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Aptamer Selections Against Bacterial Toxins and Cells

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Aptamer Selections Against Bacterial Toxins and Cells

by

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Dedication

For my mother and father.

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Aptamer Selections against Bacterial Toxins and Cells

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In vitro selection of functional RNA molecules has formed the basis for a new class of molecules termed "aptamers." Aptamers have been selected against a wide range of molecules, ranging from simple chemical compounds to multi-cellular living organisms. The majority of selections are carried out against targets, such as proteins, that are typically composed of one type of molecule. Targets composed of multiple types of molecules (lipids, proteins, carbohydrates, etc.) are termed "complex," and examples of successful selections against them include parasites, virions, and red blood cell ghosts. Through various properties inherent in their composition, aptamers have the potential to play a role in everything from therapeutics to broad based detection platforms.

Bacterial toxins are a means by which pathogenic bacteria are able to exert an effect on a host organism. Although there are a few aptamer selections that have been carried out against toxins, there have not been any successful selections against whole bacterial cells. As some bacteria are easily grown in laboratory conditions, the possibility

of their use as a biological threat agent is relatively high. Therefore, there is a need develop rapid and reliable technologies for the detection of such threats.

This work details two aptamer selections carried out against both a bacterial toxin, *Bacillus. anthracis* protective antigen (PA), and a *Bacillus subtilis* vegetative cell. The selection against PA resulted in a high affinity aptamer that is capable of inhibiting the cleavage of PA. This cleavage step is the first in the pathway whereby anthrax toxin is able to exert its effect. The selection against *B. subtilis* vegetative cells is a proof of principle selection. *B. subtilis* is meant to be a surrogate for *B. anthracis*, which has long been regarded as a potential bio-weapon. Aptamers selected against these vegetative cells are shown to discriminate between bacterial vegetative cells of the same genus, bacteria of a different genus, and also spores produced by *B. subtilis.* With these selections as examples, it is hoped that the role of aptamers can continue to be expanded into viable detection systems for biological threat agents.

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Chapter 1: Introduction

IN VITRO SELECTION

Systematic Evolution of Ligands by EXponential enrichment, or SELEX, is a powerful method wherein combinatorial chemistry is used to generate diverse pools of nucleic acids (Ellington and Szostak 1990; Tuerk and Gold 1990). These pools are then subjected to iterative rounds of selection to select functional nucleic acid species. These functional nucleic acids may take the form of ribozymes (discussed above), deoxyribozymes (catalytic DNA), or aptamers.

Aptamers, taken from the latin word aptus meaning "to fit", are defined as a target specific binding species with a high affinity for their selected target molecule. In some cases the affinity of an aptamer for its target may rival that of an antibody. As an example of the specificity an aptamer may display toward its target, consider the theophylline aptamer. This RNA aptamer possesses a binding affinity toward theophylline 10,000 times greater than it does for caffeine, which contains a single additional methyl group (Jenison, Gill et al. 1994). In addition, an inherent advantage of aptamers over antibodies is that large amounts of selected aptamers can be synthesized chemically with minimal cost and effort, thereby avoiding two of the major problems inherent in antibody generation. Chemically synthesizing nucleic acids also allows for the insertion of various chemical moieties (amines, biotin, fluorphores, etc.) in a very simple and straightforward fashion.

Regardless of the type of nucleic acid chosen for pool composition, SELEX begins with the chemical synthesis of a ssDNA pool. This pool consists of a random region with a given number of randomized bases flanked by two constant regions that are used as primer binding sites for PCR amplification. There is some conjecture that pools with a random region of between 50 and 70 residues provide the optimal length for

aptamer selection (Legiewicz, Lozupone et al. 2005). Regardless of the size of the random region, the pool is purified on a denaturing polyacrylamide gel following chemical synthesis in order to separate full-length product from the various aborted synthesis products which are present in any chemical synthesis. Following purification, the number of sequences is calculated generally by determining the absorbance at 260 nm and the utilization of Beer's law. Generally the initial diversity of the pool should be anywhere from 1×10^{14} to 1×10^{16} different molecules. Once the number of starting molecules has been determined, the number of extendable sequences must be calculated in order to gain a true accounting of the number of useful molecules in the starting pool.

The fully extendable single stranded pool is then PCR amplified to generate multiple copies of the beginning pool. Following PCR amplification, the pool is transcribed with an RNA polymerase to generate either the starting pool for an RNA aptamer selection or the RNA template for reverse transcription in the case of ssDNA aptamer selection. It is in these steps that modified bases may be inserted if the beginning pool is to have chemical modifications not present in native nucleic acids. Alternatively, if the additionally desired chemical diversity cannot be incorporated enzymatically, modifications can be incorporated after the pool has been generated.

As mentioned above, the process of SELEX is an iterative process consisting of multiple rounds. The process begins when the starting pool is combined with the target molecule and allowed to bind. The conditions of this binding reaction (temperature, buffer composition, pH, etc.) are set to favor the stability of the target molecule while providing certain essential elements to facilitate nucleic acid folding (i.e. Mg^{2+}, Na^{+}, etc.). In certain selections, such as the Protective Antigen selection detailed in Chapter 2, the conditions of the binding reaction can be altered to achieve conditions that facilitate the interaction of target and nucleic acid pool. In this particular selection, the protein

target can tolerate a wide pH range without losing stability, but at a pH of 6.0 it acquires a more favorable charge for nucleic acid binding. The reduction of the overall negative charge helps to mediate interaction between the negatively charged nucleic acid pool and the target. Ultimately, the precise conditions entailing a selection are determined by the nature of the target and the downstream application of the selected aptamer. For instance, aptamers to be used *in vivo* should be selected using conditions that approximate the internal environment to which they will be applied.

Once buffering conditions have been decided and the nucleic acid pool has been allowed to bind, the next step is to sieve bound and unbound nucleic acids from the target molecule. This is accomplished through the utilization of a wide variety of separation matrices. The most commonly used matrix is a filter of some form, although it is generally composed of nitrocellulose. As this is the most commonly used method in general, and is the method of choice for all selections in this work, further detailed description of selection procedures will assume this methodology. In this method, the binding reaction is passed over the filter, which should prevent the passage of the target while allowing the nucleic acid pool to pass through. The filter is then washed with some amount of binding buffer for additional separation of unbound or weakly bound species from the target. Here it must be noted that in any given selection there will be separation matrix binders. In the example above, these binders will possess an affinity for the filter. There are several different points in SELEX where these molecules can be disposed of and the washing step is one of them. Further washing, or washing with a buffer containing a higher salt concentration may prevent the accumulation of these particular species (Cox and Ellington 2001). Other opportunities to rid a selection of matrix binders are in subsequent rounds either pre- or post-addition to target but always pre-amplification of pool. In both of these instances, the pool is passed over the particular

matrix and thus removed before amplification takes place. In some selections where an accumulation of these background binders has been allowed to occur, or when dealing with a particularly "sticky" pool, these steps may have to be repeated multiple times before the selection can continue.

Following binding and subsequent washing of the reaction, the nucleic acid pool must now be unbound from the target. There are multiple ways to achieve this and the effectiveness of each depends primarily on the selection methodology and separation matrix chosen. For filter separation selections this is generally accomplished by eluting the target:pool complex off the filter and away from each other using a buffer containing 7 M urea. Following elution from the target, the nucleic acid is concentrated and made ready for enzymatic amplification. If the pool is composed of RNA the first step is reverse transcription followed by PCR amplification. Upon completion of the PCR cycle, the double stranded DNA product can be transcribed back into RNA and the next round of selection can begin. Figure 1-1 shows a simple diagram of a typical *in vitro* selection. As the full name of this process suggests, this is, in a sense, evolution; it simply takes place in laboratory rather than in nature. Those members of a population displaying the desired behavior, in the case of the selections to be presented to you later, binding to a target, are separated from those that do not. These "winners" are then selectively amplified and the cycle is repeated. As rounds pass and the population is further winnowed, the stringency of the selection can be increased through a variety of methods (increased washing, decreased target concentration, etc.) to ensure that only those displaying the best ability to perform the chosen task remain. The utility of this technique is highlighted by observing that aptamers have been generated against a wide variety of targets. These targets include, but are not limited to, proteins, parasites, viruses, and a plethora of small molecules, for a variety of purposes (Huizenga and

Szostak 1995; Pan, Craven et al. 1995; Schneider, Feigon et al. 1995; Homann and Goringer 1999; Berens, Thain et al. 2001).

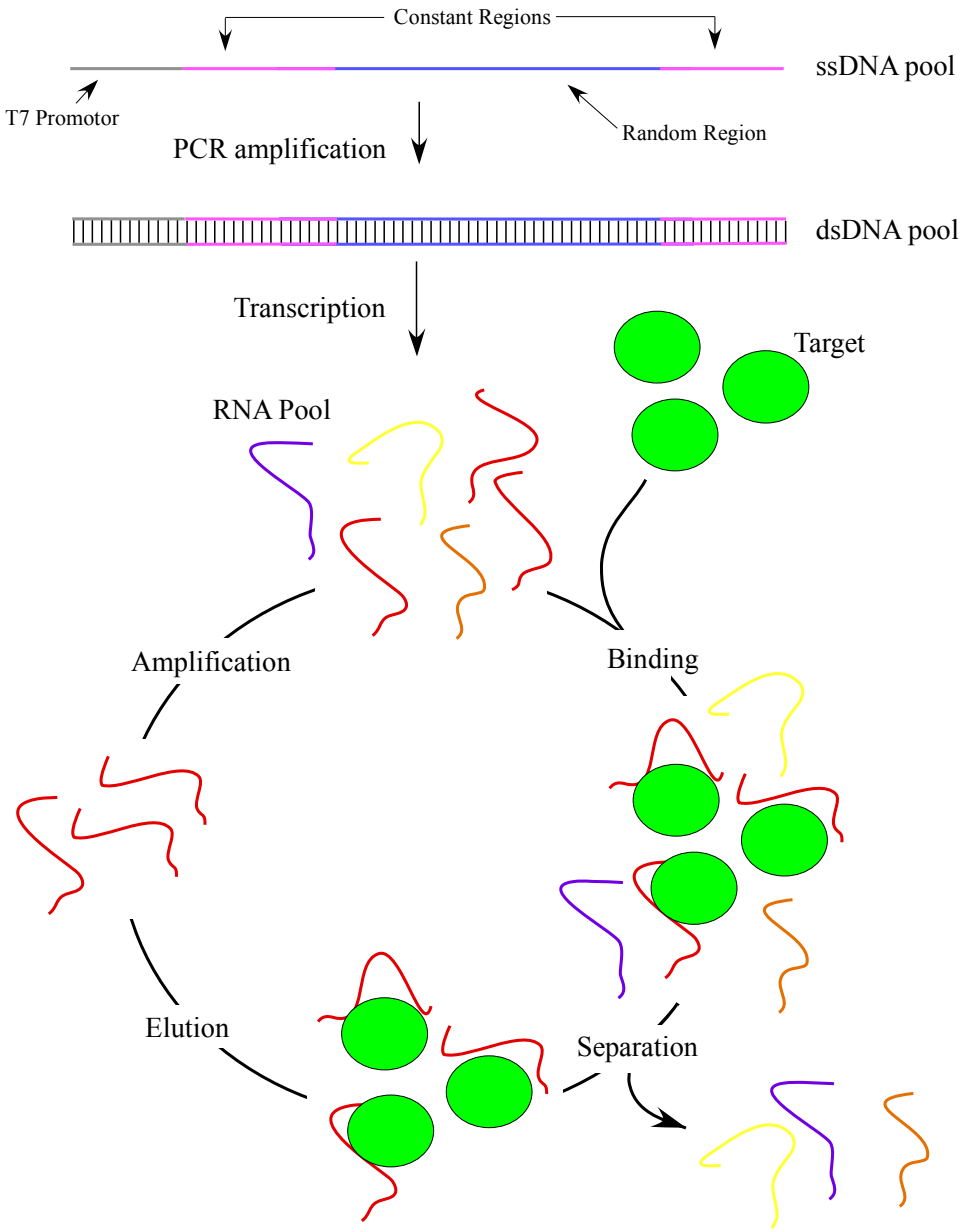

Figure 1-1. Schematic of a typical *in vitro* RNA selection. An RNA pool is generated from a chemically synthesized ssDNA pool. The pool is then allowed to incubate with its target molecule. A portion of the RNA pool will be able to bind the target through a combination of shape, charge, and hydrogen bonding. Those RNA molecules that cannot bind will be separated from those than can through a variety of separation matrices (filter, column, gel, etc.). The bound RNA will then be eluted and amplified to regenerate the RNA pool. This process continues until "winning" sequences can be identified.

RNA OR DNA?

Prior to beginning any *in vitro* selection, a decision must be reached about the nucleic acid composition of the initial pool. There are really only three considerations and each has certain advantages and disadvantages over the others. ssDNA is largely resistant to degradation, although this may not true for therapeutic applications *in vivo*. Additionally, ssDNA is inexpensive and easily amplified through common lab protocols. However, ssDNA can be difficult to work with in selection experiments, and the absence of a functional group at the 2' position of the ribose ring reduces the potential for the nucleic acid strand to interact with its target. At the same time, while RNA has a higher capacity for hydrogen bonding, it is also much more susceptible to cleavage by any number of sources, one of the most common being RNase A. An additional hindrance to working with RNA is the extreme care, in both handling and storage, required to maintain the stability of the molecule. The primary reason for this molecular frailty is the very 2' hydroxyl facilitating nucleic acid to target interactions.

These concerns are especially valid when attempting to select aptamers against a biothreat agent regardless of the intended downstream purpose. The issues involved in generating a successful therapeutic aptamer have already been discussed. While detection systems in a laboratory setting are not necessarily fragile, what happens when they are deployed outside of a laboratory setting? One must think about the environment in which this platform will be situated. Harsh climates, such as deserts with extremely high daytime temperatures and extremely low nighttime conditions, make for a particular challenge. Or envision the additional stability constraints placed on a detection system deployed underwater to ward against potential threats to a water supply. These

considerations are in addition to the inherent difficulty in selecting a specific binder against what is very often a complex target. What is needed is a molecule combining the stability of DNA with the molecular flexibility of RNA. There has been a great deal of research directed toward finding such an alternative, with the primary effort aimed at finding modifications that confer additional stability to the RNA molecule.

MODIFIED RNA

Modified RNA is becoming a common component in both *in vitro* and *in vivo* experiments. The addition of nucleotides with modifications, regardless of where the modification is placed, confers certain properties to the nucleic acid strand that will not be found in native RNA. These properties include but are not limited to nuclease resistance, increased thermal stability, greater possibilities for hydrogen bonding, potential for unusual chemical interactions, and simpler methods for functional group addition (i.e. NHS-fluorophore). There are three places where a nucleotide may be modified and they are listed and discussed below. Each of these of these modifications in turn has certain advantages and disadvantages and, again, it is important to remember the downstream purpose of the nucleotide in choosing a suitable alternative to unmodified RNA or DNA. However, for our purposes there are three primary considerations in choosing a modification. The first is retention of the capability for enzymatic incorporation. Without the use of enzymes, *in vitro* selection becomes impossible. Fortunately, there has been a great deal of research directed toward polymerase evolution and so there are now polymerases capable of incorporating certain unnatural nucleotides (Padilla and Sousa 1999; Padilla and Sousa 2002; Chelliserrykattil and Ellington 2004). The second consideration is that the modification enhances the ability of the RNA to

withstand enzymatic degradation from a nuclease. Finally, for the majority of laboratories, including ours, to consider incorporating a modified base it must be available commercially. While there are many published protocols detailing synthesis routes for a variety of modified bases, unless an individual has the knowledge, experience, necessary chemical components, and equipment this is not a feasible option. Many synthesis routes also yield various side products, reducing yield, and diastereomers that are unusable. These must be purified away from the desired product, a process requiring a great deal of time and effort.

Nitrogenous Base

Modification of the nitrogenous base is an area which has shown much promise. The majority of reasearch utilizing modifications of this nature has been carried out with dNTP's and so is not appropriate for this discussion. However, there are examples of base modified RNA nucleotides in the literature. The primary focus of this research involves modifying the pyrimidine nucleotides and there are several examples where these bases have been successfully incorporated into nucleic acid pools and subsequently used to generate unique functionalities (Vaish, Fraley et al. 2000; Schoetzau, Langner et al. 2003). It should be pointed out that there are also examples of successful purine modification as well (Ito 1997).

Halogenation (chloro-, iodo-, bromo-) of the pryrimidine ring is a common modification. Through this alteration, the nucleobases are able to crosslink with proteins in close proximity. This crosslinking is light mediated; causing a transfer of electrons from the halogen to a suitable amino acid side chain on the protein (Dietz and Koch 1987). The usefulness of this property has limited value in *in vitro* selection owing to the

need to keep the reaction in the dark at all times, and the need to digest the target following the selection. There is, however, an example of a successful selection using this type of base in the literature (Golden, Collins et al. 2000).

Other common modifications to the nitrogenous base include various additions and even substitution of amines. There are also a few commercially available substitutions using sulfur as the substituting atom. The only real concern here is ensuring whatever modification is placed here does not perturb base pairing with the complementary base for the purposes of enzyme amplification. The primary benefit from using these modified bases lies in the chemical diversity that may be conveyed to the RNA strand.

However, modifying the base portion of the nucleotide does nothing to mitigate the effects of nuclease activity and so fails to appreciably enhance the stability of the RNA both *in vitro* and *in vivo*. It thus falls short of the criteria established above when considering which modified nucleotides to use. While there is an example of a nucleotide modified on both the base and the sugar ring (Schoetzau, Langner et al. 2003), this is not a commercially available reagent and so, again, for the above reasons it is not a feasible alternative.

Phosphodiester Backbone

As the phosphodiester bond interlinking the individual nucleotides in a nucleic acid strand is the target of nuclease activity, which is the primary cause of RNA degradation, it became an early focus for modification. In addition, by replacing the negatively charged oxygen in this position it is possible to reduce the overall negative charge of the oligonucleotide chain. The implications for such a substitution hold more

importance for those considering using nucleic acids for therapeutic purposes, as a reduction in the anionic charge may allow for a more favorable passage across the cell membrane (Agrawal and Iyer 1995) . Various substitutions for the non-bridging oxygen in the interlinking phosphate such as methyl, amine, and sulfur groups have been incorporated; an excellent review of which has already been given (Micklefield 2001). The majority of the backbone modifications available, however, result in a molecule that cannot serve as a substrate for enzymatic amplification, or cleavage. These modifications must be incorporated post-selection during chemical synthesis. While this strategy may be effective in some cases, it is more likely that whole scale replacement of the backbone following selection will cause a marked reduction in binding affinity owing to the severe disruption of electrostatic interactions, which were described above as one of the contributors to target recognition.

Of those derivatives that may be incorporated enzymatically, phosphorothio and boranophosphate, only one (phosphorthio triphosphate) is available commercially. Although this modification displays nuclease resistance, it also displays a tendency to increase nonspecific binding to proteins (Stein 1996). Once specificity is lost, or decreased, the selected nucleic acid loses its usefulness. The boranophosphate derivatives show promise for future *in vitro* selections as they have several desirable properties over natural nucleotides, including nuclease resistance and chemical diversity, while maintaining the desirable features of native RNA. It will be interesting to see the results should anyone begin to utilize them in an *in vitro* selection scheme.

Sugar ring

This leaves the sugar ring as being the most amenable portion of the NTP for modification. Indeed this has been explored with a great deal of success. Several aptamers with substitutions of the 2' hydroxyl, including amine (Lee and Sullenger 1997), fluorine (Rhie, Kirby et al. 2003), and methoxy replacements (Burmeister, Lewis et al. 2005) have been selected. Substitutions at this position convey an absolute resistance to RNase A degradation. Further, by placing a functional group here the capacity for hydrogen bonding is somewhat maintained. This is not the case when the methoxy replacement is considered, but this replacement does help to stabilize the tertiary structure of the RNA and so binding affinity can be maintained. These bases can also be incorporated enzymatically using a modified polymerase. Further, these bases are also commercially available through a variety of companies. This, of course, increases their accessibility and circumvents the need for specialized knowledge in synthetic organic chemistry.

There are several considerations to take into account when choosing which of the above bases to use in a selection. Ease of use, chemical synthesis of the aptamer following selection, and the ability to hydrogen bond are all very important. For instance, to chemically synthesize an amine substituted nucleic acid, additional protection and deprotection steps must be taken to protect the nascent oligonucleotide from degrading on the synthesizer. A fluorine or O-methyl substitution has no need for these additional steps. In terms of hydrogen bonding, a methoxy substituent is functionally inert whereas amine and fluorine substations all retain this capacity. Additional consideration must also be give for ease of use. In the experience of our laboratory, although methoxy substitutions can be incorporated enzymatically, the efficiency of incorporation is lower than that for other modified pyrimidines (Chelliserrykattil and

Ellington 2004). Amine substitutions on the other hand tend to be "sticky" when it comes to gel purification. Because of this, yields following purification are greatly reduced in comparison to other substitutions. Since gel purification is repeated throughout an aptamer selection, this becomes rather inconvenient. With all of these considerations in mind, 2'-fluoro derivatives become the clear choice as a modified base for nucleic acid pool incorporation.

One other sugar-modified nucleotide deserves some discussion. In addition to the replacement of the hydroxyl on the sugar ring, there has been some success with replacing the oxygen atom with a sulfur atom at the 4' position (Kato, Minakawa et al. 2005). This replacement has been shown to convey additional thermal stability and nuclease resistance over unmodified RNA, although the amount of nuclease resistance conveyed by this substitution does not begin to approach that of RNA modified at the 2' position. A selection carried out using these bases was able to generate an aptamer against thrombin possessing a lower Kd than that of an unmodified RNA aptamer previously generated. This seems to imply that this replacement allows for a wider range of chemical binding capabilities and perhaps this particular substitution may find future use in aptamer selections. The primary limiting factor for this base lies in the lack of commercial availability, necessitating individual preparation. Should this base become commercially available it may deserve serious consideration for *in vitro* selection experiments.

Complex targets

There have been a variety of aptamer selections carried out against what are termed "complex" targets. This term complex is used in describing a target wherein the outer surface is composed of different types and multiple classes of molecules such as

proteins, carbohydrates, and lipids. These types of selection are often more difficult than those where the targeted molecule presents a single, dominant epitope, as in a purified protein. These added difficulties may include carrying out a higher than "normal" number of selection rounds and extra care in target preparation. Extreme care is necessary in terms both of handling and, in the case of living cells, ensuring growth conditions remain identical between rounds. Red blood cell ghosts (Morris, Jensen et al. 1998), glioblastoma cells (malignant tumor cells) (Hicke, Marion et al. 2001), human plasma (Fitter and James 2005), virions (Pan, Craven et al. 1995; Gopinath, Misono et al. 2006), and two different types of live trypanosomes (Homann and Goringer 1999; Ulrich, Magdesian et al. 2002) have served as targets for these complex selections. Often, but not always, during these selections very diverse aptamer sequences will be generated. This is in comparison with single target selections where the tendency is to generate very few related aptamer families. This diversity can be attributed to the various epitopes being presented simultaneously to the aptamer pool. In a sense, one can carry out multiple selections for various targets in parallel, and this can be a useful, although not necessarily intended, benefit. Regardless of how many different binding epitopes are found, the ability to select an aptamer against a living cell has the potential to generate interesting and useful aptamers that could be directed toward a variety of purposes. The aptamers selected against the targets above have been used to block viral production, specifically target tumor cells, and prevent parasitic cell invasion. These end uses more than demonstrate the viability of this technique and the feasibility of attempting selections against difficult targets. There is, however, a notable exception to the list of complex targets, bacterial vegetative cells. Although there have been selections reported that target bacterial spores (Bruno and Kiel 1999; Zhen, Song et al. 2002), specifically

those of *B. anthracis*, these reports are dubious and have not been repeatable in the hands of other researchers, including my own (Ellington lab; data not shown).

BIODEFENSE APPLICATIONS

The threat of an attack, whether terrorist or military, using biological weapons cannot be ignored. Such weapons may cause immediate effects or may lay dormant for extended periods of time prior to revealing their presence. In the case of anthrax spores, the spores are regarded as such a long term danger that an island where their release was tested had to be sterilized with seawater and formaldehyde 45 years after their release (Manchee, Broster et al. 1994). However, beyond the immediate health implications of these weapons, the danger of these types of weapons lies in the difficulty in identifying them. There are few truly rapid and reliable means to detect such weapons (Lim, Simpson et al. 2005). Their very nature (easily mutable, complex in nature, etc.) resists most attempts to put such detection systems in place. Even should detection be possible, in most scenarios detection equates to exposure. There are few safe and effective treatments for the majority of biothreat agents. Those that do exist, such as the anthrax vaccine, have short durations of efficacy, necessitating frequent boosters (Little 2005). In addition, this vaccine is expensive to generate, precluding its distribution among the general population prior to exposure to the spores (Inglesby, O'Toole et al. 2002; Fowler, Sanders et al. 2005). There is a need for a recognition agent that is inexpensive to generate, able to recognize its target molecule quickly and discriminately, and amendable to suit a variety of purposes. Aptamers have the potential to meet all of the above criteria.

There has been some effort directed to raising aptamers against a variety of relevant biodefense targets. The majority of these efforts have been directed toward the detection of biothreat agents, although there are examples of aptamers that could be adapted to therapeutic purposes (Hesselberth, Miller et al. 2000; Hirao, Madin et al. 2000). The next few paragraphs will discuss these two major areas of biodefense where aptamers may play a significant role. Generalized examples will be given to show the areas where aptamers have already been demonstrated as effective. Examples of aptamers that have been directly raised against biodefense targets and used in these capacities will be discussed. However, as there has not been much progress in this area, the forthcoming discussions will be largely theoretical.

Therapeutic Potential

A number of aptamers have been selected against targets that play a role in either various diseases or biological processes (Griffin, Tidmarsh et al. 1993; Jellinek, Green et al. 1994; Rhodes, Deakin et al. 2000; Watson, Chang et al. 2000). Some of these aptamers have shown an ability to inhibit or greatly reduce the function of their target molecule. This may convey the potential to be developed into viable therapeutics. However, the development of a therapeutic is a challenging and difficult task. One of these issues that is central to whether or not aptamers will ever develop into commonplace therapeutic agents is their ability to stay present in the body long enough to exert an effect. This issue can be resolved into two sub-groups: stability and clearance.

The stability issue can be addressed easily enough by simply carrying out the original aptamer selection utilizing a modified base, which will be discussed below. Additional protection, if needed, can be obtained through selective modification of other

bases post selection (Eaton, Gold et al. 1997). Although this method may reduce binding affinity, it is also possible to alter only those bases that do not directly contribute to the aptamers ability to bind. By leaving these bases unchanged it is possible to increase stability while only slightly, if at all, lowering the affinity of the selected aptamer. This is a time-consuming process as each base should be checked, although there can be somewhat of a rational approach taken though various minimization and structure probing studies. Rarely is it possible to alter all the bases, but any further enhancement of stability is valuable in terms of *in vivo* drug development.

As aptamers are relatively small molecules, they can be cleared from a patient's system in a very short time (Brody and Gold 2000). This obviously dramatically lowers the probability of exerting any type of pharmaceutical effect. Various strategies have been employed to slow down this process. The simplest method involves simply attaching a polyethyleneglycol molecule to the end of the aptamer (Floege, Ostendorf et al. 1999). This approach can double or even triple the molecular weight of the aptamer to which it is attached and thus increase time to clearance. Another approach is to insert the aptamer into a liposome (Willis, Collins et al. 1998). A liposome is a large molecule that contains a fluid filled space enclosed by walls composed of phospholipids. Normally this space contains a drug, such as insulin, to be delivered into a system. However, it becomes possible to use the phospholipid layer itself as the drug containing portion through the exploitation of the hydrophobic portion formed when the phospholipids come together, as they do in a cell membrane. By attaching a lipid to either end of the aptamer, it becomes possible to anchor it into this bilayer. Of course, for either of the above methods to work the binding affinity and specificity of the aptamer must not appreciably change. However, testing the effect of these conjugations is a relatively simple process. The added advantage of the above methods lies in their previous use for

drug delivery. This somewhat ameliorates the potential for harmful side effects that may be associated with developing new and unproven methods.

Those are two of the primary issues needing to be overcome in order for aptamers to be effective for *in vivo* therapeutic applications. However, as mentioned above, there have been several aptamers displaying therapeutic potential *in vitro*. There have even been some aptamers directed against potential biothreat agents with these properties. The most success has been with selecting aptamers involved in the prevention of adhesion to the host cell. Preventing adhesion to the host cell membrane is the fist step toward preventing cellular infectivity in pathogens. Pathogens come in essentially three different types bacterial, viral, and parasitic, and there has been success in preventing this interaction with all three types.

Trypanosoma cruzi is a parasite responsible for Chagas disease. Chagas disease is a chronic disease infecting an estimated 16-18 million people, resulting in approximately 23,000 deaths each year (Prata 2001). Aptamers selected against this parasite are capable of reducing parasite infectivity in host cells by preventing the parasite from attaching to the cell surface (Ulrich, Magdesian et al. 2002). Infective state *Tyrpanosoma cruzi* has binding sites on its surface that recognize certain molecules present on the host cell extracellular matrix. Following binding to the infective stage parasite, aptamers able to prevent attachment to the host cell were rationally selected from those that do not. This was accomplished by displacing those aptamers attached to these binding sites with high concentrations of their natural ligands. In this manner, all other aptamers were discarded and the pool was enriched for aptamers exhibiting one particular function, recognition of the parasites host cell ligand receptor. Although these aptamers do not convey full protection to the host, they do reduce the infectivity of these parasites, and perhaps more importantly demonstrate that it is possible to choose which

portion of an organism the aptamers bind. While parasites are an unconventional bioweapon at best, they are also capable of producing a devastating effect. Witness the ongoing battle to eradicate malaria, another parasitic disease responsible for over a million deaths per year with over 300-500 million infections (Parry, Hien et al. 2002; Suh, Kain et al. 2004).

Salmonella enterica serovar Typhi is a bacterium that is easily spread throughout the food and water supply. This is a dangerous and widespread pathogen causing severe typhoid fever epidemics in developing countries. There are an estimated 16 million new cases per year with 600,000 deaths (Parry, Hien et al. 2002). Although not traditionally thought of as a biothreat agent, this is a pathogenic bacterium capable of causing widespread harm even in a developed country. Moreover, the increasing antibiotic resistance displayed by this and other such bacterial pathogens only increases the probability that an attack carried out using such bacteria will be devastating. With this in mind, an aptamer selection was specifically engineered to select an aptamer to prevent cell adhesion, and thus reduce the infectivity of this organism (Pan, Zhang et al. 2005). Essentially the authors generated a GST fusion protein with the non-GST portion being a protein known to mediate cell adhesion. By carrying out selections specifically against this protein, and the requisite negative selections against the GST portion, they were able to select specific binders to this protein, and then use the aptamers thus generated to prevent adhesion using whole bacteria. Although one cannot always expect this strategy to work, it does provide a nice proof of principle that such things are possible. In addition, it demonstrates that bacterial components are suitable targets for selection and that aptamers can be retained on the surfaces of multi-component, changing surfaces such as bacterial cell membranes.

Influenza is a dangerous pathogen that is responsible for approximately 36,000 deaths in the United States per year (Thompson, Shay et al. 2003). Other than the possibility of death, influenza is a major cause of illness with several hundred thousand people seeking hospital treatment each year (Thompson, Shay et al. 2004). There is also the possibility of an influenza pandemic arising, such as the 1918 Spanish flu pandemic that killed an estimated 40-100 million people worldwide (Johnson and Mueller 2002). In actuality, it is widely acknowledged that it is not even a question of if another pandemic will arise, but more a matter of when (Donaldson 2006). Although there are currently influenza vaccines available, the desired level of efficacy has not been reached, due to the easily mutatable nature of the virus and the genetic differences between strains that this implies. A recent *in vitro* selection was carried out showing that it is possible to select aptamers that will selectively recognize one strain of influenza over another (Gopinath, Misono et al. 2006). In this selection, a positive selection against whole viral particles was followed by a negative selections against viral particles of a different strain. Again, the resulting aptamers were then able to block viral-host cell membrane fusion, resulting in a decrease in infectivity.

The above examples of specific selections are meant to emphasize two things. One, by tailoring selection conditions carefully it is possible to select an aptamer capable of performing a specific function. Two, it is possible to select aptamers against living organisms, or so called complex targets. This last point will be covered in more detail in Chapter 3. Although the targets above are not traditional bio-weapons, they are all certainly capable of causing wide-spread harm, especially if altered in some way to convey resistance to current therapeutic options. Even should genetic alteration reduce the ability of an aptamer to bind, a reselection to the modified target would be a relatively rapid task.

One final thought on aptamers thought to be capable of providing a therapeutic benefit. Although the above aptamers and the potential uses are all valid as demonstrated by *in vitro* experimental techniques, it is important to remember that valuable information is gained simply by studying the effect aptamers have on their target molecules. By elucidating the exact mechanisms an aptamer may use to block cell adhesion, prevent cell signaling, specifically bind to targets, and any other such behaviors we are able to learn more and more about how such interactions are mediated. Ultimately, this knowledge may lead to development of conventional (non-aptamer based) medicines or vaccines to bring about better therapies for toxins or pathogenic infections.

Detection Platforms

As detection agents, aptamers have found a number of uses. One of the characteristics making aptamers so amenable to this particular function, as mentioned above, is the ease with which chemical modifications can be added during the synthetic process. More importantly, many of these modifications involve the addition of small molecules to either the 5' or 3' end. As opposed to internal modification of the base or phosphodiester backbone, these modifications rarely impose any significant hardships on ability of aptamers to bind the correct target. A notable exception to this may occur when this modification involves subsequent attachment to an extremely large molecule. Here the difficulty may lie in steric hindrance resulting in an inability of the aptamer to fold into the proper three-dimensional shape (personal observation).

The detection systems to which aptamers may be applied are quite varied. The primary reason for this diversity is the lack of constraints placed on both the aptamer and the platform in which it is to be deployed. As opposed to an aptamer for use in

therapeutics, an aptamer to be used in detection need only bind the target. There is no need for an effect, whether direct or indirect, to be exerted. In addition, as detection systems are generated for *in vitro* use there is no need to worry about potential side effects or the immunogenic consequences of either the aptamer or the device itself.

Aptamers are nicely suited for production in a microarray format (Collett, Cho et al. 2005). Microarrays potentially allow for the simultaneous detection of several different analytes. Using this technology, aptamers are bound onto a solid matrix, most often a streptavidin coated slide, through the chemical attachment of a linker on either the 5' or 3' end. Once attached, the aptamers are allowed to assume the proper configuration through immersion in a suitable buffer. Analytes are then passed over this and are bound by the aptamer which signals this through a variety of means (fluorescence, SPR, etc). Additionally it is also possible to label the substrates themselves prior to passing over the aptamer array. A large advantage of aptamer arrays over similar techniques employing antibodies is that they are reusable. Following detection of the target, the array may be washed in a urea-containing buffer to denature the aptamer and cause release of its target. The slide may then be washed, re-natured, and reused.

Capillary electrophoresis (CE) is another technology that has recently been utilized in conjunction with aptamers to detect ricin (Haes, Giordano et al. 2006). CE is really a broad term that encompasses many varying applications (Guijt-van Duijn, Frank et al. 2000). Generally though, CE involves the separation of molecules by passing them a narrow tube containing a gel matrix and applying a current. Separation is achieved through differences in charge, shape, and size. For the detection of ricin A chain, fluorescently labeled aptamer previously selected against ricin A chain is allowed to bind its target. The aptamer protein complex is then injected into a capillary electrophoretic device, and ricin is detected through a fluorescent signal. Using this format detection of

ricin A chain is possible down to 14 ng/mL. Aptamers are becoming a more favorable alternative to antibodies for use in this format as the fluorescent labeling step is easier to control and so only one labeled product is visualized (Haes, Giordano et al. 2006). Antibodies, on the other hand, tend to present multiple sites for label incorporation. This is then detected as a broad band or sometimes multiple bands that may limit sensitivity (Krull, Strong et al. 1997).

Aptamers generated against *Francisella tularensis*, the causative agent of tularemia, have recently been used in what is called an Aptamer-Linked Immobilized Sorbent Assay (Vivekananda and Kiel 2006). This assay is very similar to an ELISA with the exception that an aptamer takes the place of an antibody in the sandwich assay. In this assay, a "capture" aptamer is immobilized in a microtiter plate and the antigen is added. The aptamer is allowed to bind the antigen and then a "reporter" aptamer with a biotin molecule on the 5' end is added. A streptavidin horseradish peroxidase conjugate is then added and HRP substrate is added. These experiments were carried out in parallel with a conventional ELISA and indicate that in this particular format aptamers are able to generate a signal superior to that of antibodies. In addition, this assay was able to detect quantities of the antigen down to 25 ng.

The past few examples have demonstrated the viability of aptamers in taking the place of antibodies in platform-based detection devices. However, it is also possible to use aptamers to detect substances in a solution-based format. The following techniques have been demonstrated to be compatible for use with aptamers.

The proximity ligation assay (PLA) is a powerful technique for detection with a threshold in the zeptamolar range (Fredriksson, Gullberg et al. 2002). In this particular assay, two aptamers previously selected against a protein target, in this case PDGF, are synthesized with extensions on either the 5' or 3' end. They are then placed in solution

with the protein target and allowed to bind. An oligonucleotide connector is then added and this connector is able to act as a template for DNA ligation using a DNA ligase. Once ligated together the extension is free to act as a template for real time PCR amplification.

Another solution-based detection system was developed in which a change in fluorescence is used as the reporting signal (Heyduk and Heyduk 2005). In this model, two aptamers are extended with sequences complementary to each other. These sequences are also chemically attached to two different fluorophores. The net result being that subsequent to binding to the target, the complementary oligonucleotide sequences will find each other and bring the different fluorophores into close proximity resulting in a change in Fluorescence Resonance Energy Transfer. One can envision several variations on this method employing various quencher and dye combinations. The real value in this technique, and those like it, lies in the lack of an amplification step for signal generation.

The above are just a few of the examples to which aptamers may play a role in the detection of biothreat agents. Other detection techniques such as SPR (Hwang and Nishikawa 2006) and microfluidic cells (Farokhzad, Khademhosseini et al. 2005) have shown themselves to be compatible with aptamers. It has even been shown possible to select aptamers that function as molecular beacons (Rajendran and Ellington 2003). As aptamers selected against biological threat agents grow more numerous, there will be more technologies adapted to suit them. Aptamers have already been shown to be capable of replacing antibodies in most assay formats. With several inherent advantages over antibodies (synthesis, labeling, reusability, expense) and the issue of stability beginning to be resolved (see below), aptamers are capable of rising to the forefront of molecular recognition molecules in detection based platforms.

CONCLUSION

In vitro selection of functional nucleic acid has become a very powerful method to study a variety of interactions. Modification of natural nucleotide bases confers additional stability and chemical diversity to a molecule already suited to a wide range of applications. Regardless of whether they take the form of ssDNA, RNA, or modified RNA, aptamers have shown themselves to be adaptable to a variety of applications. As detection agents, aptamers are becoming an increasingly useful tool in the biodefense realm. Although currently there are no viable *in vivo* therapeutic aptamers selected against biodefense targets, some have been shown to be therapeutically effective *in vitro*. Through selections against three forms of pathogenic organisms (parasites, bacteria, viruses), aptamers have again demonstrated a wide range of diversity. The remainder of this work with deal with various efforts to select specific aptamers for both single protein (simple) targets and multi-protein, carbohydrate, lipid containing (complex) targets. In addition, potential downstream uses of these aptamers in both therapeutic and detection platforms will be discussed.

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CHAPTER 2: *In vitro* selection of anti-PA 83 aptamers

INTRODUCTION

Anthrax

Bacillus anthracis is a spore forming Gram-positive bacteria that is the causative agent of the disease anthrax. Primarily a disease found in herbivores, it nonetheless is able to exert an effect on all mammals, including humans. Anthrax is spread through the spores *B. anthracis* forms during times of limited nutrients. These spores are able to enter a host mammal in one of three ways, each of which results in both different symptoms and probability of lethality. Cutaneous infection is the most common form of anthrax disease. Also known as "wool sorter's disease" this type of infection occurs when a spore is enters a small abrasion, or cut on the skin. The primary symptom associated with this type of infection is the formation of a black lesion at the site of spore entry. This type is relatively easy to diagnose with 20% of infections resulting in death when untreated (Inglesby, O'Toole et al. 2002). Treatment with appropriate antibiotics will usually prevent death. The second type of anthrax infection is known as intestinal. This type occurs when the spores are ingested, primarily when contaminated meat is consumed. This is a very difficult type of anthrax to diagnose as symptoms of this type include nausea, vomiting, abdominal pain, and severe diarrhea. Owing to the difficulty in diagnosis, this form of anthrax has a high death rate (Inglesby, O'Toole et al. 2002). The final and most fatal type of anthrax infection is inhalational. This occurs when spores are inhaled into the lungs. Even with treatment this type of anthrax infection is generally fatal (Dixon, Meselson et al. 1999). Symptoms are difficult to positively identify, generally being described as "flu-like." While this is the rarest from of the disease, it is

also the form most likely to occur should anthrax spores be deployed as a biological weapon (Meselson, Guillemin et al. 1994).

Anthrax disease is dependent upon the formation of three proteins, Lethal Factor, Edema Factor, and Protective Antigen. These three proteins are collectively referred to as anthrax toxin. Upon germination of the spores, the bacterial cell will begin generating these toxins, probably as a means to avoid the host immune response (Duesbery and Vande Woude 1999; Sanchez and Holmgren 2005). The toxin itself is a typical AB toxin composed of a binding portion and an enzymatically active portion. Two part toxins are relatively common in the world of bacterial protein toxins. *Vibrio cholerae* (Sanchez and Holmgren 2005), *Corynebacterium diphtheriae* (Collier 2001), and *Clostridium botulinum* (Barth 2004) all use a similar mode of action during the delivery of their respective toxins. This mode of action involves the binding portion attaching to the host cell membrane and creating a binding site for the enzymatically active portion. Once the binding and enzymatically active portions are bound, the now complete toxin complex is endocytosed in the host cell. Although the exact mechanisms and the complete effects of the various anthrax toxin components have not been entirely elucidated, the next few paragraphs will briefly detail what is known.

The Components

As its name implies, Lethal Factor (LF) is the portion of the toxin whose enzymatic activity is most responsible for the lethal effects of this disease. LF has a molecular weight of 90,0000 Daltons and is known to be a zinc metallo-protease. LF has been shown to be highly specific for mitogen activated protein kinase kinases (MAPKK's) (Duesbery, Webb et al. 1998). Once cleaved, MAPKK's can no longer

phosphorylate, and thus activate, members of the MAPK family (p38, ERK, JNK, etc.). Deactivation of this pathway leads to inhibition of several key regulatory pathways in a variety of eukaryotic systems (i.e. immune, endocrine, circulatory) (Agrawal and Pulendran 2004). The immune system and macrophages in particular, seem to be susceptible to LF. The full implications of which systems LF can affect is still being investigated.

Edema Factor (EF) is an 89,000 Dalton protein. It has been identified as a calmodulin dependent adenylate cyclase (Leppla 1982). The result of this adenylate cyclase activity is to significantly increase the levels of cAMP in the cytoplasm of eukaryotic cells. This in turn results in either a modification or complete inhibition of the cellular functions of the targeted cell. It appears as though the cells primarily targeted for this activity are those possessing functionality in the immune system (O'Brien, Friedlander et al. 1985). Lymphocytes are very sensitive to the effects of this toxin, typically becoming apoptotic, or at least down regulating the immune response they normally display. Even macrophages and neutrophils can have their phagocytotic response inhibited due to the increased levels of cAMP (Rossi, McCutcheon et al. 1998). Therefore, by targeting these cells involved in the immune response, EF may be able to reduce the ability of the host immune system to respond to the bacterial infection and increase the probability of *B. anthracis* survival, and thus replication, in the host.

Protective Antigen (PA) is synthesized as an 83,000 (PA 83) Dalton protein. However, in order to participate as a toxin component, it must be first cleaved by a host protease to a 63,000 (PA 63) Dalton protein. The role of PA is to bind to the cell membrane of its target cell and form a binding site for both LF and EF. Once bound PA will translocate these two toxic components into the host cell and allow for their introduction to the cell cytoplasm. Two of the cell surface receptors for PA have recently

been identified. They are Tumor Endothelial Marker 8 (Bradley, Mogridge et al. 2001) and Capillary Morphogenesis Gene 2 protein (Scobie, Rainey et al. 2003). Although these proteins are conserved across a wide variety of species, little is known about their natural function.

Mode of Action

Upon expression in the host, PA 83 binds to a cell surface receptor. Once bound, it is cleaved into PA 63 by a transmembrane associated protease that has been identified as furin or a related protease (Klimpel, Molloy et al. 1992). Once cleaved, PA 63 will heptamerize on the cell surface (Milne, Furlong et al. 1994). It has been reported that a molecule of either LF or EF may bind to the site created between two monomers of PA 63. This will allow multiple monomers of either LF or EF to enter the cell at a given time (Mogridge, Cunningham et al. 2002). EF and LF share a homology on the amino terminal end of the protein, and this is why either of the proteins may bind to the same site created on PA 63. Once bound to EF or LF, the PA 63 heptamer undergoes clathrin mediated endocytosis into the cell (Abrami, Liu et al. 2003). Following endocytosis, the interior of the endosome is subjected to a drop in pH; falling to at least 5.5. This allows the PA 63 heptamer to undergo a conformational change that will allow it to interact with the endosomal membrane. Recently solved crystal structures of the heptamer at a neutral pH suggest that this conformational change involves the formation of a transmembrane domain from a portion of each monomer that was previously involved in an interaction with a neighboring monomer (Lacy, Wigelsworth et al. 2004). It is unclear what the precise trigger for this change is, although it has been speculated that abundance of the histidine residues precipitated by the drop in pH as a sort of trigger. Regardless, a change

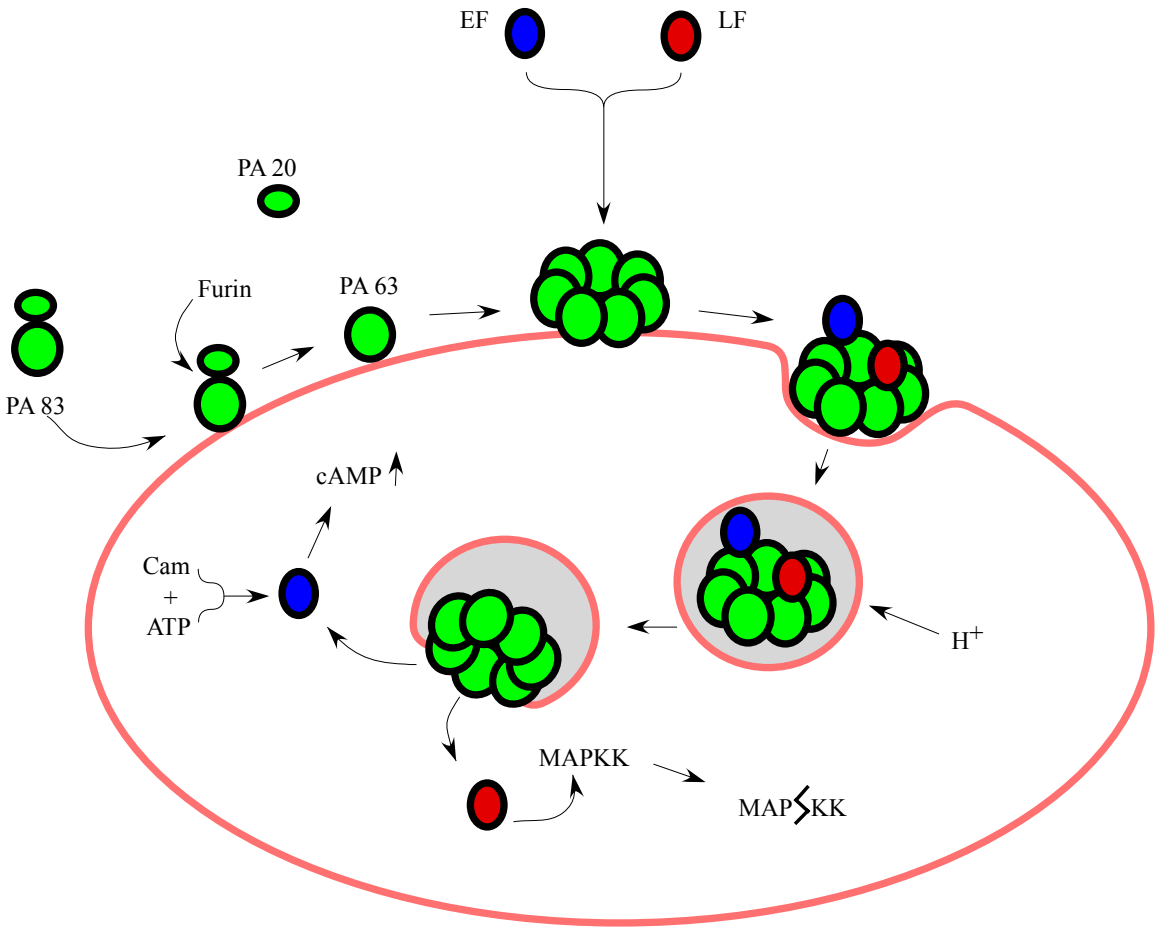

Figure 2-1. Schematic of anthrax toxin mode of action. PA 83 is expressed by the germinated bacteria upon entrance into the host cell. PA 83 will then bind to its cellular receptor on the host cell. Here it is cleaved by furin into PA 63, which will then heptamerize. The PA 63 heptamer forms a docking site for EF or LF. The toxin complex is then endocytosed into the host cell. An acidification of the endosome allows the PA 83 heptamer to form a pore in the side of the endosome, releasing EF and LF into the cytoplasm. EF is a calmodulin (Cam) dependent adenylate cyclase that increases the level of cAMP. LF is a zinc-dependent metallo-protease specific for MAPKKs.

in the conformation does occur and enables the PA 63 heptamer to open a pore in the endosomal membrane. This pore then enables EF and LF to enter the cytosol and interact with their respective targets. Figure 2 –1 illustrates the mechanism by which anthrax toxin enters the cell and takes effect.

Protective Antigen is the Key

Although antibiotic therapy is useful in the treatment of inhalational anthrax it must be administered immediately upon exposure to be truly effective. When treatment is begun too late, even if the antibiotics succeed in killing the bacteria themselves, there is often too high of a toxic load present in the host to stave off death (Duesbery and Vande Woude 1999). Complicating this situation is that the early symptoms of anthrax are very similar to that of a number of different illnesses. Clearly what is needed is a means to either rapidly detect the presence of the toxin in the blood or to find a way to combat the toxin itself. Fortunately, PA presents itself as a target equally amendable to both of these goals.

The importance of PA in the role of anthrax toxicity cannot be underestimated. There are any number of ways in which a therapeutic directed toward PA may prevent the toxic effects of anthrax. There has been a recently engineered antibody found to compete with the cellular receptor for PA binding. Through this competition, this antibody has been shown to neutralize the toxin when administered to rats *in vivo* (Maynard, Maassen et al. 2002). Work is currently underway to amend this antibody for use in humans. Along similar lines, phage display libraries have also been employed to select antibodies which may inhibit toxin action *in vivo* (Wild, Xin et al. 2003). Other strategies include using phage display libraries directed against the heptameric form of PA 63 to prevent EF or LF from interacting with it. The selected peptide could then be linked to a backbone molecule to allow for multiple copies to act in concert (Mourez, Kane et al. 2001). One target that has remained elusive is the generation of somthing that prevents furin from cleaving the protein in the first place. A hexa-arginine peptide has been shown to inhibit the function of furin (Sarac, Peinado et al. 2004), and so provide

protection against anthrax toxin, but as furin is an important cellular protease, this approach may prove problematic for the host downstream of the immediate threat.

As stated above, antibiotic therapy is a viable means to treat *B. anthracis* infection, assuming it is caught in time. As the bacteria begins to produce the toxin within an hour after germination from the spore (Cote, Rossi et al. 2005), a rapid, reliable, and sensitive means to detect the presence of the toxin in host serum would be an invaluable tool to determine when antibiotic treatment should begin. There are several assays reported in the literature that will detect the presence of protective antigen. However, many of this assays work by detecting the host antibodies that form upon the host immune system mounting a response against the toxin (Quinn, Semenova et al. 2002; Biagini, Sammons et al. 2006). While this is of some value, a better assay would be to detect the presence of the PA molecule itself. Recently there has been a report of an antibody based immunoassay capable of detecting PA in the serum of guinea pigs before onset of toxic symptoms (Mabry, Brasky et al. 2006). Although this is a promising detection method, it still requires the use of antibodies, which for reasons already listed in the introduction to this work have disadvantages to other reagents. Additionally the ELISA format this assay takes is somewhat time-consuming and may not be amendable for use in a field setting. Another approach detects PA through a multiplex PCR based approach targeting the PA gene (Selvapandiyan, Stabler et al. 2005). This method allows for the detection of *B. anthracis* in human blood 24 hours after incubation with *B. anthracis* spores. Although this is very interesting and potentially very useful, it still requires an amplification step and the use of equipment that may or may not be readily available at any given time.

The remainder of this chapter will detail the efforts to develop an aptamer capable of functioning in one of the above roles. Through the utilization of SELEX, an aptamer

shown to bind to PA with a Kd of 238 nM was selected. The anti-PA 83 aptamer is shown to inhibit cleavage of the toxin by furin, and so one day may function as a viable therapeutic. However, even if this does not come to pass, through its capacity as a PA 83 binder, this aptamer has the potential to be adapted to a rapid, specific, and renewable detection device.

RESULTS AND DISCUSSION

In vitro selection of anti-PA 83 aptamers

The target used for selection was recombinant Protective Antigen 83 (PA83) obtained from List Biological Sciences. This protein has been shown to possess full activity *in vitro*, i.e. is capable of acting as a cleavage substrate for a trypsin-like protease and, subsequent to cleavage, translocating edema factor or lethal factor into the cytosol. *In vitro* selection experiments were carried out using a pool containing a region of 45 randomized bases with an initial complexity of approximately 1.6×10^{14} unique sequences. This pool was also designed to efficiently incorporate pyrimidine nucleotide triphosphates with a 2'-Fluorine substitution. No modifications were present on the purine bases. Modified pyrimidine residues were chosen for incorporation into this selection because of the desire for added resistance to nucleases common *in vivo*. Although not fully protected, the purine residues still retain hydroxyls on the 2' carbon, this modification has been shown to increase the lifetime of RNA in serum (Heidenreich, Benseler et al. 1994). This was a consideration because the most likely downstream use for this aptamer would either be as a therapeutic or a serum screening agent.

A pH of 6.0 was chosen to reduce the negative charge present on PA 83. At a physiological pH of 7.4, PA 83 has a negative charge of 10.2. Although at a pH of 6.0

there is still a negative charge, it is reduced to 1.1 as determined by an online protein calculator (http://www.scripps.edu/cgi-bin/cdputnam/protcalc3). This helps to increase the chance of the nucleic acid pool binding the target as there is a lessened degree of repulsion between the negatively charged pool and the negatively charged protein. Magnesium and sodium salts were included to enable folding of the nucleic acid pool into 3-dimensional shapes. EDTA was added in an attempt to chelate out any divalent cations that are non-critical for folding of the RNA to occur.

Seven rounds of selection were carried out using a nitrocellulose containing filter as the separation matrix. In order to prevent the accumulation of filter binding species, the RNA used to initiate each round was passed over a filter before protein was added. This is called a negative selection and the only rounds exempt from this process was the Round 0 pool where the RNA was entirely unselected, and Round 1 which is after the initial incubation with the target. These rounds are exempt because of the risk of artificially winnowing the pool prior to addition of target.

Following each round, and prior to beginning the selection, the pool RNA was assayed for its ability to bind both PA 83 and the filter (Figure 2-3). This assay was performed to help determine the number of negative selections needed and if the selection parameters needed to be made more or less stringent. The assay is carried out by combining radiolabeled aptamer with target, or without in the case of a background assay, and passing it over a nitrocellulose/nylon filter sandwich. This is then washed with binding buffer to remove unbound or weakly bound species.

During the course of the selection, it was necessary to repeat round 4. This was because the round 4 originally selected displayed a marked increase in background (filter) binding affinity (data not shown). Round 3 RNA was then subjected to 2 negative selections and the selection was continued with one negative selection per round. Round

6 also displayed a marked increase in filter binding, but instead of reselecting this round the decision was made to subject the next round, Round 7, to 2 negative selections. This was because although background increased, there seemed to be a higher signal to noise ratio than was present when Round 4 was reselected. Additionally it was thought that if there was no improvement then it would be simple enough to return to Round 5 RNA and reselect from there. A summation of the amount of RNA to protein and the negative selections done to each round is given in Figure 2-3

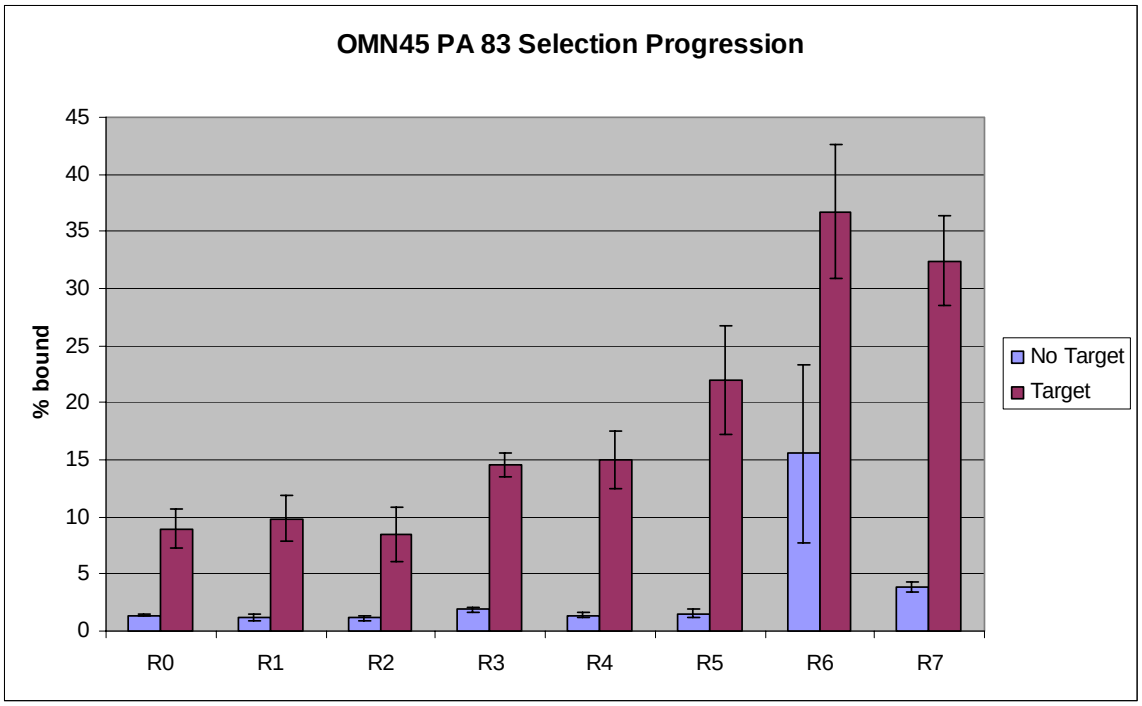

Figure 2-2. Progress of anti-PA 83 aptamer selection. 2 pmols of radiolabeled pool RNA was combined with 10 pmols of target and allowed to incubate for 30 minutes. The binding reaction was then passed over a nitrocellulose/nylon filter sandwich and washed 3 times with binding buffer. Filters were then exposed to a phosphorimage screen and radioactive counts quantitated.

Round	Negative	RNA:Protein
0	0	500:500
1	0	500:500
2	1	500:500
3	1	500:500
4	2	500:500
5	1	200:200
6	1	200:200
7	2	500:500

Figure 2-3. Summation of negative selections and RNA to protein ratios in anti-PA 83 selection

No further rounds of selection were performed following Round 7 as the background binding between round 6 and 7 decreased by 12% while the binding of the pool to PA 83 only reduced 4 % (Figure 2-2). Thus it was determined that while it may be possible to further increase binding, there was good differentiation between filter binding and target binding. Additionally the Round 7 pool had been enriched for binding PA 83 over that of the unselected pool.

Identification of Individual Aptamers Binging to PA 83

Round 7 dsDNA template was cloned and 39 sequences were successfully obtained (Figure 2-4a). Although attempts were made to group these sequences into families based upon similar groupings of nucleic acid residues (motifs) (Figure 2-4b), there was not a great deal of homology between clones. A possible reason for this lack of sequence homology is that the selection was terminated prematurely. Had more rounds of selection been carried out, then it is probable that there would have been less

diversity, and thus a greater probability of clustering individual sequences into defined familial motifs.

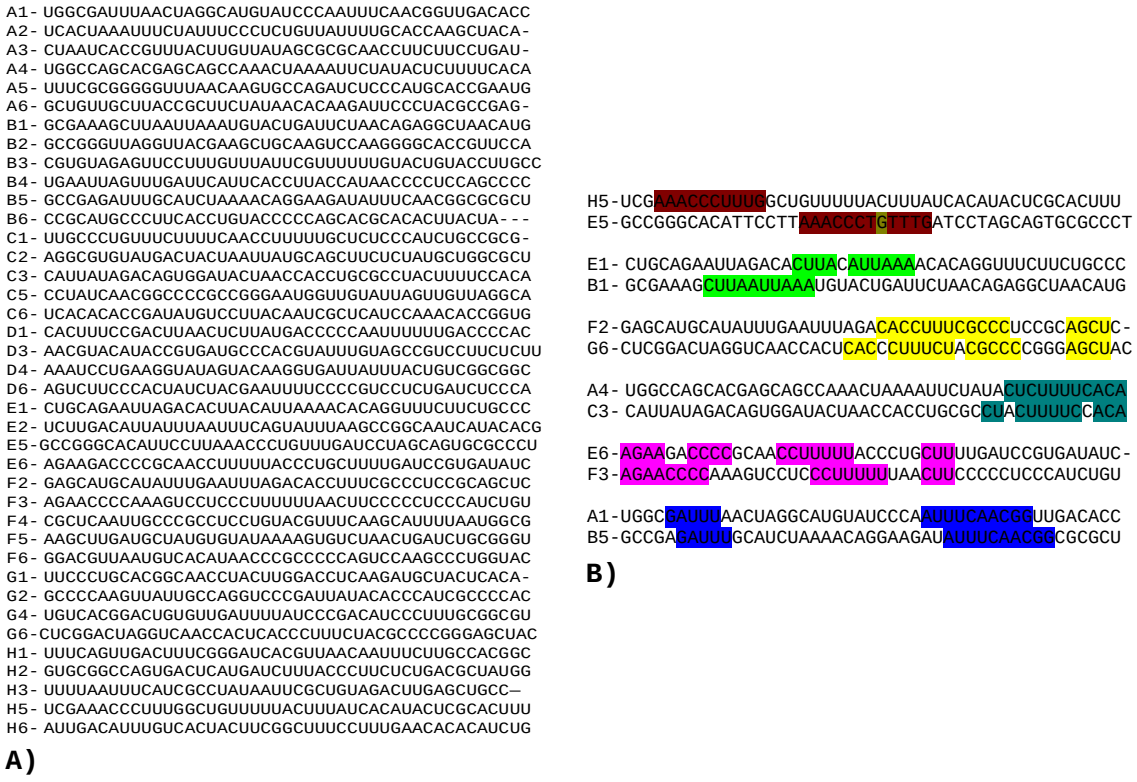

Figure 2-4. Sequences of clones derived from Round 7 of the anti-PA 83 selection. dsDNA from Round 7 was ligated into a cloning vector and transformed into a competent cell line. Following transformation, colonies were selected and subjected to colony PCR. The dsDNA template generated was then sequenced using standard dideoxy methods by the ICMB Core Facility (University of Texas at Austin)

Individual clones were assayed for ability to bind PA 83. Results of these assays are shown in Figure 2 –5. Only those clones displaying binding affinity (including tolerance determined by error bar analysis) higher than or equal to that of Round 7 pool RNA were selected for further experimentation. Additionally, any clone exhibiting an affinity (again as determined by error bar analysis) for the filter greater than that of Round 7 RNA was dismissed from further experimentation.

It was somewhat interesting to note that although the majority of the sequences did not fit the above criteria used to determine which clones would be subjected to further experimentation, only one of the clones displayed an affinity for PA 83 lower than that of the Round 0 pool. This attests to the ability of *in vitro* selection to quickly and efficiently rid a population of all those members not displaying the desired characteristic, in this case ability to bind PA 83. Additionally, filter binding for many of the clones was also low, being at or below Round 7. While some of the retained clones do display an increased affinity for the filter, this is not unexpected given that Round 6 of the selection bound the filter to a very high degree and there is only 2 negative selections separating Round 6 from Round 7.

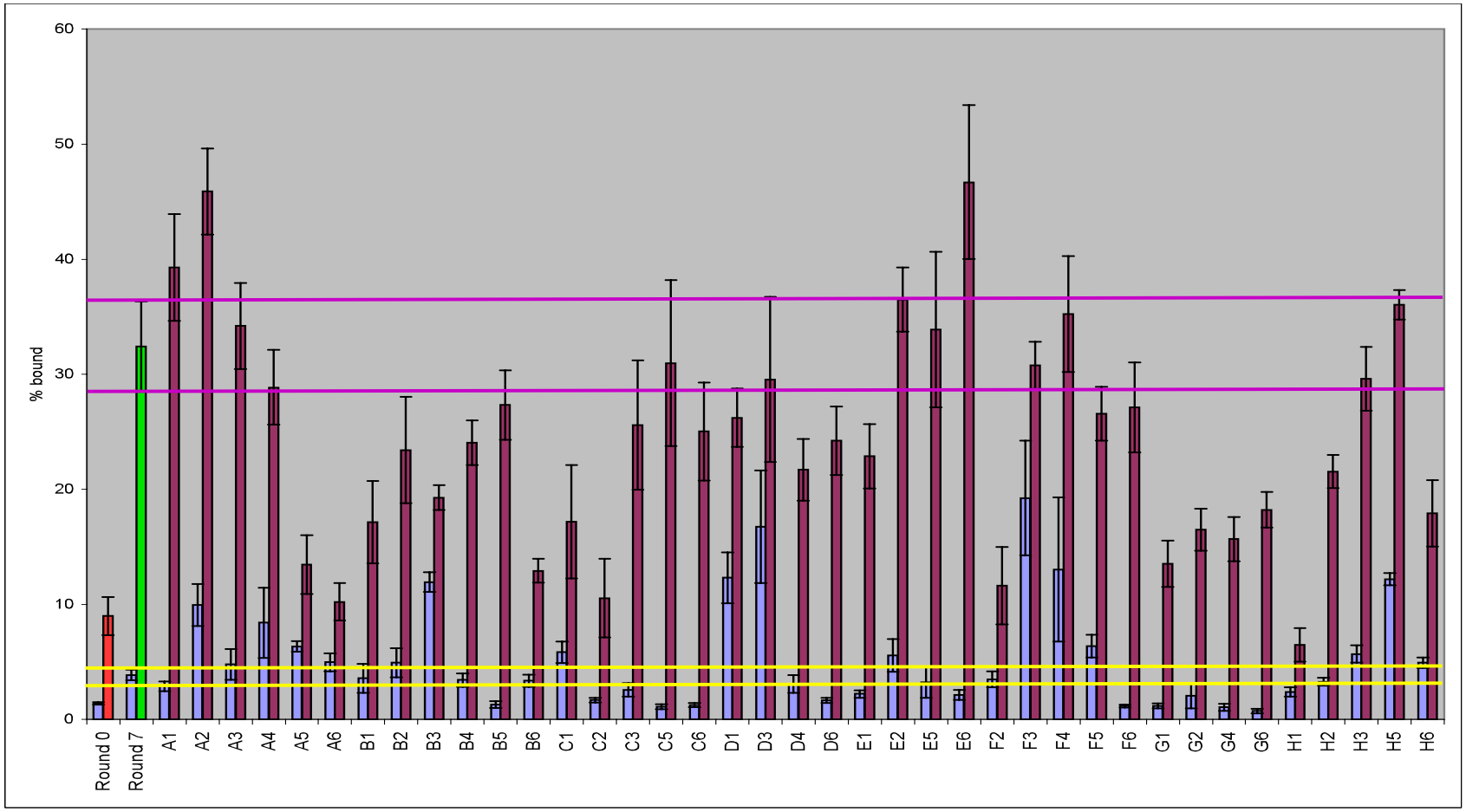

Figure 2 - 5 Binding assay for individual clones obtained from sequencing Round 7 of the anti-PA83 selection. The clones below are grouped according to arbitrary placement on a sequencing plate. Round 0 binding to PA 83 is shown in orange. Round 7 binding to PA 83 is shown in Green. For all other clones, red represents percent binding of the clone to PA 83, while blue represents binding of the clone to the nitrocellulose filter. Only those clones displaying an affinity for PA 83 greater than or equal to Round 7 (those falling in between the purple bars), while simultaneously displaying an affinity for the nitrocellulose filter lower than or equal to Round 7 (between the yellow bars) were selected for further analysis.

Selected Clones Bind PA 83 with High Affinity

The interactions between selected clones and the PA83 target were further investigated. Using the parameters established above, 7 clones were assayed for the ability to bind varying concentrations of PA 83. These assays were performed in the same manner as were the binding assays described above. The only difference being the varying amounts of PA 83 used in the binding reaction. It was found that best clone, E6, bound with a Kd of 238 ± 61 nM (Figure 2-6). This is not unexpected as clone E6 also displays the highest binding affinity in the initial assay used to determine which aptamer clones were candidates for further analysis. This clone also binds the filer in approximately the same way as the Round 0 unselected pool, indicating that it has no greater affinity for the separation matrix than does random 2'-fluorine pyrimidine substituted RNA. A summary of the other selected clones, and their respective Kds is given in Figure 2 –7. Although the calculated disassociation constants for these aptamers are not as low as would be hoped for in an aptamer selection directed against a single protein, it must be remembered that this is a negatively charged target. There is therefore some electrostatic repulsion between the pool, which is also negatively charged, and the target. Because of this it may be that a truly low disassociation constant cannot be achieved.

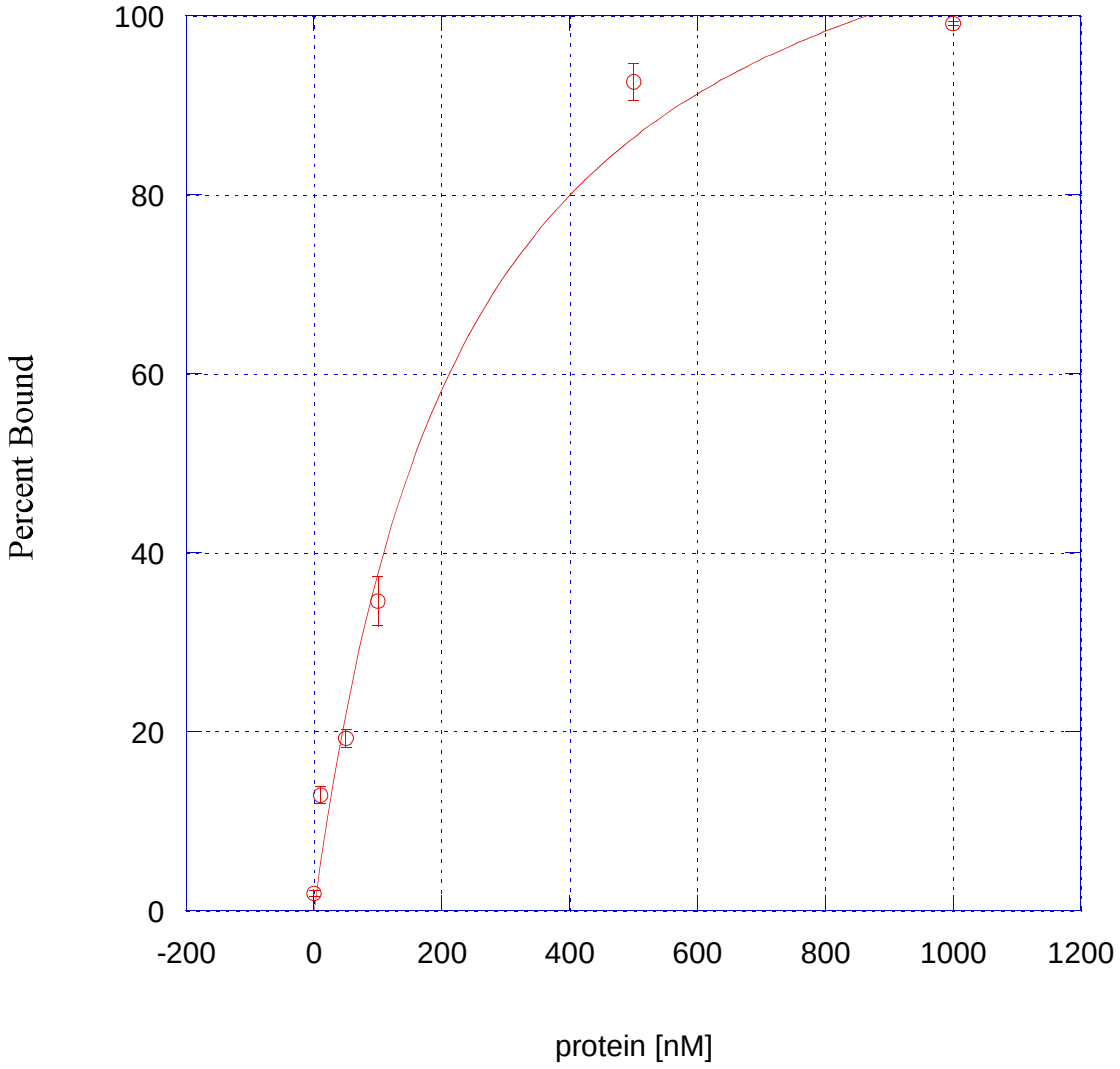

Figure 2-6. Disassociation constant determination for clone E6. Different concentrations of PA 83 were mixed with 2 pmol of body labeled clone E6. The binding reaction was allowed to come to equilibrium over 30 minutes and the binding reaction was then passed over a filter to separate bound from unbound species. This curve was fit assuming a single binding site for the aptamer on the protein. Binding affinity on the Y-axis refers to percent bound. Kd was calculated to be 238 ± 61 nM

Interestingly enough, only 4 of the selected aptamers were initially grouped based on potential motifs. Only two, A1 and B5, were actually in the same predicted family. The other potential family members for E6 and H5 (Figure 2-4b) displayed very high background binding. The other 3 clones (A3, C5, and F6) were unable to be grouped with any other similar sequences. Thus it must be considered that the aptamers originally grouped based on potential familial ties, are in fact not related to each other. Further

Clone	Kd
E6	238.08 ± 61 nM
B5	244.84 ± 64 nM
A3	593.56 ± 240 nM
F6	605.98 ± 300 nM
E5	890.16 ± 509 nM
C5	1062.6 ± 457 nM
A1	6214.7 ± 377 nM

Figure 2-7. Calculated disassociation constants for selected clones. Kds for these aptamers were determined through filter binding assays. Binding affinities were plotted on KaleidoaGraph software and disassociation constants determined through a curve fitting algorithm

evidence for this comes from looking at the two aptamers which can be placed, A1 and B5. These two clones display vastly different disassociation constants. When folded using the RNA folding program M-fold (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/) these sequences bear no resemblance to each other (Figure 2-8). One would expect a similar predicted secondary structure if these aptamers were truly related. At the least there should be a commonly formed structural element. The only true similarities are on

the 5' region formed by the constant region, which is not unexpected. Although this portion may play a role in binding, it would be more convincing if a common structural element could be found in the random region, indicating selectivity for that element. The only thing resembling a common element in these two structures is that the bases surrounding position 40 appear to be involved in a protruding loop structure; however the surrounding elements are very different from each other.

There are two potential explanations for this inconsistent performance. One, the positioning of the motif within the random region is critical for optimum performance. For instance, in A1 the GAUUU sequence is shifted one base over while the AUUUCAAACGG sequence is shifted 2 places over when compared with clone B5 (Figure 2-4b). There are examples in the literature indicating that proper positioning of certain bases in an aptamer are imporant for function (Gal, Amontov et al. 1998; Robertson, Harada et al. 2000; Sekiya, Nishikawa et al. 2003; Carothers, Davis et al. 2006) .

The other potential explanation is that while not closely related, the two families are distantly related. It could simply be random chance that these clones display these identical base runs, and this is a plausible explanation when it is considered that of all 39 clones these are the only two displaying these sequences in that approximate position in the random region. However, it must be noted that A5, H1 and H3 (Figure 2-4a) also display a portion of this motif (GAUUU) at the beginning of the random region; however the first two bases, GA, are found in the constant region. This seems to lend support to the notion that this is a motif that is in the process of being selected. The binding affinities of those clones show that two of the clones, A5 and H3, display binding affinity greater than Round 0, and H3 displays an affinity approximately that of Round 7 (Figure 2-5). H3 also displays elements of the second motif found in A1 and B5, although in a

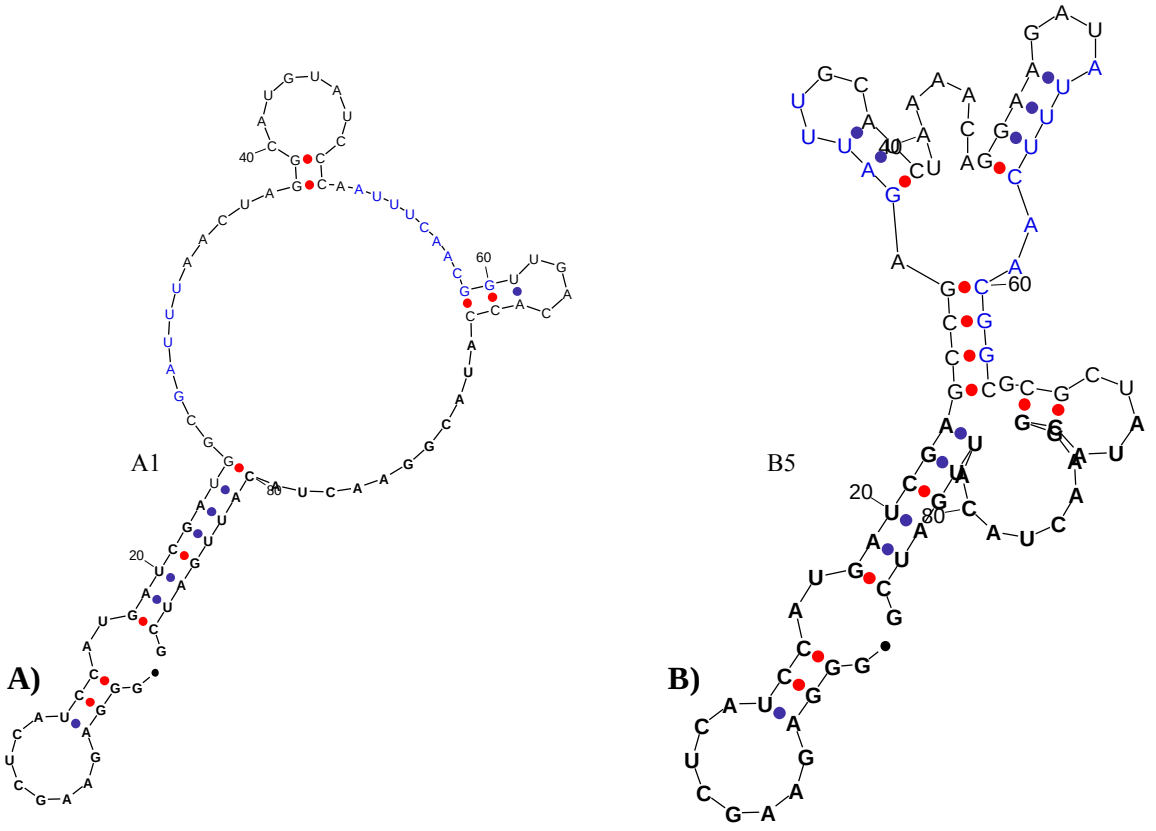

Figure 2-8. Comparison of aptamer clone predicted secondary structures. The RNA folding program, M-Fold, was used to determine the most likely structure these clones assume upon folding. Bases in blue represent identical bases in similar positions on the two clones. Primer binding regions are in bold. A) clone A1 B) clone B5

very different position. Taken all together, this information indicates that these may be favored motifs for binding, and should more selection pressure be applied then perhaps these motifs, in some position, would be found more prevalent in the selection.

Clones A1 and B5 were used as examples to consider what is potentially a structural motif in this selection because they were the only pre-analysis grouped sequences where both members met the criteria established for further analysis. Because of this they could be directly compared. That they have very different properties only

makes the discussion more interesting. However, the same conjecture on what is or is not a motif could be applied equally across all those sequences that were selected for further examination. Ultimately the difficulty in such a discussion lies in that a five base run is barely a motif, and can only be considered as one when placed in the same position along with another conserved sequence element. The fact there is are not any such wholly conserved sequence runs in these sequences prevents anything other than broad based theoretical discussions. As aptamer selections tend to generate very few similar sequences from what is a very diverse starting population, this retention of diversity, characterized by absence of prevalent sequence motifs, may again provide evidence that the selection was halted prematurely.

Aptamer clone E6 is able to delay PA 83 cleavage by Furin.

As clone E6 displays both the highest individual binding affinity for PA 83 and the lowest overall Kd, it was chosen as a candidate to inhibit the cleavage of PA 83 by furin. The cleavage recognition site for Furin is Arg-Xaa-(Lys/Arg)-Arg (Molloy, Bresnahan et al. 1992). As this is a positively charged amino acid sequence it was thought it could be a good potential binding site for the negatively charged aptamer. Aptamer was added in a four fold excess over PA 83 and allowed to bind. Following binding, a time point 0 was taken and furin was then added. Additional time points were taken at various intervals and the results are shown in Figure 2-9. E6 is able to prevent significant accumulation of cleavage product (PA 63) for 9 minutes (Figure 2-9c). At the 12^{th} minute, the intensity of the PA 63 band increases from what it was at time 0. This intensity remains approximately the same until the 45^{th} minute. The amount of PA 83 at the 45^{th} and 60^{th} minute time points are also the same, perhaps indicating the protease

loses functionality somewhere between 30 and 45 minutes. In the furin and PA 83 only control (Figure 2-9a), immediate degradation of PA 83 is observed at 1 minute with complete degradation achieved by 3 minutes. This is very similar with the furin, PA 83, OM45 pool control (Figure 2 –9b). Although here it appears cleavage of PA 83 is not complete until the 6th minute. For the furin only, and furin/pool experiments, time points were stopped after 45 minutes because it appeared as though degradation was complete by then. Taken together, these data indicate that aptamer E6 is able to convey some resistance to PA 83 from furin. Through this ability, aptamer E6 may be adaptable for use as a therapeutic.

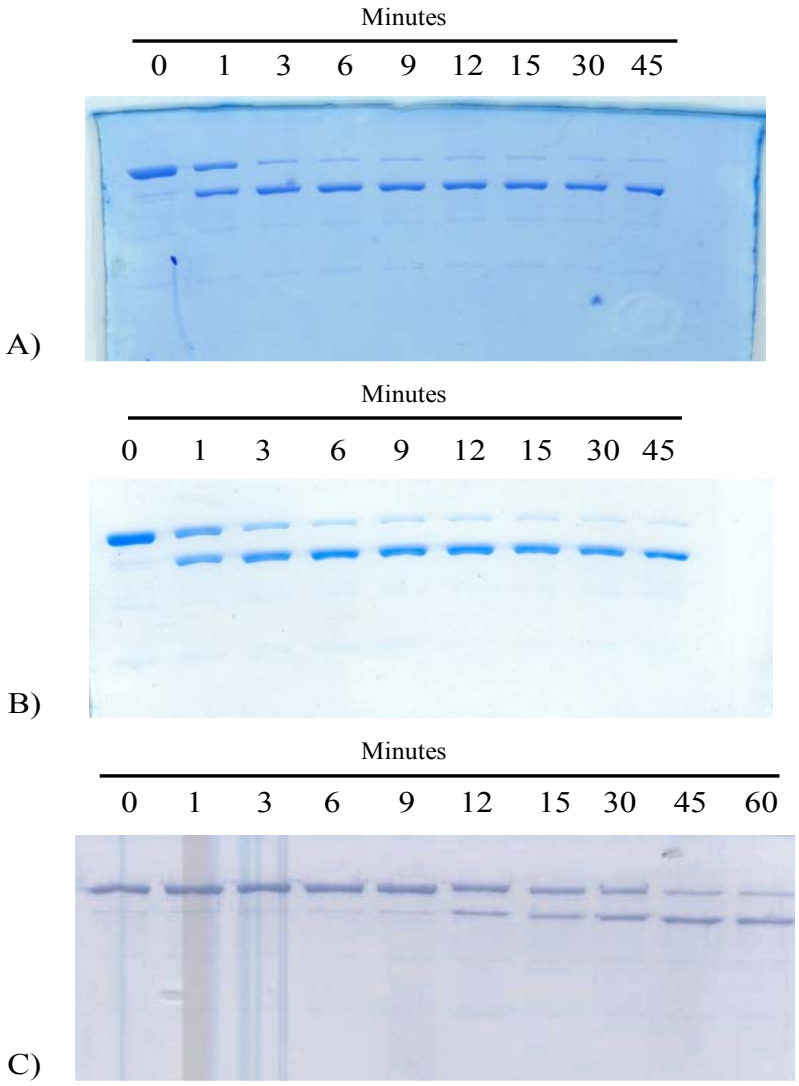

Figure 2-9. Furin cleavage inhibition by aptamer E6. RNA was heat denatured and allowed to refold in the prescence of binding buffer. PA 83 was then added and allowed to bind. Upon completion of binding, furin was added and time points taken. A) PA 83 with furin alone. Cleavage appears to be complete after 3 minutes. B) Furin cleavage of PA 83 in the presence of unselected pool RNA. Non-specific RNA was added in a four fold excess over PA 83. This does not appear to significantly affect the ability of furin to cleave the protein of interest. C) Furin cleavage of PA 83 in the precence of a four fold excess of aptamer E6. Cleavage appears to be completely delayed until 12 minutes. Following 12 minutes, the cleavage rate is still reduced with a significant portion of PA 83 still present at 30 minutes. Numbers above all 3 parts refer to minutes following addition of furin.

CONCLUSIONS

A successful aptamer selection was carried out utilizing PA 83 as a binding target. Following 7 rounds of *in vitro* selection, the pool was cloned and individual clones were assayed for binding. Ultimately 6 clones were chosen for further experimentation. One clone, E6, was shown to bind with a Kd of 238 nM, and subsequently shown to inhibit cleavage of PA 83 by furin. This aptamer was selected with modified pyrimidine residues to convey additional stability and resistance to cleavage by RNase A. A high degree of sequence diversity was retained in the sequenced pool. A mitigating factor thought to contribute to this diversity is premature halt of the selection. However, as an aptamer able to bind with high affinity was selected from the pool, this retention of diversity is irrelevant.

B. anthracis is the causative agent of anthrax, a potentially fatal disease. The direct cause of this disease is a three part toxin, in which PA 83 plays an integral role. By developing reagents to function in either a therapeutic or detection platform, it becomes possible to lessen the threat posed by this disease. Currently there are few truly rapid and reliable means to detect anthrax toxin. Those that do are either expensive to generate, time consuming, or require specialized equipment or knowledge. By adapting the above selected aptamer to already existing technologies, it may be possible to circumvent these limitations. Additionally, by preventing the furin mediated cleavage of PA 83, a key step in toxin mode of action, this aptamer may find use as a therapeutic. *In vivo* experiments are currently underway to determine this.

EXPERIMENTAL DESIGN

Synthetic Pools and Oligonucleotides

A synthetic oligonucleotide pool containing a 45 base random region was constructed for these experiments. To facilitate incorporation of 2'-fluoro 2'-deoxy pyrimidine bases, a region of 8 purine residues was placed immediately adjacent to the T7 RNA polymerase promoter (Padilla and Sousa 1999). The two constant regions, 40.45 (5'-GATAATACGACTCACTATA*GGGAGAAG*CTCATCCATGATCGA -3') and 20.45 (5'- CGATCAATGTAGTTCCGTAT -3'), flanking the 45 base random region were used as primer binding sites for PCR amplification. The underlined region in the 40.45 primer indicates the T7 promoter, while the italicized bases indicate the purine residues necessary to efficiently incorporate sugar modified pyrimidine nucleotides. All oligonucleotides and primers were synthesized in the Ellington lab using an Expedite 8909 chemical synthesizer (PerSeptive Biosystems).

Deprotection/purification of oligonucleotides

Following chemical synthesis, the oligonucleotides were deprotected for 16 hours at 55°C in the presence of 8.55 M ammonium hydroxide. Immediately after deprotection, 1-butanol was added to the deprotected DNA in ammonium hydroxide. This solution is placed at -80°C for 1 hour, and then precipitated by centrifugation at 15,000 x g for 45 minutes. The resulting pellet was washed with 1 mL of cold 95% ethanol and centrifuged as above for 5 minutes. The remaining ethanol allowed to evaporate. The pellet is then resuspended in 200 microliters of TE buffer (Tris pH 8.0, 1 mM EDTA). 200 microliters of 2x denaturing dye (1x TBE, 0.1% bromophenol blue, 7M urea) is added to the DNA and subjected to denaturing (7M urea) polyacrylamide gel

purification. A 6% gel was used for pool purification while a 10% gel was used for primer purification. Electrophoresis was carried out using 1x TBE (89 mM Tris base, 80 mM Boric acid, 1 mM EDTA pH 8.0) as the running buffer. Synthetic DNA was located through UV shadowing on a TLC plate, and only full-length bands were excised. The DNA was then eluted overnight in TE buffer at 37°C. DNA was recovered through standard ethanol precipitation.

Double Stranded DNA Pool Generation

The concentration of ssDNA following recovery from gel purification was determined to be 72 pmol/microliter by measuring absorbance at 260 nm. This equates to 18000 pmol of ssDNA (72 pmol/microliter * 250 microliter), or 1 x 10^{16} unique sequences. Reverse transcription (RT) containing 1 microgram of ssDNA, 75 pmol 40.45, 2 mM dNTP, 10 mM DTT, 5x First Strand buffer (Invitrogen), 200 U SuperScript II Reverse Transcriptase (Invitrogen) with 5' radiolabled 40.45, was carried out according to manufacturers instructions. This step is to determine the amount of extendable ssDNA. Radiolabel was incorporated through T4 Polynucleotide kinase (Invitrogen) in 5x forward reaction buffer (Invitrogen) with γ-P^{32}-ATP (ICN radiochemicals) as the radiation donor according to enzyme manufacturers instructions. Using this protocol, it was determined that 6% of the pool was extendable (6.5 x 10^{14} molecules). One quarter of the pool able to be fully extended (270 pmol) was amplified. Large scale reverse transcription (LS-RT) containing 4500 pmol ssDNA, 9024 pmol 40.45, 6000 units SuperScript II Reverse Transciptase in a 600 microliter final volume (final concentrations of dNTP's, DTT, and buffer is unchanged) was carried out as would a normal reverse transcription (according to manufacturers instructions) with the

following revisions. Heat denaturation of starting template was done at 70°C for 30 minutes, 4° cooling step subsequent to addition of buffer and DTT was done on ice for 45 minutes, preheating of reaction mixture prior to addition of enzyme was done for 15 minutes at 42°C, and finally the enzymatic reaction was allowed to run for 3 hours at 42° C. Double stranded DNA product was recovered through ethanol precipitation and the resulting pellet resuspended in 100 microliters.

RNA Pool Generation

Large scale transcription was carried out using the entire resuspended double stranded DNA (from the LS-RT step) as a template. Final transcription reaction contains 1x buffer (40 mM Tris-HCl pH 8.0, 30 mM $MgCl_2$, 20 mM spermidine), 10 mM DTT, 1.5 mM NTP final concentration, and 4 x 10^{-5} M modified T7 RNA polymerase. The reaction was allowed to proceed for 8 hours 30 minutes at 37°C. Ethanol precipitation was used to as a means to recover RNA. The resulting pellet was resuspended in 350 microliters of ddH_2O and combined with 50 microliters of 10x DNase I buffer (Invitrogen) and 5000 – 37500 units DNase I (Invitrogen) in a 500 microliter final volume. Reaction was allowed to proceed at 37°C for 30 minutes. The RNA pool was then purified on a 8% denaturing polyacrylamide gel, imaged, excised, and recovered as above. Using A_{260} readings and Beer's law, it was determined that 14.6 copies of the initial pool had been generated.

***In vitro* Selection**

Seven rounds of *in vitro* selection were carried out against PA 83 (List Biological). Five hundred thirty two pmols of pool RNA (2 copies) was used to initiate

the selection. RNA from the previous round (or initial RNA) is placed into a 1.5 mL Eppendorf tube with 10x PA83 Binding Buffer (300 mM MES pH 6.0, 200 mM NaCl, 10 mM $MgCl_2$, 10 mM EDTA) and ddH_2O to a final volume of 50 microliters. The RNA was then heat denatured at 70°C for 3 minutes, and then cooled to room temperature for ~ 10 minutes. Equimolar amounts of PA 83 is combined with 5 microliters of 10x PA83 Binding Buffer and added to the RNA containing tube to make a final volume of 100 microliters. Binding is allowed to proceed for 30 minutes at room temperature with rotation. After 30 minutes the binding reaction]was passed over a 0.45 uM nitrocellulose containing filter (Millipore) pre-wet with 1x PA83 Binding Buffer. The filter is then washed 3 times with 500 microliters ofs 1x PA83 Binding Buffer. The filter is then placed in a 1.5 mL Eppendorf tube and incubated with 200 microliters of Elution Buffer (100 mM NaOAc pH 5.2, 7 M urea, 1 mM EDTA) at 100° C for 5 minutes. The buffer is transferred to a clean 1.5 mL tube and the elution step repeated. Following completion of the elution, the binding reaction is phenol/chloroform/isoamyl alcohol extracted. The aqueous phase was retained and the RNA was ethanol precipitated using glycogen as a carrier. The pellet is used as a template for RT as above with the exception that 1000 pmol 20.45 is used as the primer. The RT reaction is placed into a 2 mL PCR reaction containing 10x PCR buffer (Tris-HCl pH 8.0, 500 mM KCl, 10 mM $MgCl_2$), 400 pmol 40.45, 400 pmol 20.45, 0.2 mM dNTPs (final concentration), and 50 units Taq DNA polymerase (NEB). PCR cycle courses are run to determine the optimal number of cycles per round. Cycles are run on a 4% agarose gel in 1x TBE to elucidate the correct number of cycles. Remaining PCR mix is cycled according to the cycle course determination. Once PCR amplification is complete the contents of the individual tubes were pooled, ethanol precipitated, and quantitated on a 4% agarose gel using a quantitation standard. Transcription is carried out as above except 1 microgram of

dsDNA is used as template and the reaction is run for 6 hours. Additionally RNA is ethanol precipitated, resuspended, and subjected to DNase I treatment. RNA is then purified on a 8% denaturing polyacrylamide gel, excised, eluted, and precipitated as above. Following round 1, all subsequent rounds were subjected to negative selections prior to addition of PA 83. Amounts of RNA and target, in addition to number of negative selections per round can be found in Figure 2-3.

Cloning and Sequencing

Round 7 RNA was chosen for sequencing as it gave the best ratio between the binding of the RNA protein target and the nitrocellulose background. dsDNA from round 7 pool was ligated into pCR 2.1 cloning vector (Invitrogen) using manufacturers protocol. Ligation product was used to transform TOP10 competent cells (Invitrogen) expressing an ampicillian resistance gene on the plasmid. Colonies were grown overnight on ampicillian containing LB plates pre-spread with X-gal at 37° C. Bacterial colonies for PCR amplification were chosen based on results of a blue/white screen. Selected colonies were PCR amplified using the M13 Forward (5'-GTTTTCCCAGTCACGA-3') and M13 Reverse (5'-GTTTTCCCAGTCACGA-3') primers. Following amplification, unused primers and dNTP's were removed from the reaction using a Montage PCR clean up plates (Millipore). PCR products were quantitated on a 4% agarose gel and prepared for sequencing. Standard dideoxynucleotide sequencing was done utilizing the M13 reverse primer (ICMB DNA sequencing facility, University of Texas, Austin, TX). Resulting sequences were tested for binding affinity.

Filter Binding Assay

Filter binding assays were performed after every round and for the initial starting pool. Round 0 pool was labeled using T4 polynucleotide kinase (Invitrogen) as above with the exception that the 5x exchange buffer was utilized. For all other rounds, RNA was body labeled during the transcription step through addition of α-P^{32} GTP (Perkin Elmer). Regardless of labeling method, RNA was gel purified and processed as above. Two pmols of labeled RNA was incubated with 10 pmol PA 83 in binding buffer for 30 minutes. Binding reactions were then vacuum filtered on a Minifold I 96-well dot-blotting array (Schleicher and Schuell) and washed 3 times with 500 microliters ofs 1 x Binding buffer. Contained within the 96 well dot-blotter is a pure nitrocellulose filter (Schleicher and Schuell) on top of a Hybond nylon (Amersham Biosciences) filter. Following washing, the radioactive RNA retained on the filters are quantitated on a phosphorimager (Molecular Dynamics) and percent bound determined by the following formula: (nitrocellulose)/ (nitrocellulose + nylon)*100. Control reaction containing no protein were run in parallel to determine the amount of RNA binding to the nitrocellulose filter.

Kd Determination

Disassociation constants for selected clones were determined using filter binding assays as described above. Binding reactions were done with 2 pmol body labeled RNA and varying amounts (0, 10, 50, 100, 500, 1000 pmol) of PA 83. Following quantitation of binding affinity, disassociation curves were plotted using a curve fitting algorithm and the software Kaleidagraph (Adelbeck Software, Reading, PA).

Furin Cleavage Assay

Furin cleavage assays were performed as follows. Four hundred pmol of aptamer was incubated with 10 microliters of 10x binding buffer supplemented with 1 mM $CaCl_2$. The RNA was heat denatured and allowed to cool as above. One hundred pmol of PA 83 was added to the aptamer and allowed to bind for 30 minutes. Upon completion of binding, a sample 0 was taken. Immediately thereafter, 6 units of recombinant human furin (Sigma) was added and samples were taken at 1, 3, 6, 9, 12, 15, 30, and 45 minutes post addition. For the aptamer containing experiment an additional time point at 60 minutes was taken. After removal from reaction mixture, samples were immediately placed in 5x SDS loading dye (250 mM Tris pH 6.8, 2.5 % SDS, 50 % glycerol, 0.001 % β-mercaptoethanol, 0.0002 % bromophenol blue) supplemented with 1 microliters of 100 % β-mercaptoethanol and a 2.5 mM final concentration $ZnCl_2$. Samples were electrophoresed on a 12 % SDS gel and stained with Simply Blue Safestain (Invitrogen). No aptamer and Round 0 RNA controls were also performed.

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CHAPTER 3: *In vitro* selection of anti-*B. subtilis* aptamers

INTRODUCTION

B. subtilis as a target

B. subtilis was thought to be a good potential target for aptamer selection for three primary reasons. The first is that it is in the same genus as *B. anthracis,* although not as closely related as some other members of this genus. Since 2001, the threat of an attack using biological weapons (spores) derived from *B. anthracis* has been raised. It is an easily grown bacterium that naturally produces lethal spores as a part of its life cycle. These spores have the potential for easy dispersion, are very hardy, and are not difficult to purify. Although a selection targeted against vegetative cells is unlikely to produce an aptamer that will detect spores, there is the possibility that spores will retain vegetative cell material on their exteriors. A so called "dirty prep" of spores would be the most likely spore preparation used in a biological weapons attack. While it is also true that aptamer selected against *B. subtilis* will most likely not recognize *B. anthracis* vegetative cells, it will serve as a nice proof-of-principle experiment.

The second reason for choosing *B. subtilis* is because it is the most extensively studied member of this genus. Indeed, *B. subtilis* has come to be regarded as the experimental model for research into Gram-positive bacteria. It was thought that this collective research would prove to be invaluable for the successful completion of this project, for the purposes of both starting the selection and evaluating the results upon completion. Additionally, various strains, including those that have been genetically modified, are readily available. The *Bacillus* Genetic Stock Center houses many of these genetically modified strains in a centralized location.

The final reason for selection of *B. subtilis* is the ease of which it can be grown. It is amendable to relatively wide temperature ranges. Although an aerobe, oxygen can be present in lower than ideal concentrations and still support growth. There are also several different media formulations using different carbon sources that have been shown to support growth. Most importantly, however, is that growth is in a predictable and reproducible manner, implying that whatever is displayed on the surface should be relatively unchanged from culture to culture.

B. subtilis vegetative cell surface

The surface of a *B. subtilis* vegetative cell is a complex and dynamic mixture of protein, glycoprotein, carbohydrates, anionic polymers, lipids, and peptides (Harwood 1990). These types of molecules are found loosely organized into stratified layers on the outer surface. These layers include a capsule, cell wall, S-layer, and a cell membrane (Figure 3-1). Also, as *B. subtilis* is a spore forming bacteria, there will be spores and lysed cells present in any given culture. Finally there are motile flagella also present on the exterior of the vegetative cell. Any and all of these superstructures are capable of serving as suitable targets for aptamer selection.

The remainder of this chapter will detail efforts to identify aptamers capable of binding to the surface of *B. subtilis* vegetative cells. Once identified, aptamers are shown to bind vegetative cells using two different assays. Additionally, it will be shown that selected aptamers are capable of recognizing a strain of *B. subtilis* other than the one which was used in the selection. Structural characterizations will then be carried out, and predicted secondary structures will be analyzed.

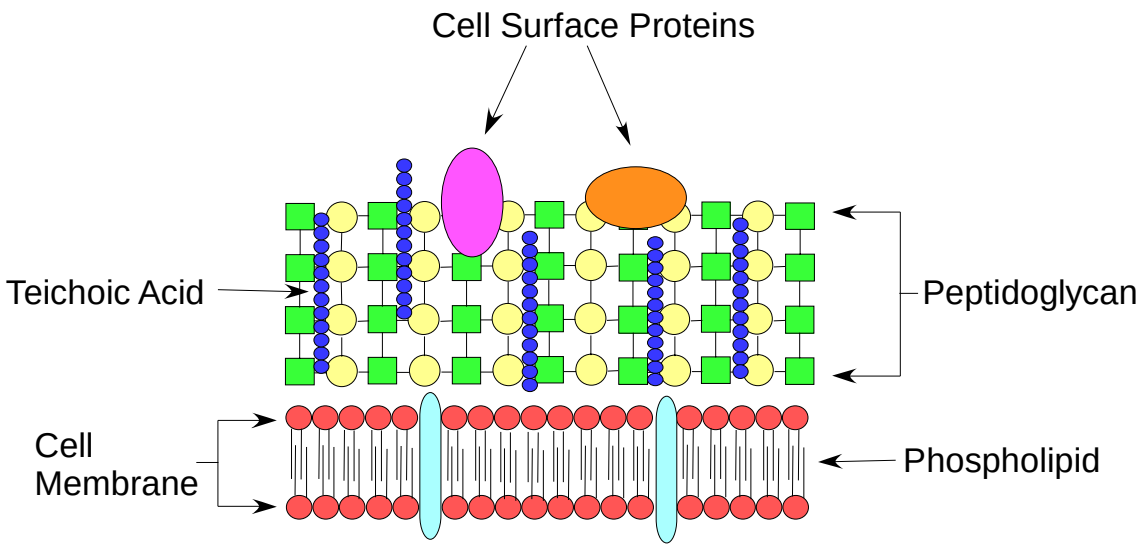

Figure 3-1. Diagram of the composition of *B. subtilis* extracellular matrix

RESULTS AND DISCUSSION

In vitro selection of anti-Bacillus subtilis aptamers.

Bacillus subtilis vegetative cells were grown to an optical density between 0.5-0.6 when measured at A_{600}. Following growth, cells were washed twice with binding buffer. 1x PBS supplemented with $MgCl_2$ was chosen as the binding buffer because it was deemed to be a suitable media for working with bacterial cells. After washing was complete, a modified RNA pool (~10^{14} variants) composed of 2'fluoropyrimidine residues to enhance stability and containing a 42 base random sequence region was applied to the cells. The pool was allowed to equilibrate with 10^7 *B. subtilis* vegetative cells for 30 minutes. Binding species were sieved from non-binding or weakly binding

species by passage over a nitrocellulose containing filter followed by washing with binding buffer. To prevent the accumulation of filter binding species, a negative selection was performed prior to addition of target on every round following the first. Selected RNA molecules were amplified by a combination of reverse transcription, PCR, and *in vitro* transcription. After each round of selection the pool was assayed for its ability to bind *B. subtilis, E. coli,* and the nitrocellulose containing filter (Figure 3-2). Following seven rounds of selection it was determined that round 5 gave the best ratio of *B. subtilis* vegetative cell binding to background binding. To ensure binding was specific for *B. subtilis* and not for bacterial cells in general, pool RNA was tested against *E. coli* cells to ensure that the aptamer pool was not binding bacterial cells indiscriminately. The binding in these assays was negligible, being at or slightly above background.

Thirty six individual aptamer sequences were cloned and sequenced (Figure 3-3a). These sequences were analyzed for the presence of a consensus sequence. It is interesting to note that the majority of cloned sequences contained a preponderance of pyrimidine bases. This may be a reflection of evolutionary pressure driving the incorporation of RNase resistant bases to protect the RNA strand from RNase degradation. It would not be unexpected to find some amounts of various RNases present in cell culture due to either autolysis of bacterial cells, or as secretion products from the bacteria themselves. The analyzed aptamers were grouped into 5 potential families on the basis of sequence homology (Figure 3 –3b). There is an apparent lack of sequence homogeneity in the clones sequenced. However, this can be explained by the relatively low number of rounds performed and the complexity of the target.

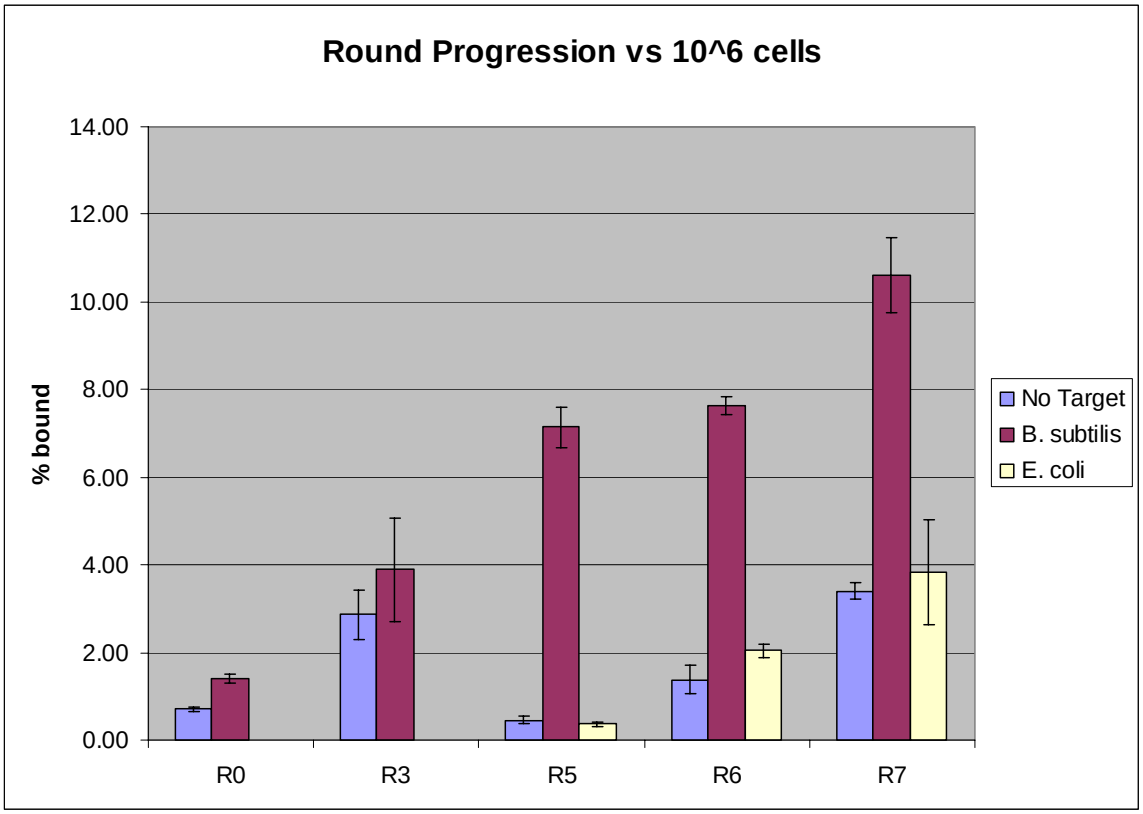

Figure 3-2. Progress of the anti-*B. subtilis* selection. 2 pmols of radiolabeled pool RNA was incubated with 10^6 B. subtilis vegetative cells. Following binding, the cells were passed through a nitrocellulose/nylon filter sandwich on a 96-well dot blot manifold and washed with binding buffer. Filters were then exposed to a phosphorimage screen and retained radiation was quantitated.

Filter binding assays

To determine the optimal binding species, individual members of the families were tested for their ability to bind *B. subtilis* vegetative cells. On the basis of filter binding assays, where the concentration of target cells was reduced to 10^6, ten fold lower than during selection, three individual aptamer sequences (B6, C3, C5) showed high affinity binding and were selected for further study (Figure 3-4). These three clones also displayed an affinity for the filter equal to or less than that of the unselected pool

(MN42). The concentration of bacteria was reduced to allow the use of a 96-well dot blotting filter. This enables many clones to be screened at once, but flow rate is limited, and if too many bacterial cells are used in the binding reaction then the filter will become blocked. This prevents accurate values for binding affinity from being obtained. Clones showing filter binding in excess of target binding, or above that of unselected pool were disregarded. Examples of clones deemed unsuitable for further experimentation are also given in Figure 3-4.

A)

```
R5-Bs-A1-UCCCUAGGAUCGCCCGUUUUACUGUUUNACCUUACCAUUGG-
R5-Bs-A3-CUUGCUCUUGAUCGAGUCCUCUUCACUCAUAAUCCACACCCA
R5-Bs-A4-GAGAAGACCGGCCGCUAUACUAAUACGUUUCCUAACAGGGCA
R5-Bs-A6-GCCGGACCGAUGAUAUCUAUUGCACUUCGCCAGCUUUACACAG
R5-Bs-B1-UUACACUCUUCCGACCGGUAUUAUAUGGCCCAGCAUGUGCA-
R5-Bs-B2-UUGUGUGUGUAUCUUCUACCUCUGUCCACGAUACCCGCUCGC
R5-Bs-B3-CUCGACGUCGCUUAAUUACCCGUCCUACAUACAACCGGACAG
R5-Bs-B4-ACUACCCAUUUUACUACUUCUGCUACGCCCUCCACUCCGCCA
R5-Bs-B6-ACUGACAAGCGCUACACUUGCUCCCCCGUUCUGCGCCGCUUA
R5-Bs-C1-UAACAGCGCUCACAAAUCAUUAACUCUCUGUCCUUCCCACCA
R5-Bs-C3-AGGGUGGGCGAACGAUAACAAACGACUAUUAACUCUUGGGCG
R5-Bs-C4-CCGUGCUUGUUGUGCUUAACUGUCUCUUUAUGACUACUUACG
R5-Bs-C5-CACCAUGAUUCAAACCAGUGCGUCCCUAUUACUCUUUCUCUU
R5-Bs-C6-UAGCCGCCUUGUUAUUCCUCAUCUUUUAACUCUCUAGGCCG-
R5-Bs-D1-CUUAAACCCACUAUCACAACUUGAGAUUCACAUAUAGCGCCG
R5-Bs-D2-ACCCGCCUCAAUCAUCUUUAUUCCUUAUUACCGUAUUCAGCG
R5-Bs-D3-UUGAUGACUAAAUCUUUACUCCUUACAUCCUUGGCCUCUGCC
R5-Bs-D5-AUCAGGCCCCGGCUGAACGAUCUCUUCGUCUAUAAUUACCUG
R5-Bs-D6-CGCAGUCCAUUCUUCACUUCCCACUGACAGCCCACGUACACCG
R5-Bs-E1-UAGCUUUGAUAUACCGAUAUCCAACCUUACACUUUUCCCGCA
R5-Bs-E3-CGUGUUCUCCCUAAGGCGCCGCCAUCAAGAUCGCUACCCCC-
R5-Bs-E4-ACCUUGAAGCUUCGUUAGACUAGCCCUUCGCUUCCCAUCCCG
R5-BS-E5-ACGCCCCUACUCCUCUGACAUCGUUACCUAUUAUCCUAACUG
R5-Bs-F1-ACACUGGUGAAUGCGCCCUUAGACUCAUGAUCGAGCAUUCGC
R5-Bs-F2-GCGAUGGCCGCUCUAUGAUCAUUUGCUAUACCAUCACACUCG
R5-Bs-F4-CAACCUCUGAAACUUAAUAACUGUUUUUAACCCCUGCGCUCG
R5-Bs-F5-AUACUGCCUUCGCUUCUUCAAUCCAGGACAAUUCUCAUGCC-
R5-Bs-F6-UCUCGAACAAUAUCUCGUGAAUGUUACAACUACAAGACCCCA
R5-Bs-G2-UCGUCAUUAAUGCCUUUCCAUUGAGUUCGCGACAUGCCUGGA
R5-Bs-G5-AUCCAACAUUCAUAGCCAACUCGUUGCACACCAGCGGCGCCA
R5-Bs-G6-UCCCGAAUUCGAUUUUUUCAGUUACCAUCUCCUUAUGGCGUGG
R5-Bs-H1-GCCACCAGUUAUUCCCUUGUCACUGCUCCCUUCUACAUUCCA
R5-Bs-H2-CAUCAUUUAUCCGCAGCCCACCUUACAGGCCAUCCACCUGCA
R5-Bs-H3-GACAAACAUAGCAGAACAACUAUUCAAAUACGUGGUUGAGUC
R5-Bs-H4-CAAGACUAGAUAUCGUAGAAUUAGAAGCCUUAAUCGACGGAG
R5-Bs-H5-UUGGGACGUGUUAACGACUUACGAUAUUUCACCUCAGGGACA
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B)

Grouped Motifs

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R5-Bs-A1-UCCCUAGGAUCGCCCGUUUUACUGUUUNACCUUACCAUUGG-
R5-Bs-E3-CGUGUUCUCCCUAAGGCGCCGCCAUCAAGAUCGCUACCCCC-
R5-Bs-B4-ACUACCCAUUUUACUACUUCUGCUACGCCCUCCACUCCGCCA

R5-Bs-C3-AGGGUGGGCGAACGAUAACAAACGACUAUUAACUCUUGGGCG
R5-Bs-C5-CACCAUGAUUCAAACCAGUGCGUCCCUAUUACUCUUUCUCUU

R5-Bs-D2-ACCCGCCUCAAUCAUCUUUAUUCCUUAUUACCGUAUUCAGCG
R5-Bs-D3-UUGAUGACUAAAUCUUUACUCCUUACAUCCUUGGCCUCUGCC

R5-Bs-F4-CAACCUCUGAAACUUAAUAACUGUUUUUAACCCCUGCGCUCG
R5-Bs-C6-UAGCCGCCUUGUUAUUCCUCAUCUUUUAACUCUCUAGGCCG-
R5-Bs-B3-CUCGACGUCGCUUAAUUACCCGUCCUACAUACAACCGGACAG

R5-Bs-C1-UAACAGCGCUCACAAAUCAUUAACUCUCUGUCCUUCCCACCA
R5-Bs-F2-GCGAUGGCCGCUCUAUGAUCAUUUGCUAUACCAUCACACUCG
```

Figure 3-3. Sequences of clones derived from Round 5 of the anti-B. subtils vegetative cell selection. dsDNA from Round 5 was ligated into a cloning vector and transformed into a competent cell line. Following transformation, colonies were selected and subjected to colony PCR. The dsDNA template generated was then sequenced using standard dideoxy methods by the ICMB Core Facility (University of Texas at Austin) A) All clone sequences B) Sequences that could be grouped based on similar sequence motifs.

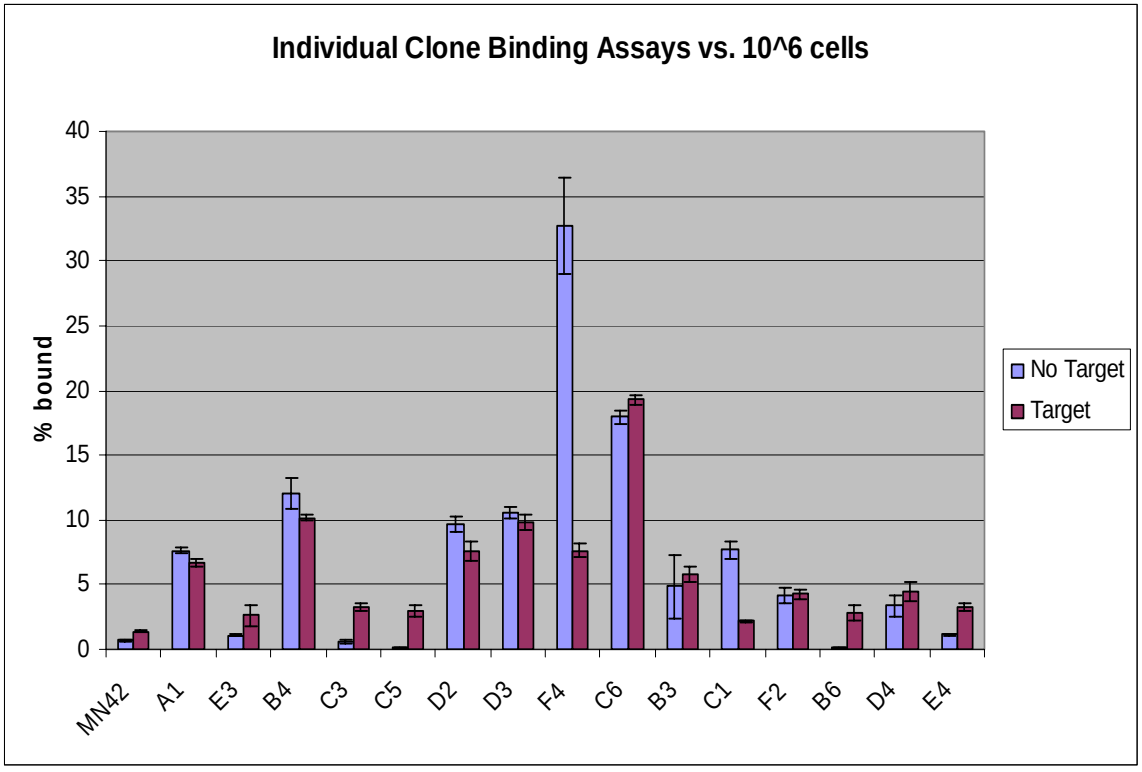


Figure 3-4. Individual clone binding assays. 2 pmols of radiolabeled pool RNA was incubated with 10^6 B. subtilis vegetative cells. Following binding, the cells were passed through a nitrocellulose/nylon filter sandwich on a 96-well dot blot manifold and washed with binding buffer. Filters were then exposed to a phoshporimage screen and retained radiation was quantitated.

Following the initial identification of the above clones as good potential binders, it was decided to repeat the binding assays with the same concentration of cells as was used in the selection. To avoid the problem of clogged filters, these assays were carried out in the same type of individual filter holders used for the selection. The results of these assays are shown in Figure 3-5. Again these clones display binding much higher than that of the unselected pool. Clone C3 displays a higher affinity for the background than do the other clones and the pool. However, as the affinity for *B. subtilis* is also very high; this is not deemed to be problematic.

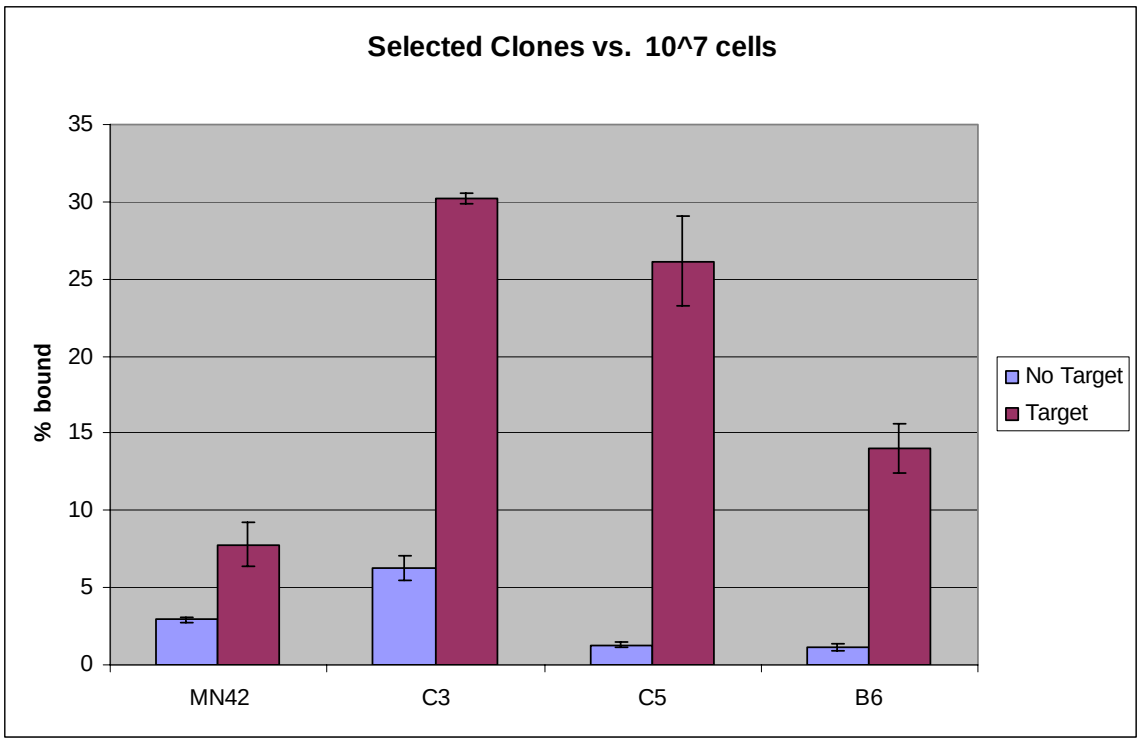


Figure 3-5. Selected clone binding assays with 1 x 10^7 vegetative cells. Two pmols of radiolabeled pool RNA was incubated with 10^7 B. subtilis vegetative cells. Following binding, the cells were passed through a nitrocellulose/nylon filter sandwich in an individual filter holder and washed with binding buffer. Filters were then exposed to a phosphorimage screen and retained radiation was quantitated.

Through these assays, it was determined that Clones C3 and C5 are the best vegetative cell binding species both, overall and in relation to background. This is not unexpected as they share the same motif, although C3 has an additional adenine residue in the motif. B6 shows the lowest degree of binding. There is no apparent homology between clone B6 and other clones. Again, this is probably due to the low number of selection rounds. All clones showed significant enrichment over the unselected pool.

FACS analysis of aptamers

As a means to confirm binding of aptamer sequences, FACS analysis was carried out utilizing two different methodologies. In one method, aptamers were labeled with an

NHS-ester containing fluorophore. This fluorophore was incorporated through a primary amine chemically added to the 5' end of the nucleic acid sequence. This method of labeling has been shown to be extremely efficient, going to >95% completion (Qin and Pyle 1999). Using this methodology, binding of the aptamer to vegetative cells was observed with all three clones (Figure 3-6a). As a control, the unselected pool also was fluorescently labeled to ensure signal was due to selective binding of the aptamer to the cells and not to non-specific interaction of RNA with the surface of the cells. As a further control, the directly modified aptamer was analyzed with *E. coli* as the target (Figure 3-6b). No binding was observed, indicating the selected sequences were not indiscriminately binding bacteria regardless of genus. These clones appear to follow the same trends in binding as they do in the radiological binding data.

The second method utilized involved the synthesis of a DNA oligonucleotide complementary to the 3' end of the RNA aptamer. This oligo was chemically synthesized with a 5' primary amine and subsequently reacted with an NHS-ester fluorophore. The aptamer and a 10-fold molar excess of fluorescent oligo were heat denatured and then allowed to anneal at room temperature. The excess oligonucleotide was added to ensure saturation of all aptamer sequences present. Once annealed, aptamer oligo pairs were combined with vegetative cells and analyzed using a FACS machine. (Figure 3-7). These results also confirmed binding of the aptamer. An additional control, where the fluorescent oligo alone was combined with cells, bound the same as unselected pool. The apparent decrease in binding affinity using this method can be accounted for by considering potential perturbations of tertiary structure caused by the complementary base pairing of 3' end to the fluorescently labeled oligonucleotide. As these bases are no longer free to base pair as in the actual selection it is very possible the structure of the aptamer is not in the preferred form for optimal binding.

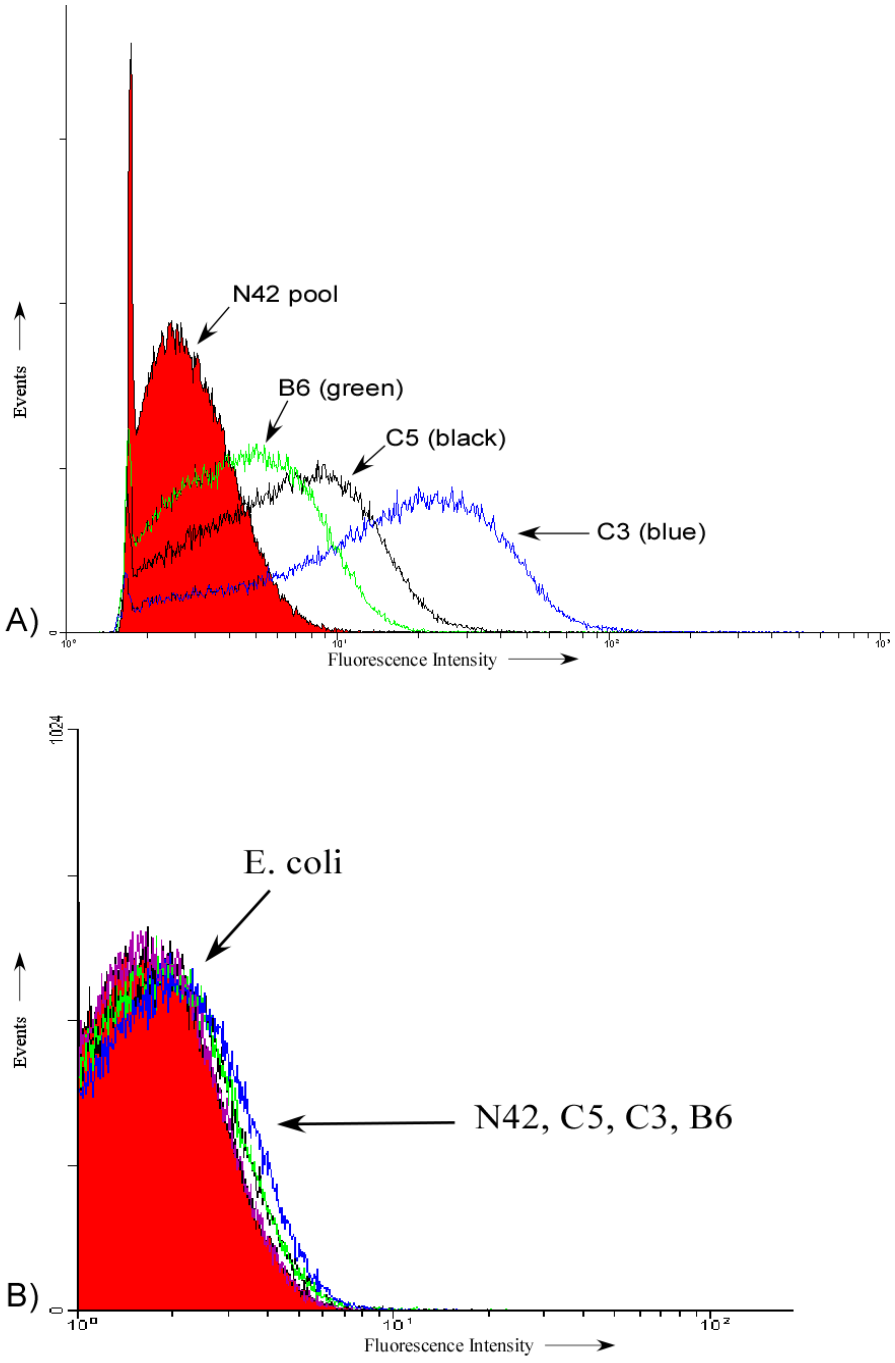


Figure 3-6. FACS assay of binding to bacterial vegetative cells with labeled aptamer. Two pmols of aptamers fluorescently labeled on the 5' end were incubated with 10^7 bacterial cells. Following the binding reaction the cells were centrifuged to remove non-bound fluorescent aptamer. Cells were then resuspended in 150 microliters of binding buffer. 20 microliters of this was then diluted into 200 microliters of binding buffer and scanned on a FACS machine. A) clones vs. *B. subtilis*. B) clones vs. *E. coli*.

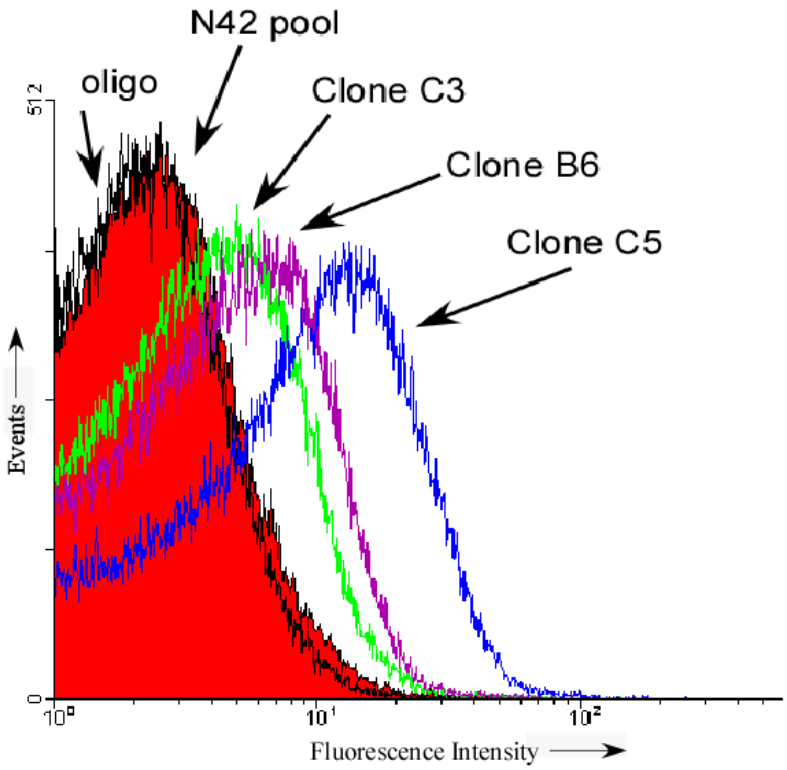


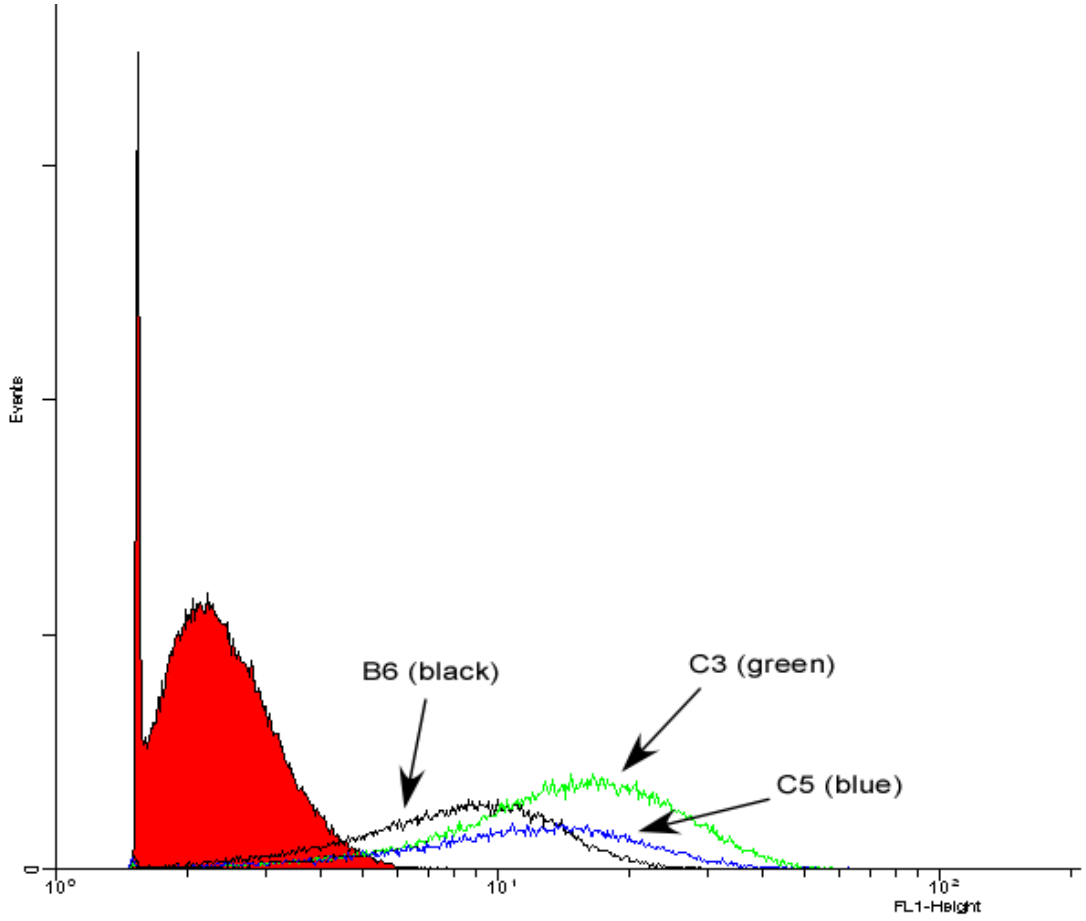
Figure 3-7. FACS assay of binding to bacterial vegetative cells using labeled oligonucleotide. A 10 fold excess of an oligonucleotide is denatured and annealed at room temperature with the aptamer clone or pool. The oligonucleotides are then incubated with 10^7 B. subtilis vegetative cells. Following binding, cells are pelleted and the supernatant discarded. Cells were then resuspended in 150 microliters of binding buffer. Twenty microliters of this was then diluted into 200 microliters of binding buffer and scanned on a FACS machine. Unselected pool and labeled oligonucleotide were used as controls.

Aptamer clones are able to bind other strains of *B. subtilis*

As it is possible to acquire mutations or contamination of the culture in any number of ways during the course of experimentation it was decided to re-order the original strain of *B. subtilis* directly from the ATCC. This also made sense as the original strain with which the aptamers were selected against was a gift from a collaborator, and so we were never involved in the initial handling of the bacteria prior to it being sent to us. Although cultures were routinely checked for characteristic *Bacillus* morphology through growth on LB agar plates, we could not be certain that the strain we were

working with was in fact *B. subtilis.* It was also decided to order a different strain of *B. subtilis* to check whether or not the aptamers are specific for the particular strain they were selected against. FACS scanning was used to determine whether or not these strains bind. Figures 3-8a and 3-8b show the results of the binding assays. From these figures it can be determine that the aptamers are able to bind both the re-ordered original strain (ATCC #: 6051) and the new strain (ATCC #: 29056). This indicates the potential robustness of the aptamer as a detection agent for various strains of *B. subtilis.*

A)



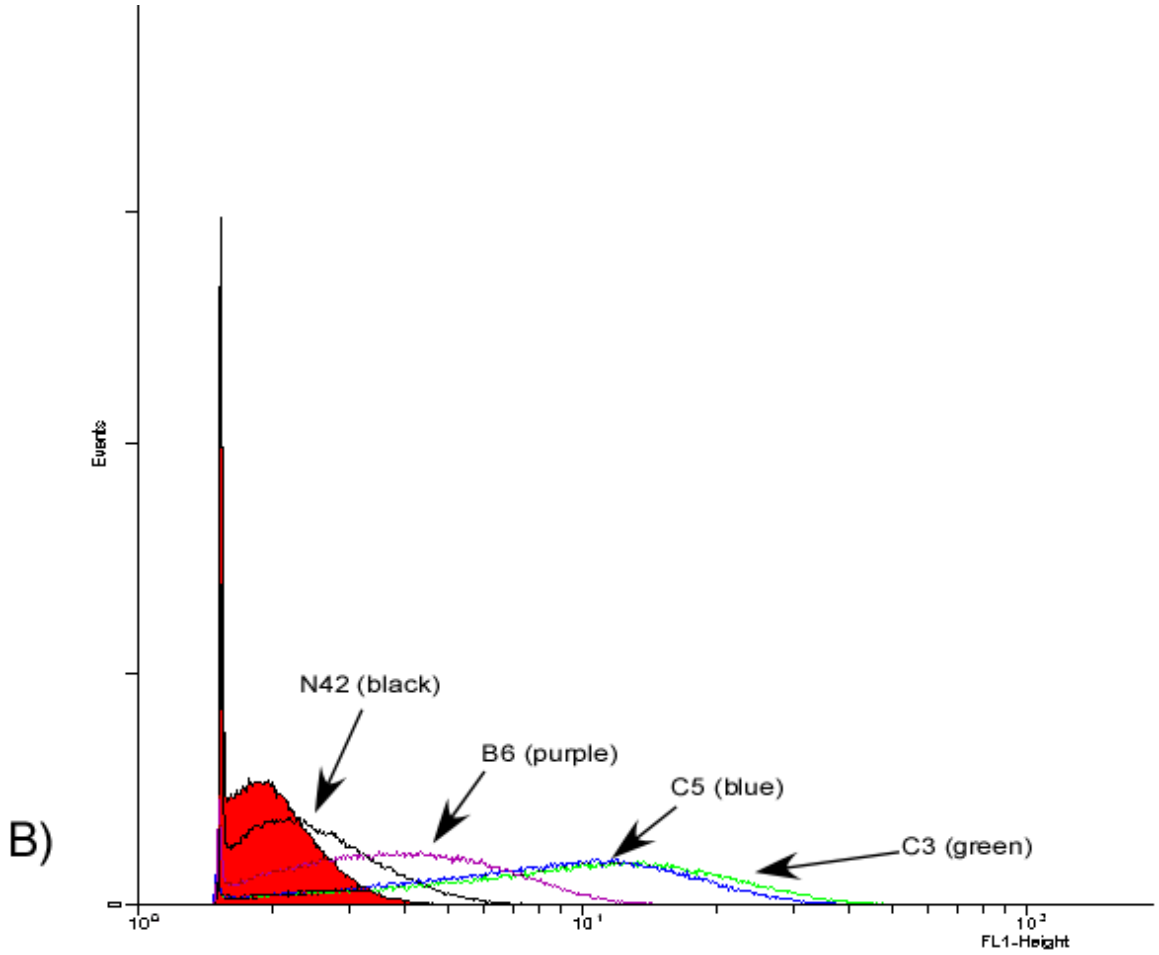


Figure 3-8. FACS analysis of aptamer binding to different strains of *B. subtilis*. Two pmols of 5' labeled RNA aptamer are incubated with 10^7 B. subtilis vegetative cells. Following binding, cells are pelleted and the supernatant discarded. Cells were then resuspended in 150 microliters of binding buffer. Twenty microliters of this was then diluted into 200 microliters of binding buffer and scanned on a FACS machine. A) re-ordered stock of strain 6051 B) strain 29056. Strain identification corresponds to ATCC catalogue numbers

Structure probing of aptamer C5

To determine putative structures of selected aptamer clones, the sequences were folded using the M-Fold folding program (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/). Once the structures were obtained, experiments were carried out to determine which specific individual nucleic acid bases were critical for maintaining binding ability. C5 was chosen as the aptamer to be analyzed for two reasons. First, C5 was deemed to have

the best combination of binding ability and low background. Second, the predicted structures showed a large 20-mer region of the aptamer which was unpaired (Figure 3 – 10). It was thought that this lengthy unpaired region, located on the 3' end of the aptamer, could be exploited for use as a "handle." A series of oligonucleotides of various lengths which are complementary to the 5' and 3' sections of the aptamer were chemically synthesized. These oligonucleotides were then annealed to the C5 aptamer which had been chemically labeled with a 5' fluorescein and assayed using the FACS caliber as above. The results of the assays are shown in Figure 3-9. The percent reduction in binding was calculated by taking the geometric mean of each peak as determined by WinMDI 2.8. Once the numerical value for each peak was established; percentage bound was calculated using unmodified C5 as the standard for binding.

Based on the experimental results, it can be concluded that the 5' end of C5 is very tightly bound to the structure. There is no reduction in binding even when the complementary oligonucleotide is extended to 50 bases. This is very surprising even though it is known that RNA:RNA interactions are stronger than RNA:DNA. To gather more information about the residues important for binding, the same approach was attempted with the 3' end of C5. Here it is apparent that the 3' end is not nearly so tightly bound to the structure. This is also in agreement with the FACS binding data above where a 20 base pair complement was attached to the 3' end. A reduction in binding is present, but yet binding can still be observed. By increasing the length of the complementary oligonucleotide by 5 bases per experiment, the portion of the aptamer necessary for binding can be roughly mapped. This is indicated by the marked reduction in binding between the 45 and 50 base oligonucleotides. On C5 this area is indicated by the blue bases on Figure 3 –9.

5′-GGGAAGAGAGCAUACAGUGCACCAUGAUUCAAACCAGUGCGUCCCUAUUACUCUUUCUCUUUACCUCACCGUCUUACGCCA-3

3′-CCCTTCTCTCGTATGTCACGTGGTACTAAGTTGCCTCACGCAGGGATAAT-5′	**No Reduction in Binding %**
3′-CCCTTCTCTCGTATGTCACGTGGTACTAAGTTTGGTCACGCAGGG-5′	**No Reduction in Binding %**
3′-CCCTTCTCTCGTATGTCACGTGGTACTAAGTTTGGTCACT-5′	**No Reduction in Binding %**
3′-CCCTTCTCTCGTATGTCACGTGGTACTAAGTTTGG-5′	**No Reduction in Binding %**
3′-CCCTTCTCTCGTATGTCACGTGGTACTAAG-5′	**No Reduction in Binding %**

56 % Reduction in Binding	3′-TTGGTCACGCAGGGATAATGAGAAAGAGAAATGGAGTGGCAGAATGCGGT-5′
35 % Reduction in Binding	3′-CACGCAGGGATAATGAGAAAGAGAAATGGAGTGGCAGAATGCGGT-5′
35 % Reduction in Binding	3′-AGGGATAATGAGAAAGAGAAATGGAGTGGCAGAATGCGGT-5′
35 % Reduction in Binding	3′-TAATGAGAAAGAGAAATGGAGTGGCAGAATGCGGT-5′

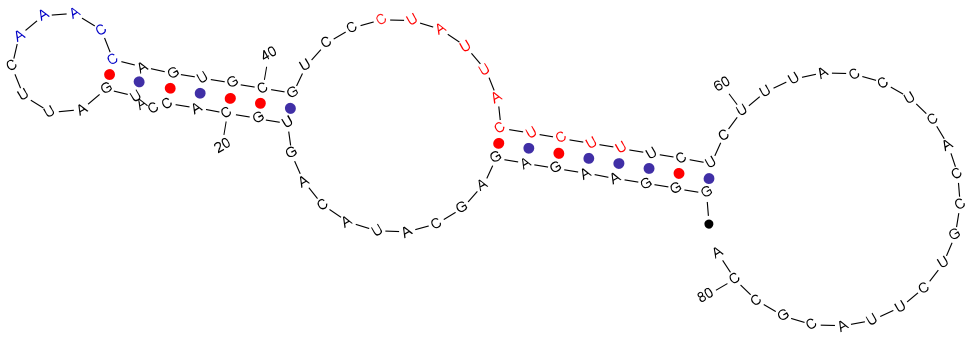


Figure 3-9. Structural probing analysis of aptamer clone C5. Aptamer clone C5 is incubated with a 100 fold molar excess of above oligonucleotides. After a 30 minute annealing time, the reaction is incubated with 10^7 *B. subtilis* vegetative cells. Binding is then assayed through FACS scanning. Oligonucleotide sequence boxed in red is aptamer clone C5. Oligonucleotides complementary to clone C5 are listed below. Reduction in binding affinity following annealing of the oligonucleotides to the aptamer is given next to complementary oligonucleotide. Structure below is aptamer clone C5 as predicted by M-Fold RNA folding program. Bases in blue on this structure are correlated to the bases where those where an abrupt drop in binding affinity occurs

Motif analysis of aptamer clones shown to bind

Although the only aptamer for which experimental data was gathered was C5, by analyzing structure similarities between the predicted structures of the clones, some educated guesses can be made in regards to which residues, or structural features, may be important for binding. This is especially true when comparing aptamer clones C3 and C5, which share the same motif. Structural predictions are based on those generated by the M-Fold RNA folding program. Folding conditions are set at 37° C and 1 M NaCl.

Although these are not the same conditions under which the aptamer was selected, the predictions may serve to indicate the potential folding pattern of the RNA.

When looking at the predicted structures of clone B6, the immediate commonality that is noticed is the first 40 bases of the aptamer form the exact same stem loop structure (Figure 3-10). The only slight deviation from this is in structure 2. Here, the small bulge formed by bases 4 through 7 is absent. This is due to an alternative base

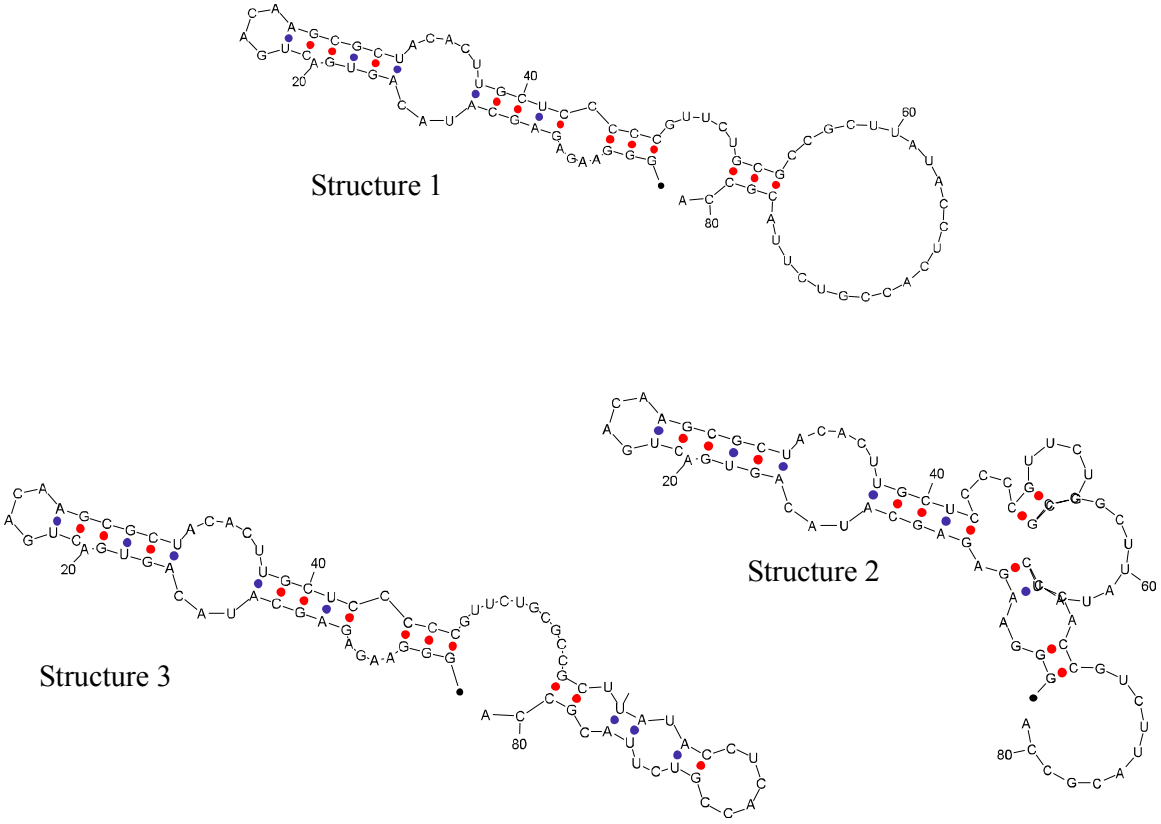


Figure 3-10. Predicted secondary structures of aptamer clone B6. M-Fold RNA folding program was used to predict these secondary structures. The structures are named in no particular order

pairing where bases 5 and 6 pair with bases 65 and 66. In the other two predicted structures this does not occur, with these 4 bases being unpaired. Regardless of the perturbation, the stem loop formed by the first 40 bases is defined. When this clone is

folded without the last 39 bases present, this same structure is formed (Figure 3-11). As this appears to be a conserved structural element, I believe this would be an excellent candidate for a minimization study. Since there is no noted motif in this clone that is shared by another, there can be no comparison to determine if this structure is conserved within another aptamer.

The predicted structures of C5 also seem to share some common elements with one another. The first 8 bases of the aptamer are involved in base pairing with bases 51 to 58 (Figure 3 –12). The only exception to this appears in structure 3 where the first 9

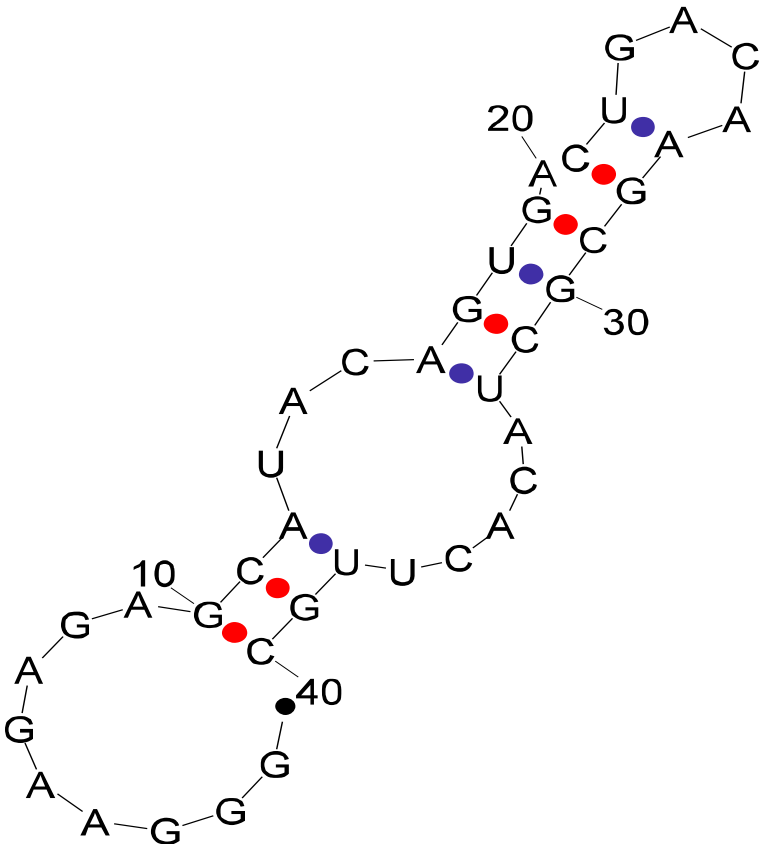


Figure 3-11. Predicted secondary structure of the first 40 bases of clone B6. Structure was predicted using M-Fold RNA folding program

bases are paired with bases 56 through 65 with the omission of base 63. Regardless, the same structure is formed. This becomes even more apparent when compared with the positioning of the conserved motif common to both C3 and C5 (red bases Figures 3-12 and 3-13. In C5 this motif appears to always be present to some degree in a loop, although with the exception of structure 3, a portion of this motif is found paired with bases 4 through 8. These base pairings appear to make up a portion of what appears to be a long stem structure. This is not surprising given that the anti-sense oligonucleotide experiment above resulted in no loss of binding due to complementarity to the 5' region.

Another common structural element is the lack of structure on the 3' end of the aptamer. None of the predicted structures show anything other than an unpaired stretch of 23 bases in 3 of the structures or a stretch of 16 in structure number 3. Another commonality between these clones is the interaction of bases 30 to 35 (Structure 1 and 2) or bases 32 to 35 (Structure 3 and 4) to form a loop structure. This is interesting because this is where the major reduction in binding affinity was observed during the structure probing experiments above (Figure 3–9). Finally, this aptamer is predicted to fold into one of two basic structures. One of these structures features a stem as the predominant feature, interrupted by a couple of loop like bulges, as in structures 1 and 2 In the other there are several stem-loop like structures culminating in the characteristic loop identified as important for binding affinity, as in structures 3 and 4. All this information taken together paints a nice picture of an aptamer with a very stable 5' end, an unstructured 3' end, and the involvement of the conserved motif in maintaining the structure through interaction with the initial bases to form this very stable initial stem. More importantly, this can be correlated with the experimental data observed above.

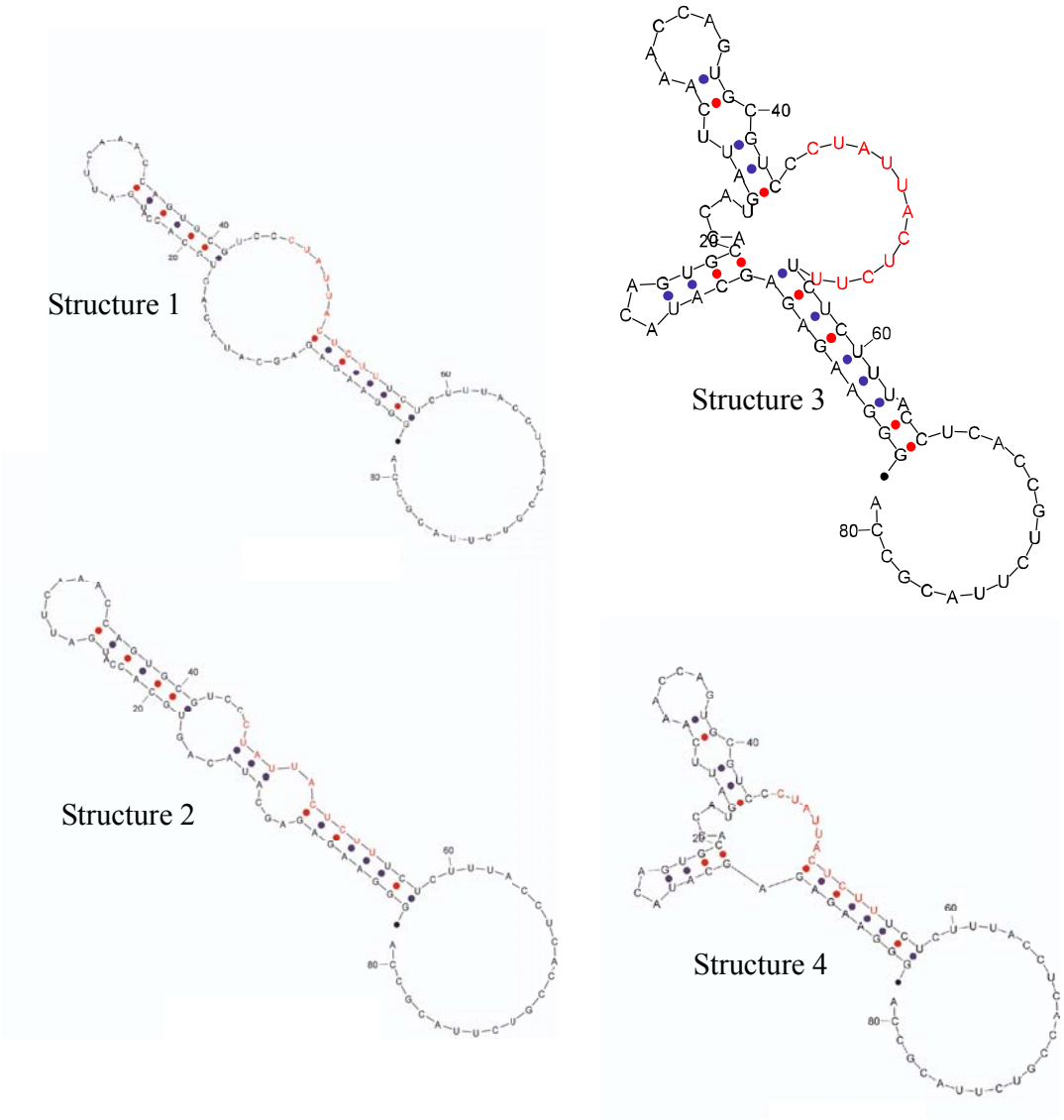


Figure 3-12. Predicted secondary structures of aptamer clone C5. M-Fold RNA folding program was used to predict these secondary structures. The structures are named in no particular order

When the structures for C3 are observed, the immediately noticeable feature is the presence of a CAAAC region in a loop that is also present in the same configuration in C5 (Figure 3 – 12 and Figure 3 -13). In clone C5 these are bases 30 through 34, and in clone C3 these are bases 38 through 43. This is extremely interesting because this is a common element in C5, but is not predicted to be part of the conserved motif. More

importantly, these are the residues in clone C5 that were determined to be critical for efficient binding as determined by the structure-probing experiments above (Figure 3-9). That these bases are found in a loop in two clones sharing a motif suggests that this loop structure is an important element for binding.

An additional feature, which can be correlated with the FACS binding data shown above, is that the 3' end of this clone seems to be involved in the maintenance of the overall structure (Figure 3-7). The correlation is the marked reduction in binding affinity when the oligonucleotide complementary to the 3' primer is labeled (Figure 3-7). The primary form this aptamer seems to take is that of a relatively unperturbed stem structure followed by a series of loops. There is really only one exception to this rule, structure 1, which follows the stem portion to the formation of a single loop. Although the majority of the first bases in structure 3 do not form as long a stem structure as in the other predicted structures, this element is preserved by the 3' region folding back onto itself.

The primary information gained from this analysis is that there may be a conserved second motif in clones C3 and C5. There is further evidence for this when it is considered that there is a common GAU element present before this element. This second motif would then take the form GAU $(N)_x$ CAAAC, where N is any base and X is either 1 or 2. This is relevant because of the potential for this conserved sequence to be involved in the binding of the aptamer to the vegetative cells. A selection using a pool doped to either one of these clones may provide further evidence of this. A doped selection is one in which a percentage of the bases in the random region of the pool are synthesized with the same base as in the originally selected aptamer and the other percentage is randomized. For example, a pool that has been doped 85:15 to C5 would contain 85% A (the original base present there), 5% C, 5% U, and 5% G at position 21. Obviously the other bases in the random region are doped similarly. This allows for the

retention of some binding affinity while allowing further selection to take place. This may result in either a better binding aptamer, or at least confirmation of which structural elements are important for binding.

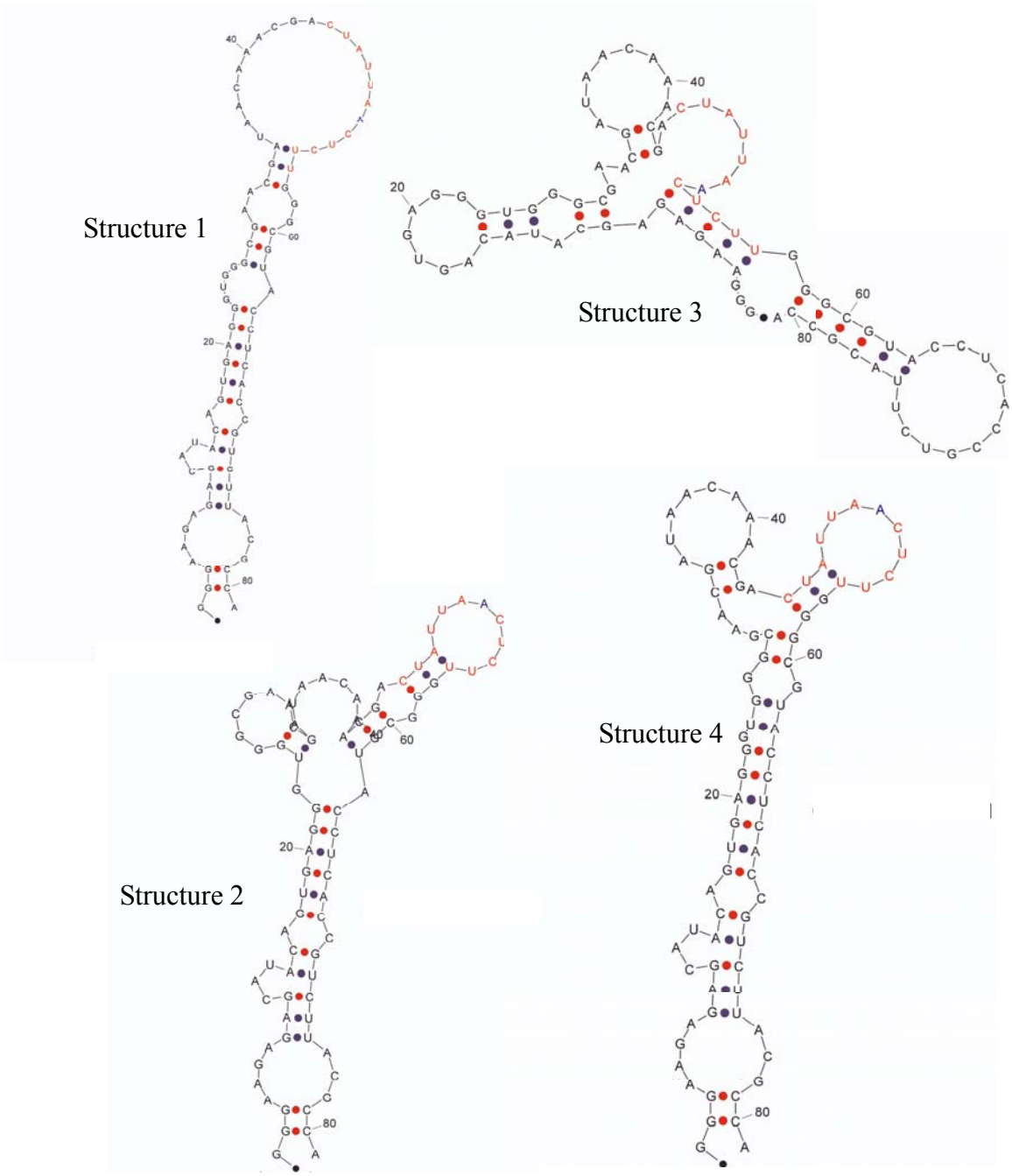


Figure 3-13. Predicted secondary structures of aptamer clone C3. M-Fold RNA folding program was used to predict these secondary structures. The structures are named in no particular order

CONCLUSIONS

An aptamer selection utilizing 2'-F modified pyrimidines was carried out against *B. subtilis* vegetative cells. Three aptamer clones were identified that show a high degree of binding to the vegetative cell. This was determined through binding assays carried out using both radioactive and fluorescent label. These clones were also shown to be capable of recognizing a strain of *B. subtilis* different from that used in the selection. The clones showed no affinity for *E. coli* strain K-12. Structural probing experiments were carried out on aptamer clone C5 to determine which portions of the aptamer were critical for binding. These studies indicated that residues 30 through 35 are critical for efficient binding to the target molecule. All three aptamer clones were folded using the M-Fold RNA folding program, and an analysis was carried out. Surprisingly, this revealed the existence of a second structural motif in aptamer clones C3 and C5. These clones were previously grouped together on the basis of a single motif present at the same location in the clones. This second motif does not occupy the same base positions in both clones. However, it is present on a loop structure in both clones. This new motif contains the consensus sequence GAU (N)x CAAAC. Interestingly, the bases composing this motif were the same bases found to be critical for binding in clone C5. Based on this information and the experimentally determined similarities between these clones, I believe this second motif to be important for binding in clone C3 as well. To further understand the binding capabilities of these clones, additional characterizations is described in Chapter 4.

EXPERIMENTAL DESIGN

Bacterial strains/growth conditions

B. subtilis strains corresponding to ATCC 6051 or ATCC 29056 were used in these experiments. 6051 was obtained from Walther Ellis at the University of Utah. An additional stock of 6051 and 29056 was obtained from the ATCC. Bacterial cultures were grown overnight at 37° C in LB. The *E. coli* strain K-12 (ATCC 29425) used in these experiments was obtained from Eric Davidson, University of Texas at Austin. Overnight cultures were then diluted 1:100 into 5 mL of LB in a 15 mL culture tube which was then grown to an $O.D._{600}$ of 0.5-0.6. One mL of this culture was then centrifuged at 1500 x g for 10 minutes. The supernatant was removed and the cell pellet was then washed twice in phosphate buffered saline (PBS) supplemented with 5 mM $MgCl_2$ (Binding Buffer) and spun down again. The cell containing pellet was then resuspended in 100 uL of binding buffer. For experiments involving fluorescent studies, the cell pellet was only washed once, prior to experimental use.

Oligonucleotides

A nucleic acid pool containing a 42 base random region flanked by constant priming regions of sequence 5'- GATAATACGACTCACTATA*GGGAAGAGAG*CATACAGTG-3' (38.42) and 5'-TGGCGTAAGACGGTGAGGTA-3' (20.42) was chemically synthesized on an Expedite 8909 chemical DNA synthesizer (PerSeptive Biosystems). A region of 8 purine bases was included in primer 41.42 to allow for efficient incorporation of 2'-fluoro 2'-deoxypyrimidine residues. The underlined region in 41.42 corresponds to the T7 promoter sequence and the italicized bases indicate the strech of 8 purine residues

included to facilitate modified pyrimidine incorporation. All oligonucleotides were deprotected and purified as in Chapter 2.

Oligonucleotides complementary to either the 5' or 3' end of aptamer clone C5 were synthesized by IDT. The sequences of these oligonucleotides are given in Figure 3-9

Double Stranded DNA Pool Generation

The amount of chemically synthesized ssDNA pool recovered following gel purification was determined to be 27,480 pmol. This equates to 1.65 x 10^{16} unique sequences. Of this, it was determined that 7.25 %, or 1992.3 pmol were extendable using the method outlined in Chapter 2. One-quarter of this, or 498 pmol, was aliquoted for PCR amplification. Following a cycle course to determine the optimum number of cycles for amplification, 113 mL of PCR reaction mix was cycled 14 times using standard PCR protocols. These reactions were carried out in 96-well plates to reduce handling time. PCR amplification resulted in 3.6 additional copies of the pool being generated. This was determined by quantitation on an agarose gel using a quantitation standard. Trancription using 2'- modified pyrimidine bases to generate the starting RNA pool was carried out exactly as in Chapter 2. Following, gel purification and subsequent recovery of the RNA it was determined that 54 copies of the pool had been generated. This was determined by A_{260} readings and Beer's law.

In vitro selection

Seven rounds of *in vitro* selection were carried out against *B. subtilis* vegetative cells. Five hundred pmols of R0 pool, representing 1 x 10^{14} unique sequences, was placed

into a 1.5 mL Eppendorf tube with 10 microliters of 10x Binding Buffer (10x PBS w/ 50 mM $MgCl_2$) and water to 50 microliters. The RNA was then heat denatured at 70°C for 3 minutes and allowed to cool to room temperature for ~10 minutes. Bacterial vegetative cells, prepared as above, were then added to the reaction vessel for a final volume of 150 microliters. The binding reaction was allowed to proceed for 30 minutes at room temperature with rotation. Following binding of the RNA to the bacterial cells, bound RNA was separated from unbound species by passing through a 0.22 uM nitrocellulose containing filter which had been pre-wet with binding buffer (Millipore). The filter was then washed three times with 500 microliters of 1 x binding buffer to further eliminate non-specifically bound RNA sequences. The filter was then heated to 100°C for 5 minutes in the presence of 200 microliters of Elution Buffer (7M urea, 100 mM NaOAc pH 5.2, 1 mM EDTA). The eluate was removed and the process repeated for a total of 400 microliters of RNA containing elution buffer. To remove bacterial proteins, the eluate was phenol/chloroform extracted twice. To ensure complete phenol removal the final extracted aqueous phase was chloroform extracted. The aqueous phase was then ethanol precipitated with 300 mM NaOAc (final concentration) used as the salt. The RNA containing pellet was then resupended in water and reverse transcribed with SuperScript III (Invitrogen). The reverse transcription was then placed into a PCR reaction and a cycle course was performed to determine the optimal number of cycles per round. DNA cycles were electrophoresed on a 4% agarose gel. Once PCR was complete the individual reaction tubes were pooled and ethanol precipitated. DNA was then quantitated using a 200 bp quantitation standard (Gensura). Five hundred nanograms of PCR product was then placed into a 20 microliter Durascribe transcription reaction (Epicentre) which uses 2'-deoxy-2'-fluoropyrimidines as bases. The transcription was then ethanol precipitated and subjected to DNase I (Invitrogen) in the presence of DNase

I reaction buffer. The RNA was then loaded onto an 8% polyacrylamide gel and electrophoresed for purification. The resulting RNA band was excised and eluted overnight in water. Following Round 1, and for all subsequent rounds, a negative selection was performed by passing the renatured pool (subsequent to heat deanturation) through a 0.22 uM filter pre-wetted with binding buffer. This is to further remove those RNA sequences showing binding to the filter.

5' end labeling

The procedure for labeling the 5' end of RNA molecules has been described previously (Qin and Pyle 1999). Briefly, transcribed RNA was reacted with Antarctic Phosphatase (NEB) at 37° C for 30 minutes and heat deactivated at 65° C for 10 minutes. The reaction was phenol/chloroform extracted to remove protein and the aqueous phase was then desalted by passage through a Centri Sep column (Princeton Separations). A single phosphate is then incorporated using T4 PNK (Invitrogen) at 37° C for 30 minutes followed by heat inactivation at 65° C for 10 minutes. Protein is removed and the reaction desalted as above. Mono-phosphate RNA is combined with 0.5M EDC (N-(3-dimethylaminopropyl)-N'-ethyldicarbodiimide) and 1.0 M Imidiazole pH 6.0. The final concentration in solution of both EDC and Imidiazole is 0.1 M. The reaction is allowed to proceed at 25° C for 1 hour. The reaction is then diluted 10 fold and reacted with 0.75 M EDA (ethylene diamine; final concentration 0.25 M) for 4 – 8 hours. The reaction is then ethanol precipitated and the resulting pellet is resuspended in 0.1 M sodium tetraborate pH 8.5 and reacted with an NHS-ester (*N*-hydroxysuccinimide) fluorophore (Invitrogen) overnight. Following covalent attachment of the fluorophores to the RNA strand, the labeled RNA is ethanol precipitated and purified on an 8% polyacrylamide

gel. Alternatively, a DNA oligonucleotide complementary to the 3' end of the RNA aptamer is chemically synthesized with an amine on the 5' end. The amine is then reacted directly with the NHS-fluorophore, and purified as above.

Cloning/Sequencing

RNA generated from Round 5 was chosen for sequencing as it gave the best ratio between the binding of the RNA to the bacterial target cells and the nitrocellulose background. All cloning and sequencing steps were carried out exactly as in Chapter 2.

Radioactive binding assay

RNA sequences were transcribed as above in a reaction supplemented with P^{32}-α-GTP. Resulting RNA sequences were ethanol precipitated, the remaining DNA was enzymatically degraded , and gel purified as above. Following gel purification, 2 pmols of radiolabeled RNA (either pool or clone) were placed into a binding reaction with vegetative bacterial cells. Upon completion of the binding reaction, the RNA was passed through a filter holder (Milipore) containing a nitrocellulose/nylon sandwich. Alternatively, a Minifold I 96-well dot-blotting array (Schleicher and Schuell) was used to hold the nitrocellulose/nylon filter sandwich. In either case following initial filtration, the filters are washed 3 times with 500 microliters of binding buffer. The retained radioactivity was quantitated on a Molecular Dynamics Phosphoimager using the Imagequant software. Binding affinity values were calculated using the equation: nitrocellulose binding/ (nitrocellulose binding + nylon binding) * 100.

FACS assays

FACS scanning was performed on a FACS Calibur (Becton Dickinson). Cells were centrifuged and resuspended as above and added to a 50 microliter solution containing 5 microliters of 10x Binding Buffer, water, and 2 pmols of 5' Alexa 488 labeled RNA prepared as above. Following binding, the cells were pelleted by centrifugation for 10 minutes at 1500 x g to separate bound from unbound RNA. The pellet was then resuspended in 150 microliters of 1 x binding buffer and 20 microliters were removed and added to 200 microliters of 1x binding buffer. Binding was then assayed. WinMDI 2.8 (Verity Software house) was used to visualize the raw fluorescence data.

Alternatively, the aptamer and a 10 fold molar excess of labeled oligonucleotide complementary to the 3' constant region were heat denatured together in the presence of binding buffer. The reaction was then allowed to cool to room temperature for 10 minutes and combined with 10^7 bacterial vegetative cells. The reaction was centrifuged at 1500 x g for 10 minutes and the supernatant containing unbound fluorescently labeled oligonucleotide was discarded. The bacterial pellet was then resuspended in 150 microliters of binding buffer. Twenty microliters of this was added to 200 microliters of binding buffer and analyzed as above.

For structural analysis studies, 5' labeled aptamer was combined with unlabeled oligonucleotides of varying lengths complementary to either the 3' or 5' end. The aptamer oligonucleotides were denatured and annealed in the presence of the complementary oligonucleotide for 30 minutes at room temperature. Binding to bacterial cells was as above. The oligonucleotides are in a 100 fold excess to the aptamer.

REFERENCES

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Qin, P. Z. and A. M. Pyle (1999). "Site-specific labeling of RNA with fluorophores and other structural probes." Methods **18**(1): 60-70.

Chapter 4: Characterization of anti-*B. subtilis* aptamers

INTRODUCTION

Following selection of aptamers capable of binding to *B. subtilis* additional characterizations of this binding capability were performed. There were several reasons for this. One, it was desired to determine the robustness of the aptamers. That is, would they be capable of binding to *B. subtilis* propagated under varying conditions? We also wanted to know whether or not the aptamers were specific for *B. subtilis* or if they would recognize any member of the *Bacillus* family. At this point it also had not been determined whether or not the aptamer clones are capable of binding spores. Finally, the identification of the target to which the aptamer binds also would be a useful piece of information that we would like to discern. Answers, even partial ones, to these questions would greatly help in identifying the capabilities of these aptamers to bind *B. subtilis* and therefore have an impact on potential downstream uses.

Environmental Effectors of *B. subtilis* Growth

Bacteria are frequently grown in an environment that is subject to change. At times, these environmental changes will induce a stress response in the bacteria. These stress inducers may take various forms including, but not limited to, temperature fluctuations, deprivation of certain nutrients, and oxygen limitation (Petersohn, Brigulla et al. 2001). When these situations arise they frequently trigger the expression of previously silent genes designed to ensure the survival of the bacteria. Activation of these genes may also result in the repression of previously active genes. This differential gene expression naturally results in an alteration in the levels of normally expressed

proteins and an emergence of previously unexpressed proteins. Changes in the availability of certain critical nutrients, such as phosphate or nitrogen, provides an excellent example of this differential protein expression. Entire gene regulatory systems are activated based on fluctuations in these two compounds (Hulett 1996; Fisher 1999). This should come as no surprise considering that an organism able to adapt to differences in its surroundings would have an immense selective advantage over those that cannot. Additionally, the ability to selectively regulate this expression provides another competitive advantage as valuable resources are not wasted in the synthesis of unnecessary gene products. Those proteins translated in response to these stress induced regulatory pathways are collectively referred to as general stress proteins (Hecker and Volker 2001). Although the full impact of varied growth conditions on the physiology and genetic regulation of *B. subtilis* is well outside the scope of this introduction, an attempt to summarize the aspects relevant to this work will be given.

One of the most well studied aspects of this stress response deals with temperature fluctuations. *B. subtilis* is recognized as being able to support growth in a temperature range from 11 - 53° C (Harwood 1990). However, bacteria grown at these vastly different temperatures would be expected to have differences in their physiologies. In fact, two of these differences can be characterized by protein expression and membrane fluidity. The proteins specifically expressed as a result of growth during non-ideal growth temperatures are referred to as either cold shock proteins (CSPs) or heat shock proteins (HSPs). Although typically studied under conditions where the temperature is altered in a dramatic fashion, hence the term “shock”, these proteins are expressed continually as long as the final temperature is sustained. The primary function of HSP’s appears to be either molecular chaperoning of proteins that have become misfolded as a result of the temperature increase, or as a protease to remove those

proteins that cannot be salvaged (Schumann 2003) . CSP's on the other hand seem to be involved primarily in alteration in the membrane fluidity and modification of the rate of metabolically important protein synthesis (Weber and Marahiel 2002). This alteration in membrane fluidity is of interest in exploring the effects of temperature alteration on the cell surface. In order to effect these changes in membrane fluidity, *B. subtilis* begin to increase the amount of unsaturated fatty acids in the lipid bilayer. This has the effect of altering the state of the membrane from liquid like (disordered) to gel-like (ordered) (Mansilla, Cybulski et al. 2004). It is unclear what changes to the extracellular matrix accompany this transition, but it would be reasonable to assume that some differences are induced.

Although long considered an obligate aerobe, *B. subtilis* has been shown to be a facultative anaerobe. Under conditions of oxygen limitation or strict anaerobic conditions, *B. subtilis* is able to use nitrate as an electron acceptor instead of the usual oxygen (Glaser, Danchin et al. 1995). The availability of oxygen has been shown to govern various components of cellular metabolism, although these studies do not necessarily involve strict anaerobic conditions. Interestingly enough when the change to absolute anaerobic growth is made it is accompanied by a marked difference in cell morphology (Hoffmann, Troup et al. 1995). This difference involves the structure of the vegetative cell changing from the usual rod-shaped form to a long filamentous shaped cell. Another major difference in physiological properties of anaerobically grown *B. subtilis* is the inability to form spores (Hoffmann, Troup et al. 1995). Although the full implications of anaerobic or oxygen limiting conditions in *B. subtilis* have yet to be fully established, it is clear that there are marked difference both in terms of cell physiology and metabolism

The nutrients to which growing *B. subtilis* has access also has a dramatic effect on both the cellular metabolism and the physiology of the vegetative cell. The best example of this is the sporulation pathway embarked on during times of nutrient starvation. In laboratory conditions nutrient availability is determined by the composition of the growth media. Indeed, many studies have been carried out exploring the effects of various media formulations on the extracellular composition of *B. subtilis* and other Gram-positive bacteria (Hancock 1988). To underscore this point, a study utilizing pyrolysis mass spectrometry found that varying media formulation for identically grown bacterial cultures of the same *B. subtilis* strain resulted in different experimentally derived mass spectroscopic values (Shute, Gutteridge et al. 1988). Clearly the changes evoked by various nutrient formulations may have a profound effect on the final composition of the growing bacterial culture.

Phenotypic Differences Between Members of *Bacillus* Genus

Differentiating members of the *Bacillus* genus is a fairly difficult task. Original classification of this genus relied primarily on the ability of the bacteria to form spores and grow aerobically. Eventually other characteristics were included and a numerical classification based on phenotype was established and used to define the genus (Priest, Goodfellow et al. 1988). Eventually genetic sequencing, based on the reliability of 16 S rRNA as an evolutionary marker, was used to make this task of classification simpler. Using genetic analysis to classify *Bacillus* has resulted in the creation of several new genera and allowed some members to be more appropriately placed with related members (Rossler, Ludwig et al. 1991; Shida, Takagi et al. 1996; Waino, Tindall et al. 1999). In some cases, though, this intensive genetic analysis has blurred the lines between what it

means to be related. For example, *B. anthracis, B. cereus,* and *B. thuringensis* can be classified into one very closely related family genetically (Helgason, Okstad et al. 2000), and yet they display very different phenotypes. These phenotypic differences are primarily attributed to the type of toxin each of these bacteria expresses, or does not express in the case of *B. cereus*. An additional study comparing the extracellular proteomes of these three species also found differences in the number and types of proteins expressed (Gohar, Gilois et al. 2005). Although some of these proteins appear to be very similar, when examined by 2-dimensional gel electrophoresis, many of them are not, i.e. some of these similar proteins are displaying different properties such as isoelectric points and molecular weights. Although these differences may be slight, they still may effect enough of a change in the extracellular matrix to prevent recognition by molecules targeting this portion of the vegetative cell. This same study also found the type of proteins secreted into the environment by these bacterial species to be vastly different. These differences were found in three closely related species, so how similar or different are the extracellular proteomes of other members of this genus who are not so closely related? Moreover, this does not even include the expression of lipids, carbohydrates, or other anionic polymers present on the cell surface. So the true phenotypic differences between members of the *Bacillus* genus are unclear. Part of the difficulty in establishing an absolute answer to this question lies in the varied effects growth conditions have on the bacteria, as mentioned above. Although genetic classification is a valid, and more accurate, tool for the classification of species, in some cases phenotypic distinction may be more useful. In the case of aptamers, it is the phenotypic differences that provide the key to molecular recognition, and thus differentiation, of the target.

The remainder of this chapter will detail efforts to further characterize the binding of these aptamer clones to *B. subtilis* vegetative cells. Varying growth conditions, and growth phase of *B. subtilis* was assayed for the effect on the ability of the aptamer to bind. The aptamers were also tested for binding to other species of *Bacillus*. Additional experiments were carried out in an attempt to define the potential binding target of the aptamer.

RESULTS AND DISCUSSION

Phylogeny studies of related *Bacillus* sp.

Thus far, it has been demonstrated that the aptamers selected against *B. subtilis* are capable of binding this bacterium. This has been confirmed with nitrocellulose binding assays, FACS data, and also confocal microscopy (data not shown). However, what has not been determined is whether or not these aptamer are specific for *B. subtilis* or if they are capable of binding any bacteria. With the exception of an *E. coli* control in the initial selection, these aptamers had not been tested against any other bacteria. Although there are a wide variety of bacterial genera, both Gram-positive and Gram-negative, from which to select, it was thought that the best test of specificity should involve members of the same phylogenetic background. Therefore, two additional species in this genus, *B. cereus* and *B. thuringensis,* were chosen to test whether or not the aptamer clones would bind them as well. These two species were chosen because they are very closely related was to *B. anthracis*. In fact *B. cerus* and *B. anthracis* are virtually identical genetically with the exception that *B. anthracis* possess two plasmids, pX01 and pX02, that convey the toxic effects associated with the disease anthrax. If this aptamer, or one selected against *B. anthracis*, would ever be used in a detection device, it

would be important to be able to screen against other, related, strains of bacteria. Obviously if an aptamer could not discriminate between a pathogenic bacteria and a non-pathogenic strain, then it would not be of much use in a biodefense detection device. However, there may be potential for use in a crude assay to immediately distinguish between bacterial genera, or as another means to discriminate between Gram positive and Gram negative bacteria.

To carry out these experiments, aptamer clones were body labeled with α-P^{32} during transcription. The bacteria were grown to an O.D. of 0.5-0.6 as measured by A_{600}, were washed twice with binding buffer, and placed together with the aptamer clones. After allowing the aptamer to bind with the target, the cells were passed through a 0.22 uM nitrocellulose filter.

The selected aptamers all appear to be capable of binding to other species in the *Bacillus* family (Figure 4-1). This is not entirely unexpected, as one would expect members of the same genus to share a similar extracellular composition. However, it is obvious that the aptamers show a preference for binding *B. subtilis* over the other two species. This indicates that while the surface of these bacterial species may be similar, there is enough of a difference, perhaps in expression of extracellular proteins, for the aptamers to bind *B. subtilis* with a higher affinity. Again, binding assays with *E. coli* show no binding, with any signal being near background. It also noteworthy to point out that binding to *B. cereus* and *B. thuringensis* is virtually identical, with perhaps only a slight preference shown for *B. thuringensis*. This is most likely due to the close genetic relationship shared by these two species.

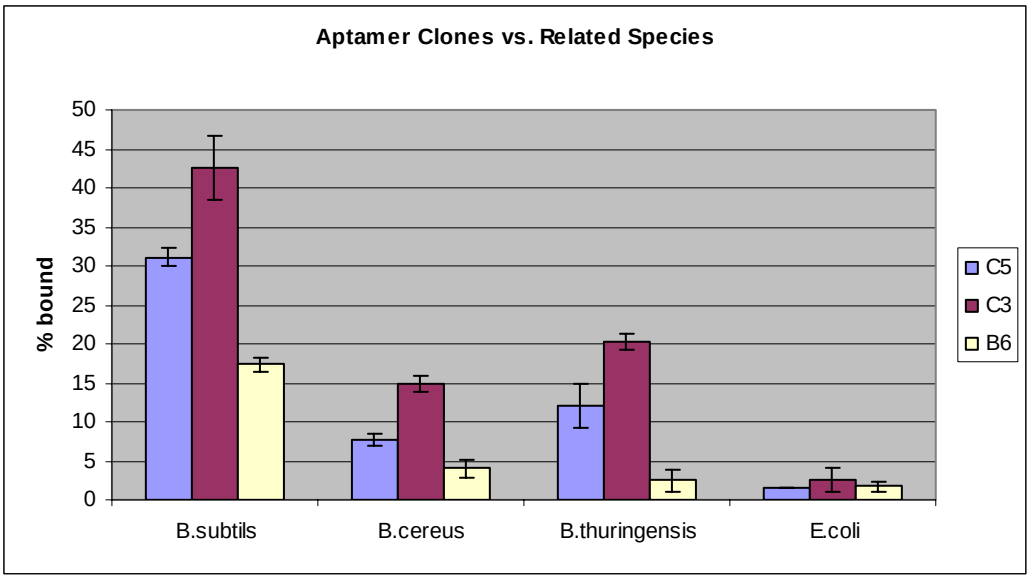


Figure 4-1. Clones vs. related species. 2 pmol of radiolabeled aptamer clones were incubated with 10^7 bacterial cells of either B. subtilis, B. cereus, or B. thuringensis. Following incubation cells were passed through a nitrocellulose/nylon filter sandwich in an individual filter holder and washed 3 times with binding buffer. Filters were then exposed to a phosphorimaging screen and the retained radioactive counts quantitated.

Growth Conditions

B. subtilis is capable of growth under a number of varied conditions. This includes a tolerance for temperature range, various carbon sources, and the amount of oxygen available for growth. As a result of this capability, there is an inherent uncertainty in determining how a given bacteria found in its native environment was grown. As stated in the introduction, the effect exerted by the growth media and conditions can produce profound changes in the extracellular make up of a given bacteria. The bacterial cultures used for the selection were grown at 37°C in LB and while this may be typical for laboratory cultures, this exact environment is unlikely to be reproduced in a naturally grown bacteria. So cultures of *B. subtilis* were grown under a number of different conditions and the aptamer was assayed for binding capability.

The first condition to be assayed involved the temperature at which the bacteria were grown. Laboratory cultures are typically grown at a 37°C whereas in a natural environment temperature will fluctuate. These temperature fluctuations are often significant enough to generate a stress response in the bacteria that will cause a differentiation in the expression of various surface components. To test whether growth temperature would significantly effect binding of the aptamer clones to the bacteria, cultures of *B. subtilis* were grown at 42° C, 37° C, 30° C, and 23° C. Bacterial cultures were grown in the same volume, 5 mL in a 15 mL culture tube, to the same optical density range, 0.5 – 0.6 A_{600}, as in the initial selection. Following growth at these temperatures, cells were washed and nitrocellulose binding assays were carried out as described (Figure 4-2). It is immediately apparent that temperature has a great influence on the ability of the aptamers to bind. Interestingly, the ability to bind is negatively affected regardless of whether or not the temperature is increased or decreased. That some affinity for binding is retained in both the 42° and 30° C experiments indicates that the target is still present to some degree in cultures grown at these temperatures. However, at 25° the binding target doesn't appear to be present as % binding has fallen to right at or slightly above that of the unselected pool. This set of experiments indicates that the temperature at which these bacteria are grown plays a pivotal role in the ability of the aptamers to bind.

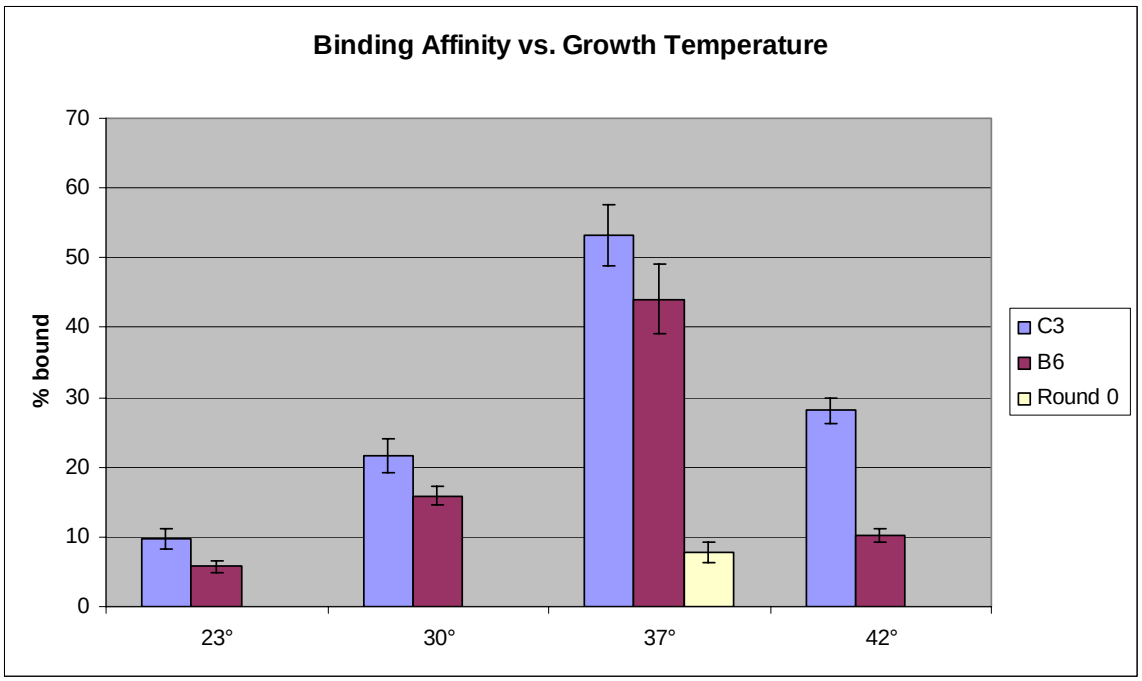


Figure 4-2. Effect of temperature. 10^7 *B. subtilis* vegetative cells were grown at a temperature of 23°, 30°, 37°, or 42° C were incubated with 2 pmols of radiolabeled aptamer. Following binding, the bacteria containing reaction was passed through a nitrocellulose/nylon filter sandwich in an individual filter holder and washed 3 times with binding buffer. Filters were exposed to a phosphorimage screen and retained radiation was quantitated. Round 0 pool was incubated with 10^7 bacterial cells grown at room temperature to provide a binding affinity comparison.

The second growth condition to be assayed concerned the carbon source the bacteria were grown on. As mentioned in the introduction to this chapter, media composition may have a profound effect on the physiology of bacteria. Thus far, all bacteria cultures have been grown in LB, considered to be a complex, undefined media. To test the effect of media composition, bacterial cultures were grown using chemically defined minimal medium. Additionally, to determine whether or not the carbon source used make a difference in the ability to grow, two different carbon sources, glucose and glycerol, were utilized. It appears as though the neither the carbon source, nor the defined media, make much of a difference in the ability of the aptamer to bind (Figure 4-3). There is a reduction in binding in both the glycerol and glucose containing minimal

media cultures from that of the LB culture, but the binding affinities are still very high. Therefore, while there may be minor differences in binding affinity, these are not considered to be significant.

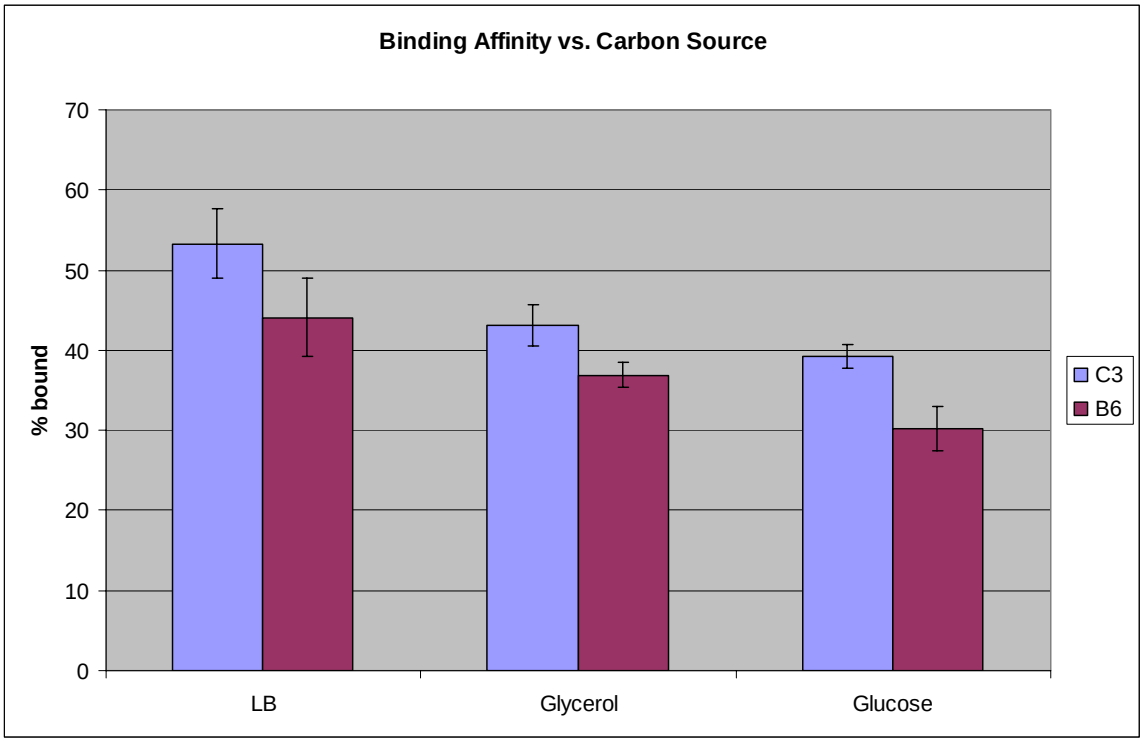


Figure 4-3. Effect of carbon source. 10^7 B. subtilis vegetative cells grown on minimal media with either glycerol or glucose as the carbon source were incubated with 2 pmols radiolabeled aptamer. Following binding, the bacteria containing reaction was passed through a nitrocellulose/nylon filter sandwich in an individual filter holder and washed 3 times with binding buffer. Filters were exposed to a phosphorimage screen and retained counts of radiation were quantitated. Assays with cells grown on LB are included as a reference for binding affinity under selected conditions.

Aptamer clones preferentially bind cells in mid-log phase

Once the effect of varied growth conditions on binding affinity had been established, it was decided to investigate if binding was a function of the growth phase itself. Optical density is a convenient method to measure the growth of cells as a

function of tubidity. That is, as cells grow they reproduce, and the more cells present in a given culture, the more light they are able to scatter, and thus the higher the optical density of a culture. Although optical density gives no information about the physical or metabolic state of a cell, or even if the cell is still viable, it does give information about the number of cells present. Used in conjunction with other experimental techniques, this information can help define which factors are important for the aptamer to recognize, or at least can narrow down what factors are not relevant for aptamer binding.

In order to explore the favored conditions for aptamer binding to the vegetative cells, binding affinity as a function of optical density was measured. Cells were grown to varying optical densities (A_{600}) and prepared as in the original selection. Aptamers labeled either with α-P^{32}-GTP or with a fluorescein molecule on the 5' end were then added in a typical binding reaction. Radiation studies were carried out in the 96-well dot blot format described in Chapter 3. Fluorescent aptamer binding to cells were assayed using FACS scanning, again as described in Chapter 3.

The results of these experiments are given in Figure 4 –4. All three clones seem to display the same trend. This trend being the ability of the aptamer to bind the cell appears to be related to an increase in optical density. This information is correlated by filter binding assays for clone B6 (Figure 4-5). In interpreting this data, it is possible that the target of the aptamer is expressed in higher amounts at a growth phase correlating to mid-log phase. This is because of the shift in fluorescent intensity as measured by FACS. Aptamer binding is most likely not correlated to the number of cells present. This is because FACS scanning measures the fluorescent intensity of individual members of a given population, and so is concentration independent (Hewitt and Nebe-Von-Caron 2004). If aptamer binding to cells was dependent on the number of cells present, then one would expect the fluorescent intensity shift given to remain static. That is, an O.D.

of 0.1 should theoretically cause the same shift as an O.D. of 0.5. As the results do not indicate this, cell concentration is most likely not the cause of the intensity shift. This is further reinforced when the radiation time course of clone B6 is examined. As these cells are not normalized, there are more cells present at higher optical densities than at lower optical densities.

The drop in binding affinity for clone B6 at higher optical densities is interesting when it is considered that the binding of the other two clones either remains the same or slightly increases (data not shown). A possibility for this would be that B6 binding to its vegetative cell target is influenced somewhat by the formation of spores. It would be expected that at higher optical densities the number of spores present in culture would also be higher. Regardless, this may provide evidence that clones C3 and C5 are binding the same cellular target, while clone B6 may be binding a different one.

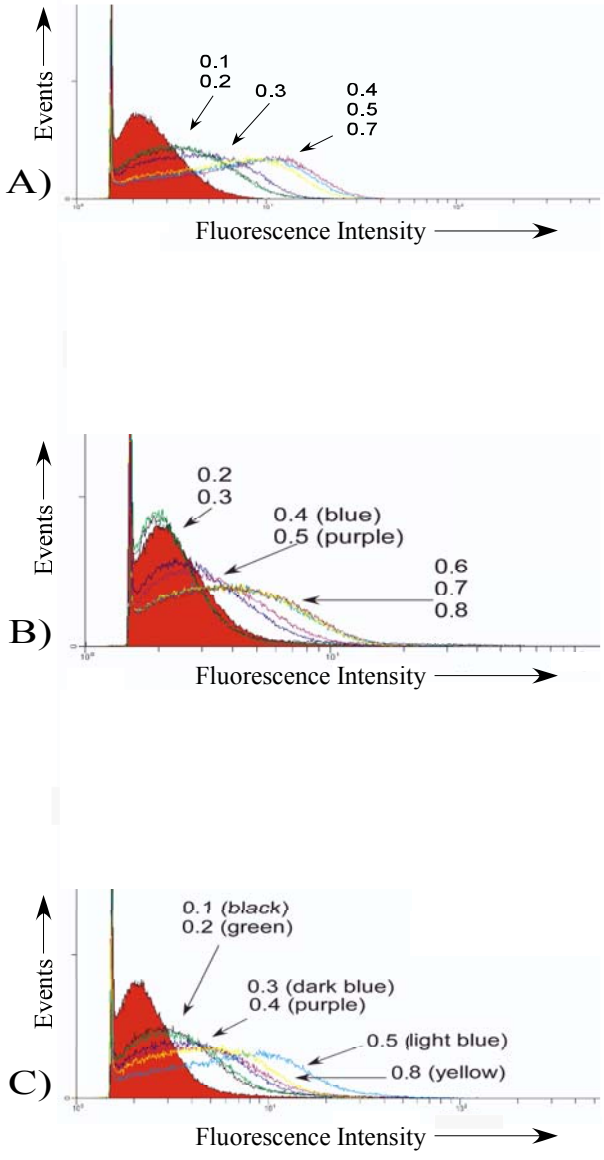


Figure 4-4. Optical density time course assays. B. subtilis vegetative cells were grown in LB at 37° C for various times. 1 mL aliquots were taken at various optical densities and washed as appropriate for the assay methodology. FACS assays were carried out using 2 pmols of aptamer labeled with fluoroscein A) clone C3 B) clone C5 C) clone B6.

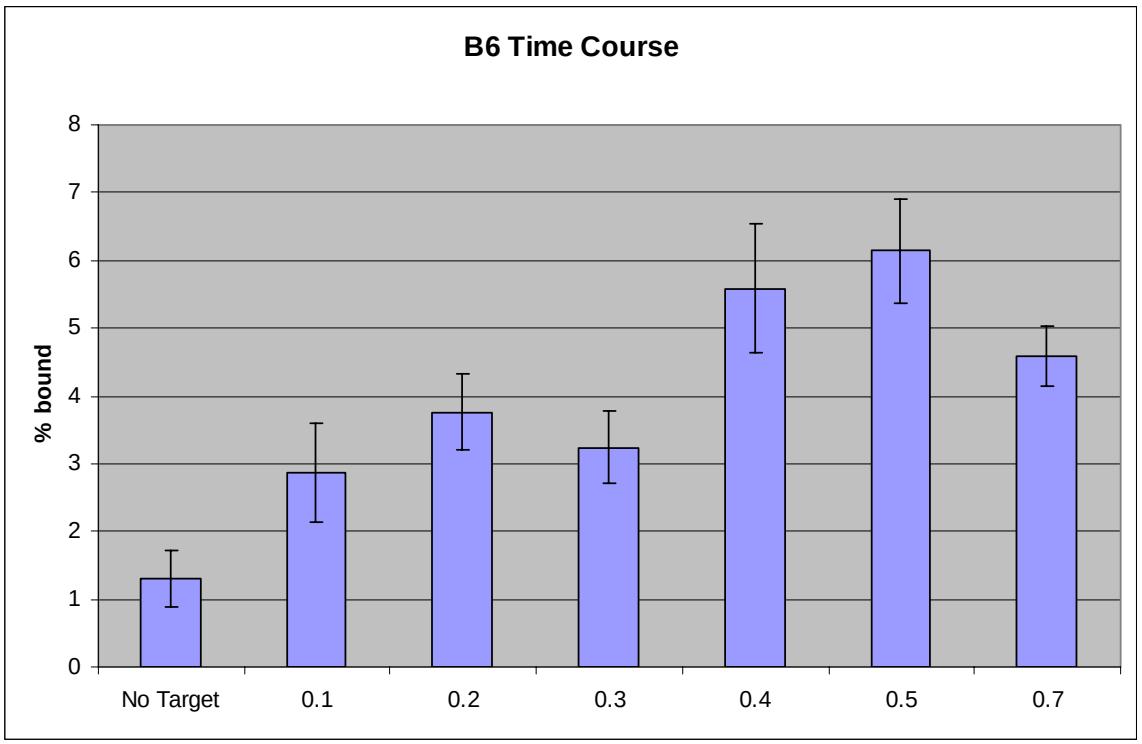


Figure 4-5. Clone B6 time course filter binding data. *B. subtilis* vegetative cells were grown to appropriate optical densities and incubated with 2 pmols radiolabeled aptamer. Following binding, the bacteria containing reaction was passed through a nitrocellulose/nylon filter sandwich in a 96 well dot blot manifold and washed 3 times with binding buffer. Filters were exposed to a phosphorimage screen and retained counts of radiation were quantitated.

Selected aptamer clones are unable to bind *B. subtilis* spores

As *B. subtilis* is a spore-forming bacterium, part of the natural end to the vegetative cell life cycle is the formation of a spore. In a given culture a certain number of bacterial cells will form spores. To ensure the selected aptamers are binding to the vegetative cells and not any spores formed in culture, binding assays were carried out against purified spores themselves. A *B. subtilis* spore preparation was generated, and examined under a phase contrast microscope to ensure there was little or no contamination of vegetative cell debris (Figure 4-6). Once it was determined that the

spores prepared were suitable for experimentation, a binding assay was carried out using both FACS and filter binding methodology. In both types of experiments, 1 x 10^8 spores(as determined by counting on a hemocytometer) were used. This is in a 10 fold

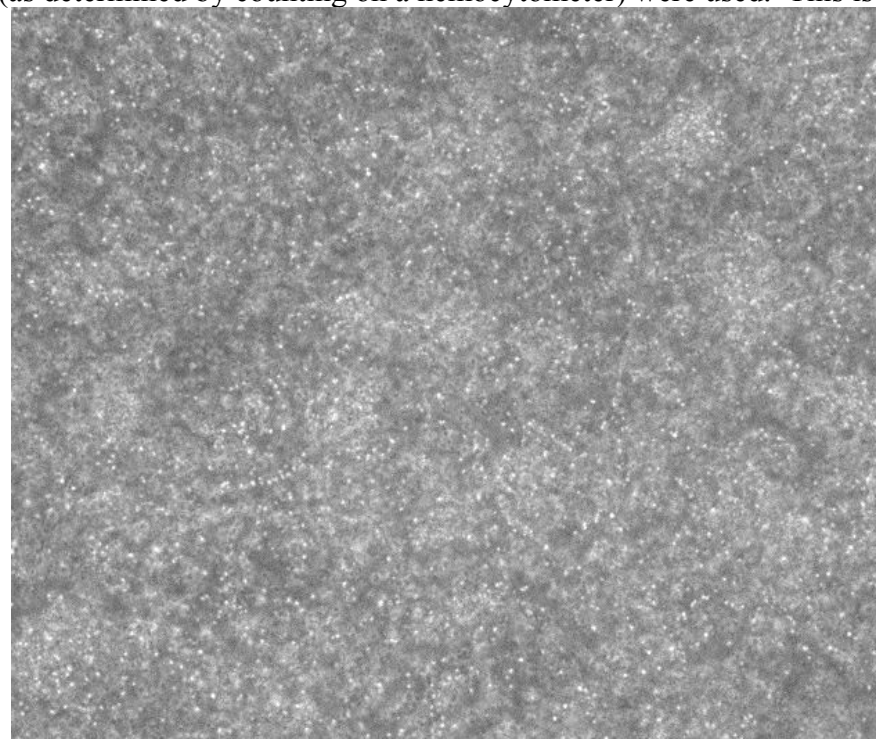


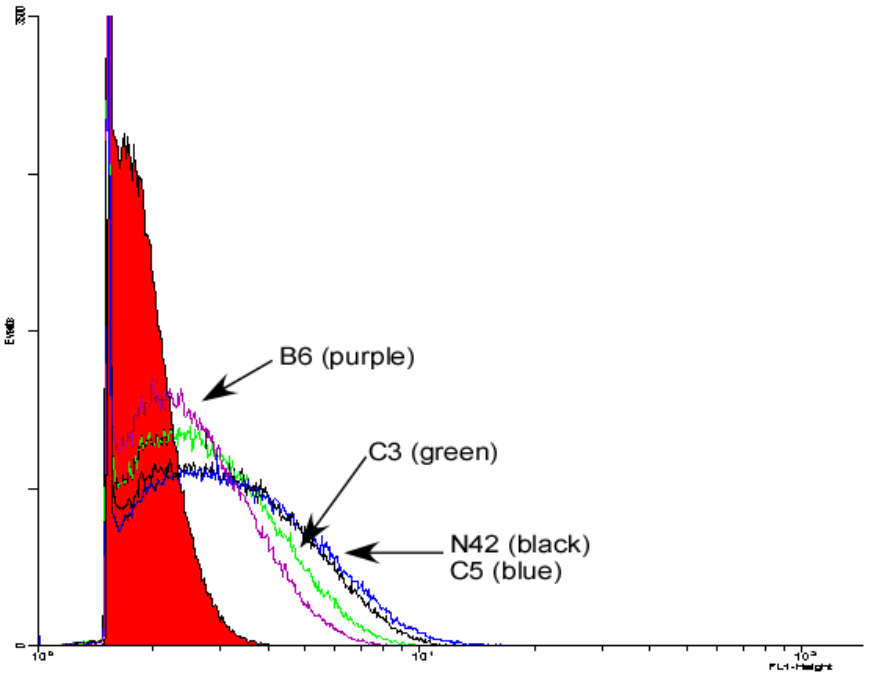
Figure 4-6. Microscope photo of spore preparation. Sporulation was induced in B. subtilis vegetative cells. Vegetative cell debris was the separated from spores through the use of a density gradient. Purity of the spores were then checked with phase contrast microscopy using a 40x objective.

excess of the vegetative cells used during both the original selection and binding assays. This was done to ensure there was no possible signal from these spores. These data indicate that the aptamer clones do not bind the spores (Figure 4-7).

However, there was still the possibility that the aptamers were binding to a sporulation molecule present at some stage in spore development. The formation of a spore from a vegetative cell is a multi-stage process involving a number of different alterations in both vegetative cell morphology and expression of surface structures (Margolis, Driks et al. 1991). Additionally, the developing spore possesses its own

unique morphology and surface composition. As the exact nature of the vegetative cell to which the aptamer was selected was not known, any of the above changes could provide a site for binding. To narrow down the list of possible targets, the asporulation strain 1S1 was again used in a binding assay. The results of these assays demonstrate that the aptamer binds the asporulation mutant with a high degree of affinity (Figure 4-8). This means that the target is definitely not a spore, nor is it a protein or other such extracellular component associated with the initiation of the sporulation process. This also means that changes to the cell surface due to sporulation are not the target of the aptamer.

A)



B)

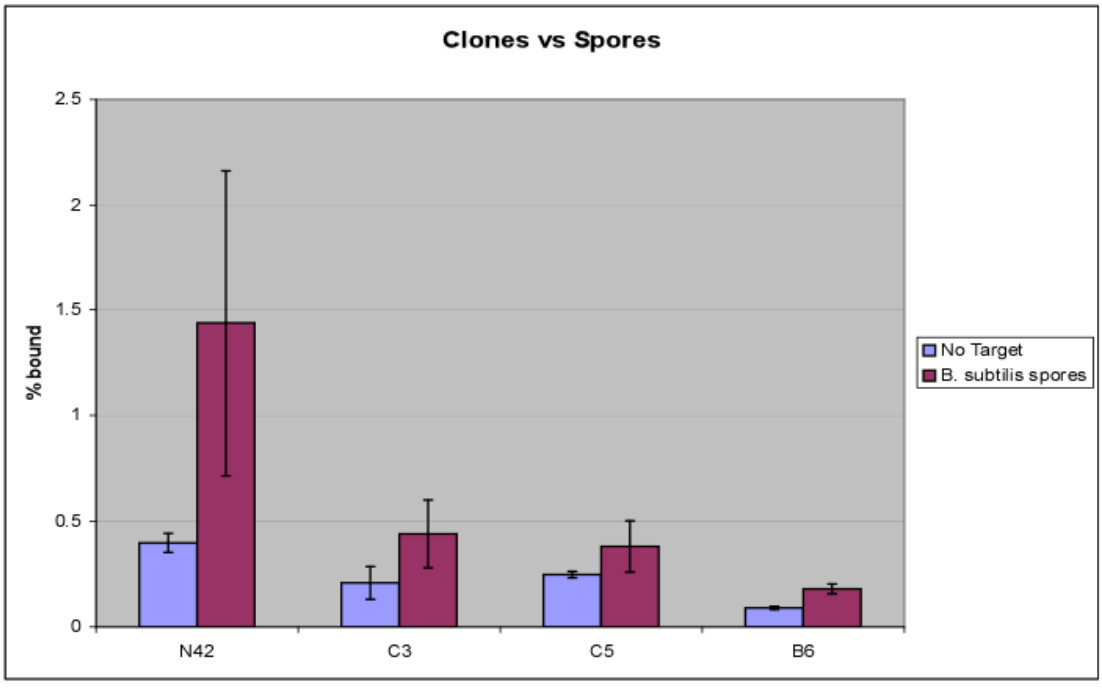
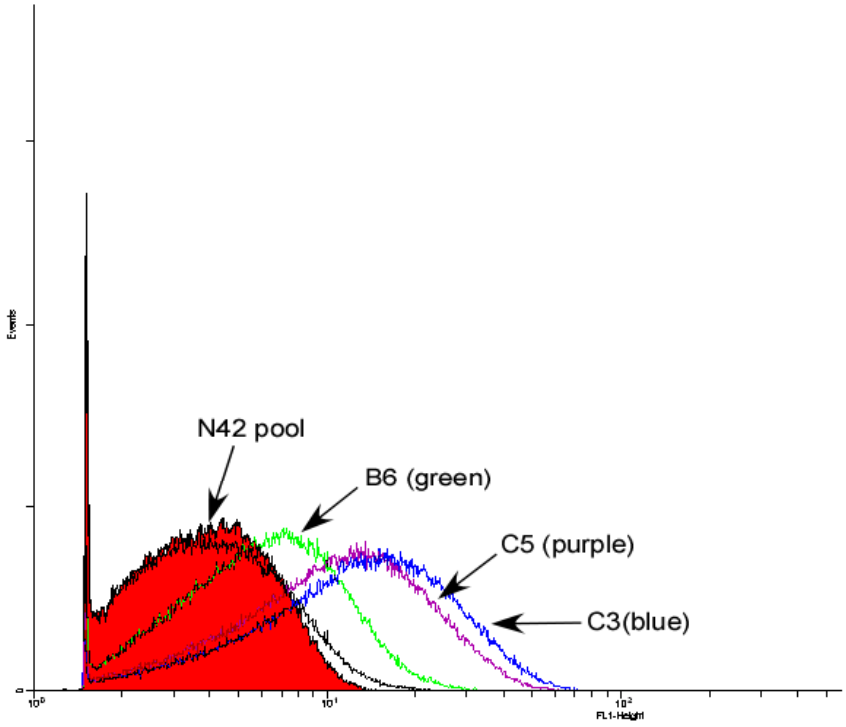


Figure 4-7. Binding assay vs. spore. 2 pmol of labeled aptamer were incubated with 10^8 B. subtilis spores. Binding was assessed either through A) FACS scanning or B) nitrocellulose/nylon filter in a 96-well dot blot manifold. Labeled N42 pool was included as a control

A)



B)

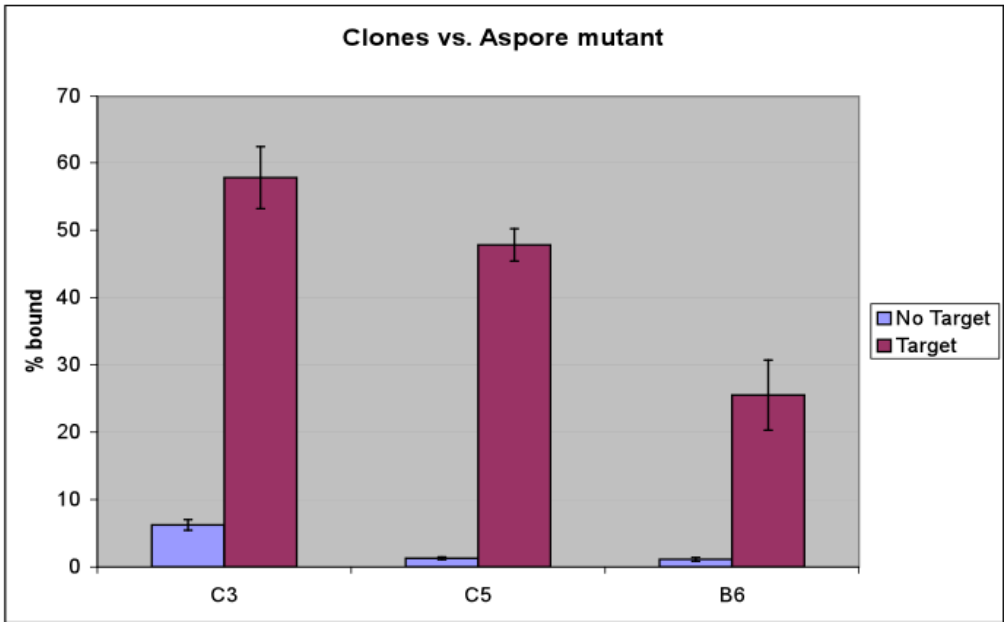


Figure 4-8. Binding assay vs. asporulation mutant. 2 pmol of labeled aptamer were incubated with 10^7 of B. subtilis vegetative cells from a strain deficient in sporulation. Binding was assessed either through A) FACS scanning or B) nitrocellulose/nylon filter in individual filter holders. Labeled N42 pool was included as a control for the FACS analysis.

Aptamer clones are able to bind *B. subtilis* mutants

The potential targets for the aptamer to bind on a surface as complex as a bacterial vegetative cell are very numerous. In order to help identify a binding target, it is sometimes helpful to eliminate what it does not bind to. By eliminating potential targets, an approach to successfully identifying the actual target can be tailored. Two extracellular structures present on *B. subtilis* immediately come to mind when thinking about potential binding targets, the cell wall and flagella. The cell wall primarily consists of peptidoglycans (about 40% by dry weight) and anionic polymers such as teichoic acid. There are other attached surface proteins and lipids as well, but those are the primary constituents of the cell wall. The role of teichoic acid contributes significantly to the formation and assembly of the cell wall, by acting as an anchor for the cross-linking of the peptidoglycans (Bhavsar, Erdman et al. 2004). Additionally, as teichoic acids contain charged groups, (phosphate for teichoic acid) polymers are able to contribute to the net charge of the cell wall. Flagella are the primary means for locomotion in these bacteria. Flagella are the primary means for locomotion in these bacteria. They are composed of flagellins, which are small globular proteins with a molecular weight of approximately 50,000 Daltons. One reason to suspect that flagella may have been a target of the whole cell selections is that the flagella of various bacteria species (including the *Bacillus* genus) have been shown to be highly antigenic (Motzel and Riley 1991; Murakami, Hiraoka et al. 1993), and antibodies have been raised against the flagellar fractions of various bacteria (Tanaka, Hirayama et al. 1987; Simonson and Siebeling 1988; Ricci, Torosantucci et al. 2005). Additionally, aptamers have been successfully selected against pili from *Salmonella enterica* serovar Typhi. Following selection, these aptamers were found to be able to bind to the whole bacteria and inhibit cell invasion (Pan, Zhang et al. 2005).

To test whether the above structures are the target of the aptamer or perhaps influence the ability of the aptamer to bind, two mutants deficient in the production of flagella and teichoic acid were obtained from the Bacillus Genetic Stock Center (The Ohio State University, Columbus, OH). These bacteria were grown as all other strains and filter binding assays were carried out using body labeled RNA.

The lack of either of these two portions of the cell apparently does not result in a loss of binding affinity (Figure 4 –9). However, this really only eliminates flagella as a potential binding candidate. Teichoic acid cannot be completely eliminated as a target, although this is not a very likely candidate as it is a negatively charged molecule and so would tend to repel nucleic acids, because it is doubtful that the teichoic acid deficient mutant is completely deficient in teichoic acid synthesis. Bacteria where production is completely blocked are not viable. The real value of this mutant lies in that the deficiency in teichoic acid likely causes other anionic polymers, such as teichuronic acid, to take on teichoic acids role in cell wall assembly. As these two polymers are functionally distinct, it was hoped that this would result in an altered morphology (Bhavsar, Erdman et al. 2004), and if a binding deficiency occurred perhaps given additional insight into the potential target.

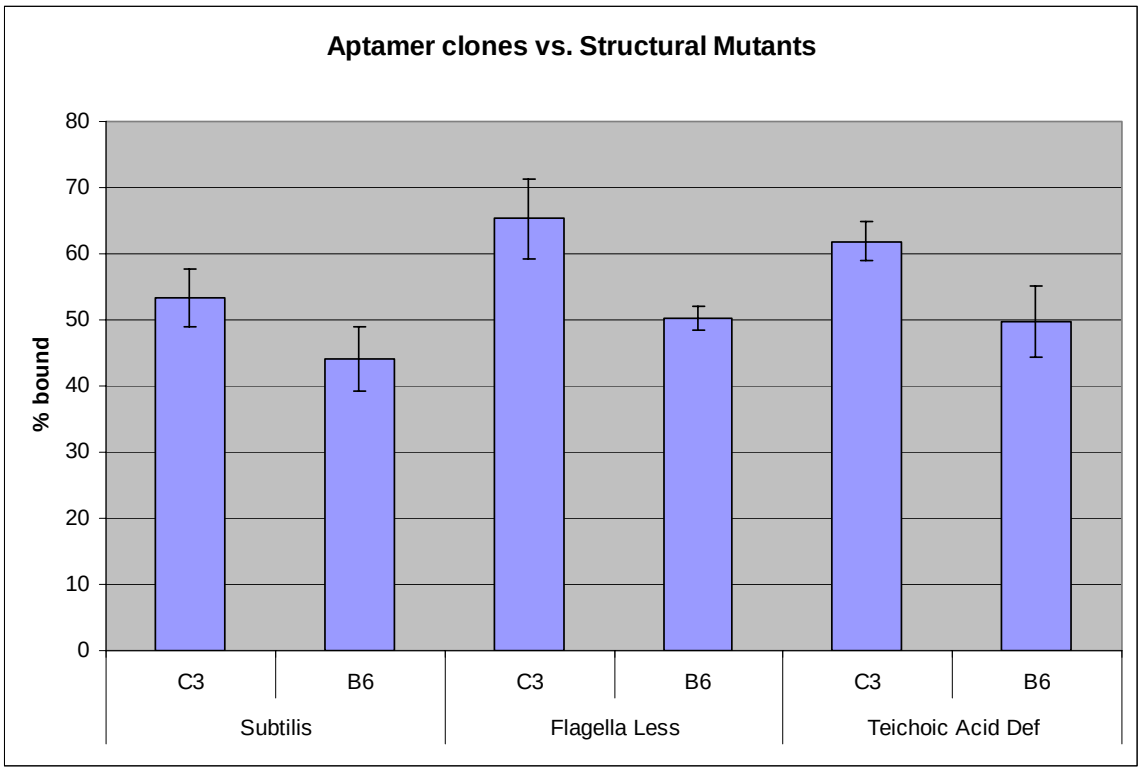


Figure 4-9. Binding assays with structural mutants. B. subtilis strains deficient in the production of either teichoic acid or flagella were obtained from the BGSC. 10^7 of these bacteria were incubated with 2 pmols of radiolabeled aptamer. Following binding, the bacteria containing reaction was passed through a nitrocellulose/nylon filter sandwich in an individual filter holder and washed 3 times with binding buffer. Filters were exposed to a phosphorimage screen and retained counts of radiation were quantitated. Assays against the selected strain were included to serve as a comparison

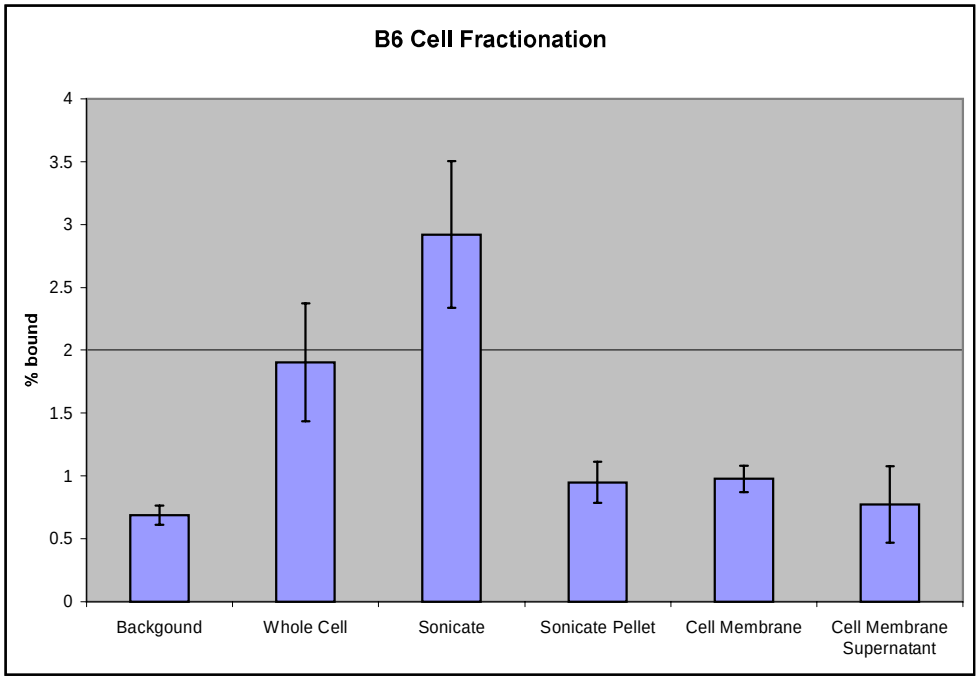
Further Attempt to Identify the Binding Target

To further define the binding target of the aptamer clones, a cell fractionation study was carried out. By partitioning cells into separate fractions, the aptamers may be assessed for their ability to bind various, crude portions of the vegetative cell. This will then give more information about the identity of the binding target. Also, by separating portions that have little or no affinity for the aptamers there will be less potential for these portions to interfere with the binding of the aptamer for its target, although this is probably more important for affinity separation experiments.

Body labeled aptamers were combined with vegetative cells grown and fractioned as in the experimental section. Each fraction was assayed for binding on a 96-well dot blotter. The results of these assays indicate that both aptamer clones are able to bind the fraction consisting of sonicated whole cells with a higher affinity than they do the other fractions (Figure 4-10). This may indicate the binding target is a cell wall associated protein. That the cell membrane fraction does not bind indicates that binding target is probably a loosely attached component. If it was an integral part of the cell wall or membrane, then the binding affinity should be retained in that portion.

To examine the possibility that the binding target of the aptamers is a protein, the sonicated fraction of the cells were subjected to trypsin mediated protease digestion (Figure 4-11). The resulting drop in binding affinity of both clones for that of the sonicated fraction indicates that the binding target is a protein. Although C3 does not show a decrease in degree of binding equivalent to that of B6, there is still a sufficient drop in binding to logically conclude that the target is a protein. As a control, a portion of the sonicated fraction was kept and not subjected to trypsin digestion.

A)



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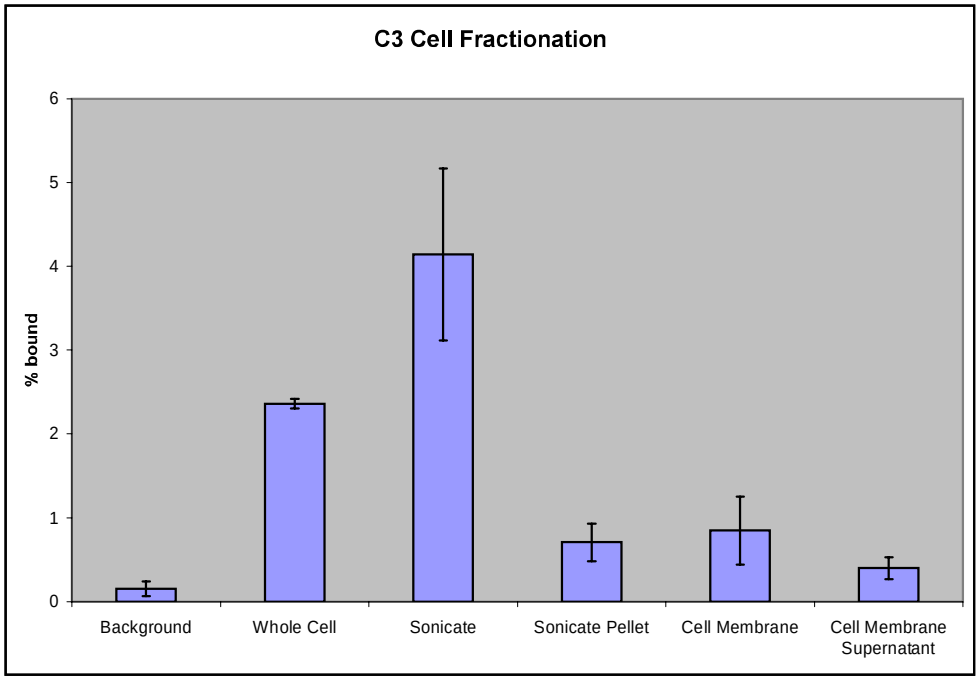
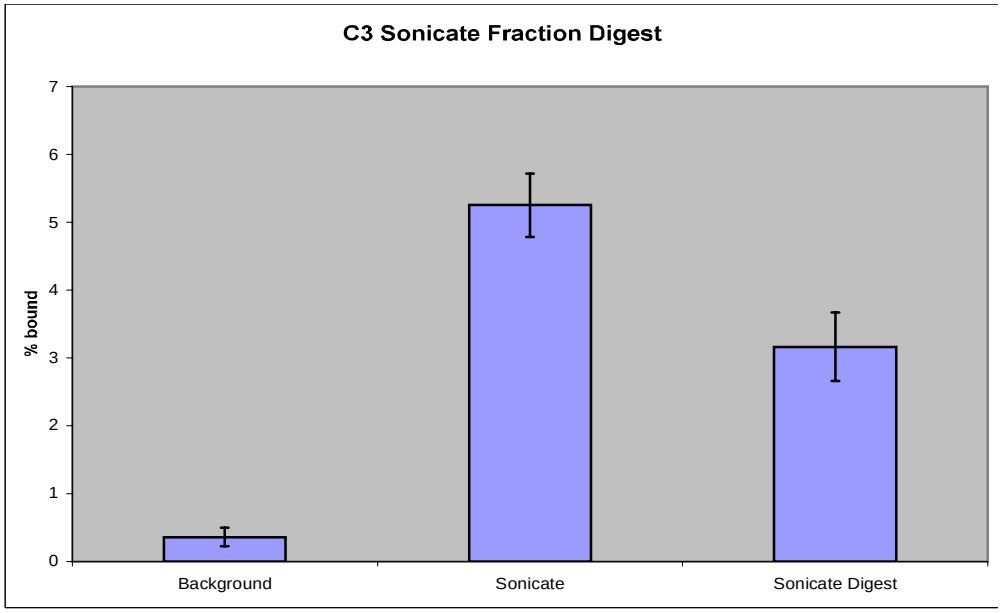


Figure 4-10. Assays with cell fractions. B. subtilis vegetative cells were grown and fractionated as described in the Experimental Design section. 2 pmols of radiolabeled aptamer were then incubated with the various fractions. The binding reaction was then passed through a nitrocellulose/nylon filter sandwich in a 96 well dot blot manifold and washed 3 times with binding buffer. Filters were exposed to a phosphorimage screen and retained counts of radiation were quantitated. A) clone B6 B) clone C3

A)



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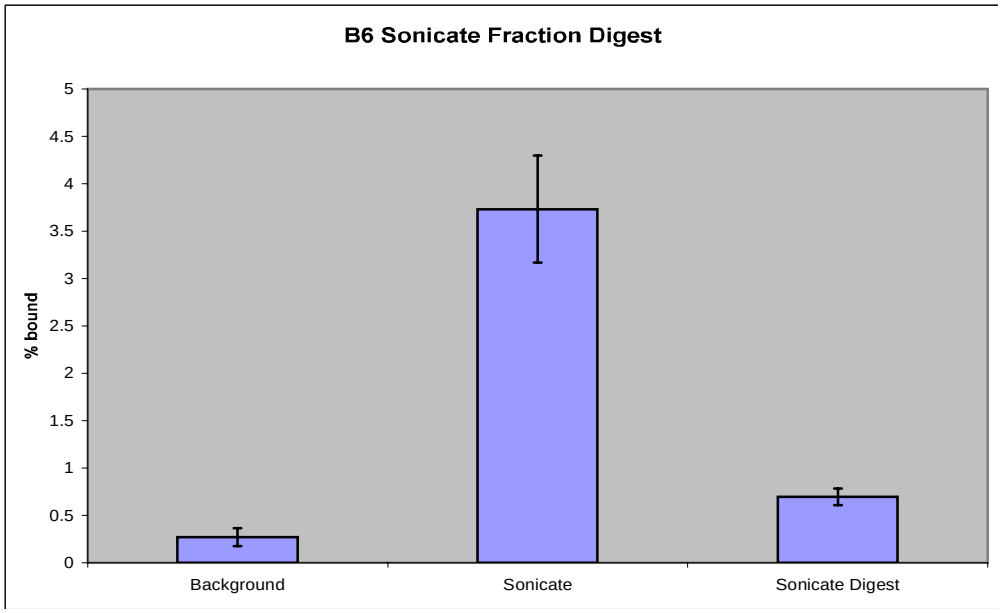
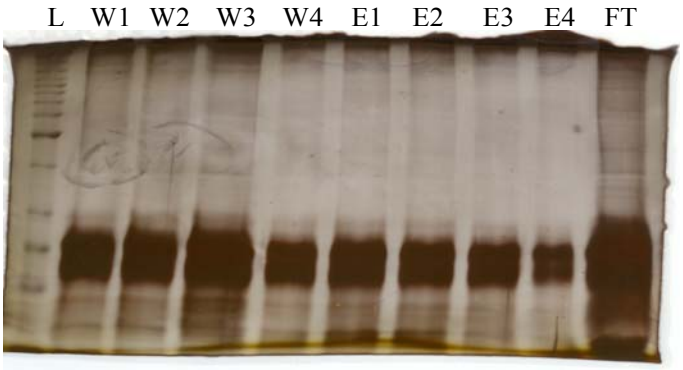


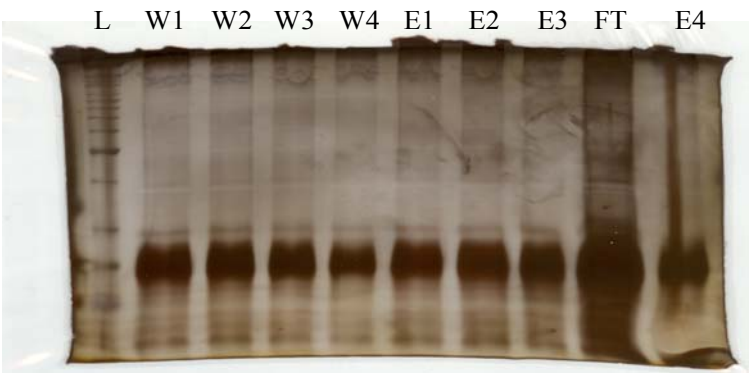
Figure 4-11. Aptamer clones vs. trypsin digested sonicated fraction. Bacillus subtilis vegetative cells were grown and fractionated. The sonicated fraction was then incubated at 37° C for 1 hour in the presence of tyrpsin. The resulting digested fraction was then incubated with 2 pmol radiolabeled aptamer. The binding reaction was then passed through a nitrocellulose/nylon filter sandwich in a 96 well dot blot manifold and washed 3 times with binding buffer. Filters were exposed to a phosphorimage screen and retained counts of radiation were quantitated. A) clone C3 B) clone B6

In an attempt to pull down the binding target, the sonicated fraction was passed through an aptamer affinity column in a manner analogous to an antibody immunoaffinity column. This method has been successfully used in previous studies to identify aptamer binding targets (Romig, Bell et al. 1999; Daniels, Chen et al. 2003). To carry out these experiments, biotinylated aptamer was combined with streptavidin conjugated agarose beads and sonicated *B. subtilis* vegetative cells. Unfortunately this approach did not reveal the binding candidate (Figure 4-12). This could be for a couple of different reasons. One, and most importantly, it could be that this technique is not conducive for determining the binding target. That there are only two examples of this methodology in the literature indicates that it may not be applicable to all targets (Romig, Bell et al. 1999; Daniels, Chen et al. 2003). Another possibility is that the aptamer is unable to assume the proper binding structure when it becomes attached to the streptavidin. Although the biotin molecule itself does not interfere with binding (data not shown) it is possible that the streptavidin molecule could disrupt this.

A)



B)



C)

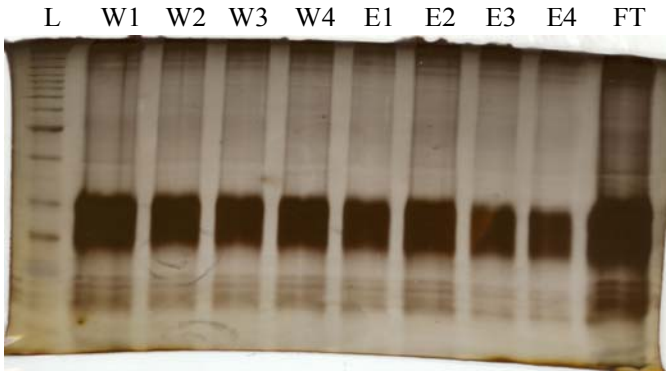


Figure 4-12. Results of the aptamer affinity column. 500 pmols of biotinylated aptamer was incubated with streptavidin coated agarose beads and the sonicated fraction of B. subtilis vegetative cells. The reaction was allowed to bind at room temperature for 45 minutes, at which time it was passed through a column. The beads were washed with 4 resin volumes of binding buffer in 4 separate washes and the fractions were collected. Following washing, the beads were washed with an elution buffer and the fractions were collected. L = ladder, W= wash, E= elution. The numbers following the letters correspond to the fraction order. FT = flow through and refers to the initial flow through following application of the binding reaction. A) clone C3 B) clone B6 C) negative control. The gel was silver stained for visualization of protein bands.

CONCLUSIONS

The above experiments were designed to more fully define the capabilities of the selected aptamers to bind *B. subtilis* vegetative cells. Several pieces of useful information were determined, not the least of which was that the aptamers do in fact bind vegetative cells and not spores. This is indicated with photographic evidence, sporulation deficient mutants, and assays against pure spore preparations. It was also determined that the aptamers display a reduced affinity for genetically related *Bacillus* genus members. Although aptamers do display some binding to related bacteria, it may still be possible to distinguish *B. subtilis* from these other species.

It was also established that the aptamers preferentially bind to cells at optical densities of 0.5 to 0.6. FACS scanning data indicates that this is not a concentration dependent phenomenon. Therefore, the increased binding as a function of optical density is may be due to increased expression of binding target on the cell surface. This could be due to several different factors, including changes in membrane surface. This possibility may be correlated to the dramatic effect temperature has on binding affinity. It is clear that significant changes occur in bacteria grown at less than ideal temperatures including changes in membrane fluidity and differential gene expression.

Although these experiments did not yield a positive identification of the binding target, several pieces of information were gained. It was determined that the binding target is not a spore, nor a target associated with the sporulation process. This is indicated by lack of binding to spores, and retention of binding to asporulation mutants blocked at stage 0 of the sporulation process. Since the aptamers are still able to recognize *B. cereus* and *B. thuringensis* it is probably that the target is expressed to some degree on these two species, although not as highly as it is on *B. subtilis.* Alternatively, the binding target for these two species may not be an identical copy of what is found on

B. subtilis, but something related. If this is so, it indicates something that is conserved.

Ultimately, the identity of the target is not of critical importance. It would be an interesting thing to know and correlate to experimental data, but in the end what is relevant is that the aptamers are able to recognize the vegetative cells of *B. subtilis.* This provides proof of principle that it is possible to select aptamers against bacterial cells. Future selections may be adapted based upon what was learned in these experiments to further increase specificity for species, and decrease sensitivity to temperature to generate an aptamer more viable for use in a detection device. This was the ultimate purpose of these experiments.

EXPERIMENTAL DESIGN

Bacterial strains/Growth Conditions

Bacterial strains used in these experiments are *B. subtilis* ATCC 6051, *B. cereus* ATCC 14579, and *B. thuringensis* ATCC 10792 obtained from Walther Ellis at the University of Utah. Mutant strains of *B. subtilis* used in this study were obtained from the Bacillus Genetic Stock Center (The Ohio State University, Columbus, OH) and are teichoic acid deficient BGSC 1A136, flagella deficient BGSC 1A764, and sporulation deficient BGSC 1S1. All bacterial strains were grown as 5 mL overnight cultures (15 mL culture tube) in LB at 37° C with shaking. Overnight cultures were then used to start fresh LB cultures and growth was continued at 37° C until the appropriate optical density as determined by A_{600} measurements was reached. These conditions were used for all bacterial cell preparations unless otherwise noted. Fifteen mL culture tubes were used for culturing all bacterial strains. Unless otherwise noted, prior to experimental use all

bacterial cultures were centrifuged at 1500 x g for 10 minutes. They were then washed 2 x in Binding Buffer (1x PBS supplemented with 5 mM $MgCl_2$) and centrifuged as above.

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Variable Growth Conditions

For temperature studies, frozen stocks were used to start overnight cultures which were then grown at the requisite temperature 23° C, 30° C, or 37° C. These stocks were then used to initiate growth of bacterial cells in fresh 5 mL LB cultures at the appropriate temperature.

For experiments using different carbon sources, growth conditions were as follows. Minimal medium was prepared as follows 1 g of $(NH_4)_2SO_4$, 7 g of K_2HPO_4, 3 g KH_2PO_4, and 0.5 g of sodium citrate were combined into 490 mL of ddH_2O and autoclaved on liquid cycle for 30 minutes. Upon cooling to room temperature, 400 microliters of a 1 M $MgSO_4.7H_2O$ and 5 mL of filter sterilized 5 mg/mL L-tryptophan were added. The media was then split into 2 equal volumes and 2.5 mL of filter sterilized 50% glucose was added to 1 aliquout and 2 mL of 60% autoclaved glycerol was added to the other aliquot for a final volume of 250 mL of each varied carbon source containing medium. Both aliquots were then filter sterilized by passing over a 0.22 uM nitrocellulose filter. 5 mL of minimal media thus prepared was used to start a culture of *B. subtilis* from a frozen stock. This overnight culture was then used to start a fresh 5 mL culture of bacterial cells using the appropriate glucose or glycerol containing minimal media.

Spore Preparation

Preparations of *B. subtilis* spores were prepared as previously described (Jinks, Guthrie et al. 1985). Briefly, overnight bacterial cultures were grown and then diluted to an optical density of 0.4 – 0.6 in SRSM – 1. SRSM – 1 was prepared as follows 5.8 g of K_2HPO_4 and 9.05 g KH_2PO_4 were added to 970 mL of ddH_2O and autoclaved for 30 minutes on liquid cycle. Upon cooling to room temperature, 5 mL of a filter sterilized metal mixture (0.14 M $CaCl_2$, 0.01 M $MnCl_2$, 0.2 M $MgCl_2$), 0.5 mL of 2 mM $FeCl_3$ in 0.1 N HCl, and 20 mL of a filter sterilized 0.5 monosodium L-glutamate were added and the final volume brought to 1000 mL. The final pH is 6.5. The final volume of liquid media in the culture flask was kept to below 25% of the total flask volume. The culture was incubated at 37° C for 24 hours and progress of sporulation was checked by phase contrast microscopy (Olympus). To purify pure spores from vegetative cell debris, the sporulation culture was first centrifuged at 10,000 x g for 10 minutes. The resulting pellet was then purified utilizing a density gradient. Briefly the pellet was resuspended in 2 mL of 20% sodium diatrizoate. This 2 mL was then layered on top of a 20 mL preparation of 50% sodium diatrizoate and centrifuged at 18,000 x g for 30 minutes. The spore containing pellet is found at the bottom of the tube while the vegetative cell debris remains at the top. If necessary this was repeated to achieve a higher degree of purity. Once the desired level of purity was obtained as determined by phase contrast microscopy the spore pellet was washed 3x in ddH_2O to remove trace amounts of sodium diatrizoate. Cells were then quantitated by counting on a hemocytometer.

Nitrocellulose/Nylon Filter Binding Assays

As described in Chapter 3.

FACS Binding Assays

As described in Chapter 3

Cell Fractionation

Cells to be fractionated were grown to an O.D. A_{600} of 0.5-0.6 in LB at 37° C. Upon reaching this optical density, the cells were transferred to a Sardest tube and centrifuged at 1500 x g for 15 minutes at 4°C. Following centrifugation the supernatant was discarded and the pellet resuspended in 5 mL of Solution A (0.1 M sodium phosphate buffer pH 7.0, 1 mM EDTA, 200 microgram/mL chloramphenicol, and Roche Complete EDTA-free protease inhibitor (Roche). One mL of this was aliquoted for later use in a binding assay. The remaining 4 mL was then sonicated on ice until cell disruption was ~80 % as determined by light microscopy (Olympus). 1 mL of the sonicated cells was aliquoted for later use in a binding assay. The remaining 3 mL of sonicated cells were then centrifuged at 5000 x g for 15 minutes. The resulting pellet was resuspended in 300 microliters of Solution A. The supernatant was then removed and centrifuged at 212,000 x g for 1 hour and 30 minutes in a Beckman L7-55 Ultracentrigue (Beckman-Coulter). The resulting pellet is resuspended in 300 microliters of Solubilization buffer (1 x PBS, 1 mM EDTA, 0.1% Triton-X 100, Roche Complete EDTA-free protease inhibitor (Roche). One hundred microliters of the two pellets resuspended in 300 microliters was retained and diluted to 1 mL in their respective buffers. All fractions collected were then subjected to nitrocellulose/nylon binding assays on a 96-well dot blotter as previously described. Cell concentration per fraction was kept constant throughout.

Trypsin digestion of the sonicated fraction was achieved as follows. Cells were grown and sonicated as above. One mL of this sonicated fraction was then combined

with 20 micrograms of sequence grade modified Trypsin (Promega) resuspended in Resuspension buffer as per manufacturers instructions. The resulting suspension was then incubated at 37° C for 1 hour and 30 minutes. The digested fraction was then placed on ice and subjected to radioactive filter binding assays with labeled aptamer in a 96-well dot blotter as previously described. To provide a control to assess binding, 1 mL of the sonicated fraction was also removed from the initially sonicated cell suspension and immediately placed on ice.

Aptamer Affinity Column

Aptamer clones were subjected to chemical biotinylation on the 3' end. This was carried out as described (Qin and Pyle 1999). Briefly, RNA is oxidized with 0.1 M $NaIO_4$ in 0.1 M NaOAc pH 5.2 (final concentration). The reaction is allowed to proceed for 90 minutes in the dark. Upon completion, the reaction is passed through a NAP 5

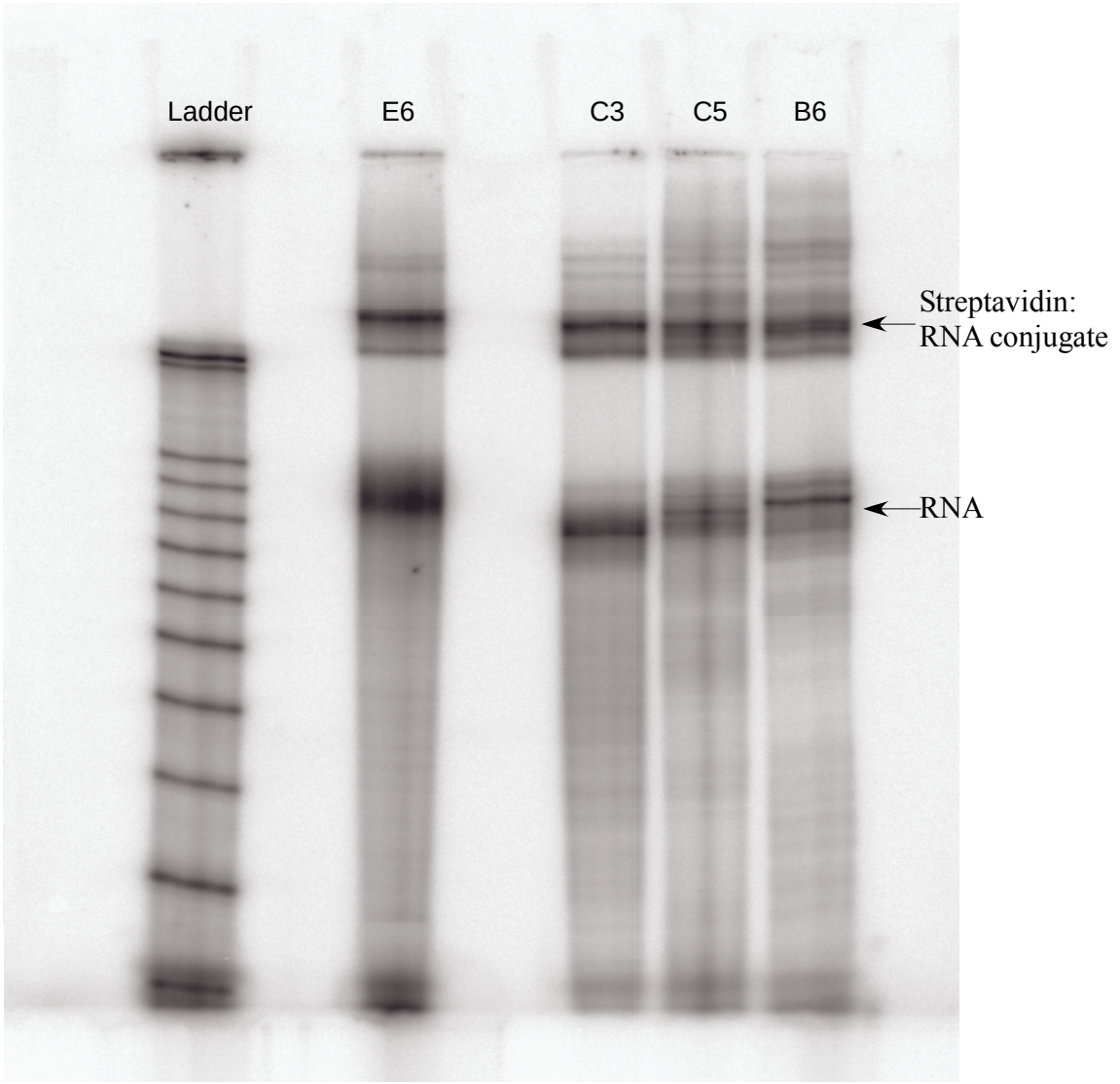

Figure 4-13. Test for biotinylation of RNA. To test if the aptamer were successfully conjugated to a biotin molecule, 50 pmols of radiolabeled biotinylated RNA was incubated with 50 pmols of free streptavidin protein in 1 x PBS with $MgCl_2$. Following incubation for 30 minutes, the reaction are electrophoresed on an 8% denaturing polyacrylamide gel. The gel is then dried and exposed to a phosphorimage screen. A ladder is run as a size standard. Bound RNA is shifted upward on the gel and is indicated by an arrow labeled as such. Free RNA runs according to its size and is confirmed by the ladder.

Desalting column (Amersham Bioscience) and ethanol precipitated. The pellet is resuspended in 0.1 M NaOAc pH 5.2, and a solution of biotin hydrazide in DMSO is added (50 mM final concentration). The reaction is allowed to proceed for 3 hours. Once

complete, the reaction is passed through a NAP 5 Desalting column and ethanol precipitated. A portion of the biotinylated RNA is radiolabeled on the 5' end using T4 Polynucleotide kinase (Invitrogen) and γ-P^{32} ATP (ICN radiochemicals). 50 pmol's of radiolabeled RNA is then combined with 50 pmols of free streptavidin protein in binding buffer. The reaction is then run on a gel to ensure successful biotinylation of the RNA molecule (Figure 4-13). Clone E6 is used as a negative control in these experiments.

Five hundred mL of *B. subtilis* vegetative cells were grown and sonciated as above. One mL aliquouts were removed and kept at 4° C until ready for use. Immediately prior to use, 100 microliters of 10x PBS and 5.5 microliters of $MgCl_2$ is added to the 1 mL aliquots of sonicated cells to ensure proper salt balance for the binding reaction. 500 pmols of biotinylated aptamer is heat denatured for 3 minutes at 70° C and allowed to cool to room temperature. Following cooling, the RNA containing solution is combined with the sonicated cells. Ten microliters of streptavidin coated agarose beads that have been washed 3 times in 1x binding buffer are also added to the reaction. Binding is allowed to proceed at room temperature for 45 minutes. The reactions are then passed through a column and the flow through collected. This flow-through is combined with a 5-fold excess of ice cold acetone and kept at -20° C for 2 hours. It is then precipitated at 15,000 x g for 30 minutes and the pellets resuspended in 20 microliters of binding buffer.

The agarose beads remaining on the column are washed with 4 10-microliter aliquots of binding buffer supplemented with Roche – EDTA free protease inhibitor. Each fraction is immediately combined with 5x denaturing protein loading dye (0.1 M Tris pH 6.8, 2.5% SDS, 50% glycerol, 0.0005% (w/v) bromophenol blue, 0.1% (v/v) β-mercaptoethanol) and placed on ice. Elution fractions are then collected by passing 10 microliters of Elution solution (1 x PBS, 1 M NaCl, 5 mM EDTA, Roche EDTA-free

protease inhibitor) through the streptavidin agarose beads. Four such fractions are taken and immediately combined with 5 x denaturing protein loading dye and placed on ice. All fractions are then electrophoresed on a 12% SDS-protein gel and silver stained (Bio-Rad) following manufacturers protocol.

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