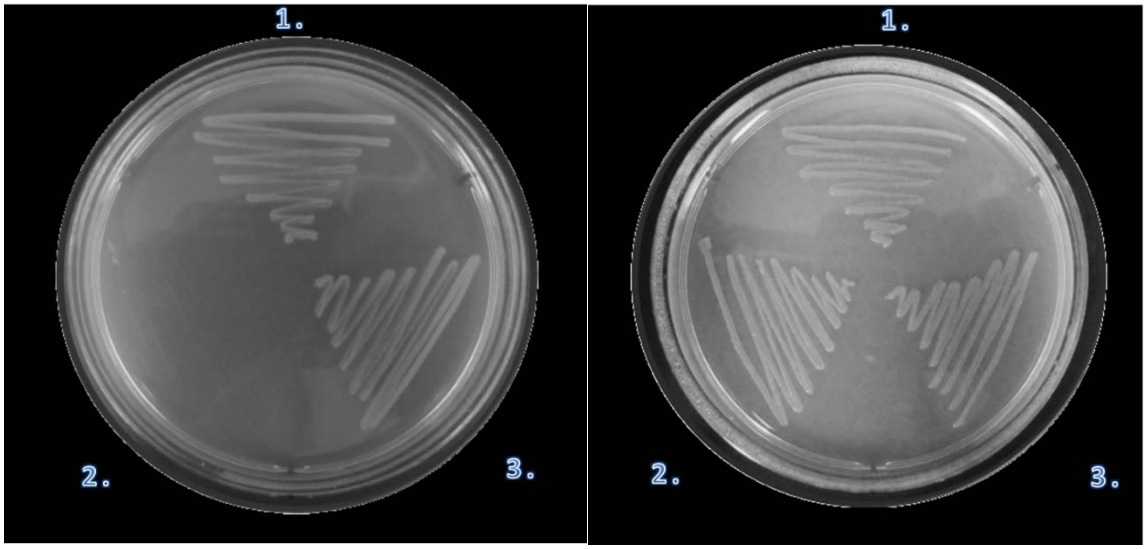


Figure 4. Crossover PCR. The oligonucleotides are 18 nucleotides long, complementary to the ends of the sequence to be truncated. 4 primers are used for each construct. P3 and P4 contain a region that is complementary to each other, which will allow them to bind to each other.

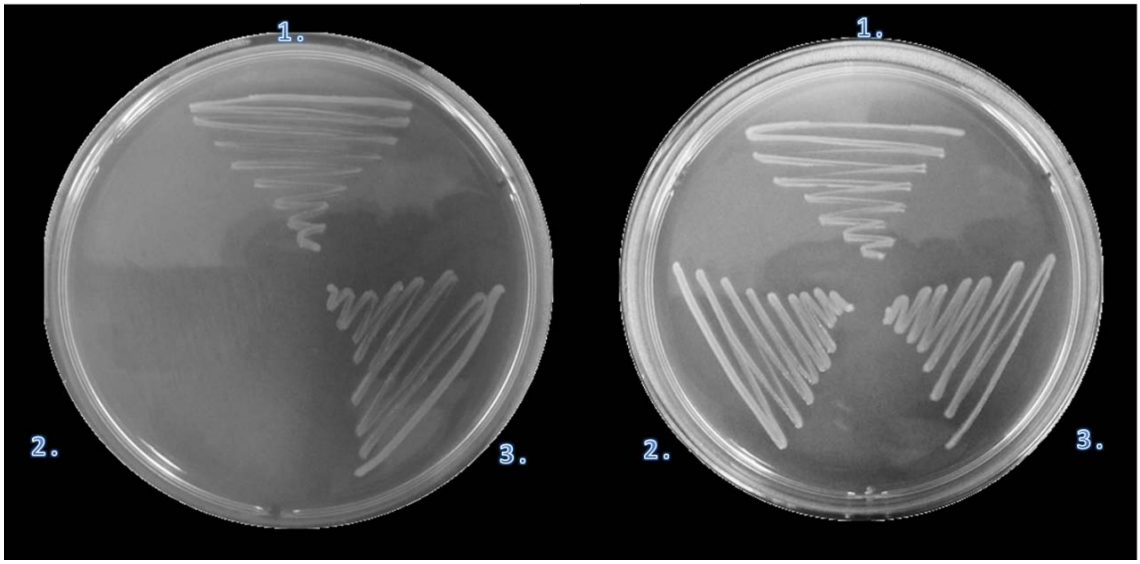
A.



B.

-aTc +aTc

1. pNYL 9 ***2.*** tGcd ***3.*** full Length Gcd with Native RBS



-aTc +aTc

1. pNYL 9 ***2.*** tGcd ***3.*** full Length Gcd with Consensus RBS

Figure 5. Plate analysis of full length Gcd. Growth analysis on solid agar in presence (right) and absence (left) of 200 ng/mL aTc after incubating at 37˚C for 16 hours. (A.) Growth of *E. coli* cells is similar to that of the pNYL 9 control when full length Gcd is expressed under the native ribosome binding site after induction with aTc. (B.) Densities of *E. coli* cells are similar to that of the pNYL 9 control when full length Gcd is expressed under the modified ribosome binding site after induction with aTc.

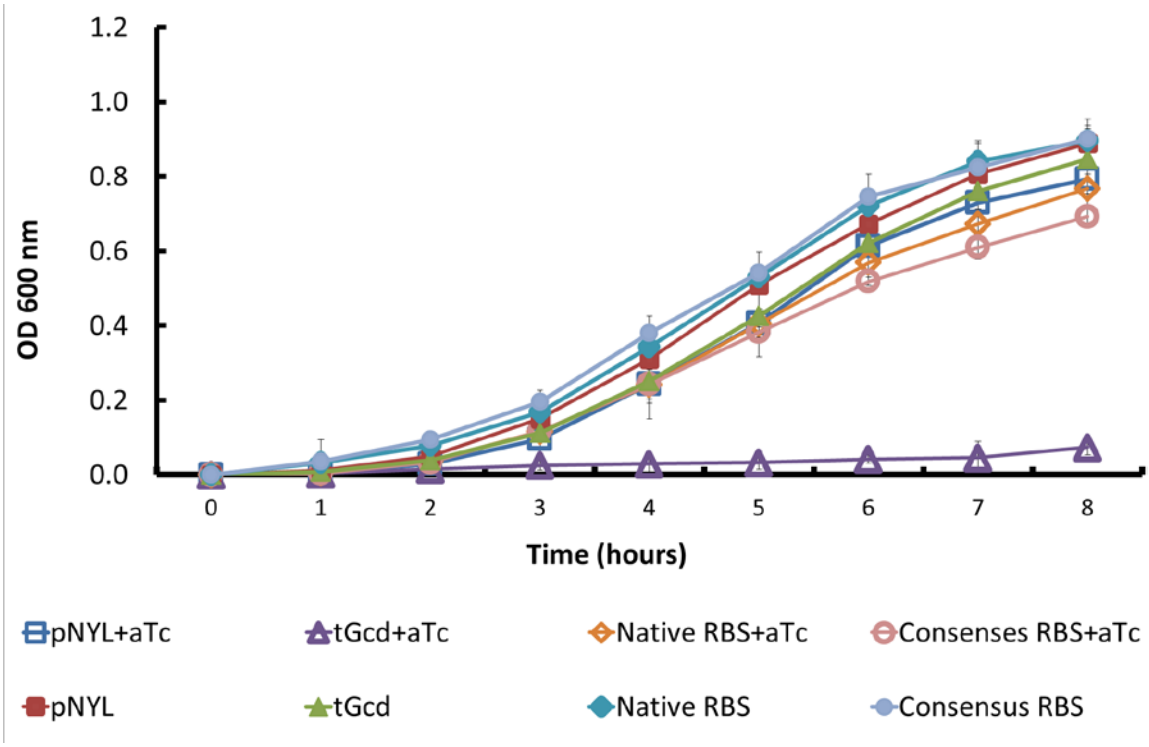
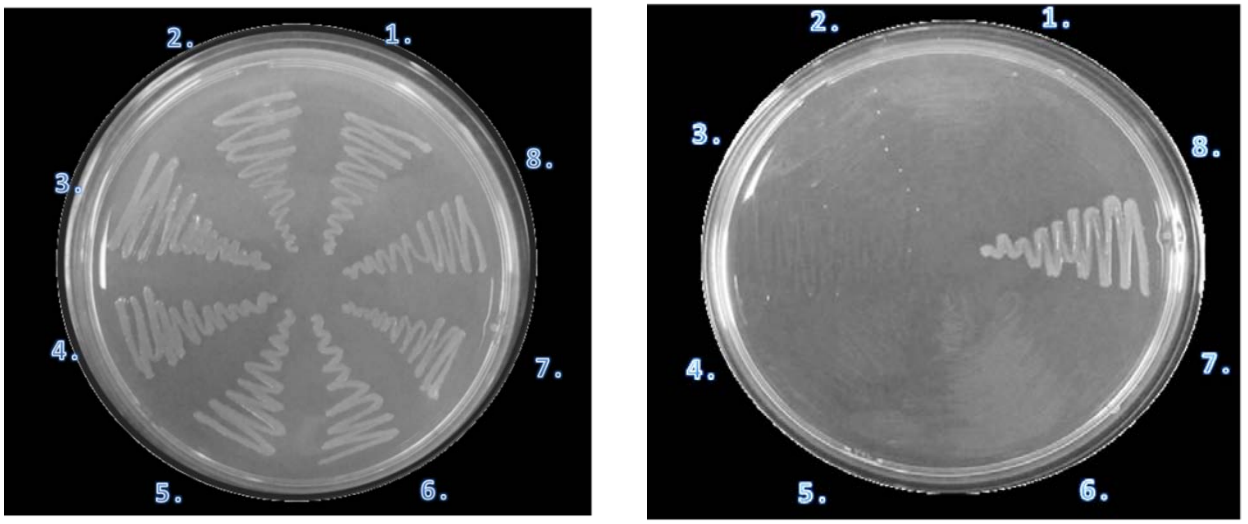
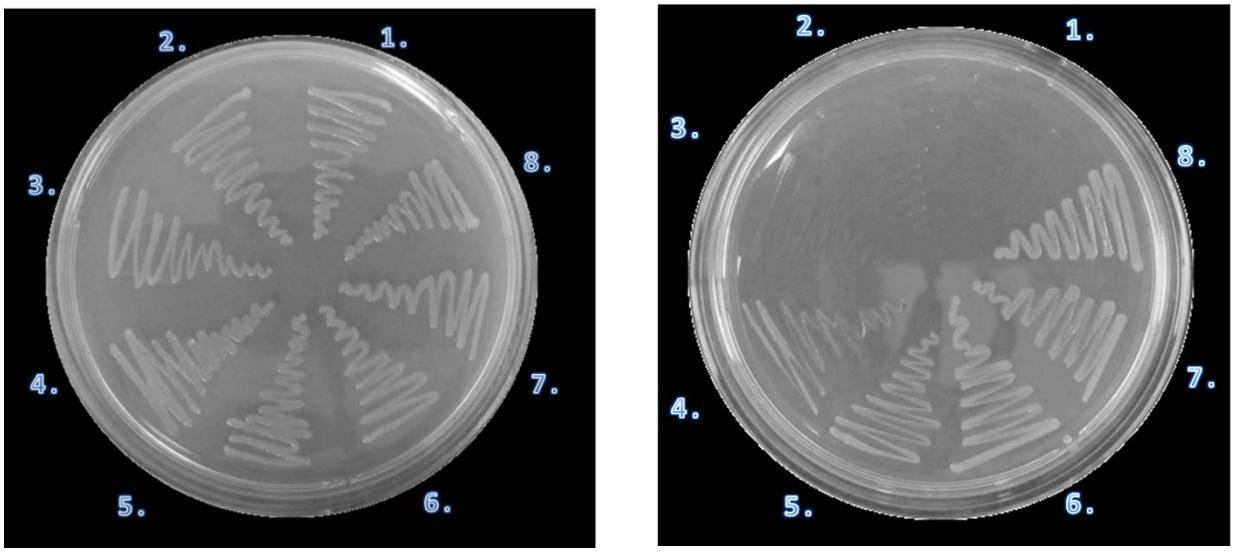


Figure 6. Growth curve analysis of full length Gcd. Growth of *E. coli* cells was analyzed in presence and absence of aTc. One can observe that only the positive control (tGcd) was able to significantly suppress *E. coli* growth. Thus, one can infer that the full length Gcd is not toxic when expressed.

A.



B. -aTc ***1.*** tGcd ***2.*** t1 ***3.*** t2 ***4.*** t3 ***5.*** t4 ***6.*** t5 ***7.*** t11 **8.** pNYL9 +aTc



-aTc +aTc

1. tGcd *2.* t12 *3.* t13 *4.* t14 *5.* t15 *6.* t16 *7.* t17 8. pNYL 9

Figure 7. Plate analysis of truncated tGcd t1-t17. Growth analysis on solid agar in presence (right) and absence (left) of 200 ng/mL aTc after incubating at 37 ˚C for 16 hours. (A.) Growth analysis of truncations 1 to 11 shows that after induction, all truncations were able to retain the some ability to suppress growth comparative to the positive control tGcd (B.) After induction, truncations 12 – 15 were able to retain some ability to suppress growth comparative to the positive control tGcd t16 and t17 grow similar to the negative control pNYL9.

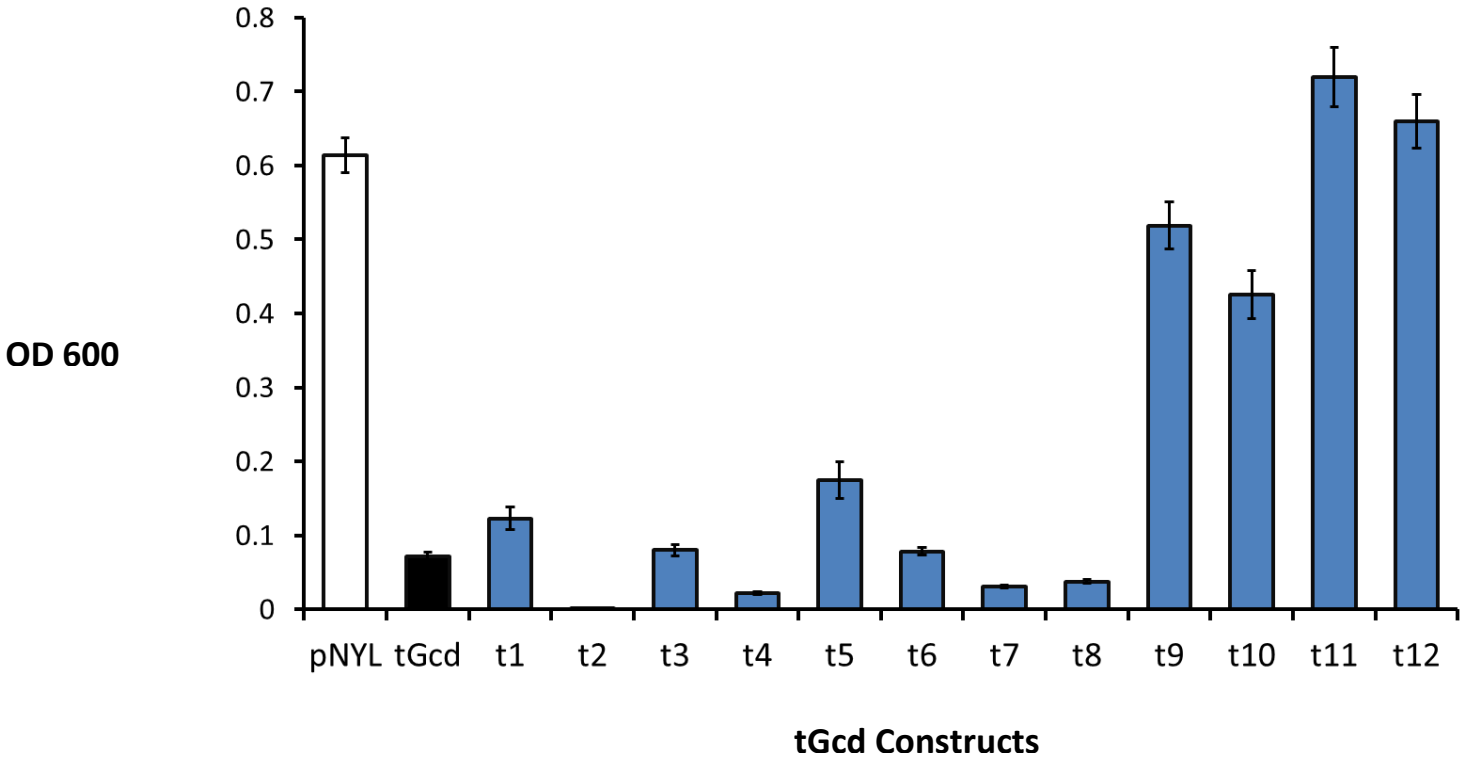


Figure 8. Growth analysis of t1-17. Growth of *E. coli* cells were analyzed in presence of 200ng/mL aTc. Optical densities were measured 6 hours at 600 nm post-induction. All truncations lost toxicity to some extent; however, truncations closer to the middle of the gene are able to suppress growth more than the truncations towards the N and the C termini.

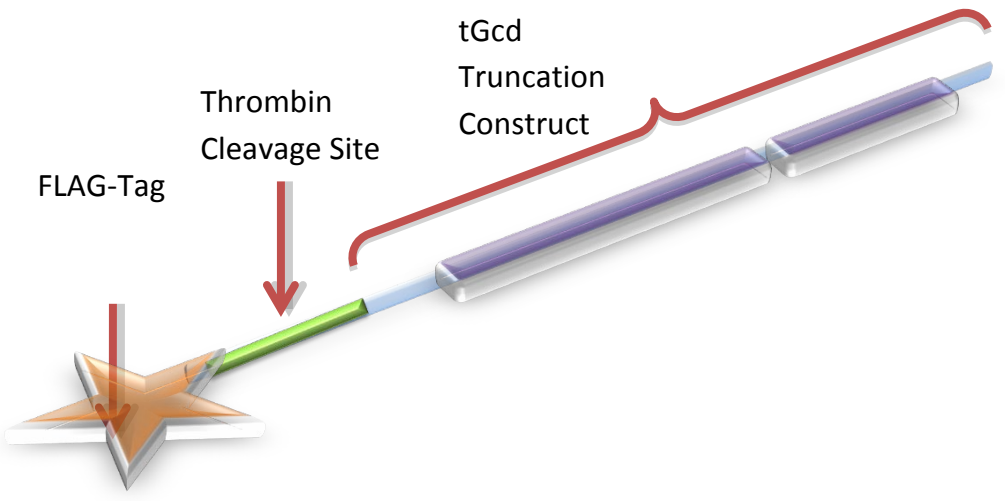
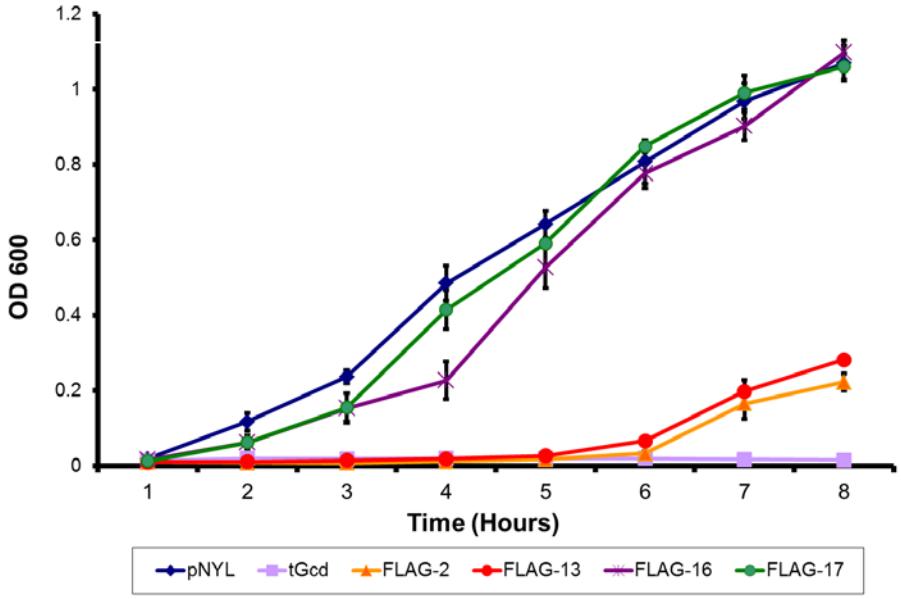


Figure 9. FLAG-Tag design. FLAG-TAG Incorporation at the N-terminus. Fusing FLAG-TAGs to tGcd truncation constructs allow one to follow the proteins with antibody against the FLAG sequence (Sigma ANTI-FLAG) on SDS-PAGE. A thrombin cleavage site is incorporated between the FLAG-TAG and tGcd truncation to allow future removal of the FLAG-TAG if needed.

A)



B)

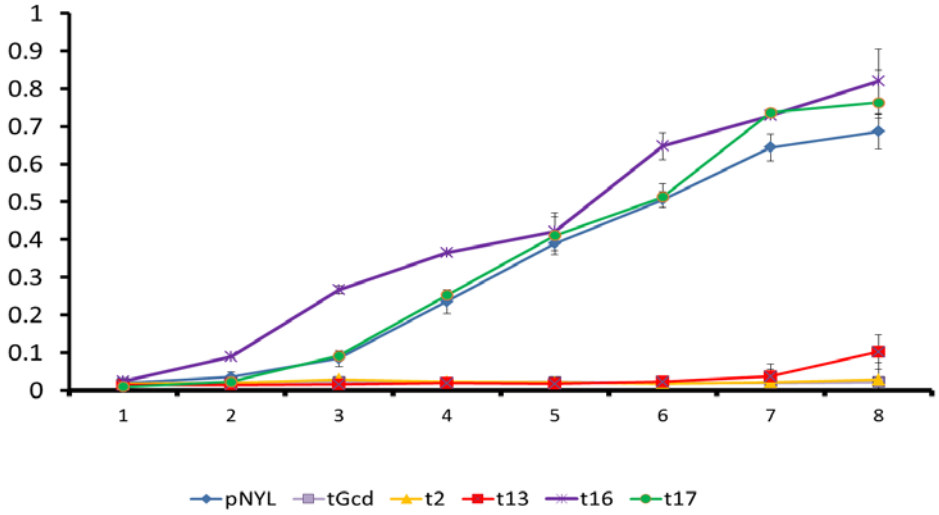
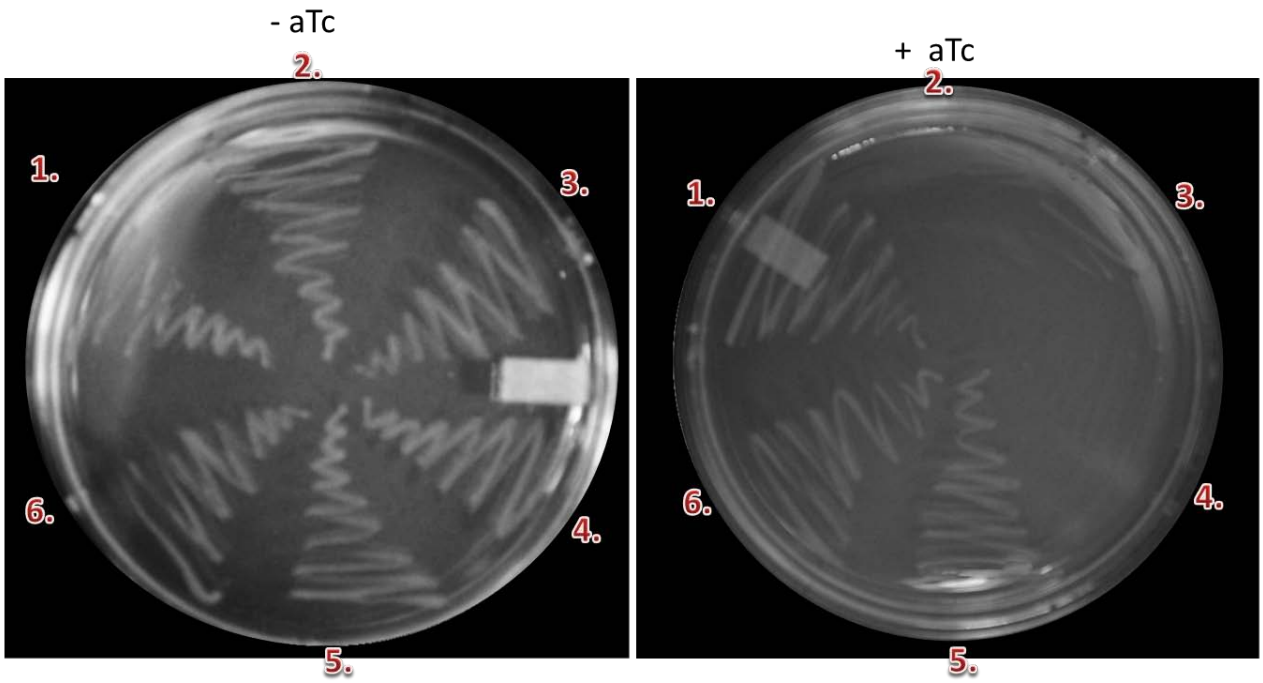


Figure 10. Growth analysis of FLAG-Tag constructs compared to untagged constructs. Growth of *E. coli* cells are analyzed in presence of 200ng/mL aTc. Optical densities were measured each hour at 600 nm for a minimum of eight hours. (A) The positive control (FLAG - tGcd) and constructions FLAG 2 and FLAG 13 were able to significantly suppress *E. coli* growth. The negative control, constructs FLAG 16 and FLAG 17 were not able to suppress growth. (B) The untagged constructs have similar trend as the FLAG-Tag constructs.



1. pNYL **2.**tGcd **3.** FLAG-t2 **4.** FLAG-t13 **5.** FLAG-t16 **6.** FLAG-t17

Figure 11. Plate Analysis of FLAG-tagged Truncated tGcd. Growth analysis on solid agar in presence (right) and absence (left) of 200 ng/mL aTc after incubating at 37˚C for 16 hour incubation period. It can be seen that after induction, only FLAG-t2 and FLAG-t13 retain the ability to suppress frowth comparative to the positive control tGcd. All other truncations grow similar to the negative control pNYL9.

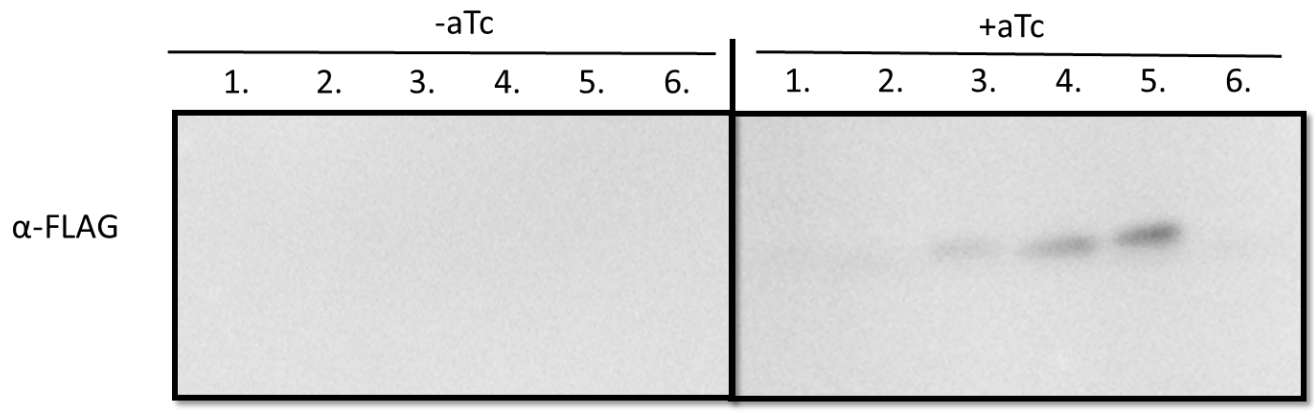


Figure 12. Western Blot Analysis Showing Different Expression Levels for tGcd Truncations. Strong signals can be seen at 7.5 kDa 3 hours following induction with aTc from tGcd-FLAG constructs: t2 (lane 3), t13(lane 4), and t16 (lane 5). No peptide could be detected in our negative control: L1R 4-4 (lane 1) and tGcd (lane 2) lacking a FLAG-tag. Western blot was conducted 3 times and similar results were observed.

5. MATERIAL AND METHODS

Plasmids and Oligonucleotides

All oligonucleotides and PCR primers were chemically synthesized by Integrated DNA technologies (Coraville, IA, USA). The plasmid pNYL9-MCS11 was used as the vector for full length Gcd and tGcd, which are cloned between the EcoRI and BamHI sites. pNYL-MCS11 was derived from pZE21-MCS1 (courtesy of H. Bujard) by digestion with restriction enzyme EcoRI to remove the ribosome binding site downstream of a tetracycline inducible promoter. New multiple cloning sites were then introduced at the EcoRI site, replacing the lost restriction sites by RBS removal.

Cloning and Reagents

Molecular cloning steps – PCR amplification, restriction digestion, and ligation - were conducted following protocols provided by suppliers. PCR amplification from *E. coli* DH5αZ1 genomic DNA was used to clone the full length Gcd and crossover PCR was used to create internal truncations. Digestion with EcoRI and BamHI restriction enzymes was conducted after amplification. Ligation of the desired gene into the pNYL9 vector was conducted with T4 DNA ligase purchased from Fermentas. The antibiotics used – kanamycin and anhydrotetracycline - were purchased from Sigma-Aldrich. High Fidelity PCR enzyme mix purchased from Fermentas was used for PCR reaction. The restriction enzymes, EcoRI and BamHI were purchased from Fermentas. Plasmid Miniprep kits were purchased from Qiagen or Promega. Chromosomal DNA/PCR products were prepared using GenElute Gel Extraction Kit purchased from Sigma.

Bacterial Strains and Growth Conditions

The *E. coli* expression strain used in this study was DH5α-Z1, as described in previous studies [22]. *E. coli* strains were grown in Luria Bertani (LB) media supplemented with kanamycine at 37˚C in a shaking incubator (New Brunswick Scientific).

As the recipient strain of recombinant plasmids, DH5α-Z1 competent cells were prepared by growing for 18 hours at 37˚C shaking at 260 rpm in plain LB media. A 1:100 dilution of the cells were created following initial growth and cells were allowed to grow at 37˚C shaking at 260 rpm until an OD600 of 0.3 to 0.4 is achieved. Then cells and all containers used in the process were chilled on ice for 20 minutes prior to the procedures. Then cells were harvested by centrifugation at 5000g for 10 minutes at 4˚C. Supernatant were removed after centrifugation. Cells were subsequently resuspended in 10% filter sterilized glycerol. Then cells were centrifuged, resuspended again until they are fairly concentrated. Finally, cells were put into centrifuged tubes with the volume of 50 microL per tube and freezed immediate with liquid nitrogen. Cells are then stored at -70 ˚C for future use.

Transformation

Recombinant plasmids were introduced into *E. coli* DH5α-Z1 via electroporation. Competent cells were first thawed on ice before adding 1 µL of gene of interest. The mixture of competent cells and desired genes were then transferred to a chilled cuvette and electroporated at 1650V using an Eppendorf Electroporator 2510. The pulsed cells were immediately suspended in 1 mL of LB broth and shaken for 45 minutes at 260 rpm and 37˚C. Cells were harvested by centrifugation at 5000 rpm for 5 minutes and plated

onto kanamycin (50 µg/mL) containing plates. After incubation for 18 hours at 37°C, colonies were collected and all clones were verified by DNA sequencing from the Mobix laboratory (McMaster University, Hamilton, ON).

Lethality Screens

Successfully transformed cells with the correct inserts were grown at 37˚C on solid LB plate supplemented with kanamycin (50 µg/mL). For uninduced condition, cells were grown in absence of aTc; for induced condition, cells were grown in presence of aTc (200ng/mL). The effect of Gcd overexpression was analyzed after 18 hours of growth.

Growth Curve Analysis

Growth curves measured in liquid LB media were used to evaluate the inhibition of cell growth. Negative controls (cells carrying pNYL-MCS11 plasmid) and positive controls (cells carrying the PNYL-MCS11 Plasmid and a tGcd insert) were grown overnight at 37˚C in the shaking incubator at 260 rpm in 3 mL of LB broth supplemented with kanamycine (50 µg/mL). Cells were then subcultured in fresh LB-kanamycine media (5 mL) containing selective antibiotics in a 1:200 dilution following the overnight growth. For induced conditions, the samples were grown in the absence of light in the presence of 200 ng/mL anhydrotetracycline (aTc, Sigma-Aldrich); for uninduced conditions, the samples were grown in presence of light and in absence of 200 ng/mL anhydrotetracycline (aTc, Sigma-Aldrich). Cells were incubated at 37°C with shaking over a time course of 8 -hrs. Growth of samples was monitored hourly by measuring the optical density at 600 nm (OD_{600}) for each 0.2 mL culture using the SpectraMax Plus 384

plate reader (Molecular Devices) after each hour of growth. This assay was conducted in triplicates.

Internal Truncation

The shortest tGcd sequence was further truncated via cross over PCR as described previously [23]. Forward and reverse internal truncation primers (18 nt) were designed such that there is a 9 nt complementary region between the two primers. This is done by including nucleotides at the 5' end of each primer that was complementary to the 3' end of the other primer. These products were then amplified using the tGcd sequence as a template with the High Fidelity PCR enzyme mix (Fermentas). Following amplification, the products were separated by electrogelphoresis and the DNA was extracted following supplier-provided protocols using GenElute Gel Extraction Kit (Sigma). These products were mixed in another 25 cycle PCR reaction carried out in an automated thermal cycler. Final products were verified by DNA sequencing from the Mobix laboratory (McMaster University, Hamilton, ON).

Western Blot Analysis

The samples were prepared by growing *E. coli* K12 cells containing the desired constructs overnight and subsequently sub-cultured into fresh LB media containing selective antibiotics kanamycin in a 1:200 dilution. Then cells were grown to an optical density of about 0.40 at 600 nm. One set of samples was then induced with 200 ng/mL aTc, leaving the other set uninduced; and all cells were then grown in the absence of light. One milliliter of cells was extracted for each sample 3 hours after induction. Then cells were

lysed by sonification and re-suspended in TE buffer (0.5 M Tris-HCl at pH 8.0 and 0.1 ml EDTA and made up with double distilled water up to 100ml). 15% SDS-PAGE was conducted and proteins were transferred to nitrocellulose membrane in transfer apparatus by running at 75V for 1 hour. Then the membrane was placed in blocking buffer containing 2 g of dry skim milk powder, 1 x TBS, and 0.05% Tween-20 on rocker. Then samples were washed in PBS + Tween and placed in a solution of primary antibody (anti-FLAG M2 IgG antibody from sigma). After thorough rinsing the blot in PBS Tween wash buffer and PBS alone, the blot was placed in a solution of 2^{nd} Antibody (anti mouse IgG from goat) in PBS for 1 hour on rocker at room temperature. After thorough washing, the proteins were detected using bio imaging system MicroChemi 4.2.

6. REFERNCES

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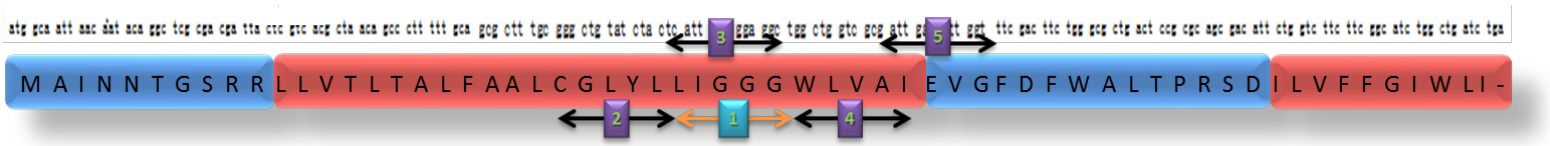
Appendix
6.1 Supplementary Figures

A.



B.

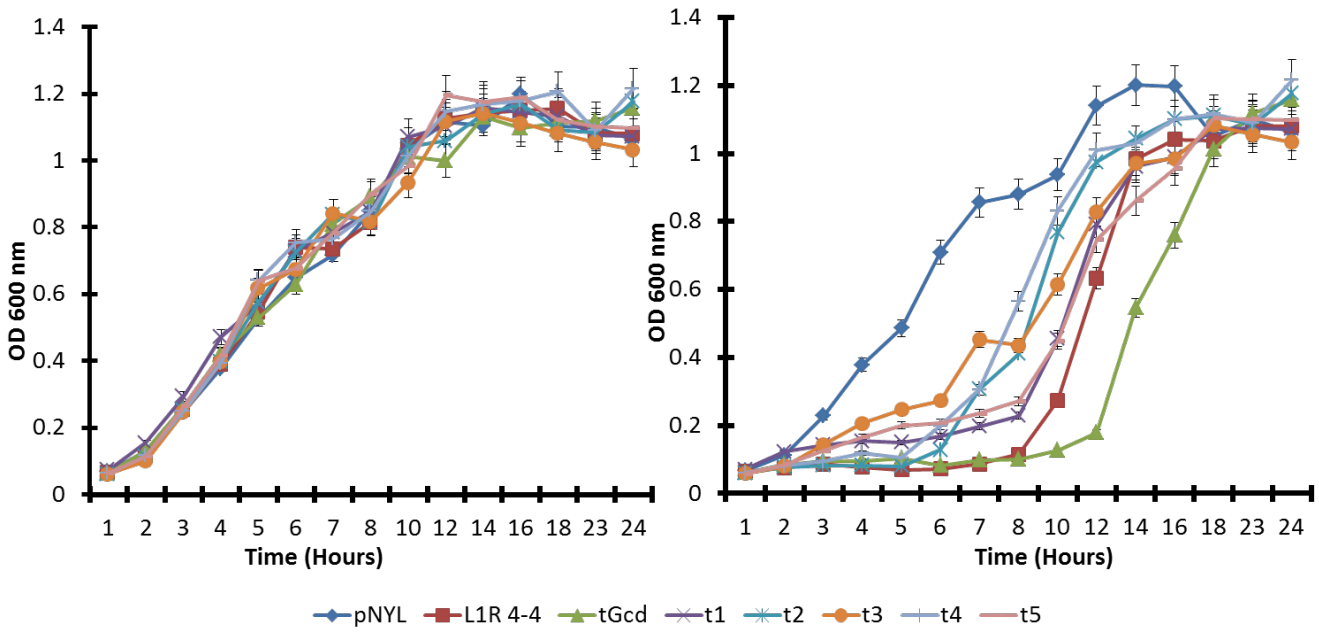


Figure 13. Growth analysis of t1-5. (A) Truncations 1-5 are positioned randomly because little is known about the sequence requirement for toxicity. (B) Growth of *E. coli* cells were analyzed in presence (right) and absence (left) of 200ng/mL aTc. Optical densities were measured each hour at 600 nm for a minimum of eight hours. The positive control (tGcd) and t1-t5 were able to significantly suppress *E. coli* growth.

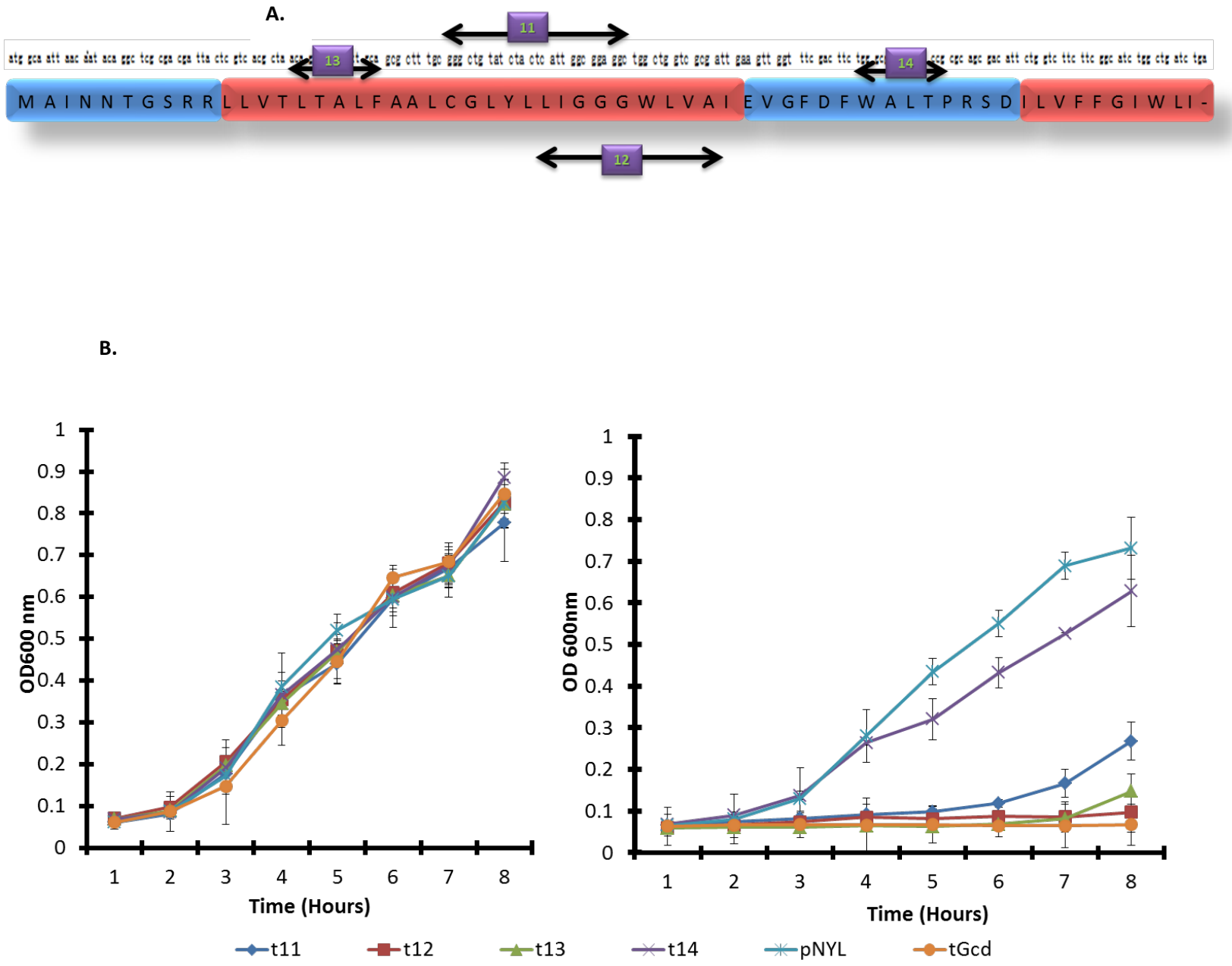
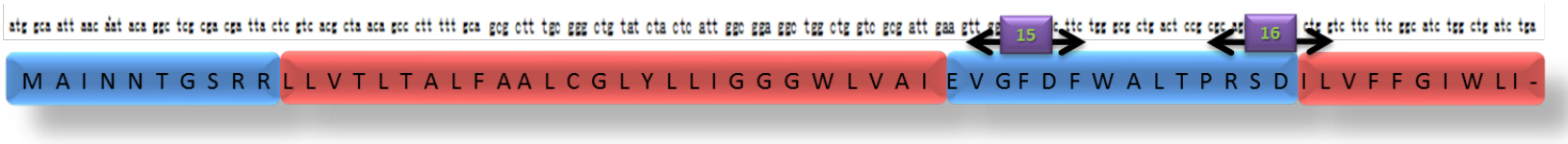


Figure 14. Growth analysis of t11-13. (A) Truncations 11-14 are designed based on the observation that t1-5 suppressed growth. (B) Growth of *E. coli* cells were analyzed in presence (right) and absence (left) of 200ng/mL aTc. Optical densities were measured each hour at 600 nm for a minimum of eight hours. The positive control (tGcd) and t11-t13 were able to significantly suppress *E. coli* growth; t14 has lost some toxicity.

A.



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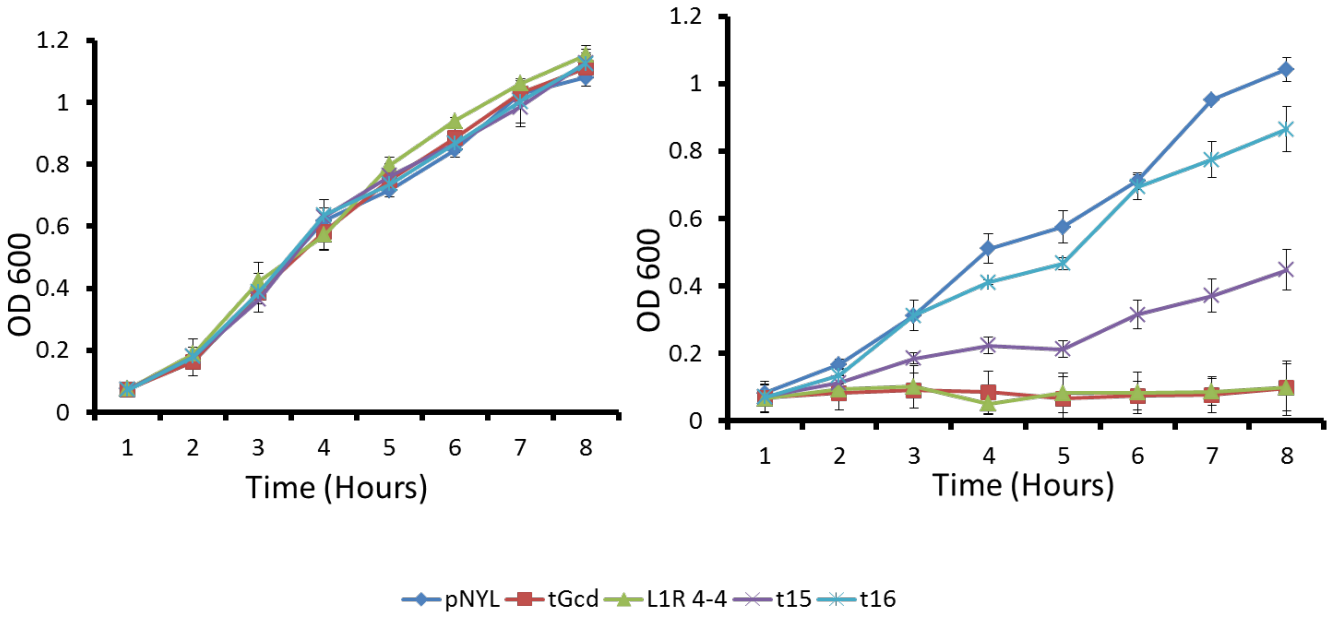
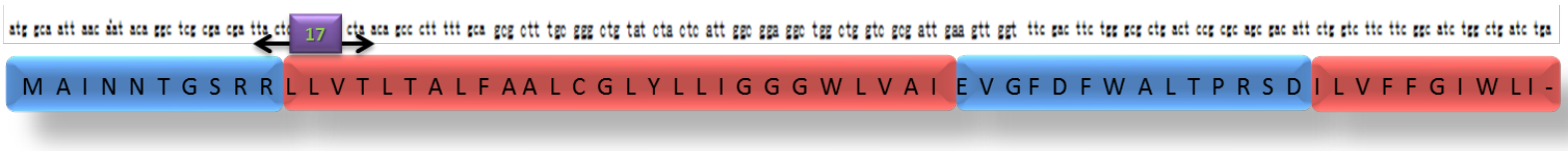


Figure 15. Growth analysis of t15-16. (A) Truncations 15-16 are designed based on the observation that t14 has limited ability to suppress growth. (B) Growth of *E. coli* cells were analyzed in presence (right) and absence (left) of 200ng/mL aTc. Optical densities were measured each hour at 600 nm for a minimum of eight hours.

A.



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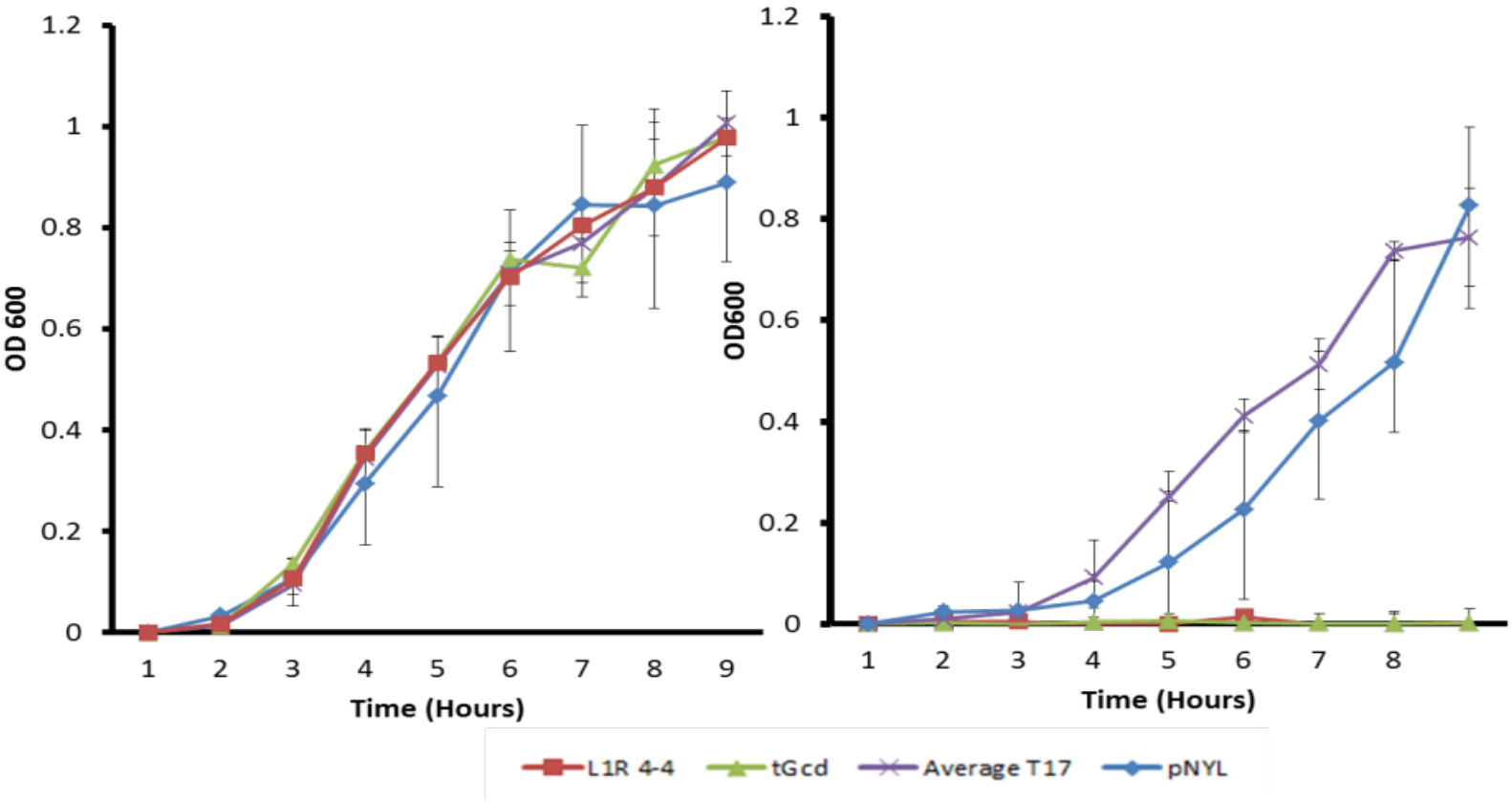

Figure 16. Growth analysis of t17. (A) Truncation 17 is designed based on the observation that truncation 13 suppresses growth. (B) Growth of *E. coli* cells were analyzed in presence (right) and absence (left) of 200ng/mL aTc. Optical densities were measured each hour at 600 nm for a minimum of eight hours. Truncation 17 was not able to suppress *E. coli* growth.

The DNA Sequence that Encodes the 186 Nucleotides tGcd Peptide

ATGGCAATTAACAATACAGGCTCGCGACGATTACTCGTCACGCTAACAGCCC
TTTTTGCAGCGCTTTGCGGGCTGTfTACTCATTGGCGGAGGCTGGCTGGTCGCG
ATTGAAGTTGGTTTCGACTTCTGGGCGCTGACTCCGCGCAGCGACATTCTGGT
CTTCTTCGGCATCTGGCTGATCTGA