Evaluation of the Knocking-down Effect of Shiga Toxins in the Enterohemorrhagic *E. coli* O157 (EHEC); a Strategy Towards RNA-based Therapeutics

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2018

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Arslan Sarwar

Dedicated to

My Grandmother, Parents and Brothers

ACKNOWLEDGEMENTS

In the name of Allah, the Most Beneficent, The Most merciful

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Abstract

Antibiotic discovery was considered as a “Miracle”, until the antimicrobial resistance developed against them became the major clinical concern. Shiga toxin producing *E. coli* (STEC) is also such kind of microbe against which misuse and overuse of antibiotics has led to the development of resistant bacterial strains. Among STEC, *E. coli* O157:H7 strain is the most notorious and have reported in many outbreaks. Few novel anti-bacterial strategies have successfully been administrated to treat the bacterial infections while many are under investigation. Use of synthetic non-coding RNA is also such kind of strategy which has the potential to become a trusted way of treating bacterial infections. For regulation of gene, synthetic sRNAs need to bind with mRNA of the target gene via Watson-crick complementary model and need promoter, mRNA binding region, Hfq protein and terminator for their proper functioning. In this study, a synthetic non-coding RNA cassette was designed against the shiga toxin 2 gene *(stx-2)* and was incorporated into *E. coli* O157:H7 with the help of pAB.001 plasmid (already harboring the promoter, Hfq and terminator regions). Effects caused by synthetic sRNA on shiga toxin production, in target bacteria, were analyzed by real time RT-PCR and MTT assay. Computational web tools based predictions indicate that synthetic sRNA against *stx-2* showed maximum repression capability. Synthetic sRNA designed against *stx-2* gene also showed significant reduction at the mRNA and protein levels after analysis in the wet lab. A comparative analysis of the effects caused by similar synthetic sRNAs designed against *stx-1* was performed and it was observed that *stx-1* gene showed no significant change at the mRNA level compared to *stx-2* which showed a significant reduction. However a clear reduction in the shiga toxin level of both genes was observed at the protein level.

Difference in silencing effect at mRNA level was observed due to different production rate of target mRNAs.

1. Introduction

During the past twenty years, advancements in the field of synthetic biology has led scientists to consider small non-coding RNAs (sRNAs) as major regulators of the genes. sRNAs can be regarded as every RNA transcript (<500 nucleotide base pairs) present in bacteria which can control gene expression at the mRNA or protein level (Liu & Camilli, 2011). RNA transcripts responsible for gene silencing in bacteria can be termed as non-coding RNA regulators (Thomason & Storz, 2010). Regulation by non-coding sRNA can be regarded as a natural phenomenon evolved in bacteria (Dinan & Loftus, 2013). A huge number of sRNAs discovered in last decade can regulate gene expression by overexpressing or repressing genes in microorganisms in which they have been identified (Saito, Kakeshita, & Nakamura, 2009). About 80 naturally occurring sRNAs have been discovered in *Escherichia coli* which do not encode for tRNA or mRNA and they have been considered as regulators of proteins (Chen *et al*., n.d.; Gottesman & Storz, 2011; Morita, Maki, Yagi, & Aiba, 2008; Saito *et al*., 2009). Analysis of total RNA in different bacteria has suggested that about 13 to 49% of bacterial genes are subjected to regulation with help of noncoding RNAs (Lasa, Toledo-Arana, & Gingeras, 2012). 14 sRNas have been identified from *E. coli* K12 strain which include *sraA, sraB, rprA, sraC, sraD, sraE, gcvB, sra G, sra H, sraK, sraL, sraI, sra J* and *sraF* (Saito *et al*., 2009).

In the last few decades developing resistance against antibiotics in bacteria has been considered as one of the major hurdles in medical sciences. Frequent use of antibiotics has induced resistance in many types of bacterial strains. Most prominent strains which have gained resistance against antibiotics include *Staphylococcus aerus, Mycobacterium tuberculosis, Neisseria gonorroheae* and various strains of *E. coli*. Discovery of new antibiotics has been reduced to a critical level

because of development of different resistance mechanisms in bacteria against such drugs (Penchovsky & Traykovska, 2015). There is a need to develop new mechanisms to counter toxic effects produced by antibiotic resistant bacteria (Lewis, 2013).

Non-coding sRNAs can be divide into *cis*-encoded or *trans*-encoded regulatory sRNAs. *Cis*-encoded non-coding RNAs originate from the same loci as of their target mRNA. Such *cis*-encoded non-coding RNAs can be regarded as antisense RNA. *Cis*-encoded non-coding RNAs can act by inhibiting transcription elongation with help of terminator hairpins or they can prevent translation initiation by occupying the ribosome binding site thus prevent translation initiation (Chappell, Watters, Takahashi, & Lucks, 2015). *Cis*-encoded non coding sRNAs form a duplex by perfect complementary base pairing with target mRNA. They develop an antisense-sense fold by following Watson Crick model. This folded duplex seizes the ribosome binding site for target mRNA and hinders the protein formation by stopping translation initiation (Waters & Storz, 2011). There is another type of *cis*-acting RNAs which originates from the 5' end of untranslated region of same gene which is being targeted. Such type of sRNA include Riboswitches (Lai, 2003). Riboswitches form hairpin loops and regulate protein coding genes (Serganov & Nudler, 2013).

Trans-encoded non-coding sRNAs are encoded at positions which are distinct from their target genes. *Trans*-encoded sRNAs have partial complementarity with target mRNA and due to this fact a simple *trans*-encoded sRNA can target multiple mRNAs at the same time (Waters & Storz, 2011). *Trans*-acting sRNAs bind with mRNA with help of short stretches, 7 to 12 nucleotides in length, which are regarded as seed regions (Storz, Vogel, & Wassarman, 2011) . Such non-coding RNAs are usually expressed under stress conditions. When *trans*-coding RNA binds with target messenger RNA different outcomes are possible. *Trans*-acting sRNAs can lead to translation activation by pairing with 5'-untranslated region and allows entry of ribosomes for translation

initiation. *Trans*-acting sRNAs can repress gene expression by occupying mRNA or by degrading mRNA (Lay, Schu, Lay, Schu, & Gottesman, 2013). *RhyB* is a small non-coding RNA that regulates the expression of six mRNAs encoding Fe-binding or storage genes in *E. coli*. Degradation of mRNA with the help of *RhyB* depends upon its binding with Hfq protein and RNase E. RNase E gathers a complex of proteins which is called degradosome. Degradosome is essential for degradation of mRNA (Massé, Escorcia, & Gottesman, 2003).

Hfq is RNA binding protein which is required by most of sRNA especially *trans*-encoded sRNAs for their stability and activity (Lenz *et al*., 2004). Hfq stabilizes the sRNA and prevents degradation of sRNAs. It also increases binding rate of mRNA with sRNA and can open folded structures present in sRNA for binding (Vogel & Luisi, 2015). sRNA forms tight bonds with hfq protein before attachment with mRNA (E. Gerhart H Wagner, 2013). Hfq can also interacts with polymerase I, polynucleotide phosphrylase and RNase E which all are involved in mRNA degradation. Hfq-binding sRNAs of *E. coli* facilitates degradation and destabilization of mRNA by RNase E-dependent mechanism. RNase, through its C-scaffold region forms ribonucleoprotein complexes with Hfq and small sRNAs. These complexes formed by RNase E can initiates degradation of target mRNAs by small non-coding RNAs (Morita, Maki, & Aiba, 2005). Hfq dependent sRNAs include *DsrA, RprA , OxyS , flhA* and *RyhB* which controls regulation of their target messenger RNA (Gottesman, 2004).

sRNAs depending upon Hfq for their binding usually requires three elementary components which include mRNA base pair region, Hfq binding site and 3’ terminator structure (Gottesman, 2004). *SgrS* is one of the best characterized sRNA which requires Hfq protein for its binding in *E. coli*. It represses the expression of a glucose transporter, encoded by *ptsG* gene, present in cell membrane (Vanderpool, 2007).

Natural sRNAs have inspired the design of synthetic *trans*-acting RNA molecules to efficiently control gene expression (Yoo, Na, & Lee, 2013). Various artificial *trans*-encoded sRNAs, which can target for specific mRNAs, have been developed (Man *et al*., 2011). Non-coding sRNAs have been synthesized to silence the porin and flagellin genes in various bacteria (V. Sharma, Yamamura, & Yokobayashi, 2012) . So far riboswitches have been developed artificially for controlling gene expression but they offer certain difficulties for their designing and insertion into 5' UTR of chromosomal genes. However, Hfq dependent sRNAs are known for effectively controling the expression of genes in *trans* (Yoo *et al*., 2013). Synthetic sRNAs are convenient, conditional, portable, tunable, and allows control of gene regulation. They can be used for high through searching of target genes along with regulation of expression of those genes. The design strategy entails developing a synthetic RNA comprising of two parts; the first is the target binding sequence and second is a scaffold sequence. The scaffold sequence provides a secondary structure that helps in the recruitment of Hfq protein to the site and helps in binding of sRNA to the target mRNA as well as mRNA degradation. Several natural sRNAs in *E. coli* were screened to establish the most efficient scaffold for gram negative bacteria, of which *MicC* has been found to have maximum repression capability (Yoo *et al*., 2013). Naturally existing scaffold region, *MicC* required for recruiting Hfq protein has been amplified by Na *et al*., (2013). The target binding sequence also known as the guide sequence is the sequence that recognizes the target mRNA, the first 24 nucleotides from AUG to +21 of mRNA sequence has been found as efficient guide sequence with maximum repression and minimum off target binding (Na *et al*., 2013; Yoo *et al*., 2013) .

Shiga toxin producing *E. coli* which are known as (STEC) has been considered as the major food born pathogen worldwide and it is responsible for diseases ranging from mild diarrhea to

complicated hemolytic-uremic syndrome (HUS) (Mainil & Daube, 2005). Not all reported strains of STEC are capable of causing infections in humans however those STEC strains which can cause diseases in humans are regarded as Enterohemorrhagic *E. coli* (EHEC). Only those STEC can make humans sick which have components required for the attachment of enterocyte (Cleary, 2004). Among EHEC, *E. coli* O157 has been reported as major cause of outbreaks across the globe (Blanco *et al*., 2004; Brusa *et al*., 2013; Sasaki *et al*., 2012). Strong pathogenicity of *E. coli* O157 is associated with release of verotoxins such as shiga toxin 1 (encoded by *stx-1* gene) and shiga toxin 2 (encoded by *stx-2* gene) (Jian-guo, Bo-kun, & Huai-qi, 1999). Shiga Toxins produced by EHEC can damage small vessels present in intestine, kidney and brain (Mohamed A. Karmali, Gannon, & Sargeant, 2010). There has been continuous debate on treatment of EHEC infections with antibiotics. Treatment of EHEC infections with antibiotics not only can develop antibiotic resistance among the targeted strains but also can cause increased production of shiga toxins. Multiple drug resistance to streptomycin, tetracycline and silfisoxazole has been observed in EHEC strains. Antibiotics may lyse bacterial cell wall resulting in release of shiga toxin in the outside environment of the cell (Mora *et* al., 2005; Sasaki *et* al., 2012).

In the current study, we have designed a mechanism to target virulent genes of shiga toxins with the help of non-coding RNA. sRNA was designed against only *stx-2* gene to avoid off target bindings because *stx-1* and *stx-2* genes share 50-60 percent of homology (Cleary, 2004). Present study involves computational designing of synthetic non-coding RNA against *stx-2* gene followed by assessing its repression capability using bioinformatics tools including ClustalW, IntaRNA, and RNAfold. The anti-shiga sRNA cassette was incorporated in pAB.001 plasmid via site directed mutagenesis PCR technique and was kindly provided to our group by Bernheim *et al.,* (2016). *E. coli* S17-1 was used as a donor strain to transform *E. coli* O157:H7 with the plasmid carrying anti-

shiga sRNA cassette. Repression of *stx-2* levels in *E. coli* was then determined by real-time RT-PCR analysis and MTT cell viability assay. Real time RT-PCR detected levels of mRNA produced in the cells while MTT assay compared the amount of shiga toxin produced by the control strain wild type *E. coli* O157:H7 (WT-0157) and the anti-shiga sRNA harboring strain (ASR-0157) through their cytotoxic effects on U87 cell lines.

2. Literature Review

2.1 History of sRNAs

MicF, the very first sRNA encoded by chromosome, was identified in *E. coli* in 1984. Its length is 174 nucleotides and it prevents the translation of *ompF* mRNA in *E. coli* (Chou, 1984). Till 2000, 13 new sRNAs were discovered with help of traditional methods such as instant separation by polyacrylamide gel electrophoresis (Massé, Majdalani, & Gottesman, 2003). Various bioinformatics tools were designed to examine intergenic regions present in genome and they are being used since 2001 (Livny & Waldor, 2007; Wassarman, Repoila, Rosenow, Storz, & Gottesman, 2001). Along with bioinformatics tools various experimental methods have been developed for identification of new sRNAs at genomic level which include microarray, comparative genomics and deep sequencing (Livny & Waldor, 2007; Cynthia Mira Sharma & Vogel, 2009). Various sRNAs have been identified in *Bacillus subtilis* (Irnov, Sharma, Vogel, & Winkler, 2010), *Staphylococcus aureus* (Geissmann *et al*., 2009), *Pseudomonas aeruginosa* (Sonnleitner, Romeo, & Bläsi, 2012), and *Listeria monocytogenes* (Christiansen *et al*., 2006). By 2006, only in *E. coli*, about 80 sRNAs were identified experimentally and there is possibility that more sRNAs would be discovered with continuous development in genomics which will help to analyze various mechanisms and their functions (Christiansen *et al*., 2006).

2.2 Classes of sRNAs

2.2.1 sRNAs controlling protein activity

Three sRNAs that can bind with proteins found to have intrinsic activity (RNase P) and they also can function as a ribonucleoprotein component such 4.5S. There are also some other protein binding non-coding sRNAs which can act antagonistically against their respective cognate

proteins. Such sRNAs include *CsrB, GlmY* and they act by mimicking structures of other nucleic acids. CsrA is a protein which can bind with RNA and whose function is to regulate bacterial motility and carbon usage during stationary phase. *CsrB* and *CsrC* RNAs are known to control activity of CsrA (Babitzke & Romeo, 2007). Dimers formed by CsrA bind with GGA motifs of target mRNAs and thus they affect stability of the mRNAs. Various GGA binding sites are present in *CsrB* (22 binding sites) and *CsrC* (13 binding sites) for CsrA. Therefore, when levels of *CsrB* and *CsrC* is in upturn, the sRNAs moves the CsrA away from the mRNA leaders. Two component regulators are responsible for induction of *CsrB* and CsrA transcription. These regulators are activated when poor nutrient growth conditions are encountered by the cell. However, it is not known that which signal is responsible for transcription initiation. CsrD protein can also regulates *CsrB* and *CsrC* RNAs. CsrD is a di-GMP binding protein which is responsible for recruiting RNase E in order to degrade sRNAs (Suzuki, Babitzke, Kushner, & Romeo, 2006).

This mechanism has reported in many bacteria such as *Erwinia*, *Salmonella*, *Pseudomonas* and *Vibrio* in which homologs of *CsrB* and *CsrC* have an impact on quorum sensing, secondarymetabolism and epithelial cell invasion (Lucchetti-Miganeh, Burrowes, Baysse, & Ermel, 2008). *GlmY*, an sRNA, also act via protein binding mechanism (Görke & Vogel, 2008). Expression within transcripts is altered when GGA hairpins present in mRNAs is occupied by CsrA however when levels of *CsrB* rises, CsrA is sequestered by sRNA which results in termination of its regulatory effect. Upon increasing the *CsrB* RNA levels, the sRNA seizes the CsrA and checks its regulatory functions as shown Figure 2.1.

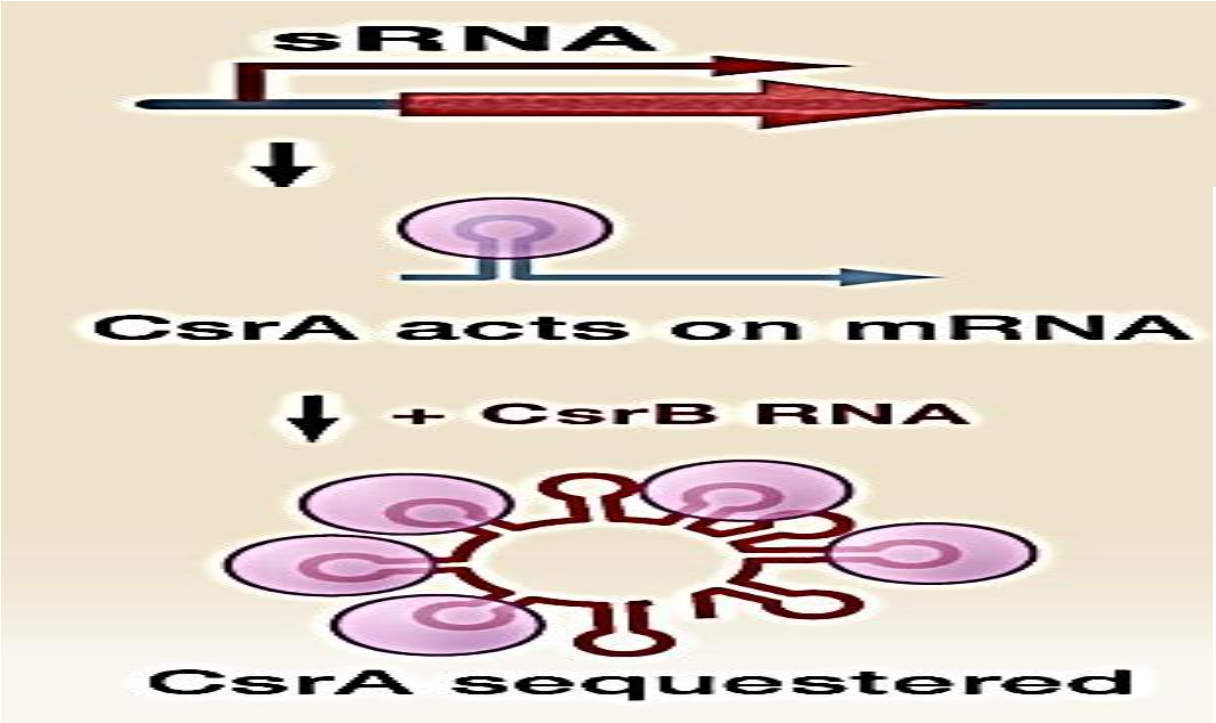

Figure 2.1: This figure illustrates sRNAs action via protein binding. Protein binding sRNA is shown in red. CsrA protein binds with GGA hairpin present in mRNAs and alters expression of transcripts. However, when levels of *CsrB* increases, the CsrA is sequestered by sRNA which averts its regulatory effects (adopted from Waters & Storz, (2011).

2.2.2 *Cis*-encoded sRNAs

Cis-encoded sRNAs are type of sRNA which originate from the antisense strand of the same DNA whose mRNA is going to be targeted. *Cis*-encoded sRNAs usually down regulate the genes at post transcriptional level and they do not require binding with Hfq protein (Caldelari, Chao, & Romby, 2013). There are some *cis*-encoded sRNAs, for example *AsdA*, which are known to stabilize their target mRNA. Although Phages, plasmids and transposons were the very first *cis*-encoded sRNAs (E G H Wagner & Simons, 1994) however by 2007 some of *cis*-encoded sRNAs, encoded chromosomally, were also identified (Sabine Brantl, 2007). *Cis*-encoded sRNAs can be classified into 3' overlapping, 5' overlapping or internally located sRNAs with respect to their originating location. They can also be classified according to their size into long or short antisense sRNA.

Long *cis*-encoded sRNAs have size reaching to several kilo bases while others are 100 to 300 nucleotides in length (Georg & Hess, 2011). Short sRNAs were discovered mainly in plasmids, transposons and phages and are encoded at the same DNA locus therefore they have complete complementary binding with their target mRNA along long stretches. They employ various mechanisms to regulate the gene which include RNA pseudoknot construction, transcriptional attenuation, primer maturation inhibition, translational inhibition or RNA cleavage and degradation (Figure 2.2 B). The antisense RNA and target mRNA hybrid is degraded by RNase III. *Cis*-encoded sRNAs usually regulate fundamental processes in cells such as replication initiation, suicide and conjugation efficiency (Sabine Brantl, 2007).

2.2.3 *Trans*-encoded sRNAs

Trans-encoded sRNA are located at the distinct positions than their target mRNAs (Figure 2.2A). *Trans*-encoded sRNAs usually act by sequestering the ribosomal binding site of their target mRNA. For this purpose they form complementary binding with Shine-Dalgarno region. Such sRNA can also form base pairing with coding sequence of target mRNAs (Wade, Li, & M. Wahl, 2013). Many characterized *trans*-encoded sRNAs are coupled with RNases and can control the regulation of genes. RNase is the enzyme which is responsible for RNA turnover via RNA cleavage (Saramago *et al*., 2014; Viegas, Silva, Saramago, Domingues, & Arraiano, 2011). Also, *trans*-encoded sRNAs do not have complete complementary binding with their target mRNAs and therefore they need an RNA chaperone protein which is called Hfq for their functioning (Vogel & Luisi, 2015). The first *trans*-encoded sRNA was *MicF*, discovered in *E. coli*, and regulate *ompF* mRNA. Hfq is the most important component in *trans*-encoded sRNA based gene regulation and absence of this protein is responsible for pleiotropic phenotypes which include loss of virulence and impaired stress regulation. Hfq is responsible for rapid formation of sRNA-mRNA duplex.

Therefore for proper functioning, sRNA or mRNA should bound with Hfq (E. Gerhart H Wagner, 2013).

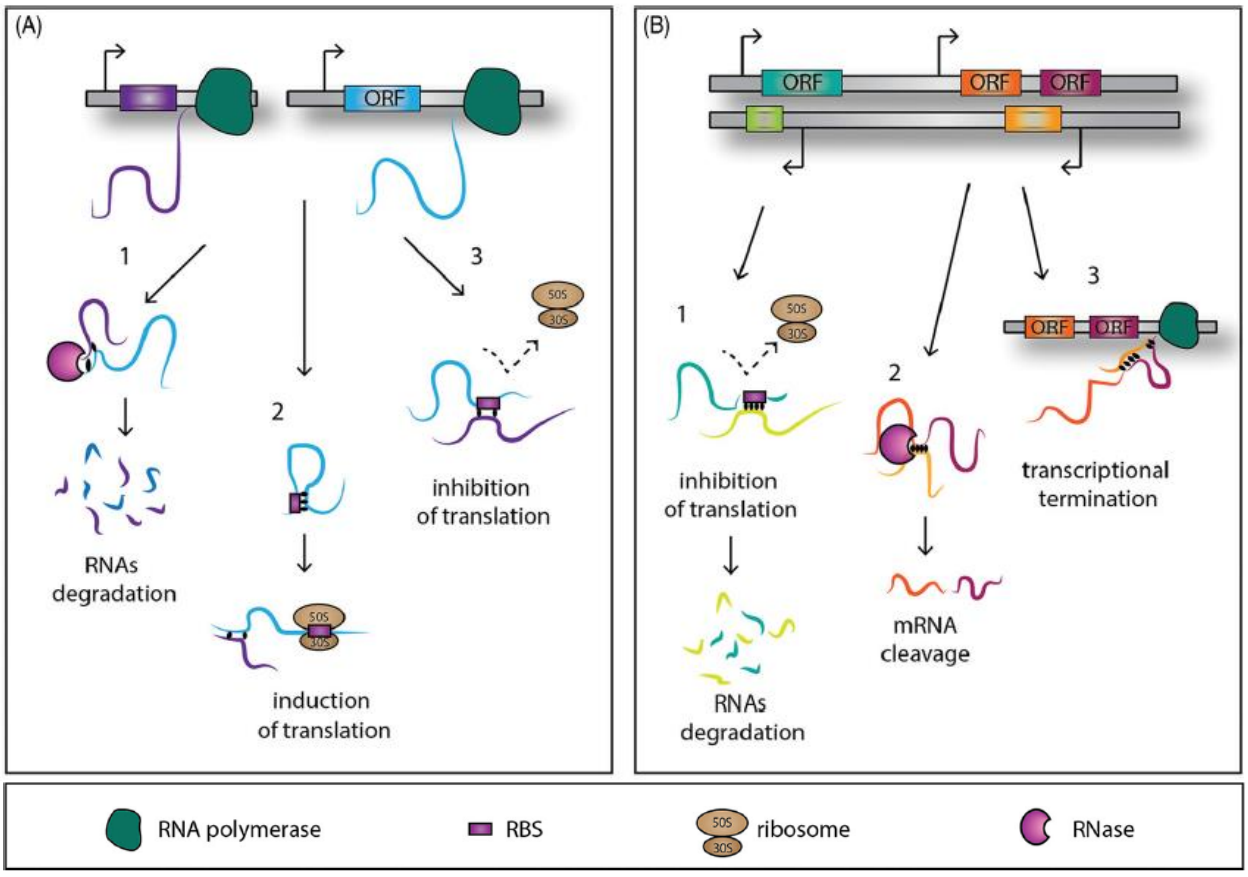

Figure 2.2: ***(A)*** *Trans*-encoded sRNAs originate from different locus as of their target gene and have imperfect base pairing with target mRNA. **(A1)** Mechanism of Translation inhibition of *trans*-encoded sRNAs via mRNA degradation promoted by RNase. **(A2)** Translation activation by binding with *trans*-encoded sRNAs **(A3)** Translation inhibition by binding with target mRNA, and occupying its RBS. **(B)** *Cis*-encoded sRNA originate from same locus as of their target gene and have perfect pairing with target mRNA. **(B1)** After complete complementary binding with target mRNA, *cis*-encoded sRNA can lead to the degradation of its target mRNA leading to translation inhibition. **(B2)** *Cis*-encoded sRNA present between two genes can lead towards mRNA cleavage. **(B3)** *Cis*-encoded sRNA can also form a hairpin loop structure causing translation inhibition (adopted from Oliva, Sahr, & Buchrieser, 2015).

2.2.4 Riboswitches

Ronald Breaker in 2002 proposed the term "riboswitch" for the first time (Winkler, Nahvi, & Breaker, 2002), after that many classes of riboswitches have been identified which are regulated by different metabolites. Riboswitches can regulate various biological processes inside the cells

which include translation initiation, mRNA processing, mRNA splicing and transcription attenuation. Riboswitches can perform these functions with minimal changes in their structures and without helper protein. This type of regulation is very important because it is involved in different cellular pathways which include production of amino acids, purines and vitamins. Structurally riboswitches contain two domains, one is called aptamer domain whose function is to bind with ligand and other is expression domain which regulates the target gene (Coppins, Hall, & Groisman, 2007). Functionality of riboswitches usually depends upon interaction between changed conformations of conserved metabolite binding domain and variable expression domain. Aptamer domain is folded into distinct structures with increase in levels of certain metabolites by showing high attraction for the some atomic groups present in ligand. This ligand-aptamer complex helps in folding of expression platform which is present downstream and usually reacts with RBS of target mRNA (Breaker, 2012).

Riboswitches can regulate gene expression either by translation ignition or by transcription termination (Figure. 2.3). However, in *E. coli*, some riboswitches have identified which can perform dual action such as *lysC*. *LysC* riboswitch, when binds with lysine, adopts such conformation that hinders translation initiation as well as exposes cleavage site present in riboswitch expression domain. However, when lysine is not present, the riboswitch adopts such conformation that stimulates translation initiation and also seizes RNase E cleavage sites (Caron *et al*., 2012).

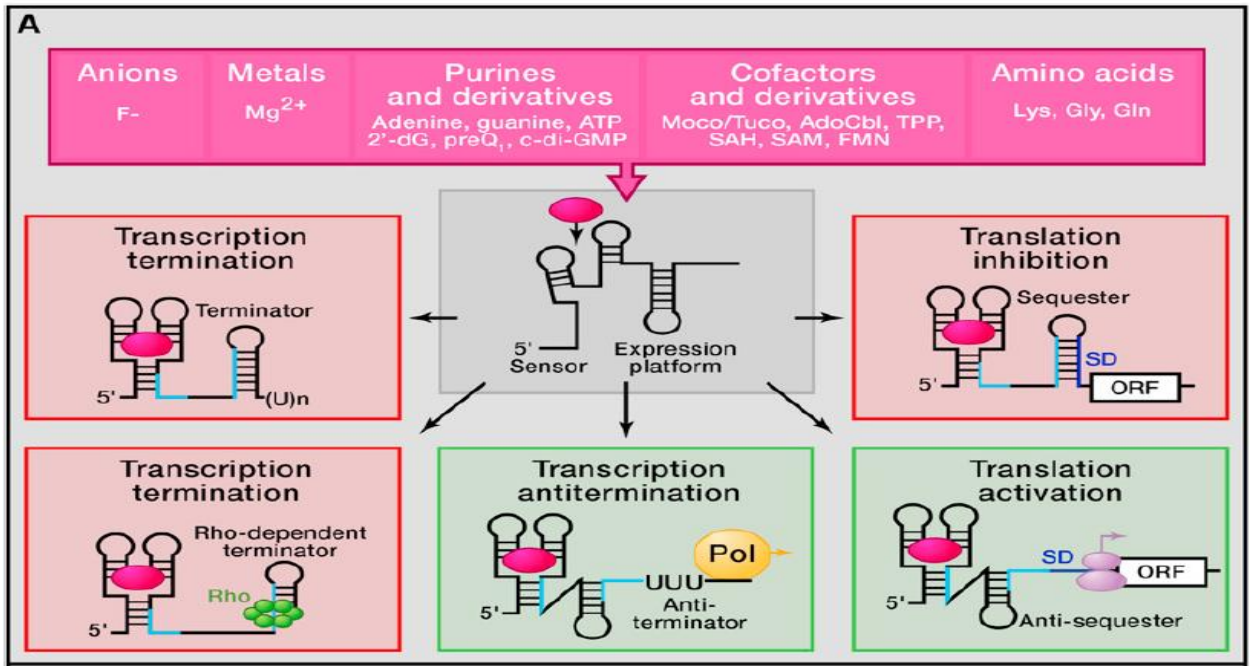

Figure 2.3: Regulation of gene expression is divergent in prokaryotes and includes control of translation, transcription, mRNA stability and splicing. Different metabolites (hot pink at top) can be recognized by sensor domains present in riboswitches (gray). Left: Binding of ligands with riboswitches mostly leads to the anti-terminator hairpin formation and can promote development of substitute Rho-independent termination hairpin structure (middle) or can cause premature transcriptional termination via Rho binding sites. Center: Some times, anti-terminator hairpin is stabilized by ligand bound riboswitches that allows RNA polymerase to successfully complete transcription of gene (bottom). Right: Sequestration of RBS entry site can lead to the repressed expression of ORF (middle). Riboswitches can also facilitate SD anti-sequester hairpin formation that can promote translation initiation (bottom) (adopted from Serganov & Nudler, 2013).

2.2.5 Clustered regularly interspaced short palindromic repeats (CRISPR RNAs)

CRISPR RNAs is a class of regulatory RNAs which have been discovered recently and they are responsible for providing resistance to bacteriophage. Eukaryotic siRNA based gene silencing have certain similar characteristics with CRISPR RNA though they also have some different features. CRISPR sequences have identified in about 90% of archaea and 40% of bacteria which indicates their wide range of functions. CRISPR sequences can be regarded as extremely variable DNA stretches that contain a leader sequence of almost 550bp. Leader sequence is usually

followed by various repeats of spacer units (Sorek, Kunin, & Hugenholtz, 2008). Length of repeated DNA in CRISPR can range from 24 to 27bp and can be repeated 2 to 249 times. Repeat sequences greatly vary between bacteria but still they can be grouped in 12 different major types. CRISPR repeats are combined with spacers of 26-27bp which are not conserved between various bacterial species. Many CRISPR-associated (CAS) genes are present nearby CRISPR DNA array (Figure 2.4A). Most of the CRISPR systems contain 2-6 CAS genes. Along with these core genes many CRISPR types also have CAS genes present in nearby region. The functions of CAS genes is still unclear it is believed that they contain a DNA or RNA binding domains and helicase motifs (Sorek *et al*., 2008).

CAS proteins process the transcribed CRISPR DNA into a repeat spacer unit which is known as crRNA (Brouns *et al*., 2008). The crRNAs are single stranded as compared to siRNA which are double-stranded. Cascade or CAS proteins along with crRNA sequence can directly base pair with plasmid or phage nucleic acid targets (Figure 2.4B) (Fitz, Cronican, Lefterov, & Koldamova, 2013).

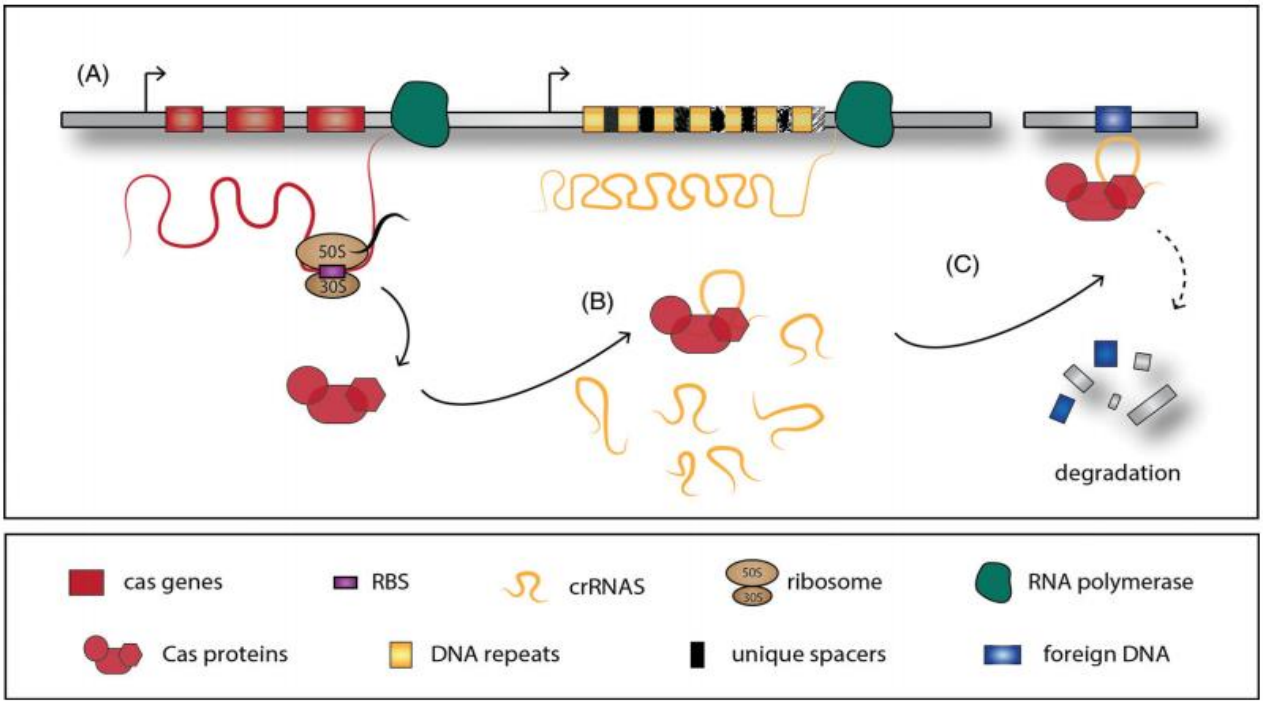

Figure 2.4: (A) Simplified model of CRISPR/*cas* system. Many *Cas* genes, which encode the Cas proteins, consist of different number of DNA repeats which is disturbed by distinctive spacer origination from external DNA. **(B)** Cas proteins controls the crRNA processing and interference with cDNA region which can lead to DNA degaradation or **(C)** silencing (Oliva *et al*., 2015).

2.3 Modes of action of sRNAs

In order to regulate the gene expression, various mechanisms are employed by sRNAs which depend upon sRNA binding site on target mRNA and on additional proteins.

2.3.1 Inhibition by occupying the ribosomal binding site

Most of the sRNAs act by targeting 5'-UTR which is present close to RBS. When an sRNA binds with the target mRNA at RBS , it inhibits the entrance of 30S ribosomes and thus leads to the blockage of translation as shown in Figure 2.5 A (Kawamoto, Koide, Morita, & Aiba, 2006). Usually, base pairing sequence overlaps by Shine Dalgarno sequence but other target sequences within range of 55nt, which can be contacted by 30S subunit, are sufficient for translation inhibition (Hüttenhofer & Noller, 1994).

2.3.2 Translational regulation via translational enhancers

In many mRNA, analytical results have shown that binding sites present at distinct position from upstream of the sequence, were occupied by 30S ribosome but still mRNA was not able to be translated into protein (Figure 2.5 B). *IstR1*/*tisB* involves binding with a site present at 100 nt upstream of *tisB* RBS. The *tisB* site is seized in stable structure which prevents translation initiation but effective initiation was permitted by binding with 30S upon transient breathing (Darfeuille, Unoson, Vogel, & Wagner, 2007). In some cases, sRNA binds with the translational enhancers. For example U/G-rich sequence is used by *GcvB* in order to bind with C/A-rich enhancers, which are usually present at distance upstream of SD (Cynthia M Sharma, Darfeuille, Plantinga, & Vogel, 2007).

2.3.3 Translational control via leader ORFs

sRNAs can also regulate the gene expression indirectly. Sometimes, reading frame's translation is obstructed by formation of secondary structures in RBS while translation linking to an ORF present at upstream leader is necessary for expression (Figure 2.5 C). RepA synthesis is indirectly prevented because *CopA* present in plasmid R1 is capable of directly inhibiting translation within leader ORF (Blomberg, Nordström, & Wagner, 1992). *RyhB* is an example of sRNA that is chromosomally encoded. This sRNA is responsible for blocking its target fur by inhibiting translation of upstream leader ORF (Veĉerek, Moll, & Bläsi, 2007). ORFs of leader peptide are also involved in up regulation of translation by sRNA. *PhrS* is an sRNA present in *Pseudomonas aeruginosa* which is capable of activating translation at an upstream ORF and then via translational coupling it activates virulence transcription factors (Sonnleitner *et al*., 2011).

2.3.4 Translation activation

Translational activation by sRNA is widespread in nature, however, it was first identified in *DsrA*/*rpoS* locus. The translation is activated usually by ant-antisense mechanism. An mRNA having longer leader sequence, which seizes the RBS in normal stable stem loop, can be activated for translation. For this purpose a sRNA can bind to 50 flank region of stem loop which in turn will liberate the RBS (Figure 2.5 D). RNAIII/*hla* are examples of this mechanism (Morfeldt, Taylor, von Gabain, & Arvidson, 1995). Mostly leader contains site for binding of Hfq which is necessary for control. Activation mechanism by sRNA is clearly understood by studying Hfq-*rpos*. *RpoS* leader has three sites which bind with distal face of Hfq. This binding results in formation of compact folding that places the sRNA target sequence near to antisense sequence which is present is sRNA (Peng, Curtis, Fang, & Woodson, 2014).

2.3.5 Regulation via transcriptional attenuation

Transcriptional attenuation achieved by antisense RNA involves binding which induces structural and conformation changes in mRNA in such a way that causes premature termination (Figure 2.5 E). This phenomenon was first observed in replication of plasmid (S. Brantl, Birch-Hirschfeld, & Behnke, 1993). This mechanism is alternative to riboswitch control and it is also present for regulating the aa-tRNA synthetase in *Bacillus subtilis*. However, an alternative conformational change, leading to transcriptional promotion, can be observed when uncharged tRNA binds with leader of aa-tRNA synthetase RNA (Henkin, 2008).

2.3.6 Target mRNA degradation as a secondary effect

Translation inhibition caused by sRNA usually associated with mRNA degradation. This occurs because the naked mRNA, unable to bind with ribosome, can easily be accessed for endoribonucleolytic attack (Figure 2.5 F). *SgrS*/*ptsG* system clearly indicates that the phenomenon

of mRNA degradation is a secondary event which is caused by the translation inhibition as the primary effect. *SgrS* causes translational blockage which leads to rapid mRNA degradation by Hfq and RNase E (Kawamoto *et al*., 2006; Morita *et al*., 2005). While in other way sRNA-activated translation leads to stabilized mRNA (Fröhlich, Papenfort, Fekete, & Vogel, 2013). Sometimes, combined effects caused by translational blockage and RNA degradation are needed for efficient down regulation of gene (Chevalier *et al*., 2010).

2.3.7 Target RNA degradation-only effects

Sometimes mRNA degradation can be observed as an only effect without effecting translation initiation (Figure 2.5G). *MicC* identified in *salmonella* can base pair with 70nt present downstream of the start codon of *ompD* mRNA. However, this base pairing have no effect on translation initiation still this binding facilitates RNase E- dependent degradation close to the RNA duplex (Chevalier *et al*., 2010). A ternary complex is formed by sRNA, RNase E and Hfq which binds with target mRNA with *MicC* seed. If a 50 monophosphate is present in sRNA then RNase E is activated for target degradation (Garrey *et al*., 2009). If the target mRNA is absent then RNase E can inactivate sRNA by cutting its seed sequence. Therefore, sRNA is responsible for guiding RNase E to the specific target and also form a complex that is capable to perform cleavage activity (Manuscript & Dysfunction, 2015) . Recent work has suggested that after binding of sRNA, Hfq and degradosome are recruited that starts cleaving mRNA far from downstream (Prévost, Desnoyers, Jacques, Lavoie, & Massé, 2011).

2.3.8 Stabilization of target RNA

sRNA upon binding with mRNA can also stabilize the target mRNA which in turn increases the protein production, although this phenomenon is less common. *RydC* is such type of sRNA present in *Salmonella* which can bind with mRNA named as cyclopropane fatty acid synthase (*CFA*).

RydC binds far upstream of the RBS and inhibits RNase E dependent cleavage as indicated in Figure 2.5 H (Fröhlich *et al*., 2013).

2.3.9 Operon-wide effects

Binding of a sRNA called *ChiX* with an operon *chiP* (chitobiose) revealed a mechanism of regulation that can be applied to those operons in which RBS of upstream ORF is going to be targeted. *ChiX* causes translation inhibition after co-transcriptional binding with chip RBS which shreds mRNA and exposes hidden Rho utilization sites downstream. Then termination factor gets attached with duplex and causes. By co transcriptional binding to the *chiP*, *ChiX* ends the translation, which exposes hidden rut (Rho utilization) sites present in downstream region. Rho function causes the premature termination and checks the transcription of the downstream region (Figure 2.5 I) (Bossi, Schwartz, Guillemardet, Boudvillain, & Figueroa-Bossi, 2012).

2.3.10 RNA-based traps and sponges

There are many RNAs that are able to bind with sRNA and can indirectly affect expression of target gene (Figure 2.5 J). Such RNAs can be called taps, sponges, decoys, competing endogenous RNAs (Vogel, 2009). Such kind of traps was first reported in a plant *Arabidopsis thaliana*. In *A. thaliana* when an ncRNA, *IPS1*, was induced, it seizes the *miR399* and then prevented this miRNA from down regulation of its target (Franco-Zorrilla *et al*., 2007).

2.3.11 Recruitment of proteins to mRNA

Most of the regulatory sRNAs bind with Hfq protein which indicates that the Hfq has regulatory effect. At least 3 sRNAs can target the *sdhC* mRNA. *Rhyb* and *RyhB* can block translation directly, while the sequence of spot 42 is not in reach to block the initiation. Therefore this sRNA binds and deposits Hfq at RBS in order to inhibit translation. Hfq is able to bind with specific sequences of mRNAs which inhibits translation and causes mRNA degradation (Desnoyers & Massé, 2012).

2.3.12 Protein sequestration

Some sRNA can specifically bind with regulatory proteins. These sRNAs can seize or block the activity of these regulatory protein and thus can affect regulation without antisense mechanism. *CsrB* type RNAs and 6S RNA comes under this category. In *Erwinia carotovora tox1* gene encodes for a precursor RNA that can be processed into many pseudo-knot RNAs of 36nt by an endoribonuclease ToxN. Active sites present in ToxN trimer can bond with three copies of *toxI*. TA systems is naturally found in bacterial chromosomes and plasmids (Blower *et al*., 2012).

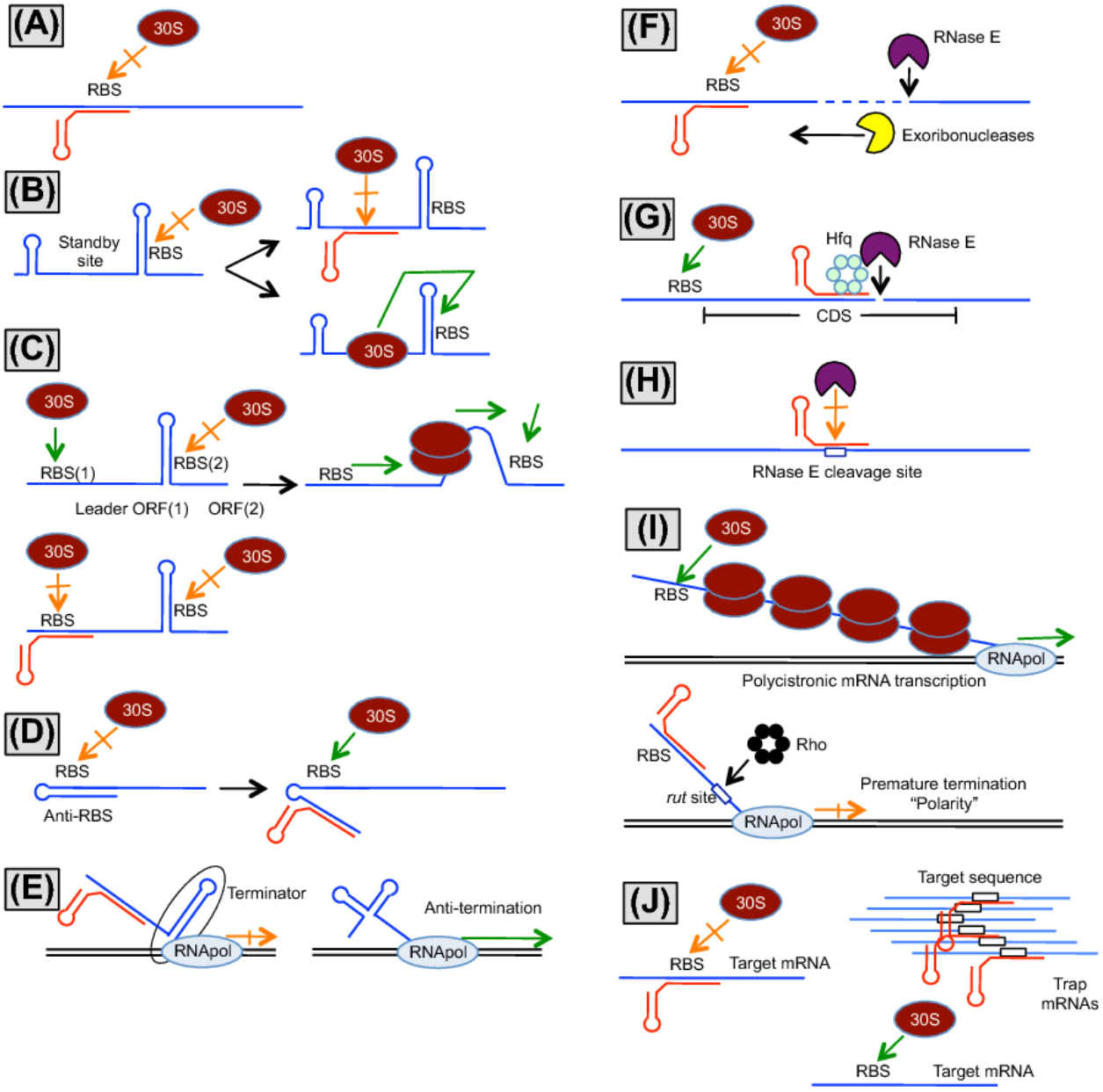

Figure 2.5: Various mechanisms engaged by sRNAs. Dark blue shows the mRNA structures and sRNAs are shown in red. **(A)** Translation inhibition by occupying the RBS **(B)** translational control with help of translational enhancers **(C)** translation regulation by forming secondary structures in RBS **(D)** translation activation by anti-antisense mechanism **(E)** premature termination of mRNA by conformational changes **(F)** translation inhibition by degrading the mRNA after occupying the RBS **(G)** mRNA degradation without effecting translation **(H)** sRNA stabilizing the mRNA **(I)** regulation via premature termination by exposing the RHO factor **(J)** RNA based sponges (adopted from E. Gerhart H. Wagner & Romby, 2015).

2.4 Designing of synthetic non-coding RNA for silencing of gene expression

Synthetic non-coding RNA is an efficient tool for performing gene knock out experiments since the conventional methods of constructing gene knockout libraries are highly time consuming and laborious. Moreover they are limited to perform on those genes that are essential for cell survival (Yoo *et al*., 2013). Synthetic riboswitches are major synthetic sRNAs, which have developed so for in *E. coli*, for up or down regulation of gene expression (Desai & Gallivan, 2004; Jin & Huang, 2011; Nomura & Yokobayashi, 2007). Use of such *cis*-acting sRNAs is difficult to apply to metabolic engineering because such sRNAs need to be inserted in 5' UTR of the target genes. Also designing of a synthetic riboswitch sequence is difficult because they have many secondary structures which can change upon ligand binding. In contrast to riboswitches, Hfq-dependent *trans*-encoded sRNAs can easily be produced through plasmid based expression for the regulation of gene. Designing of synthetic *trans*-encoded sRNA also doesn't require any direct modification of chromosomal sequence (Yoo *et al*., 2013). Natural small regulatory RNA(sRNA) are responsible for gene regulation in prokaryotes, they are basically short non-coding RNA that possess a scaffold and a target binding region that interacts with the target messenger RNA in *trans* and initiate their degradation. The scaffold structure is responsible for recruiting the Hfq protein that helps in the formation of the mRNA-sRNA duplex. The target-binding sequence guides the synthetic sRNA to bind to its target mRNA. Yoo *et al*., (2013) described the protocol for synthetic non-coding RNA based control of gene expression. The design principles include selecting a scaffold and target binding sequence that is complementary to the target mRNA to be repressed. Na *et al*., (2013) screened a total of 101 sRNA in *E. coli*, discovered to date to select the most efficient scaffold with maximum repression ability. Three scaffolds were selected that are *SgrS, MicC* and *MicF* (Man *et al*., 2011). *MicC* was selected as final scaffold with most efficient

repression efficiency. The second important region in sRNA for repression is selection of appropriate region of target mRNA as guide sequence. TIR (translation initiation region) has been found to be most responsive to sRNA mediated repression. Na *et al*., (2013) also described the correlation of sRNA and target mRNA binding energy in repression ability of sRNA.

2.4.1 Advantages of synthetic noncoding RNA

Synthetic non-coding RNAs are tunable, portable and easy to condition making them one of the best methods for metabolic engineering. The synthetic sRNA can be easily transferred using plasmid based transformation method making them highly portable moreover it does not effects the information coded on the chromosome therefore the risk of disrupting the expression of other genes is avoided, thus they can be transferred to any bacterial strain. The sRNA system is easy to be conditioned by either controlling the amount of sRNA being produced or by altering repression capability of sRNA. The repression efficiency of sRNA depends upon the binding energy of the sRNA with its target mRNA that can be tuned according to requirement (Yoo *et al*., 2013). All these advantages make synthetic sRNA a promising candidate for studying gene regulations and evaluating the effects of gene modification on physiology and metabolic activity of different bacteria. All these characteristic also enable us to design more complex and finely controlled circuits of synthetic sRNA (V. Sharma *et al*., 2012).

2.5 Shiga Toxin

Shiga toxins are group of functionally and structurally related exotoxins. Toxin from *S. dysenteriae* serotype 1, named as shiga toxin 1, and other shiga toxins which are formed and excreted by enterohaemorrhagic *E. coli* strains are included in this family. Historically these toxins can be described by interchangeable terms. Kiyoshi Shiga, a Japanese microbiologist, characterized

dysentery triggered by *S. dysenteriae* for the very first time in 1897. Konowalchuk isolated and identified some *E. coli* strains in 1977 which were able to produce a toxin that can cause death of vero cell lines (Island, Park, & Todd, 1977). He named these toxins as verotoxin and strains producing these toxins were named as verotoxin producing *E. coli.* In 1980, O' Brien and his colleagues reported few shiga-like-toxin-producing *E. coli* strains (STEC) because of their ability to produce toxins which were closely related with shiga toxin (O'Brien *et al*., 1984). In 1983, it was reported for the first time that STEC strains are responsible for hemolytic uraemic syndrome (HUS) (M A Karmali, Steele, Petric, & Lim, 1983). Researchers concluded that they were working on highly identical toxins. Various variants of shiga toxins are produced by shiga toxin producing *E. coli* which include Stx1 and its variant (Stx1c), Stx2 and its variants (Stx2c, Stx2d, Stx2e and Stx2f). STEC can also produce combinations of these variants as shown in table 2.1. Stx1 and Stx2 are the major types of shiga toxins because they target a common receptor as well as they have similar mechanism of action. However, they are only 56% similar in amino acid sequences (Jackson, Newland, Holmes, & O'Brien, 1987) . A vast number of lambdoid bacteriophages encode shiga toxins which are excreted by *S. dysenteriae* and STEC. These lambdoid phages are responsible for horizontal gene relocation. Activation of lytic phage cycle lead to the expression of *stx* genes in elevated amounts (Jackson *et al*., 1987). Phages employ various mechanisms for regulation of shiga toxins production which include activating phage gene promoters, controlling release of toxin and regulating gene amplification. Some negative bacteria have certain periplasmic conditions which are necessary for folding and assembling of toxin subunits. When toxin subunits are assembled the toxin secretion is caused by phage lytic cycle (Tyler, Mills, & Friedman, 2004).

Table 2.1: Variants of shiga toxins present in *S. dysenteriae* and STEC (adopted from Johannes & Römer, 2010)

Organism	Toxin	Sequence similarity to shiga toxin		Characteristics	Synonyms	Cellular receptors
		A subunit	B subunit			
S. dysenteriae	Shiga toxin	N/A	N/A	N/A	N/A	GB3
STEC	Stx1	97%	98%	N/A	SLT1 and VT1	GB3
	Stx1c	97%	98%	N/A	Slt1c and VT1c	GB3
	Stx2	53%	64%	Associated with severe disease in Humans	SLTII and VT2	GB3
	Stx2c	53%	61%	N/A	SLTIIc and VT2c	GB3
	Stx2d	54%	61%	N/A	SLTIId and VT2d	GB3
	Stx2e	53%	61%	Associated with piglet edema disease	SLTIIe and VT2e	GB3 and GB4

2.5.1 Structure of Shiga toxins

X-ray crystallography have shown AB5 molecular configuration of shiga toxins (Fraser, Chernaia, Kozlov, & James, 1994). A shiga toxin molecule has two subunits, one is StxA while other is StxB. StxA unit is enzymatically active monomer having 32 kDa of molecular mass which is connected non-covalently with identical pentamer fragments of B subunit. Each B unit has molecular mass of 7.7 kDa and this pentamer fragment forms StxB subunit. StxB is responsible for binding with receptors present at target cell surface.

Doughnut like structure is formed by StxB pentamer which have a central pore with which C-terminus of StxA is attached. Both fragments of shiga toxin molecule are excreted into periplasm

of bacteria (A. Donohue-Rolfe & Keusch, 1983). Both units are assembled and bound with each other non-covalently to form a holotoxin (Figure 2.6) (Hirst, Sanchez, Kaper, Hardy, & Holmgren, 1984).

StxA has a very particular RNA N-glycosidase function that cuts an adenine molecule at 4,324 position with respect to the α-sarcin present on VI domain of 28S ribosomal RNA (rRNA) of eukaryotic ribosomes, thus they interfere with aminoacyl tRNA binding and consequently inhibits chain elongation. Ribosomes of bacteria is considered as a substrate for stxA (Endo *et al*., 1988). StxB gets attached with globotriaosylceramide (Gb3), which is a neutral glycosphingolipid, on the target cell surface which leads to internalization of holotoxin into the cell as indicated in Figure 2.7 (Jacewicz, Clausen, Nudelman, Donohue-Rolfe, & Keusch, 1986). Even when StxA is not present with StxB subunit, StxB unit can still have a pentameric formation which has similar to the holotoxin in binding with receptor (A. Donohue-Rolfe, Jacewicz, & Keusch, 1989).

Crystal structure has demonstrated a conformational difference in site 2 of Stx2 from other shiga toxin and Stx1 B subunits. Binding with Gb3 receptor is preferred in case of Stx1 and Stx2c and the strongest bonding have observed for these receptor species (Fraser *et al*., 1994). Stx1 can bind with Gb3 by 10 folds more affinity than Stx2 (Head, Karmali, & Lingwood, 1991), however Stx2 has 400 folds lower LD_{50} than Stx1 when tested in mice. This contradiction may be explained by clear difference in their biodistribution (Tesh *et al*., 1993).

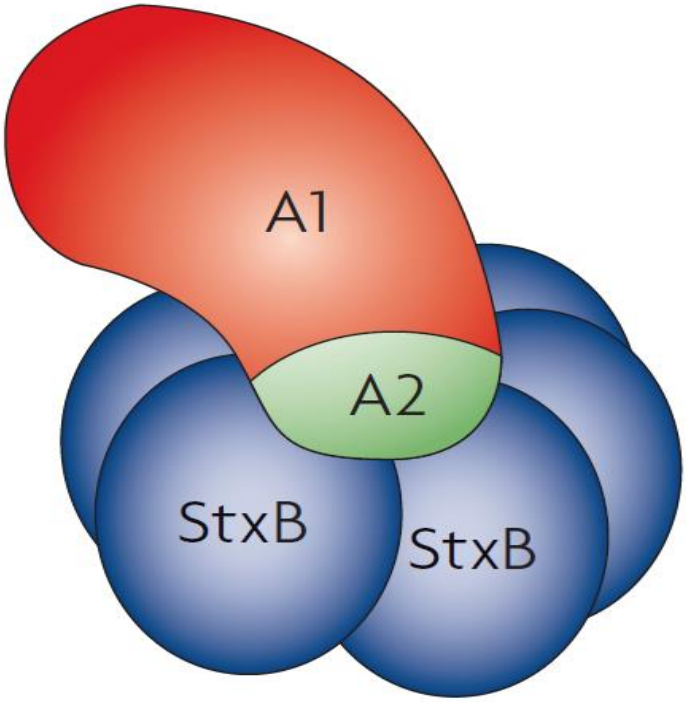

Figure 2.7: Cartoon illustrating Shiga holotoxin, that contains one A subunit, cleaved into A1 and A2, and five B fragments, forming homopentameric B subunit (adopted from Johannes & Römer, 2010).

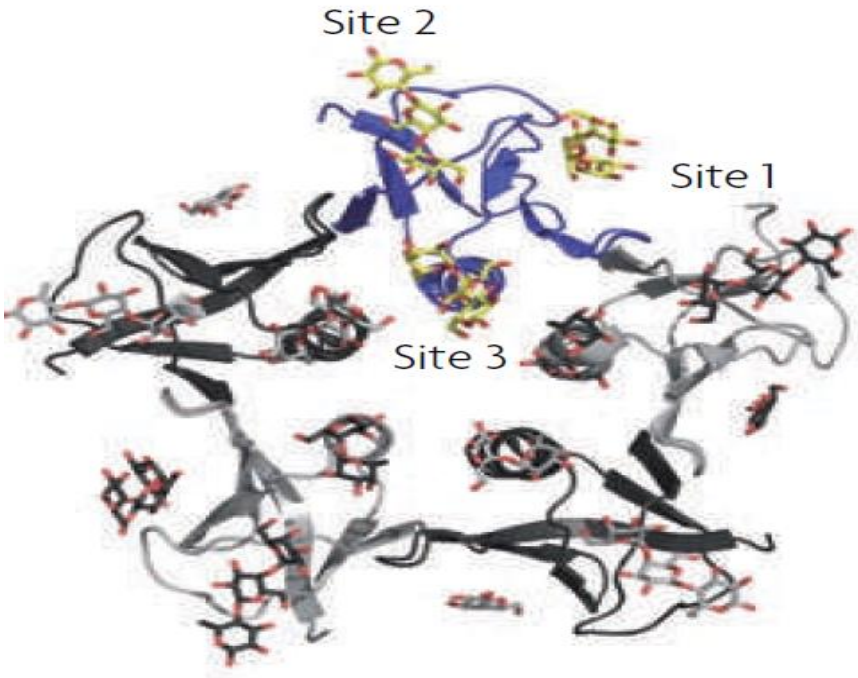

Figure 2.6: A ribbon diagram of StxB subunit from membrane-oriented surface, highlighting the three Gb3-binding sites (Johannes & Römer, 2010).

2.5.2 Trafficking of Shiga toxin

2.5.2.1 Endocytosis

Shiga toxin molecules are taken into the target cells via endocytosis after binding with Gb3 receptors at the plasma membrane. Clathrin-coated pits have shiga toxin in them (K Sandvig, Olsnes, Brown, Petersen, & van Deurs, 1989), however in the absence of clathrin dependent intake, the cells are still able to take up the shiga toxins. This clathrin independent pathway indicates that clathrin pathway is not always necessary for initial entry of toxins into the cells. Shiga toxins molecules have ability to induct invaginations in plasma membrane with or without cytosolic machinery (Lauvrak, Torgersen, & Sandvig, 2004; Römer *et al*., 2007).

2.5.2.2 Retrograde transport

Shiga toxin upon entering into the cell is localized to early endosome. Sandvig and van Feurs, in a revolutionary study, suggested that shiga toxin could be spotted in endoplasmic reticulum membranes (Kirsten Sandvig *et al*., 1992). This detection indicated that toxin was transported in a retrograde way via secretory pathway. Host of various proteins, recruit the clathrin to endosome. Espin related protein and phosphatidylinositol-4-phosphate binding protein are involved in hiring of clathrin. Retromer contains a combination of two sorting nexins subunits which act as curvutare recognition unit. Retromer also contains cargo recognition unit wich is composed of vacuolar associated proteins (Bonifacino & Hurley, 2008). Clathrin produces membrane curvature on initially produced endosomes. Clathrin adopters and cargo recognition units present in retromer are used by cargo in order to localize within the sites of transport intermediates. Retrograde tubules are converted into retrograde transport intermediates (Johannes & Popoff, 2008).

2.5.2.3 Retro-translocation

Pores are not formed by shiga toxins on the cell membrane, instead shiga toxin molecules are dependent on host cell components for translocation across cellular membrane. During the early entry stage, C-terminal of StxA, which is sensitive to protease activity, is cleaved at Arg251-met252 site residues by endoprotease furin. This cleavage results in the breakdown of StxA molecule into A1 fragment (catalytic fragment) and and A2 fragment (StxB-associated fragment) (Garred, van Deurs, & Sandvig, 1995). Disulfide bond linkage keeps the two fragments in close proximity with each other. This disulfide bond is formed between Cys242 (StxA1) and cys261 (StxA2). In lumen of endoplasmic reticulum disulfide bond is broke down which leads to the liberation of enzymatic A1 fragment. This liberated A1 fragment is translocated to the cytosol (Kurmanova *et al*., 2007; Lea, Lord, & Roberts, 1999; Olsnes, Reisbig, & Eiklid, 1981).

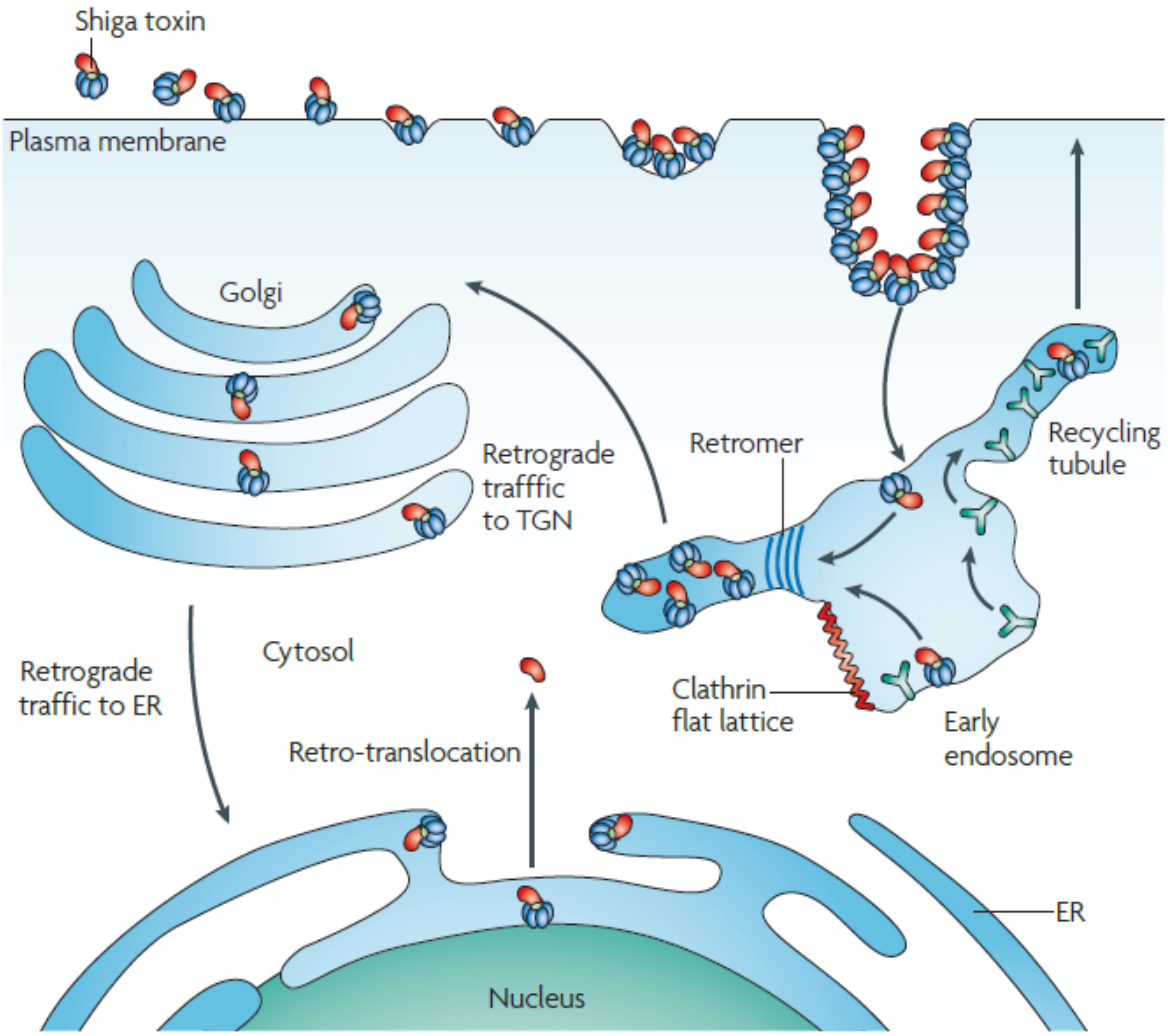

Figure 2.8: Transport of shiga toxins into the cell. Toxin upon binding to plasma membrane causes local curvature, endocytic invaginations and membrane mediated clustering followed by restrograde sorting by toxins in early endosomes which leads to the formation of restrograde tubules in clathrin-dependent manner. Shiga toxins are localized into this tubular environment. In a retromer-dependent manner, these retrograde tubules are processed by scission. Shiga toxins are transferred from the early endosome to the trans- Golgi network (TGN), bypassing the late endocytic pathway, and then on to the endoplasmic reticulum (ER). Then ER-associated degradation machinery is used by shiga toxins in order to facilitate retrotranslocation into the cytosol of host cell (adopted from Johannes & Römer, 2010).

2.5.3 Intoxication by Shiga toxins

Shiga toxins, in patients having HUS, usually affect gastrointestinal tract and kidney but sometimes clinical symptoms have shown that central nervous system and other organs can also be affected (Ray & Liu, 2001). Shiga toxins cause inhibition of protein synthesis in target cells.

In short, Shiga toxins can directly inhibit protein biosynthesis, therefore commensal bacteria present in lumen of gut can be targeted leading to proliferation of Shiga toxin producing bacteria. Along with commensal bacteria, Shiga toxins can also target the host cells. In intestinal mucosa, the initial damage is due to bacterial products other than toxins, because Gb3 is expressed in low levels by human gut enterocytes (Falguieres *et al*., 2008). However, Shiga toxins result in vasculitis by damaging the microcirculation which aggravates the mucosal damage. This infection may lead towards bleeding in the bowel and also can cause bloody diarrhea. This helps in survival of bacteria because they are provided with growth promoters in sufficient amounts.

Initially it was thought that Shiga toxins, during development of HUS, only leads to the protein synthesis, but the research has shown that Shiga toxins can trigger various signaling cascades which can influence their own trafficking as well as other function of host cells such as cytokine secretion and death of cell by apoptosis. Pro-inflammatory cytokines are released from monocytes or macrophages during toxin induction which can lead to disease progression by enhancing Gb3 expression in endothelial cells. Shiga toxins activate polymorphonuclear leukocytes which can cause endothelial injury by releasing reactive oxygen metabolites (Foster & Tesh, 2002; Iordanov *et al*., 1997).

2.6 *E. coli* O157: H7- A Food borne Enterohemorrhagic *E. coli* (EHEC)

Several categories have been used to describe diarrheagenic *E. coli* strains which include enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC) and entero-hemorrhagic *E. coli* (EHEC). *E. coli* O157:H7 is considered as major food borne pathogen acros the world. *E. coli* O157: H7 can be called EHEC with respect to its diseases, however with respect to toxins produced, it is called Shiga toxin *E. coli* (STEC). EHEC can be regarded as a group of water and food-borne pathogens which can cause diseases ranging from

mild diarrhea to acute hemolytic uremic syndrome and hemorrhagic colitis via release of shiga like toxins. If the disease prolongs, EHEC infections may also lead to the death (Mohamed A. Karmali *et al*., 2010). *E. coli* O157:H7 was first identified by Riley *et al*., (1983) during an outbreak caused by eating contaminated hamburger meat in United States of America. In 1993, after an outbreak of *E. coli* O157 caused by undercooked ground beef patties it was broadly recognized as a threatening pathogen. Largest outbreak caused by *E. coli* O157 has been reported in Japan where 10000 cases were identified in summer 1996 (Michino *et al*., 1999).

2.6.1 Epidemiology of *E. coli* O157

Main sources of *E. coli* O157 strains are animals and food. The first case of human illness due to *E. coli* O157 was caused due to the contaminated hamburger. It is known that Cattle are routinely colonized by the EHEC and these organisms can also resist the acid environment present in apple cider and other processed foods. Even, vegetables which have fertilized with cow manure can become contaminated and cause human disease. Very low number of this organisms (fewer than 100 bacteria) can cause illness and spread from person to person. Family studies have shown that when a child gets infection of *E. coli* O157: H7 then other family members have high risk of developing the infection. Swimming in polluted lakes can also cause life threatening illness via ingestion of organism (Cleary, 2004). *E. coli* O157:H7 infection is associated with relatively longer duration of excretion of organism. Karch and colleagues found that organism was excreted after about 13 and 21 days in heamorrahgic colitis and HUS, respectively (Karch, Rüssmann, Schmidt, Schwarzkopf, & Heesemann, 1995). Pai and colleagues also found the similar prolonged excretion of microorganism however some patients have no signs of bacteria after 10 days of infections. Also, a relation exists between symptoms of infection and age of patient. Children less than 6 years of age never develop clinical signs of infections. The absence of infection in infants

can be justified through trans placental immunity, lack of receptors required for colonization and protective factors present in human milk (Pai *et al*., 1988). Worst cases of HUS have observed among patients having age between 6 and 48 months.

2.6.2 Clinical manifestation

Sometimes, EHEC can cause infection in humans without showing any symptoms but most of the time they can cause watery diarrhea. Watery diarrhea may be followed by blood streaked which is termed as hemorrhagic colitis. Hemorrhagic colitis is characterized by severe abdominal pain and vomiting. *E. coli* O157 strains are more responsible for bloody diarrhea than non-O157 strains (Werber *et al*., 2004). Diarrhea can take a week, before recovery of a patient. Only few children, infected with EHEC, can have fever. However patients which are going to develop HUS can have some fever during prodromal stage of bloody diarrhea. HUS is the major complication which can be caused by EHEC infection however it is not the only problem. The other complications include intussusception, gangrene with sepsis and peritonitis, coma, seizures and hemiplegia (Lopez *et al*., 1992). 5 to 8 percent children having hemorrhagic colitis can develop HUS. 4 to 13 days of lag period usually occurs between diarrhea onset and first sign of HUS. Less than 5 percent of children can die if they are not given appropriate treatment. Fewer than 5 percent of children with HUS die, if given appropriate medical care, although a much higher percentage have some permanent residual renal impairment (Cleary, 2004).

3. Materials and Methods

3.1 Materials

3.1.1 Equipment used in this study

Table 3.1: List of equipment used in this study

Incubator	Memert
Incubator (shaking)	Heidolph InKubator1000
Refrigerated centrifuge	HERMLE
Tabletop centrifuge	Sigma
Thermo cycler Swift MaxPro	ESCO
Thermocycler (Gradient)	peQlab
pH meter	InoLab
UV illuminator	WEAL TEC
Gel electrophoresis tank ELITE 300 PLUS	WEAL TEC
Concentrator plus	Eppendorf
Spectrophotometer SP-300	OPTIMA
Micro Biological Safety Cabinet	Technico Scientific Supply
MyiQ real-time PCR	BIO-RAD
Sterile filter 0.22 μm	Nalagene
Filter paper- Whatman	Hartenstein
NanoDrop 1000 spectrophotometer Peq Lab	Peq Lab

3.1.2 Chemicals used in this study

Table 3.2: List of chemicals used in this study

Ethidium bromide (EtBr)	Invitrogen
EDTA	ROTH
Ethanol	MERCK (USA)
Glucose	Riedel-deHaen (Germany)
Glycerol	MERCK (USA)
Isopropyl-β-D-thiogalactoside (IPTG)	Invitrogen
Isopropanol	EMPLURA(Germany)
Magnesium Chloride	Scharlau (Spain)
Potassium Acetate	MERCK (USA)
Sodium hydroxide	Scharlau (Spain)
Sodium Chloride	Scharlau (Spain)

Acetic acid	Sigma-ALDRICH
Agarose	MERCK (USA)
Calcium Chloride	Scharlau (Spain)
Deoxyribonucleoside triphosphates (dNTPs)	Thermo Fisher Scientific
Sodium Dodecyl Sulphate (SDS)	Phytotechnology Lab
Tris/Hcl	Phytotechnology Lab.
Tris/Base	Phytotechnology Lab
Tryptone	OXOID

3.1.3 Antibiotics used in this study

Table 3.3*:* List of antibiotics used in this study

Ampicillin	Thermo Fisher Scientific

3.1.4 Culturing media used in this study:

Table 3.4: List of media used in this study

Nutrient agar	OXOID
LB liquid media	1% Tryptone, 0.5% Yeast, 1%NaCl
Sorbitol Mckonkey agar	OXOID

3.1.5 Bacterial strains Used in this study:

Table 3.5: List of bacterial strains used in this study

E.coli DH5α
E.coli O157
E.coli S17-1

3.1.6 Software used for this study:

Table 3.6: List of Software used for this study:

ClustalW	This tool is used for multiple sequence alignment with colored results
IntaRNA (Freiburg Bioinformatics Group)	This tool was used in our study to predict the interaction efficiencies of our designed synthetic ncRNA against Shiga Toxin *stx-2* mRNA.
RNAfold web server	This tool was used to predict the structure of synthetic ncRNA, analysis of its secondary structures, and thermodynamics.

3.2 Methods

3.2.1 *In silico* designing of anti-shiga sRNA:

In order to design the synthetic sRNA against *stx-2* gene of *E. coli* O157, the *stx-2* gene sequence was retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and start codon and stop codon of the gene was carefully indicated. The gene sequence was then compared with the library of sequencing available in all databases using nucleotide BLAST tool to study the conservation of gene sequence among all STEC strains. After studying the homology of *stx-2* gene among all strains the most appropriate GUIDE sequence was selected. The selected guide sequence was then analyzed on IntaRNA bioinformatics tool to predict its interaction efficiency of non-coding RNA with target mRNA. Binding energy of non-coding RNA with its target mRNA majorly determines the repression efficiency of a designed non-coding RNA.

The second step is the structure prediction of designed non-coding RNA using MFOLD wed server (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi), an RNA structure prediction tool. The predicted structure was assessed for the presence of secondary stem loop structures and minimum free energy of synthetic non-coding RNA as it determines the stability of a RNA structure.

3.2.2 Culturing conditions for *E. coli* DH5α:

In order to obtain fresh liquid cultures of *E. coli*, cells were taken by scrapping the cryo-stocks wit sterilized inoculating loop. Loop filled with E. coli cells was dipped in LB medium and was incubated overnight at 37°C at 150rmp. Volume of culture was kept one-third of total volume of flask in order to make ensure sufficient supply of oxygen to the cells.

3.2.3 Culturing conditions for *E .coli* O157: H7

Cryo-stocks were used to obtain fresh liquid cultures of *E. coli* O157:H7 strain. The culturing conditions for *E. coli* O157 (STEC) were same as that of *E. coli* DH5α.

3.2.4 Bacterial Cryo-stock Preparations

The bacterial strains were preserved by preparing cryo-stocks of overnight cultures. 2 ml of overnight liquid cultures were taken in centrifuge tubes and centrifuged at 5000 rpm for 5 min. Supernatant was discarded. 1ml of liquid culture was added and centrifuged again. This step was repeated until thick pellet was obtained. Then pellet was re-suspended in 1 ml of LB media. 0.5ml of 80 % sterilized glycerol was added in cryo-tubes. Resuspended pellet was added in this tube and vortexed for few seconds. These cryo-tubes were kept in liquid nitrogen for 5-10 min. Tubes were labeled with name of bacterial strain, date and relevant information and then were transferred to -20°C and -80°C for long term storage.

3.2.5 Preparation of *E. coli* S17-1 competent cells:

E. coli S17-1 cells from cryo-stock were streaked on nutrient agar plates by sterile inoculating loop and incubated overnight at 37°C. Next morning single colony was picked from overnight agar plate and incubated in LB media at 37°C overnight in a shaking incubator at 150rpm. Next morning 1ml of overnight culture was inoculated in 50ml of fresh LB media, incubated at 37°C in a shaking incubator till OD_{600} 0.5 to 0.6 was obtained. In order to harvest the cells, the culture was shifted in already chilled 50ml falcon tubes over ice and centrifuged at 4000rmp for 20 minutes at 4°C. Supernatant was discarded and rest of the pallet was resuspended in 10ml of chilled Solution 1. The cells were centrifuged again at 4000rpm for 20 minutes at 4°C. Pellet was resuspended in 1ml of chilled Solution 2. The resuspended pellet was aliquoted in 50 µl ice chilled Eppendorf tubes

and then frozen in liquid nitrogen for a while. The competent *E. coli* cells were stored in aliquots at -20°C.

Table 3.7: Composition of solutions used for competent cells

Solution 1 (autoclaved)	$Mgcl_2$ (0.5M)
	$Cacl_2$ (0.5M)
	ddH_2O
Solution 2 (autoclaved)	$Cacl_2$ (0.5M)
	Glycerol 50%
	ddH_2O

3.2.6 Isolation of plasmid DNA:

In order to isolate plasmid DNA from cells alkaline lysis method was used which involves lysis of the cells using alkaline lysis buffer that contains a detergent (SDS) sodium dodecyl sulfate and a strong base sodium hydroxide. 2 ml of cell culture was taken in an eppendorf tube and centrifuged at 10,000 rpm for 5 minutes at room temperature. The supernatant was discarded and cell pallet was dissolved in 200µl of resuspension solution and incubated for 10 minutes on bench top. RNaseA (4µl/ml) was added along with resuspention solution. Then 200µl of Lysis solution was added to the tube for 10 minutes and inverted several times to lyse the cells. In order to precipitate the chromosomal DNA and proteins of the cells, 200µl of neutralization solution was added and incubated on ice for 15 minutes. In order to separate the precipitated chromosomal DNA and cell debris the tubes were centrifuged at 13,000 rpm (4°C) for 15 minutes. The supernatant containing the plasmid DNA was transferred into a new eppendorf tube and 500µl of chilled isopropanol was added to precipitate plasmid DNA. The tubes were centrifuged again for 15 minutes at 15,000 rpm (4°C). The pallet was washed with chilled 80% ethanol and centrifuged at 13,000 rpm for 10

minutes. Pallet was dried in Concentrator *plus* to remove left over ethanol and then dissolved in 50µl ddH_2O.

Table 3.8*:* composition of solutions used for plasmid extraction

Resuspention Solution (pH 7.4)		**Lysis Solution**		**Neutralization Solution** (pH 5.6)	
Glucose	0.05M	NaOH	0.2M	KAc	5M
Tris/Hcl	0.025M	SDS	1%	Acetic acid	2M
EDTA	0.01M				

3.2.7 Restriction Digestion of Plasmid:

In order to linearize the plasmid, for confirmation of size, it was digested by using *Eco*R1 restriction enzyme. After preparing the reaction mixture by adding components given in table 3.8, in an eppendorf tube, it was mixed gently and spun down for a few seconds. Then it was incubated at 37°C for 1 and then size of plasmid was checked by running on gel electrophoresis.

Table 3.9: Components used for restriction digestion

Component	**Volume**
Nuclease free water	16 µl
10X Buffer *Eco*RI	2
DNA 0.5-1	1
Enzyme (*Eco*R1)	0.5

3.2.8 PCR Reaction:

Phusion polymerase master mix was used for incorporation of guide sequence into the plasmid via rolling circle PCR. 50 µl reaction was prepared by adding the components given in table 3.9.

Table 3.10: Components used for making reaction mixture of PCR

REAGENT	**Volume in µl**
Phusion Master Mix	25 µl
Forward Primer	1.5 µl

Reverse Primer	1.5 µl
DNA	2 µl
Nuclease Free Water	20 µl

Table 3.11: Primers used for modification of plasmid pAB.001 via PCR

Primer Name	**Sequence**	**Reference**
Stx2- F	5'- CTGGCGGTGATAATGGTTGCCCATTTAAATAATA TACACTTCATTTTCTGTTGGGCCATTGC - 3'	This study
Stx2-R	5'-GCAATGGCCCAACAGAAAATGAAGTGTATATTAT TTAAATGGGCAACCATTATCACCGCCAG - 3'	This study

Table 3.12: Optimized conditions for rolling circle PCR

Step	**Temperature**	**Time**	**Cycle**
Initial Denaturation	95 ◦C	5 min	1
Denaturation Annealing Extension	95 ◦C 68 ◦C 72 ◦C	40 s 1 min 2 min	30 cycles
Final Extension	72 ◦C	10 min	1
Hold	4 ◦C	-------	------

3.2.9 PCR Product Purification:

Thermo scientific GeneJET PCR purification Kit was used for the purification of PCR product. For the purification of PCR product binding buffer was added in 1:1 volume. This solution was then added to the GeneJET purification column and centrifuged at 12000g for 1 min. Flow through was discarded. 700 µl of wash buffer was added to the purification column and centrifuged at 12000g for 1 min. Then 50 µl of elution buffer was added, centrifuged for 1 min at 12000g and eluted DNA was stored at -20 in micro-centrifuge tubes.

3.2.10 Confirmation of insert via sequencing of modified pAB.001 plasmid:

To confirm the incorporation of correct guide sequence the modified plasmid was extracted and sent to Macrogen *Inc.* Korea. The correct modified plasmid colonies were preserved at -80°C.

Table 3.13: List of primers used for sequencing

Primer Name	Sequence	Reference
Primer F	5' -ATACTCCAATTGGCGATGGC - 3'	Samia Shafqat MS thesis (2017)
Primer R	5' -ATAAACGCAGAAAGGCCCAC- 3'	Samia Shafqat MS thesis (2017)

3.2.11 Transformation of plasmid by heat-shock method in *E. coli* S17-1:

In order to transform modified plasmid DNA (containing anti-shiga cassette) into *E. coli S17-1* cells via heat-shock method, the competent cells were taken from -80°C freezer and thawed on ice. 10µl of modified plasmid DNA was mixed very gently with 50µl of competent cells on ice. The mixture of competent cells and plasmid DNA was incubated on ice for about 30 minutes then placed in 42°C water bath for 2 minutes after which it was immediately placed back on ice for 2 minutes. 0.5 ml of LB media was added to transformed cells and incubated at 37°C for 2 hours. After incubation centrifuged at maximum speed for 5 minutes. After discarding half of the supernatant the pellet was mixed in remaining supernatant and spread on nutrient agar plates containing ampicillin as antibiotic marker. The agar plates were incubated overnight at 37°C.

3.2.12 Transformation via Plasmid conjugation in *E. coli* O157:H7:

Direct transformation of plasmid DNA in *E. coli* O157:H7 strain EDL933 is not possible, therefore di-parental conjugation is used for gene transfer purposes. In di-parental conjugation the *E. coli* S17-1 strain with the modified plasmid is used as a donor strain while the EHEC O157:H7 is the recipient strain. The donor strain contain a plasmid with *mob*- genes (for mobilization), whose gene products trigger a nicked plasmid. *E. coli* strain S17-1, used as a donor strain for diparental

conjugation, contains the *tra*-genes which encode the formation of the F –pilus, make the contact between donor and recipient.

To perform di-parental conjugation about 1 ml of an overnight culture of *E. coli* O157 cells was centrifuged at 3000 rpm for 5 min and pellet was dissolved in 100 µl of LB medium. An inoculating loop filled with cell culture of the donor strain S17-1 which was grown overnight on agar plates was mixed with the resuspended EHEC O157 cells. After gentle mixing of the two strains, the mixture was transferred on top of a sterile membrane filters already placed on nutrient agar plates. Nutrient agar allows both *E. coli* strains to grow. After incubating the conjugation mix at 37°C for 6-8 h, membrane filters were soaked in Eppendorf tubes containing 1 ml LB media. After thorough resuspension, dilution series ($100 – 100^{-3}$) was made and spread on sorbitol mackonkey agar plates containing the respective antibiotics. EHEC O157 cell colonies appear as colorless colonies on sorbitol mackonkey agar while *E. coli* S17-1 cells appear pink. Sorbitol mackonkey agar is a selective differential medium for the detection of *E. coli* O157 strains from other *E. coli* strains. SMAC agar helps in differentiating EHEC O157 from *E. coli* S17-1 cells. Plates were incubated overnight at 37°C. Single colorless colonies of *E. coli* O157 were selected and cultured for further verification to determine extent of repression of Shiga toxin production

3.2.13 Isolation of bacterial RNA:

15ml of overnight culture was taken in a falcon tube and centrifuged at 6,000 rpm for 10 minutes. Supernatant was discarded and dissolved pallet in 200-250µl of TE buffer containing lysozyme. Then it was incubated over ice for 15 minutes then Trizol was added 1 to 1.2 ml of reagent. Immediately 20-30 µl of glacial acetic acid was added and incubated for 15-20 minutes over ice. Afterwards 0.2 ml chilled chloroform was added and vortex vigorously for 5 minutes on and off. Samples were centrifuged at 13,000 rpm for 20 minutes at 4°C. Upper aqueous phase was collected

carefully in a new eppendorf tubes. Then equal volume of chilled isopropanol (500 µl) was added and inverted several times. Sample was incubated at -20°C for 20 minutes in order to precipitate RNA. Then the sample was centrifuged at 14,000 rpm for 10 minutes at 4°C. Supernatant was discarded and rinsed in 1ml chilled 80% ethanol. Ethanol was decanted and allowed the RNA to dry at room temperature for 5 minutes. RNA was dissolved in 20-25 µl of DEPC water. Sample was quantified on Nanodrop.

Note: All steps must be done over the ice to prevent RNA degradation.

3.2.14 cDNA Synthesis:

Step1: In a PCR tube 11µl of quantified RNA sample and 1 µl of OligoDT primer was added. Sample was incubated at 70°C for 5 minutes.

Step 2: 4 µl of Reverse Transcriptase buffer and 1 µl of RNase inhibitor was added and incubated the sample at 37°C for 5 minutes

Step 3: 2 µl of 10mM dNTPs mix and 1 µl of Reverse Transcriptase was added and Incubated at 42° for 60 minutes and then at 70°C for 10 minutes.

3.2.15 Real-Time RT-PCR to assess the expression of *stx-2* gene:

Real-time RT-PCR amplifies and simultaneously detects or quantifies a targeted DNA molecule made from the mRNA template. The procedure is based on the general principle of polymerase chain reaction but the key feature is that the amplified DNA is detected as the reaction progresses in “real time”. As the template used is RNA therefore in the first step a cDNA of the RNA is made by the reverse transcriptase (RT) enzymes and this transcript is amplified further as a template by PCR. The SYBR Green-I dye intercalates into the double standard DNA formed and thereby the

increase of the PCR products is monitored in real time. SYBR Green-I can be excited by irradiation with light to emit green light which is measured in turn to give the number of cycles performed. For detection of change in *stx-2* gene expression analysis in *E. coli* O157: H7 real time RT-PCR was performed on BioRAD real-time PCR system with fluorescence based SYBR green assay. To normalize the data, 16S RNA was used as a reference gene. The reaction mixture was prepared according to the amounts given in table 3.14. Thermal profile and dissociation stage was set which is given in table 3.15. The stx-2 gene expression was analyzed at 2 intervals for both control strain and transformed strain. First sample was taken at 3 hours after incubation and then 24 hours of incubation.

Table 3.14: Primers used for Real time RT-PCR

Primer Name	**Sequence**	**Reference**
STX2-RT-For	5'- CGGACAGAGATATCGACCCC - 3'	This study
STX2-RT-Rev	5'- ACGACTGATTTGCATTCCGG - 3'	This study
27F	5'- AGAGTTTGATCMTGGCTCAG- 3'	Turner *et al* (1999)
1522R	5'- AAGGAGGTGATCCARCCGCA- 3'	Turner *et al* (1999)

Table 3.15: Components used for making reaction mixture for Real time RT-PCR

Component	**Volume**
Template DNA	40 ng/ µl
Forward Primer STX2-RT-FOR	0.5 µl
Reverse Primer STX2-RT-REV	0.5 µl
Maxima SYBR GREEN	6.25 µl
Volume was raised upto 12.5 µl with nuclease free water	

Table 3.16: Optimized conditions used for real time RT-PCR

Step	**Temperature**	**Time**	**Cycle**
1- Denaturation	95 ◦C	10 min	1
2- Annealing	95 ◦C 50 ◦C 72 ◦C	15 s 30 sec 35 sec	40 cycles
3-Dissociation Stage	95 ◦C 50 ◦C	15 sec 30 sec	1 cycle

3.2.16 Toxin preparation for cytotoxic assay:

The WT-O157 strain and ASR-O157 strain was cultured in 10 ml of LB broth at 37ºC for 18 h, with shaking until OD_{660nm} of 0.5 was achieved for both strains. Same optical density of WT-O157 strain and ASR-O157 strain is crucial since it will affect the amount of toxin produced. The culture filtrates were obtained by centrifugation at 5000rpm for 10 minutes at 4°C and the toxin containing supernatants were sterilized by filtration through 0.2-µm filters (Millipore).

3.2.17 Cell lines and culture conditions:

U87 cell lines were grown in RPMI-1640 supplemented with 10% heat fetal bovine serum (FBS), 100 IU/ml ampicillin, 100 mg/ml streptomycin and 2 mm-glutamine. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 °C. The cells were sub-cultured twice each week, seeding at a density of about 2×10^3 cells/ml. Before the MTT assay, cells were washed with PBS and fresh medium was added. For final analysis, exponentially growing cells were collected and re-suspended in fresh culture medium with 10% FBS.

3.2.18 MTT Assay:

MTT assay is a cell viability assay developed for 96 wells plate, used to assess the effect the particular compounds on cell proliferation or toxicity. The cytotoxic effect of particular compounds is determined using tetrazolium reduction assay (Mosmann T., 1983). The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) is a water soluble compound that is positively charged and readily penetrates viable eukaryotic cells that is reduced to insoluble formazan. The formazan produced is quantified by determining the absorbance at 570 nm using plate read spectrophotometer. The amount of formazan is directly proportional to number of viable cells. Each step was performed in triplicates. U87 (ATCC# HTB-14) a human primary glioblastoma cell line was used to perform the MTT assay.

Step 1: **Trypsininization**: The adhered cells were trypsinized in T-75 flask by adding 3mL of Trypsin. The flask was incubated for 5 minutes in the incubator. 5mL of complete media was added to the trypsinized cells and transferred to a 15mL sterile pyrogen free falcon tube. Cells were centrifuged at 1000rpm at 4°C for about 10 minutes. The media was discarded and cells were resuspended in 1mL complete media.

Step 2: **Cell Counting**: 10µL of the cells were added in 1.5mL micro-tube and 10µL of Trypan blue stain was added to it. Then 10µL of the stained solution was placed on a Cell Countess slide. The number of viable cells and dead cells were counted per ml. The cells were diluted by adding 9mL of complete media to make $1.5x10^3$ per ml (cv=cv).

Step 3: **96 Wells plating**: 100µL of cells were added into each well and incubated overnight in the CO_2 incubator.

Step 4: **Toxin administration**: After overnight incubation different concentrations (10µL, 100µL) of WT-O157 shiga toxin and ASR-O157 toxin were added in the wells in triplicates. The volume in each flask must not exceed 200µL. the plate was incubated overnight in the incubator.

Step5: **MTT assay**: 30µL of 5mg/ml MTT was added to each well. A negative control of 30 µL of the MTT stock solution added to 100 µL of medium alone was included in the plate. The plate was incubated for 3 to 4 hours in the incubator. The media from all wells was carefully removed without disturbing the cells. 50µL of DMSO (MTT solvent) was added and left for 10 minutes. The plate was then placed in the ELISA plate reader. The readings at 590 nm absorbance were recorded with a reference filter of 620 nm. The readings gave the percentage of viable cells number according to the formula given below:

$$\% \text{ Cell Viability} = \left[\frac{O.D._{TEST/SAMPLE}}{O.D._{CELL\ CONTROL}}\right] \times 100$$

4. Results

4.1 Consensus sequence identification via BLAST and ClustalW

Designing of synthetic non-coding RNA was accomplished using various bioinformatics tools. In 1st step *stx-2* gene sequence for *E. coli* O157:H7 was retrieved from NCBI nucleotide database (ID 912807) which was 959 nucleotides in length. Then all the sequences reported in NCBI nucleotide database for *stx-2* gene were compared for assessing similarity using nucleotide BLAST tool. BLAST results showed 99% sequence similarity by giving alignment score >=200 among all the *stx-2* gene sequences reported in various organisms. In order to further confirm the similarity of *stx-2* gene among all EHEC strains ClustalW was used as basic alignment tool. *Stx-2* gene sequences present among different EHEC strains showed 100 percent similarity. Alignment results of *stx-2* variants among EHEC strains is shown in Figure 4.1.

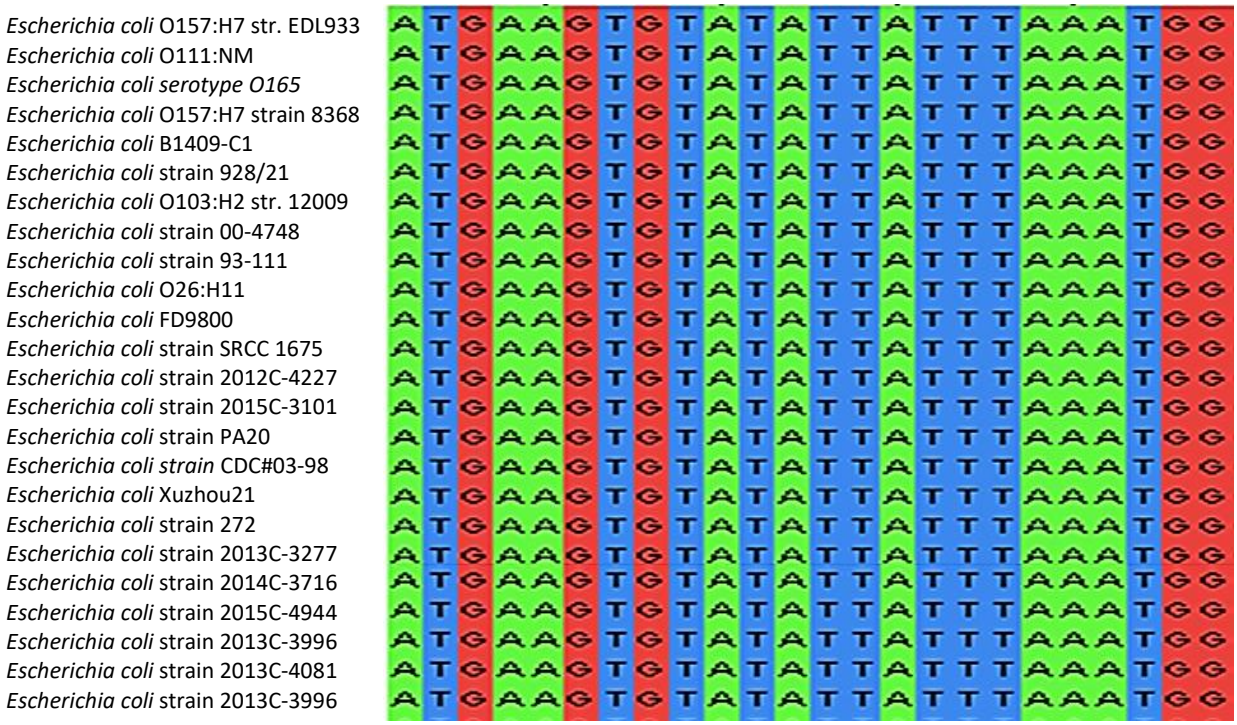

Figure 4.1: A multiple sequence alignment of 1st 24 nucleotides of several *stx-2* homologs with conserved sequences depicted with colored lines using ClustalW.

4.2 Confirmation of sequence of synthtic non-coding RNA cassette

A synthetic sRNA cassette was constructed computationally that contains all the necessary components for synthetic sRNA synthesis ; a promoter, specific guide sequence that directs the sRNA to its target mRNA, a scaffold sequence that recruits cellular Hfq proteins involved in mRNA degradation and a terminator sequence, for synthesis of synthetic sRNA as shown in Figures 4.2 and 4.3. The *MicC* sequence was selected as a scaffold for Hfq recruitment. *MicC* was selected because of its superior repression capability determined by Na *et al*., (2013). T1/TE sequences are used as a terminator. Reverse complementary sequence of 1st 24 nucleotides of *stx-2* gene was chosen as the guide sequence for synthetic sRNA. This guide sequence will directs the sRNAs on mRNA. Designed non-coding RNA was named as anti-shiga sRNA.

TAACACCGTGCGTGTTGACTATTTTACCTCTGGCGGTGATA
ATGGTTGCCCATTTAAATAATATACACTTCATTTTCTGTTG
GGCCATTGCATTGCCACTGATTTTCCAACATATAAAAAGAC
AAGCCCGAACAGTCGTCCGGGCTTTTTTTCTCGAGCTCGAG
CCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTG
GGCCTTTCGTTTTATCTGTTTTTGTCGGTGAACGCTCTCTAC
TAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGT
TTATA

Figure 4.2: Synthetic sRNA cassette containing Promoter (Yellow), Guide sequence (Red), Hfq binding region (Green) and Terminator (Grey).

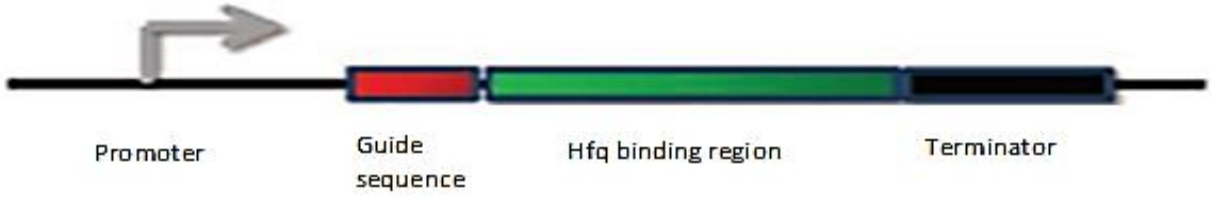

Figure 4.3*:* Synthetic non-coding RNA expression cassette containing a promoter, guide, scaffold and terminator region (adopted from yoo *et al*., 2013).

Cassette for synthtic non coding RNA was inseted into pAB.001 plasmid. pAB.001 was provided by Bernhiem *et al*., (2016) which already contains most of the components (except of guide sequence) needed for sRNA expression. The complete plasmid map of pAB.001 is shown in Figure 4.4 containing ampicillin resistance gene as a selective marker.

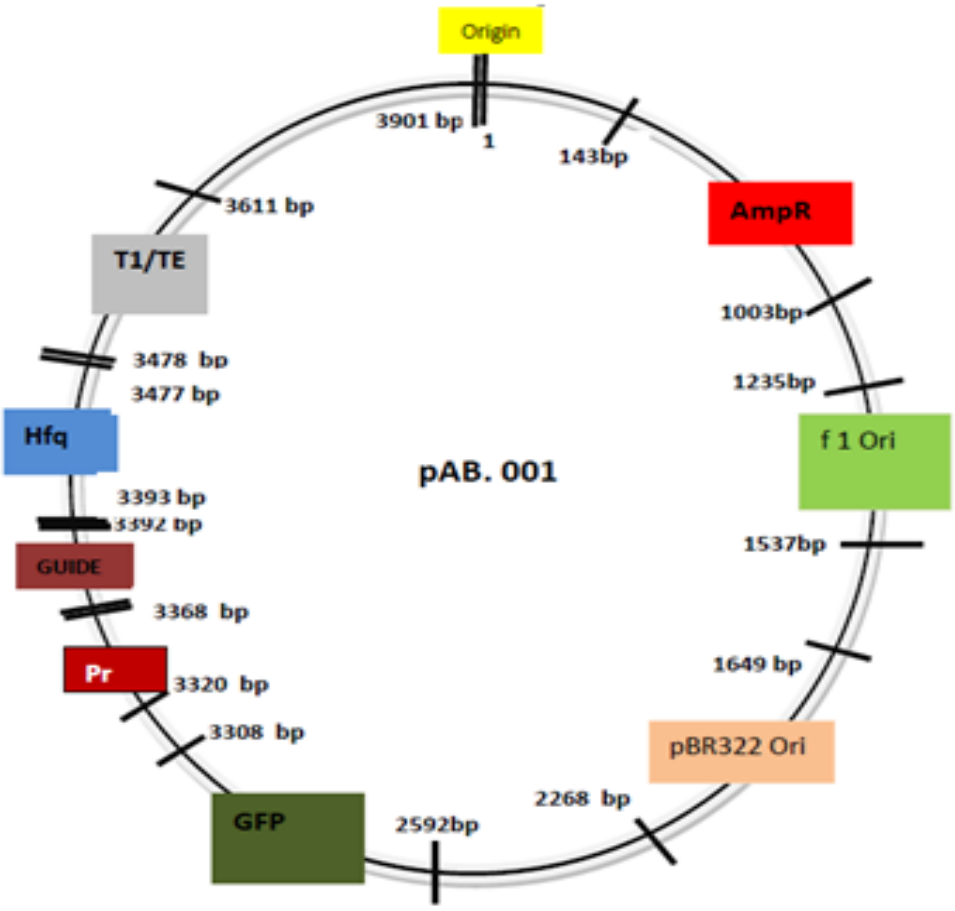

Figure 4.4: pAB.001 plasmid map, dipictting Ampicillin resistance gene (Amp^R), F1origin of replication (F1 Ori), the pasmid origin of replication as pBR322 Ori, Green Flourscent Protein gene (GFP), synthetic non-coding RNA cassette containing Pr (promotor), GUIDE region, *MicC* Scaffold and T1/TE terminator region (Adopted from Bernhiem *et al*., 2016).

4.3 Binding energy prediction by IntaRNA webtool

To assess the binding energy of synthetic sRNA with target mRNA IntaRNA tool was used. Binding energy of synthetic sRNA with its target mRNA affects its repression ability (Na *et al*., 2013). The predicted binding energy was 29.45 KJ/mol. IntaRNA shows complete complementary binding between sRNA and 1st 24 nucleotides of target mRNA of *stx-2* gene. Predicted binging energy of sRNA with target mRNA along with predicted complementary binding between sRNA with target mRNA is shown in Figure 4.5.

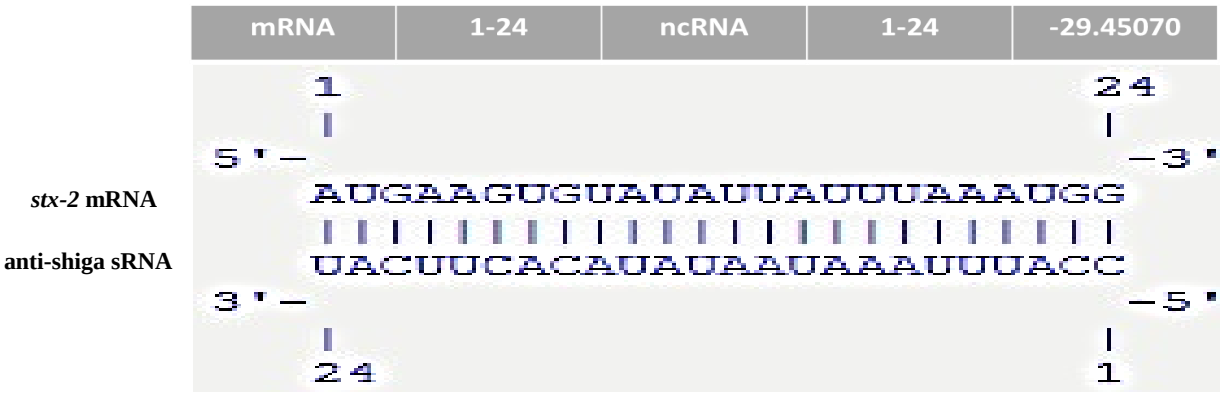

Figure 4.5: IntaRNA results predicting binding energy of -29.45070 KJmol-. IntaRNA also predicted complete complementary binding between the 1st 24 nucleotides of *stx-2* gene and synthetic sRNA.

4.4 Secondary structure prediction of anti-shiga sRNA via RNAfold

sRNA secondary structure plays a major role in its activity and stability (Shuai Man *et al.*, 2011). Two to three stem loop structures are crucial for sRNA activity determined by (Shuai Man *et al.*, 2011). RNAfold web server was used for secondary structure prediction. This tool was employed for predicting the secondary structures of sRNA mainly by using thermodynamics. The predicted structure of our designed anti-shiga sRNA is shown in Figure 4.6. The anti-shiga non-coding RNA has three stem loop structure confirming its stability *in vivo* and efficient repression capabilities. The results show optimal secondary structure in dot-bracket notation with a minimum free energy

of -102.19 kcal/mol. The first stem loop structure is formed in the guide sequence region. It facilitate in recognition of target mRNA. The second stem loop structure is formed in the scaffold region facilitating recruitment of Hfq protein while third stem loop is present in the terminator sequence.

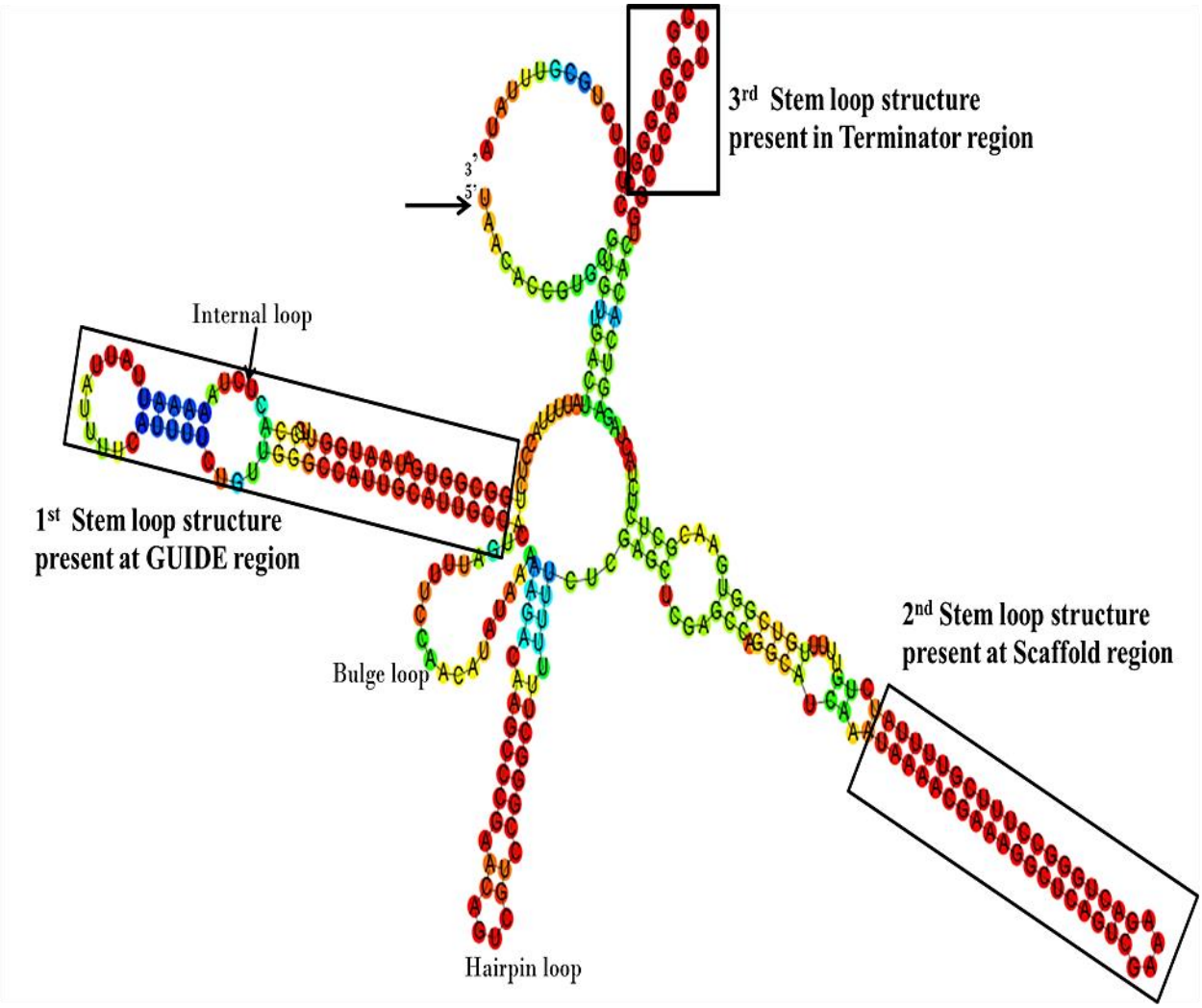

Figure 4.6: The predicted minumum free energy structure of anti-shiga sRNA using RNAfold bioinformatics tool. The first stem loop structure is formed in the guide sequence region. The second stem loop structure is formed in the scaffold region that will facilitate recruitment of Hfq protein while third stem loop is present in the terminator sequence.

4.5 Confirmation of pAB.001 plasmid via restriction digestion

Plasmid pAB.001 containing all the necessary components for synthetic sRNA expression was transformed into *E. coli* DH5α strain. It was extracted and analysed via agarose gel .

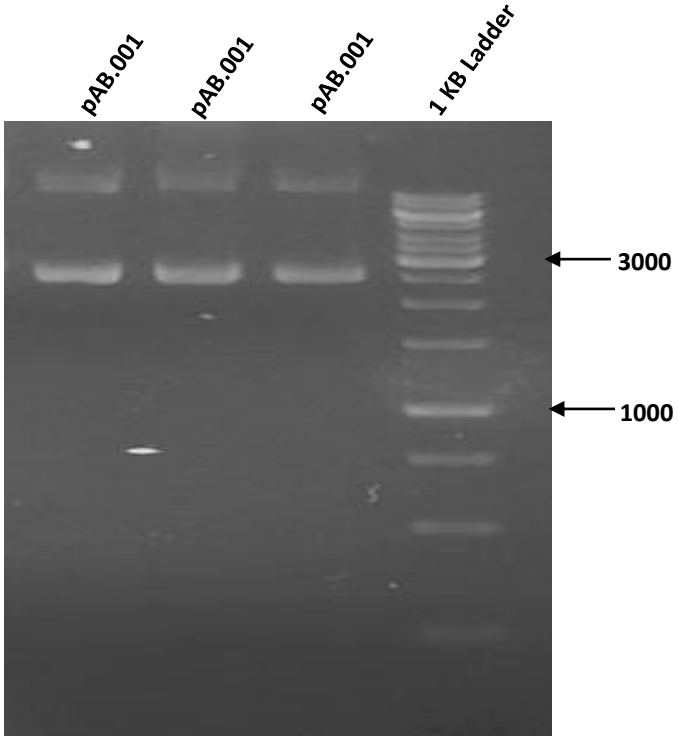

Figure 4.7: Agarose gel electrophoresis of plasmid pAB.001 extracted by plasmid miniprep method.

Size of plasmid was confirmed by restriction disgestion with *Eco*RI enzyme. Clear bands of 3.9 kb were obtained on gel electropherosis as shown in Figure 4.8.

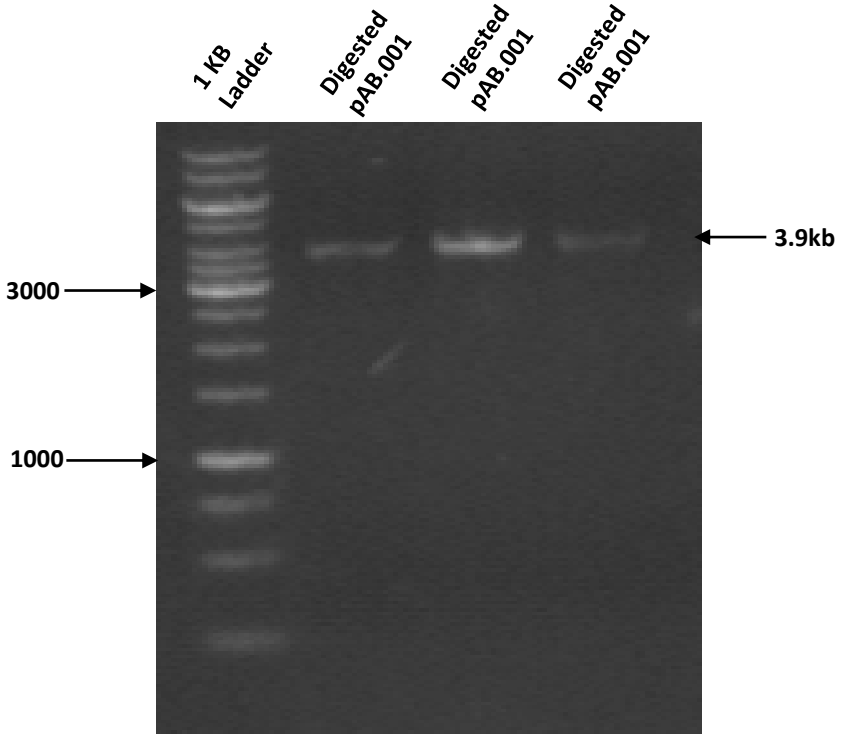

Figure 4.8: Agarose gel electrophoresis of plasmid pAB.001 after digesting it with *Eco*R1 enzyme Clear bands of 3.9 Kb were obtained confirming the size of Plasmid

2.1 Insertion of guide sequence via site directed mutagenesis

To insert our guide sequence against *stx-2* into the Plasmid, site directed mutagenesis was performed with help of PCR. PCR conditions were optimized with varying annealing temperatures of 72^0C, 70^0C, 68^0C and 66^0C. At 68^0C brighter bands were obtained depicted in Figure 4.9. After optimizing the conditions PCR was performed (Figure 4.10) and guide sequence was inserted with help of primers mentioned in table 3.11.

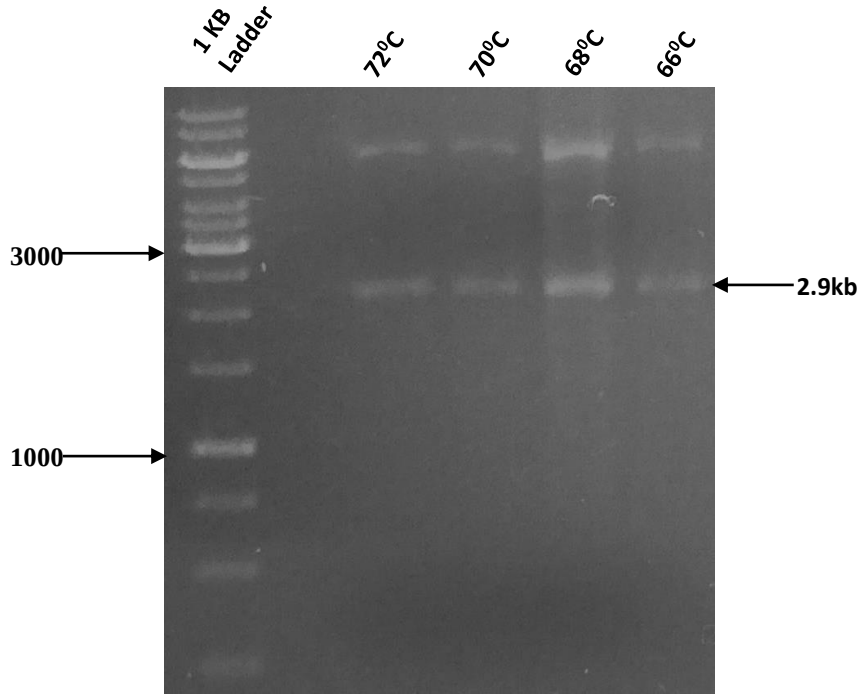

Figure 4.9: Agarose gel electrophoresis of PCR at different annealing temperatures. Annealing gradient was set at 72°C, 70°C, 68°C and 66°C. 68°C is the most optimim temperature for primer annealing giving specific results with highest yield.

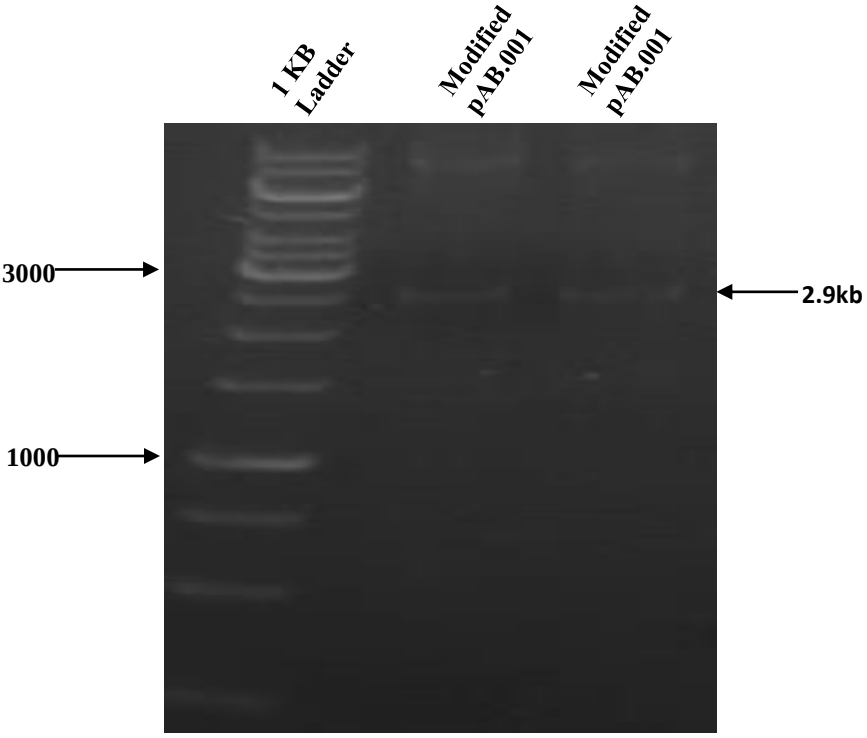

Figure 4.10: Agarose gel electrophoresis of modified plasmid pAB.001 incorporating *stx-2* gene targeting guide sequence via site directed mutagenesis PCR.

4.7 Sequence analysis of the modified pAB.001 harboring anti-shiga sRNA cassette

In order to confirm the correct insertion of our guide sequence, modified plasmid was sent to Macrogen *Inc*. Korea along with primers designed for sequencing mentioned in Table 3.13. Correct insertion of our guide sequence was confirmed as shown in Figure 4.11.

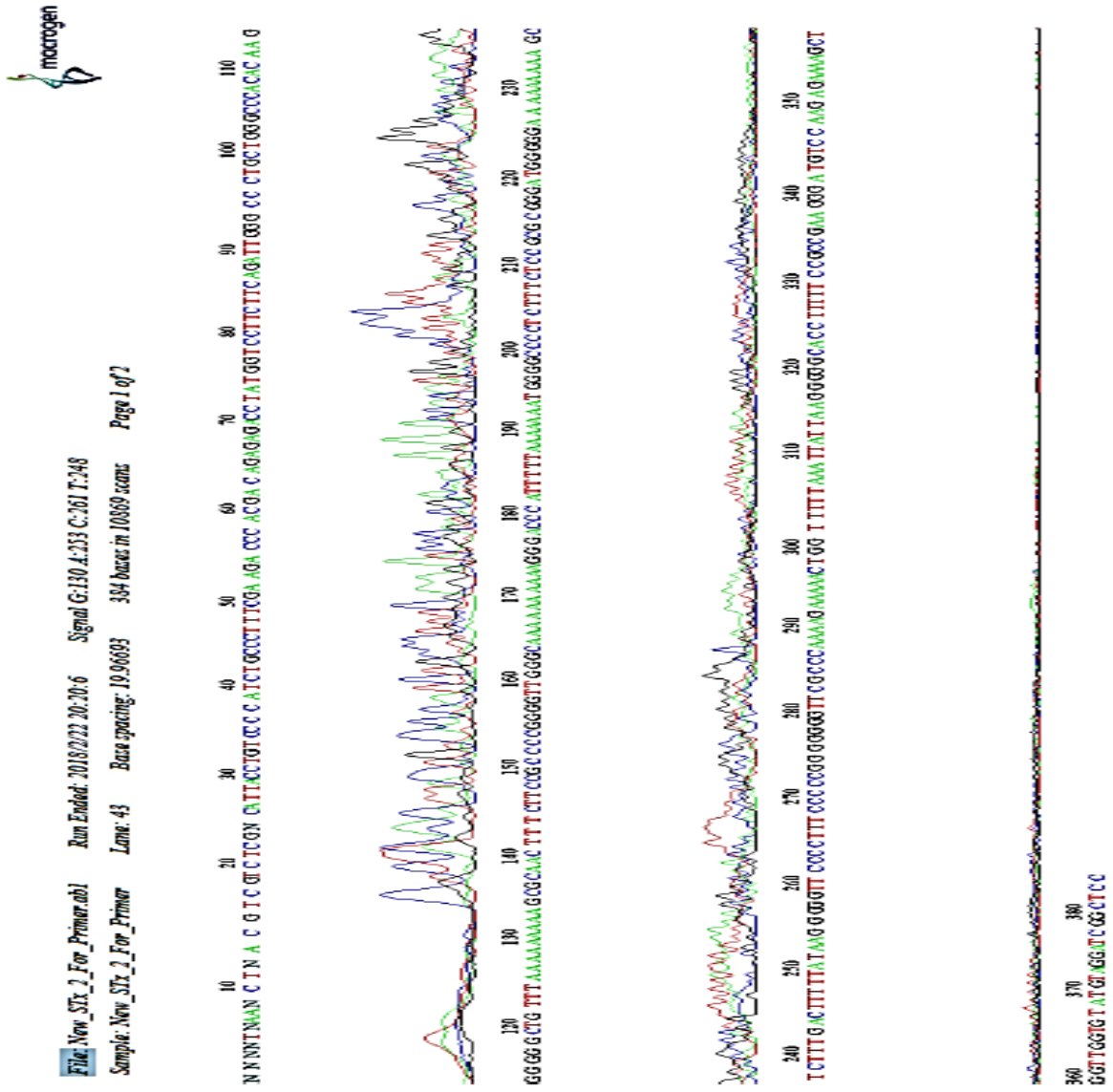

Figure 4.11: Sequencing results of modified plasmid plasmid with incorporation of *stx-2* targeting guide sequence.

4.8 Transformation of modified plasmid into *E. coli* O157:H7

Direct transformation of *E. coli* O157:H7 was not possible therefore we first transformed *E. coli* S17-1 by heat shock method and then we used *E. coli* S17-1 as a donor strain to transform the *E. coli* O157:H7. Transformed *E. coli* S17-1 grown on nutrient agar plate containing ampicillin is shown in Figure 4.12. pAB.001 contains ampicillin genes as antibiotic marker and allowed only transformed colonies to grow.

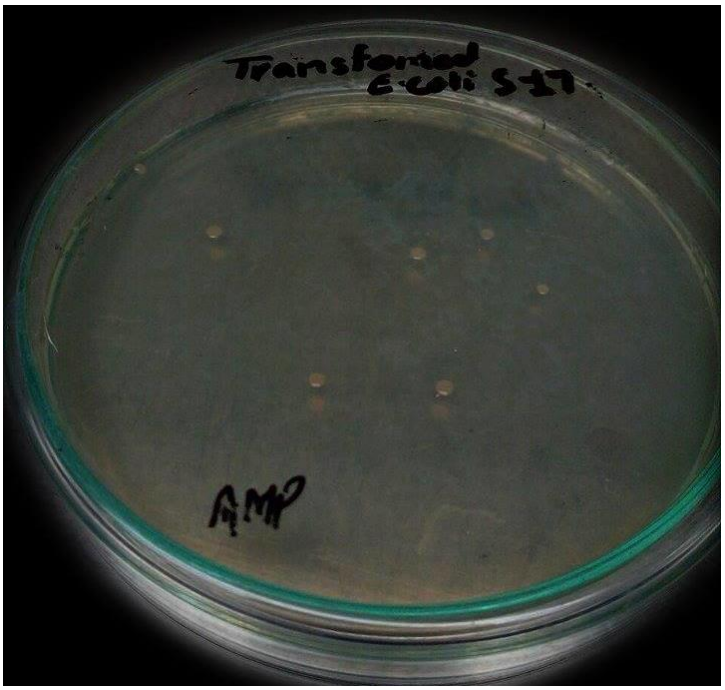

Figure 4.12: *E. coli* S17-1 transformed with pAB.001 plasmid grown on Nutrient agar plate containing Ampicillin as resistance marker.

E. coli O157:H7 was then conjugated with transformed *E. coli* S17-1 by placing both strains on membrane filters on an agar plate. S-17 *cells* contain the *tra*-genes which encode the formation of the F –pilus, which helps in making contact between donor and recipient strains. Membrane filters containing conjugation mixture of both strains was incubated for 4-6 hours as shown in Figure 4.13.

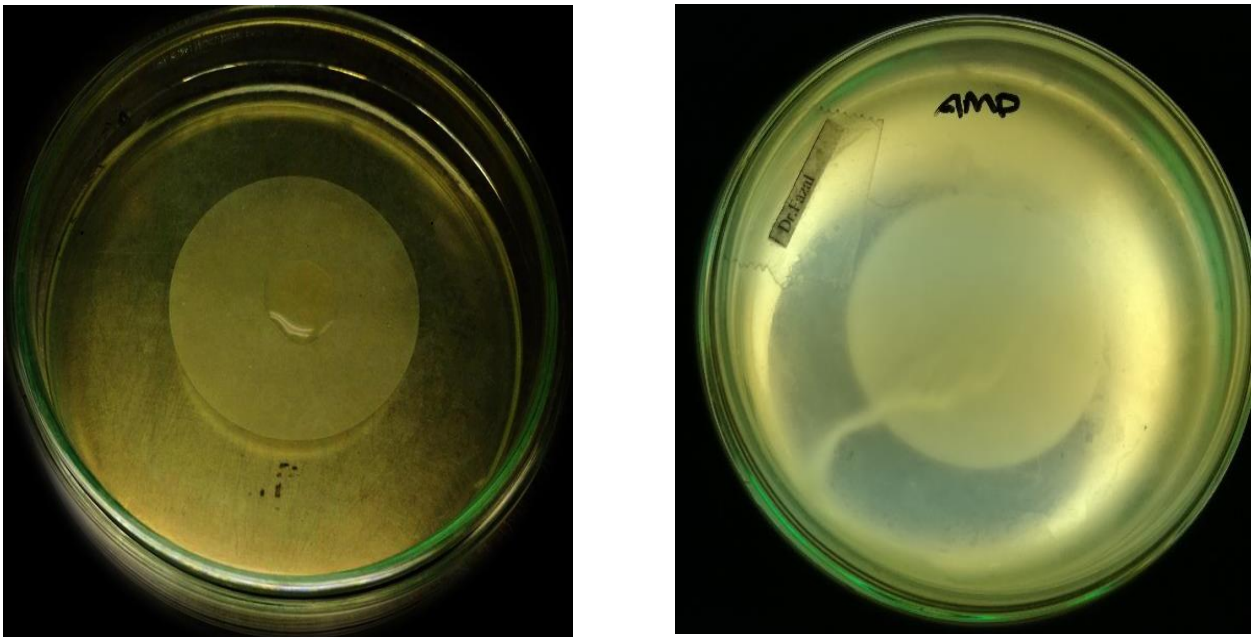

Figure 4.13: Conjugation mixture of *E. coli* S17-1 and *E. coli* O157:H7 on membrane filters placed on nutrient agar plate.

Conjugation mixture was then cultured on SMAC agar containing ampicillin. SMAC agar is a selective and differential medium for the selection of *E. coli* O157. Since *E. coli* O157 appears colorless while non-pathogenic *E. coli* strains appear pink as shown in Figure 4.14A. Transformed *E. coli* O157:H7 colonies were isolated from SMAC agar containing ampicillin shown in Figure 4.14 B and was named as ASR-O157.

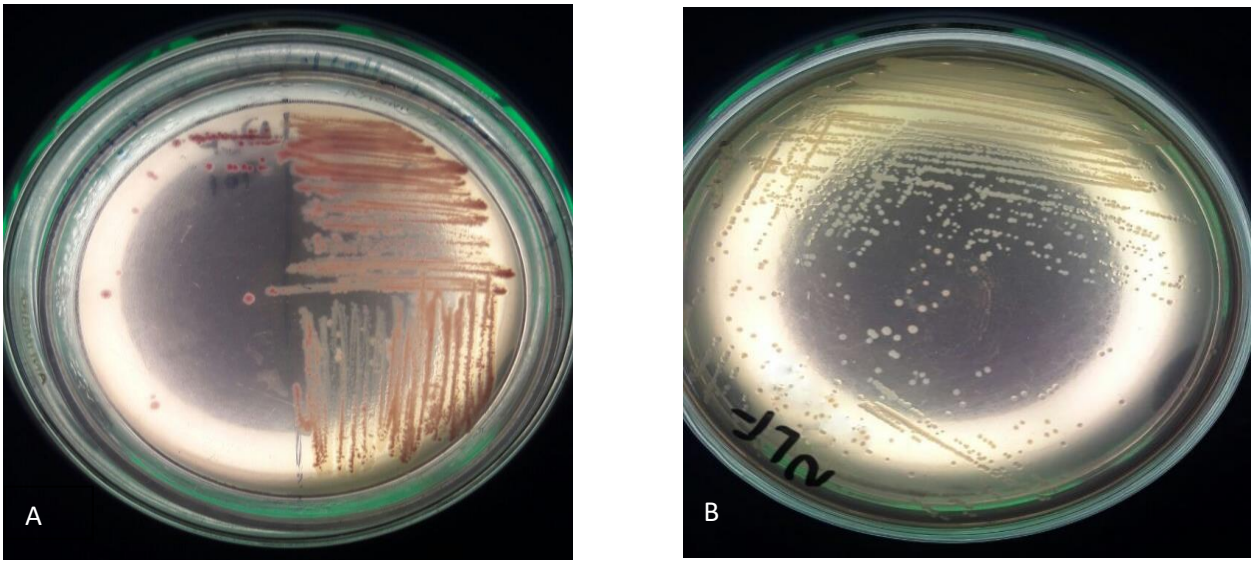

Figure 4.14: A: Growth of *E. coli* S17-1 cells on SMAC agar. **B:** Growth of anti shiga harboring *E. coli* O157 cells on SMAC agar. SMAC agar is the selective and differential medium for the detection of *E. coli* O157. Non-pathogenic *E. coli* appears pink due to fermentation of sorbitol in the medium. While *E. coli* O157 lack sorbitol fermentation and remain colorless.

4.9 Analysis of *stx-2* gene expression at mRNA level

In order to assess the repression of *stx-2* gene at mRNA level in target transformed strain of *E. coli* O157 (ASR-O157) as compared to wild type *E. coli* O157 (WT-O157), real Time RT-PCR was performed on Biorad Real time PCR system with fluorescent based SYBR green assay. To normalize the data 16S rRNA was used with help of 27F' and 1522R' primers as given in table 3.14. To compare the mRNA levels in above mentioned two strains Livak method ($2^{-\Delta\Delta CT)}$) was used. The mRNA levels were compared after 3 and 24 hours of incubation for both strains. The Real Time RT PCR shows 9.8 and 3.8 folds reduction in *stx-2* gene level in ASR-O157 strain as compared to WT-O157 strain at 3 and 24 hours respectively as shown in graphical Figure 4.15. In order to further confirm the reduction in *stx-2* gene at protein level MTT assay was performed.

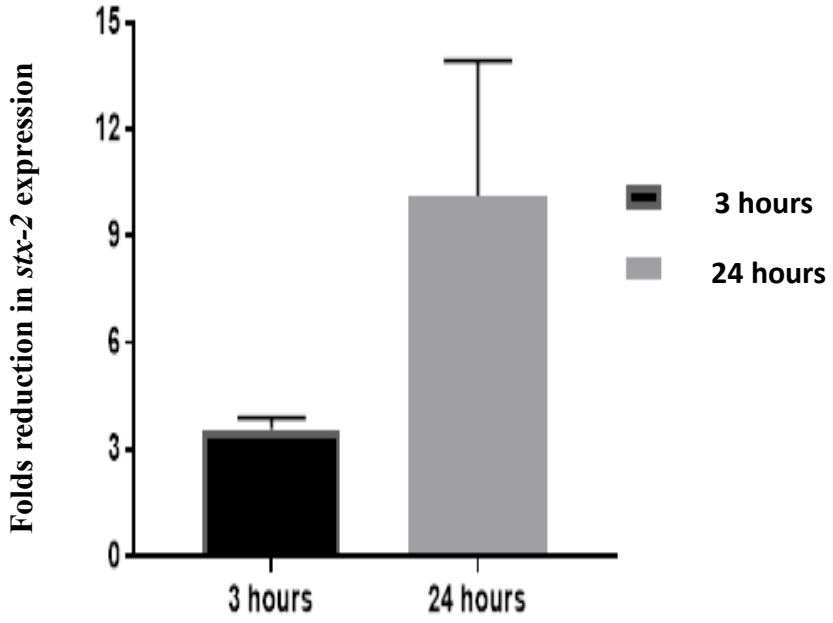

Figure 4.15*:* Graphical representation of real time RT-PCR for detection of reduction in *stx-2* gene expression at mRNA level. After 3 hours of incubation of liquid culture ASR-O157 strain showed 3.9 folds reduction in *stx-2* gene expression as compared to WT-O157. While after 24 hours of incubation 9.8 folds of reduction in *stx-2* gene was observed in ASR-O157 strain as compared to WT-O157.

4.10 Analysis of Shiga toxin 2 protein

To further confirm the change in expression of *stx-2* gene at protein level in *ASR-O157* strain, MTT cell proliferation assays was performed. MTT was converted to formazan by living cells and was detected by spectrophotometric quantification. Glioblastoma cell lines, U87 (ATCC: HTB-14) were used to determine the reduction at toxin level. Confluent monolayer of U87 cell lines cultivated in tissue culture flasks with Eagle's minimum essential medium (EMEM) supplemented with 10% (v/v) fetal bovine serum incubated at 37°C in a 5% CO_2 chamber. The cells were then transferred to 96 wells plate and treated with shiga toxins filtrate (10uL and 100uL) from ASR-O157 and WT-O157. In the next step, MTT was added to each well. A negative control of the MTT stock solution with medium alone was included in the plate as shown. The plate was incubated for 3 hours in the incubator. Then media from all wells was carefully removed without disturbing the cells. DMSO (MTT solvent) was added and left for 10 minutes. The wells containing ASR-O157 toxin appear darker as compared to WT-O157 toxin which shows that ASR-O157 toxin filtrate is less cytotoxic as compared to WT-O157 toxin. The assay plates were placed in the ELISA plate reader. The readings at 590 nm absorbance were recorded with a reference filter of 620 nm. The readings gave the percentage of viable cells. The graphical results for both cell lines as shown in the Figure 16 clearly indicate that ASR-O157 toxin filtrate has greater number of viable cells as compared to WT-O157 toxin. WT-0157 toxin has greater cytotoxic effect.

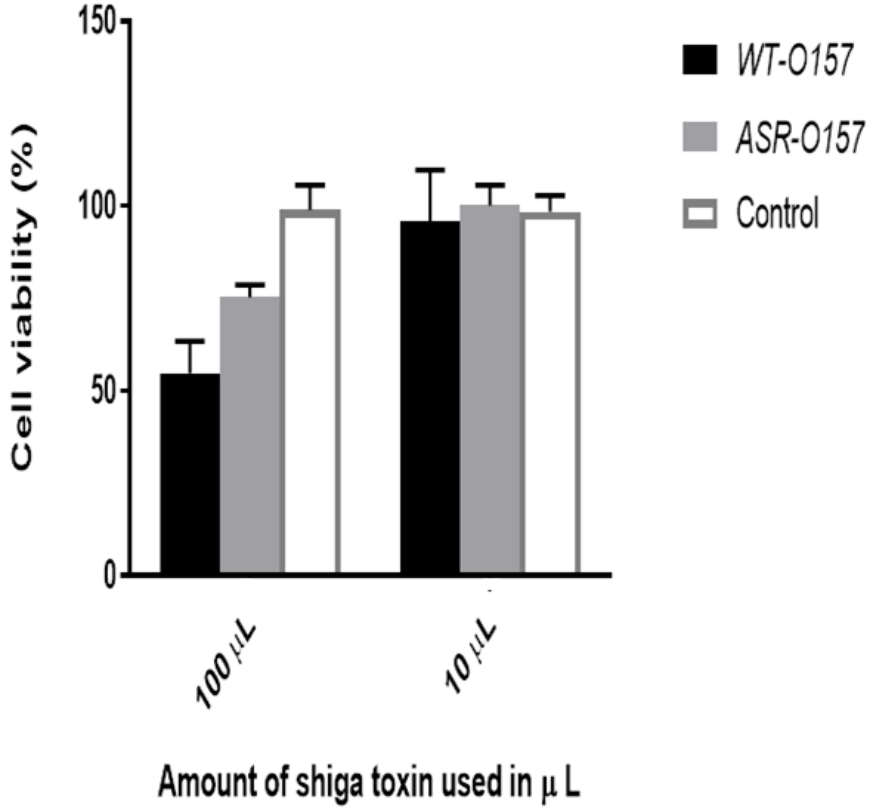

Figure 4.16: Graphical presentation of percentage cell viability and comparison between WT-O157 toxins and ASR-O157 toxins at different concentrations of 10µl and 100µl of total cell lysate for observing the knocking-down effect of *stx-2* gene. ASR-O157 toxin is less cytotoxic as compared to WT-O157 toxin.

4.11 Comparison of reduction in expression level between *stx-1* and *stx-2 at* the mRNA and protein levels

In previous study, our group has worked on silencing of *stx-1* gene via synthetic non-coding RNA. A comparison was carried out between the silencing effects caused by synthetic sRNAs designed against *stx-1* and *stx-2* genes. For silencing of *stx-2* gene a clear reduction was observed in mRNA level (Figure 4.15) and protein level (Figure 4.16). However, no significant fold change was detected at mRNA level in ASR-O157 strain compared to WT-O157 strain at 3 hour and 24 hours of incubation for synthetic sRNA designed against *stx-1* gene as illustrated in graphical Figure 4.17. Interestingly, a clear reduction was observed in shiga toxins levels for silencing of *stx-1* gene by MTT assay as indicated in Figure 4.18.

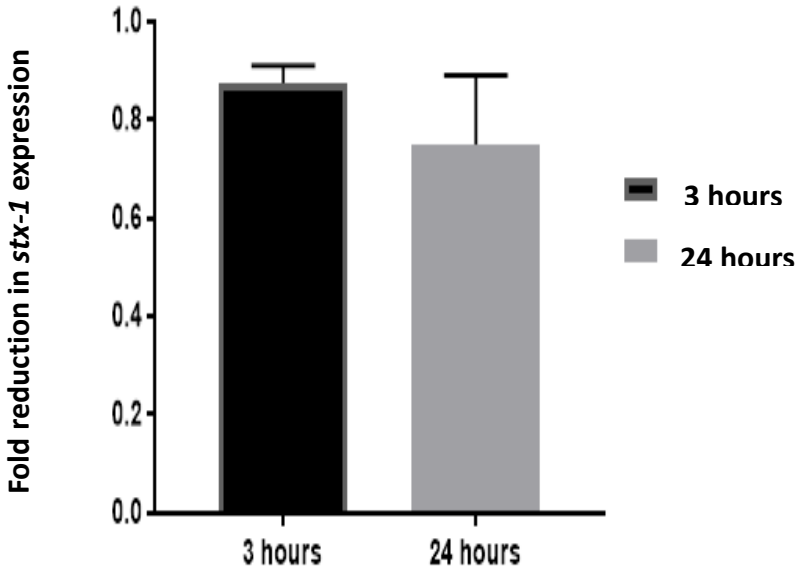

Figure 4.17: Effect of anti-shiga sRNA on *stx-1* gene expression from ASR-O157 strain at 3h and 24 h. The real time RT-PCR data shows no significant fold change in *stx-1* expression in ASR-O157 strain relative to WT-O157 strain. RNA expression was normalized to 16S rRNA housekeeping gene.

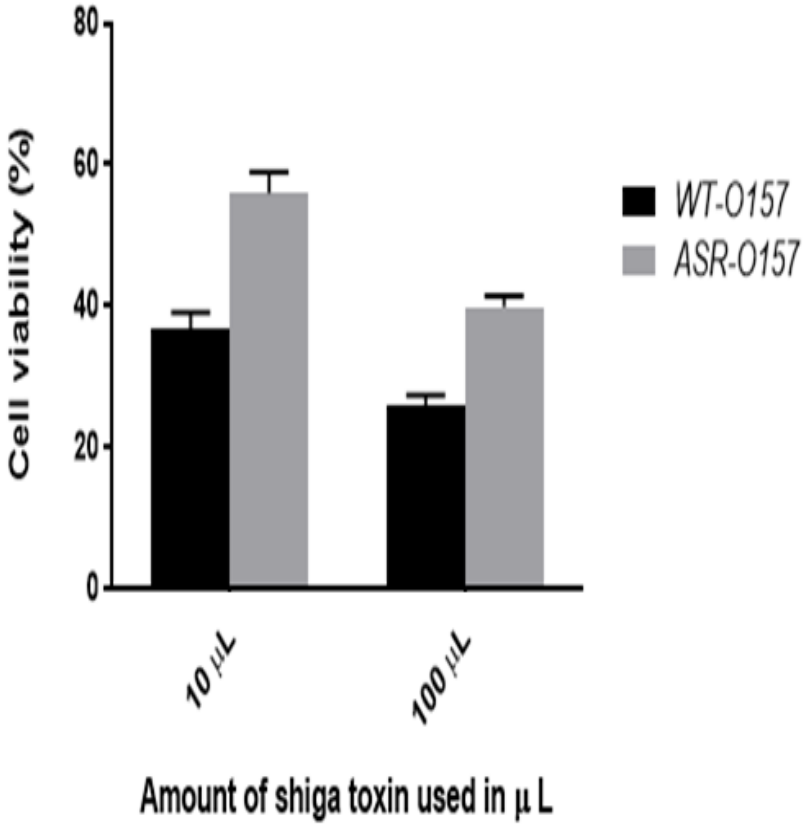

Figure 4.18: Graphical representation of percentage cell viability and comparison between WT-O157 toxins and ASR-O157 toxin at different concentrations of 10µl and 100µl of total cell lysate for observing the knocking-down effect of *stx-1* gene. ASR-O157toxin is less cytotoxic as compared to WT-O157 toxin.

5. Discussions

Various strains of Shiga toxin producing *E. coli* (STEC) have considered to be responsible for outbreaks of diarrhea, hemolytic-uremic syndrome and hemorrhagic colitis in various parts of the world. Main cause of infections through STEC is the production of shiga toxins which include shiga toxin 1 and shiga toxin 2 encoded by *stx-1* and *stx-2* genes respectively (Ibarra, 2011). *E. coli* O157:H7 serotype of STEC was selected because among various strains of STEC, *E. coli* O157:H7 is reported in a large numbers of Hemolytic uremic syndrome cases (Ibarra, 2011).

Use of antibiotics against *E. coli* O157:H7 infections is debatable because use of antibiotics not only induce antimicrobial resistance in this strain but it also increases the population of *E. coli* O157:H7. Therefore use of antibiotics against STEC infections is associated with higher amount of shiga toxins production making the situation worse (Sasaki *et* al., 2012; Schroeder *et* al., 2002). There is an immediate need to develop various strategies other than antimicrobial drugs in order to combat the global threats associated with antimicrobial resistance spreading across the world. Toxin producing bacteria have unique proteins coding genes for the production of toxins. Targeting such proteins at mRNA level can be a fruitful strategy to tackle antimicrobial resistance (Penchovsky & Traykovska, 2015).

Small regulatory RNAs (sRNAs) are non-coding RNAs of short sequence which are associated with regulation of their respective gene targets in *-trans* at the post transcriptional level in prokaryotes. RNA mediated gene regulation and their potential application in synthetic biology has been well documented (Yoo *et al*., 2013). Three distinct functional elements need to be present in synthetic sRNA: mRNA base pair region, Hfq binding site and terminator. Base pairing region of sRNA forms complementary hybrid with target mRNA to effect its regulation (Ishikawa, Otaka,

Maki, Morita, & Aiba, 2012). Synthetic sRNA offers Tunable base pair complementation and it can be used to exploit genome wide screening of effective target genes and flux (Lay *et al*., 2013; Maki, Morita, Otaka, & Aiba, 2010; Serganov & Nudler, 2013; Storz *et al*., 2011; Thomason & Storz, 2010). Synthetic non-coding RNA have been used to silence various genes in *E. coli* such as *ompF* and *fliC* (V. Sharma et al., 2012), *tyrR, csrA, pgi and ppc* to enhance tyrosine titer (Na *et al*., 2013), exogenous EGFP and endogenous *uidA* gene (Man *et al*., 2011).

Among shiga toxin producing genes selected *stx*-2 gene was selected because shiga toxin 2 is considered to be about 400 folds more virulent than shiga toxin 1 (Tesh *et al*., 1993). Shiga toxin 2 producing strains are more often associated with HUS than shiga toxin 1 producing strains and also they are associated with intra-uterine hematoma, fibrin deposition and feto-placental re-absorption (a Donohue-Rolfe, Kondova, Oswald, Hutto, & Tzipori, 2000).

Our designed synthetic sRNA contains a promoter, guide sequence, scaffold and terminator. We selected *MicC* sRNA scaffold because this scaffold shows higher repression capability (>90 %) as compared to other scaffolds. Scaffold structure recruits Hfq protein which is required for the formation and stabilization of sRNA-mRNA duplex (Na *et al*., 2013). Choice of guide sequence is the most important part because it will create short stretches of sRNA complementary to the specific region of target mRNA. sRNA against Translation Initiation Region (TIR) in mRNA is found to be most effective in sRNA mediated gene repression. Guide sequence was selected against TIR region of target mRNA. Twenty four nucleotides were selected as a guide sequence because sRNA of about 24 nucleotides is proven to have maximum repression capability (Na *et al*., 2013).

Promoter present in our sRNA cassette is lambda phage promoter because its site required for transcription initiation and it is well documented (Na *et al*., 2013). Promoter region controls the

production of synthetic sRNA. In order to efficiently terminate the synthesis of sRNA a strong terminator region was also added. Terminator sequence stops the transcription and helps in creating distinct sRNAs (Ishikawa *et al*., 2012).

The repression capability of anti-shiga sRNA against *stx-2* gene has determined using computational prediction tools, expression analysis and cell viability assay. The computational algorithms take into account the secondary structure of synthetic sRNA, secondary structure of target mRNAs, potential off-targets, binding free energies to engineer sRNA–mRNA interactions and regulation in bacteria (Rodrigo, Landrain, & Jaramillo, 2012)

For efficient repression of target mRNA synthetic sRNA should possess a binding energy between -15 to -40 Kcal mol^{-1}. Our synthetic sRNA has binding energy of -29.45 Kcal mol^{-1} which is in the range of optimal energy required for repression of gene at mRNA (Na *et al*., 2013).

Secondary structure of sRNA was determined by using RNAfold software. Secondary structure of sRNA is important in RNA-mediated silencing because folded structure recruits the Hfq protein (Na *et al*., 2013). Artificial sRNAs forming internal loops are proven to be more effective (Park, Bak, Kim, & Lee, 2013). Synthetic sRNA need to form 2 to 4 stem loops for efficient repression including one by mRNA base pair region while other by terminator sequence (Man *et al*., 2011). Our predicted sRNA has 3 stem loops: one is formed sRNA binding region, second is formed in terminator region and third loop is present in scaffold region (Man *et al*., 2011).

The designed anti-shiga sRNA was then constructed in pAB.001 plasmid according to the protocol described by Bernheim *et al*., (2016) and Yoo *et al.,* (2013). Plasmid pAB.001 that contains sRNA was transformed by heat shock method into *E. coli* S17-1 and then this strain was used to transform *E. coli* O157:H7. Transformed *E. coli* O157 was separated from donor S17-1 strain by using

sorbitol MacConkey agar because *E. coli* O157 appears colorless as they do not ferment sorbitol (De Boer & Heuvelink, 2000).

In order to analyze repression of *stx-2* gene caused by our synthetic sRNA, real time RT-PCR was performed by comparing total RNA of wild type *E. coli O157* and *ASR-O157* (Yoo *et al*., 2013). Target messenger RNA quantity decreases with production of synthetic sRNA because synthetic sRNA causes destabilization of mRNA (Man *et al*., 2011). The reduction in mRNA level was observed because sRNA can cause decay of target transcript (Good & Stach, 2011; Massé, Escorcia, et al., 2003). Binding of sRNA with mRNA depends on the production rate of both RNAs (Shimoni *et al*., 2007) therefore it was concluded that the production rate of sRNA is higher than the *stx-2* mRNA and it also gradually increases with time.

To further confirm the reduction in shiga toxin amount at protein level, MTT cytotoxitcity assay was performed on U87 cell lines after application of total cell lysate both from the control and the modified bacterial culture. U87 cell lines were chosen because shiga toxin 2 is frequently associated with neurological lesions and central nervous system disorders (a Donohue-Rolfe et al., 2000; Francis, Moxley, & Andraos, 1989). Our results clearly caused a reduction in cytotoxicity levels of ASR-O157 strain as compared to wild type strain.

An interesting behavior was observed for silencing of *stx-1* gene by synthetic sRNA using similar method in which silencing at protein level was observed however no change was detected at mRNA level by real time RT-PCR. Possible justification for this behavior might be that for detection in reduction caused by synthetic sRNA at mRNA depends on the production rate of both synthetic sRNA and target mRNA (Shimoni *et al*., 2007). It was concluded that production rate of synthetic sRNA designed against *stx-1* gene was lower than the production rate of respective target

mRNA. Whereas, production rate of synthetic sRNA designed against *stx-2* gene was higher than the production rate of target *stx-2* mRNA.

The reduction in the shiga toxin level can be a first line of defense in preventing HUS in patients infected with EHEC infections (Mohsin, Guenther, Schierack, Tedin, & Wieler, 2015). Shiga toxin is an important virulent factor of EHEC infections and strategies aimed at inhibiting shiga toxin expression are expected to reduce HUS incidence. This is especially significant considering the controversial behavior of antibiotic treatment to eradicate EHEC infections and is often contraindicated. On the basis of these findings, we suggest that synthetic sRNA could be considered a potential therapeutic candidate for the amelioration of EHEC diseases, in particular caused by strains of serotype O157:H7. Synthetic sRNA can be used to silence pathogenic/virulent genes in pathogenic bacteria. Moreover, the synthetic sRNA can be easily transferred using plasmid based transformation method making them highly portable and also it does not effects the information coded on the chromosome therefore the risk of disrupting the expression of other genes is avoided, thus they can be transferred to any bacterial strain (Yoo *et al*., 2013). However, further studies are required to elucidate the extent of gene repression by synthetic sRNA. To be used as alternative antibiotic approach, synthetic sRNA must possess 100 percent repression capability and it must be delivered to all bacterial cells to produce the desired results.

6. Conclusion

In this study we practically designed a strategy aimed for production of synthetic non-coding sRNA that can repress the expression of *stx-2* gene at post transcription level. Our designed sRNA disrupts the target mRNA by complementary binding and interferes with its stability. The strategy was inspired by the modular architectures of natural sRNA that control gene expression in prokaryotes. The key element in designing of sRNA was selection of highly specific guide sequence that will produce sRNA complementary to of *stx-2* gene. Along with guide sequence, a scaffold sequence for the requirement of Hfq protein and a terminator sequence for synthesis of correct sRNA is also necessary. Stability and secondary structures of synthetic sRNA are directly related to the rate of repression of target gene via sRNA. The predicted binding energy of anti-shiga sRNA using IntaRNA bioinformatics tool was found to be optimal for efficient repression with maximum complementarity to target mRNA. The secondary structure prediction of anti-shiga sRNA using RNAfold software depicted three stem loop structure confirming its stability in vivo and efficient repression capability. The modification of pAB.001 plasmid with desired guide sequence was acomplished using PCR based site directed mutagenesis. *E. coli* S17-1 were transformed modified plasmid and used as donor strain to transfer pAB.001 to *E. coli* O157 strain through diparental conjugation. The effect of anti-shiga sRNA on *stx-2* gene expression was determined by quantitative real time RT-PCR analysis. The results illustrated a significant fold change in *stx-2* expression because mRNA was being degraded after forming duplex with sRNA. In order to further confirm the repression in shiga toxins level MTT cell proliferation assay was performed on brain cell lines U87. MTT cell proliferation assay clearly shown the reduction in toxins level produced by ASR-O157 strain as compared to wild type O157. This effect is due to

the reduction of shiga toxins levels obtained by targeting mRNA of *stx-2* gene. Therefore, we can say that synthetic sRNA mediated silencing of bacterial genes is one of the potential tools in combating multidrug resistant bacteria. In future with our proposed synthetic sRNA cassette one can target any toxic gene in any bacteria by simply changing the guide sequence present in our sRNA cassette. Major hurdle in development of RNA-based therapeutics is the mode of delivery therefore using *E. coli* S17-1 for transformation of STEC can be the 1st step towards development of sRNA based therapeutics for STEC.

Our designed sRNA mediated silencing enables a dynamic and tunable silencing of bacterial genes that have many potential applications in medical microbiology and tailor-made medicines to treat life threatening bacterial infections.

7. Future prospects

In this study we designed a strategy to knock-down the shiga toxin 1 *(stx-1)* and shiga toxin 2 gene (*stx-2*) in *E. coli* O157:H7.

E. coli O157:H7 also has gene which can encode Shiga toxin 1 protein. In order to use this strategy effectively and to completely knock-down the shiga toxin genes, silencing of *stx-2* and *stx-1* will be carried out, at the same time, with two different sRNA expression cassettes.

Our research group is also working on phage mediated delivery of synthetic non-coding RNA in the target bacteria as the delivery of synthetic sRNA is the major hurdle in successful implementation of this anti-sense based strategies. This strategy will also be tested on mouse models via suitable delivery vehicles.

8. References

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