THE MOLECULAR BASIS OF SSRA DELETION PHENOTYPE IN DIFFERENT BACTERIA

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ABSTRACT

ssrA, a gene that encodes tmRNA is found in all bacteria that have been examined. tmRNA is a unique small RNA that has properties of both tRNA and mRNA. Using its unusual structure, tmRNA plays an important role in ribosome rescue and protein quality control through a trans-translation process. In this remarkable process, tmRNA first recognizes stalled translation complex by an unknown mechanism and enters the A site of a stalled ribosome like a tRNA. After transpeptidation reaction, the nascent polypeptide chain is transferred to the alanyl-tmRNA. tmRNA then moves from the A site to the P site in the ribosome and the translation template switches from the former mRNA to the mRNA portion within tmRNA. Normal translation continues till the ribosome reaches the stop codon within tmRNA. Release factors are recruited and the ribosomes, which were originally stalled on the mRNA, get released from the tmRNA and recycled. A peptide tag encoded by tmRNA is added to the polypeptide. This peptide tag is recognized by a number of intracellular proteases and therefore targets the tagged protein for rapid degradation.

The deletion of ssrA causes different phenotypes in different bacterial species. In Caulobacter crescentus, ssrA deletion causes a specific delay in DNA replication initiation as well as a defect in maintaining pBBR1 family plasmids. In Escherichia coli, ssrA deletion results in higher sensitivity to heat shock and lower motility on semi-solid agar. In Shigella flexneri, a closely related species of E. coli, ssrA deletion is lethal. This study has identified three genes in C. crescentus that inactivation of any of these genes in ΔssrA strain can bypass the plasmid maintenance defect. This study has also demonstrated that not only is ssrA essential in S. flexneri, some DNA element(s) can even bypass this phenotype in S. flexneri. Understanding the molecular basis of the ssrA deletion phenotypes can shed light on elucidating the physiological role of tmRNA in different species and understanding how
tmRNA is integrated into bacterial genetic network. Since tmRNA is important for bacterial pathogenesis, this study may also reveal targets for new antibiotic development.
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CHAPTER 1

TMRNA IN EUBACTERIA
Introduction

Sensing and adaptation to the changing environment is crucial for the survival of bacteria. Although sensory and regulatory proteins have been found to be important in this process, more and more evidence suggests that the contribution from small RNAs is not neglectable. Small RNAs have been found to affect gene expression through three mechanisms. First, small RNAs can complementarily pair with target mRNAs to affect their stability and translation (Gottesman, 2002). Second, small RNAs can also bind to proteins factors to affect the transcription or translation levels of their regulons. (Gottesman, 2002). Third, tmRNA, a unique small RNA molecule encoded by ssrA, can affect gene expression through trans-translation (Keiler et al., 1996). tmRNA was first discovered in E. coli in 1978 (Lee et al., 1978). Then it was subsequently found in all bacteria species that has been examined. It is highly expressed and conserved in bacteria (Keiler et al., 2000), suggesting that tmRNA plays an important role in bacterial physiology.

tmRNA structure

As its name suggests, tmRNA has properties of both tRNA and mRNA. It possesses a very unique structure with a tRNA-like domain and an mRNA-like domain (Fig.1). The 5’ end and 3’ end of mature tmRNA can partially base pair with each other and fold into a tRNA like structure with a D-arm, a T-arm and an acceptor arm. The acceptor arm contains a G:U wobble in the third base pair position just as in alanyl-tRNA (Ushida et al., 1994). In vivo and in vitro assays showed that tmRNA can be charged with alanine by alanyl-tRNA synthetase (Komine et al., 1994). The mRNA-like domain contains an open reading frame (ORF), which can be translated into a short peptide with 8 to 35 amino acids, depending on the species of the bacterium. There is some variation in the sequence of this short peptide among species but Ala and Asp are the most commonly used residues (Moore et al., 2007). There are also
three to four pseudoknots in the tmRNA molecule, depending on the species (Felden et al., 1997). The sequences that constitute the pseudoknots are not very conserved among species (Moore et al., 2007) but this structure seems to be important in tmRNA folding, stability and associate protein(s) binding (Wower, 2004).

Fig. 1 Proposed secondary structure of *E. coli* tmRNA. The 5’ and 3’ ends of the RNA are folded into a tRNA-like structure and can be charged with alanine by alanyl-tRNA synthetase. The other half of the RNA contains an open reading frame that encodes a special peptide that leads tagged proteins to proteolysis (Dulebohn et al., 2007).

The *trans*-translation model

Normal translation process in bacteria starts with the binding of ribosomes to mRNA molecule. The ribosome moves along the mRNA till it encounters a stop codon. The ribosome then dissociates from the mRNA with the help from release factors and the finished peptide is released. However, in bacteria, transcription and translation are coupled. Therefore
even if an mRNA is truncated due to premature transcription termination, partial degradation, or physical/chemical damage (Roche et al., 1999), ribosomes will still bind to the mRNA and start translation. The consequence is that ribosomes frequently reach the end of an mRNA without terminating at a stop codon and cannot be released. It is estimated that this event occurs about 13000 times per cell per generation (Moore et al., 2005) so it is obvious that there must be some mechanisms through which cells can recycle these stalled ribosomes and maintain a pool of active ribosomes for new translation.

tmRNA solves this problem by tagging the incomplete protein for rapid degradation and releasing the stalled ribosomes from the mRNA. In this remarkable mechanism, tmRNA first recognizes the stalled translation complex and enters the A site of a stalled ribosome like a tRNA. Transpeptidation reaction occurs and the nascent polypeptide chain is transferred to alanyl-tmRNA. tmRNA then moves with the nascent peptide chain at the 3’ end of the acceptor arm from the A site to the P site in the ribosome. The translation template switches from the former mRNA to the mRNA portion within tmRNA. The mRNA is released from the complex and degraded. Normal translation continues and terminates at the stop codon within tmRNA. The stalled ribosomes get released and recycled later. A peptide tag encoded by tmRNA is added to the polypeptides. This peptide tag is recognized by a number of intracellular proteases and targets the tagged protein for rapid proteolysis (Gottesman et al., 1998; Keiler et al 1996; Herman et al., 1998) (Fig.2).
Fig. 2: Current model suggested for protein degradation through tmRNA pathway. tmRNA charged with alanine enters the ribosomal A site acting like a tRNA. The nascent protein is transferred to alanyl-tmRNA by a normal transpeptidation reaction. Then the translational reading frame switches from the original mRNA to a reading frame within the tmRNA. Translation resumes using the reading frame in tmRNA and the peptide tag is added to the nascent polypeptide. The tmRNA-encoded peptide tag targets the incomplete protein for rapid degradation. (Keiler, 2007)

Data from many experiments have been found to be in accordance with this trans-translation model. For example, the N-terminal domain of namda repressor generated from an mRNA without in frame stop codon was found to be tagged at the C terminal by tmRNA and was rapidly degraded (Keiler et al., 1996). tmRNA can only associate with 70s ribosomes but not 30s or 50s subunits (Ushida et al., 1994; Komine et al., 1996). Also a cryoelectron microscopy structure has shown the association of tmRNA and ribosome (Valle et al., 2003).

The correct tag sequence is critical and sufficient for the recognition of the intracellular proteases. For example, GFP with the tag sequence in E. coli can be rapidly degraded both in
vivo and in vitro (Cheng et al., 2007). It has also been reported that after the last two residues in the tmRNA ORF were changed from alanine to aspartate, the variant tmRNAs can still recognize stalled the ribosomes and tag their substrates. However, the half-life of most of the tagged proteins is noticeably longer (Keiler et al., 1996).

To sum up, there are three outcomes after the trans-translation process. 1) The stalled ribosomes are rescued. 2) The truncated mRNA is degraded. 3) The incomplete protein is degraded. These three outcomes are important for the well-being of cells. It is estimated that the ribosome stalling events occurs about 13000 times per cell per generation (Moore et al., 2005). So if there is no ribosome recycling mechanism, all ribosomes would be stalled on mRNAs in less than one cell cycle and the cell will die (Keiler, 2007). Also, the polypeptides synthesized from truncated mRNAs are incomplete. They may have some malfunctions because of misfolding, or they may clog the chaperone and proteolysis pathways and generating detrimental activities (Keiler, 2007). Last but not least, the truncated mRNAs will keep trapping and stalling ribosomes if they are not degraded. Since tmRNA pathway solves these three fundamental problems, it should play a critical role in bacterial physiology.

**Physiological roles of tmRNA in eubacteria**

**tmRNA is important for bacterial pathogenesis**

It is found that tmRNA is important for bacterial pathogenesis. For example, in *Yersinia pseudotuberculosis*, without tmRNA the bacterium is no longer able to cause lethal phenotype in mice (Okan et al., 2006). In *Salmonella typhimurium*, deletion of *ssrA* results in lower virulence in mice (Julio, 2000). In *Neisseria gonorrhoeae*, *ssrA* is an essential gene (Huang et al., 2000). Data in this study showed that *ssrA* is also essential in *Shigella flexneri*. All of these evidences suggest that tmRNA plays an important role in bacterial pathogenesis.
tmRNA is important for bacterial stress tolerance

It is found that tmRNA is important for bacterial stress tolerance. For example, *ssrA* deletion in *E. coli* is not lethal. However, Δ*ssrA E. coli* strain exhibits higher sensitivity to heat shock (Kirby et al., 1994) and antibiotic treatment (Abo et al., 2002). It also displays lower motility in semi-solid agar (Kirby et al., 1994) and slower recovery from carbon starvation (Oh et al., 1991). Δ*ssrA B. Subtilis* also shows higher sensitivity to high temperature (over 40°C) and high concentrations of ethanol or cadmium chloride (Muto et al., 2000).

tmRNA is important for bacterial development

It is also found that tmRNA is important for bacterial development. For example, in *C. crescentus*, the deletion of *ssrA* causes a specific delay in chromosomal DNA replication initiation (Keiler et al., 2003). In *Bradyrhizobium japonicum*, the deletion of *ssrA* results in slower growth under free living conditions and a severe defect in colonizing the root nodules (Ebeling et al., 1991).

What can induce tmRNA tagging?

Figuring out what can induce tmRNA tagging is crucial for understanding its biological function in bacteria. Although initially only mRNAs without stop codons were found to be tagged by tmRNA (Keiler et al., 1996), recent studies have shown that the tmRNA pathway is far more complicated than what were originally expected.

Truncated mRNAs without stop codon

Several pathways in bacterial cells can result in truncated mRNAs without stop codon, including premature transcription termination, partial degradation, and physical/chemical
damage (Roche et al., 1999). Transcript and translation in prokaryotes are coupled so even the 3’ end of an mRNA is truncated, ribosomes will still bind to the 5’ of the mRNA and start translation. When the ribosomes reach the end of the mRNA without stop codon, they cannot get dissociated and released because release factors cannot be recruited (Keiler et al., 1996). tmRNA pathway can release the truncated mRNA, dissociate the subunits of the ribosomes and tag the incomplete peptide for degradation.

**Intact mRNAs with rare codons or inefficient stop codons**

Under stress conditions such as amino acid starvation, rare codons may trigger tmRNA tagging activity, especially when there are more than one consecutive rare codons. The ribosomes may stall at the rare codons and the mRNA will be cleaved within or beside the ribosome A site (Li et al., 2006). This process generates an mRNA molecule without a stop codon, which can trigger tmRNA tagging. Over expression of the cognate tRNA to the rare codon reduces the level of tmRNA tagging while depletion of the cognate tRNA increases the level of tagging (Roche et al., 1999). Changing the rare codon to the more preferred codon in the species can also reduce the level of tagging (Hayes et al., 2002). It has also been found that translational readthrough, which is caused by inefficient opal stop codon (UGA) can lead to tmRNA tagging too (Sunohara et al., 2002). Similarly, changing the inefficient opal stop codon to a more efficient ochre stop codon can reduce the level of tmRNA tagging (Hayes et al., 2002).

**DNA motif**

Most recent study in our lab has shown that among all the tmRNA substrates in *Caulobacter crescentus*, about 66% of them contain a conserved DNA motif “CGACAAGATCGTCG” (Hong et al., 2007). When the motif is mutated, the level of
tmRNA tagging decreases. However, the mechanism why this DNA motif can lead to tmRNA tagging is still unknown.

The C terminal sequence of some proteins

The C terminal sequences of some proteins are also critical in tmRNA tagging determination. For example, YbeL, an *E. coli* protein, can be tagged by tmRNA in full length. It has been found that the C-terminal residue, proline, of YbeL is sufficient to induce tmRNA tagging. It has also been found that the penultimate amino acid can positively affect the level of tagging if they are Asp, Glu, Ile, Pro or Val (Hayes et al., 2002).

**tmRNA associated protein factors**

tmRNA does not act alone. There are several protein factors that are important for the function and stability of tmRNA.

**SmpB**

SmpB is required for tmRNA function. It binds to tmRNA specifically and mediates the interaction between tmRNA and the ribosome (Metzinger et al., 2005, Karzai et al., 1999, Hallier et al., 2004). In *C. crescentus*, SmpB is also required for tmRNA stability (Hong et al., 2005). Deletion of the *smpb* gene results in the same phenotype as deletion of *ssrA* gene (Karzai et al., 1999, Julio et al., 2000, Keiler et al 2003).

**Alanine tRNA synthetase and EF-Tu**

Since tmRNA has properties of an Ala-tRNA, it is not surprising that Alanine tRNA synthetase and EF-Tu, the two translational factors are also required for tmRNA function. Alanine tRNA synthetase can charge the 3’ end of tmRNA with Alanine (Komine, Y., et al.,
1994). EF-Tu protects the ester bond of Alanine charged tmRNA and delivers the tmRNA to ribosomes (Barends, 2000; Rudinger-Thirion et al., 1999, Valle et al., 2003).

**RNase R**

RNase R, encoded by *rnr*, is a highly conserved exoribonuclease (Zuo et al., 2001). In *E. coli*, it is important for non-stop mRNAs degradation (Karzai et al., 2001). In *C. crescentus*, it degrades tmRNA in the absence of SmpB (Hong et al., 2005). In *S. flexneri*, it is required for the expression of virulence genes, which may have some relationship with tmRNA activity (Tobe et al., 1992).
References


CHAPTER 2

SSRA IS ESSENTIAL IN *SHIGELLA FLEXNERI* BUT NOT IN THE CLOSERLY RELATED SPECIES *ESCHRICHA COLI*
Abstract

*Shigella flexneri* and *Escherichia coli* are taxonomically indistinguishable. The sequence of the open reading frame (ORF) of *ssrA*, a universally conserved gene among bacteria, is exactly the same in these two species. Though *ssrA* deletion only results in mild phenotypes in *E. coli*, our data demonstrated that *ssrA* deletion in *S. flexneri* is lethal. It has also been found that after conjugating the *S. flexneri* 2457T with an *E. coli* Hfr strain, *S. flexneri* colonies that do not require *ssrA* were identified, suggesting that some DNA element(s) can bypass the *ssrA*-essential phenotype in *S. flexneri*. Since *ssrA* is important in *S. flexneri* and many other pathogenic species, it can be a potential target for new antibiotic development.
**Introduction**

*srrA* is found in all bacteria that have been examined (Keiler et al., 2000). Its product, transfer messenger RNA (tmRNA), is a small RNA with a very unusual structure. As its name suggests, tmRNA has properties of both tRNA and mRNA. The 5’ and 3’ ends of the RNA are folded into a tRNA-like structure, which can be charged with alanine by alanyl-tRNA synthetase (Komine et al., 1994; Ushida et al., 1994). The other half of the RNA contains an open reading frame, which can be translated into a short peptide like a normal mRNA (Keiler et al., 1996).

The functions of tmRNA identified so far are ribosome rescue and protein quality control (Keiler et al., 2007). In bacteria, unlike in eukaryotes, transcription and translation are coupled. Therefore even if an mRNA is truncated due to premature transcription termination, partial degradation, or physical/chemical damage (Roche et al., 1999), ribosomes still bind to the mRNA and start translation. The consequence is that ribosomes frequently reach the end of an mRNA without terminating at a stop codon and cannot be released. This is a serious problem because if there is no ribosome recycling, all ribosomes will be stalled on mRNAs in less than one cell cycle and the cell will die (Keiler, 2007). In addition, the polypeptides synthesized from the incomplete translation might be toxic to the cell. tmRNA solves this problem by tagging the incomplete protein for rapid degradation and releasing the stalled ribosomes from the mRNA. tmRNA first recognizes the stalled translation complex through an unknown mechanism. Then it enters the A site of a stalled ribosome like a tRNA. Transpeptidation reaction occurs and the nascent polypeptide chain is transferred to alanyl-tmRNA. tmRNA then moves with the nascent peptide chain at the 3’ end of the acceptor arm from the A site to the P site in the ribosome. The translation template switches from the former mRNA to the mRNA portion within tmRNA. Normal translation continues and terminates at the stop codon within tmRNA. Therefore stalled ribosomes get released and
a peptide tag encoded by tmRNA is added to the polypeptides. This peptide tag will be recognized by a number of intracellular proteases and target the tagged protein for rapid proteolysis (Gottesman et al., 1998; Keiler et al. 1996; Herman et al., 1998).

The sequence of the peptide tag is important for the recognition by proteases. Mutations in this tag sequence can abolish the proteolysis of some tagged proteins. For example, if the last two residues of the tag peptide in tmRNA are changed from alanines (SsrA-AA) in wild type to aspartates (SsrA-DD), the variant tmRNA can still tag its substrates, but tagged proteins cannot be recognized and proteolysed rapidly by proteases, so the half-life of most SsrA-DD tagged proteins are be much longer (Keiler et al., 1996; Keiler et al., 2003, Fujihara et al 2002; Keiler, 2007).

If tmRNA only functions in protein quality control and ribosome rescue, then most of the proteins in bacteria should be substrates of tmRNA, and they should be tagged at random locations. However, during the identification of tmRNA substrates, it is found that not all proteins are tagged at random sites with even frequency. Some proteins have higher frequency to be tagged and prefer specific tagging sites (Wiegert et al., 2001). All these results indicate that tmRNA has substrate specificity. In addition, sometimes tmRNA tags a protein at its C-terminus instead of the truncated site (Roche et al., 2001). This suggests that tmRNA may have some function in regulating gene expression.

Deletion of the tmRNA coding gene ssrA generates different phenotypes in different bacterial species. In Caulobacter crescentus, deletion of ssrA causes a specific delay in DNA replication initiation and defect in maintaining pBBR1 plasmids (Keiler et al., 2003). In E. coli, deletion of ssrA causes higher sensitivity to heat shock and lower motility on semisolid agar (Ranquet et al., 2007). In some pathogenic strains such as Neisseria gonorrhoeae, deletion of ssrA is lethal (Huang et al., 2000). The variation in the phenotype of ssrA deletion in different species further suggests that tmRNA may play a regulatory role in bacterial
physiology in addition to protein quality control and ribosome rescue. Also, since tmRNA is important in pathogenic strains, it can be a potential target for new antibiotic development.

As previously mentioned, *ssrA* deletion in *E. coli* results in mild phenotypes. On the other hand, preliminary research in our lab suggests that *ssrA* deletion in *S. flexneri* may be lethal. *S. flexneri* is an important human pathogen and responsible for the majority of endemic bacillary dysentery cases prevalent in developing countries. It is so closely related to *E. coli* that the two species are taxonomically indistinguishable (Wei et al., 2003). Therefore it is surprising to find such a marked difference in *ssrA* deletion phenotypes between the two closely related species. The goal of this project is to confirm the lethal phenotype of *ssrA* deletion in *S. flexneri* and identify the gene(s) that is responsible for the difference in *ssrA* deletion phenotypes between the two species.
Results

In *S. flexneri* 2a 2457T, the chromosomal copy of *ssrA* cannot be deleted unless a plasmid copy of *ssrA* is present.

Previous research in our lab showed that no colonies could be obtained when knocking out *ssrA* in *S. flexneri* 2a 2457T, suggesting that *ssrA* is essential in this species. To confirm this result, the Wanner method (Kirill et al., 2000) was performed to knock out the chromosomal copy of *ssrA* in *S. flexneri* 2a 2457T with and without a plasmid copy *ssrA*. A covering plasmid which expresses wild type tmRNA was first generated and transformed into *S. flexneri* 2a 2457T strain. A helper plasmid was transformed into both wild type *S. flexneri* 2a 2457T and *S. flexneri* 2a 2457T with the covering plasmid. The helper plasmid produces Red recombinase to promote homologous recombination. A linear DNA fragment which consists of a kanamycin resistance gene with 40 base pairs *S. flexneri ssrA* flanking regions on both ends was generated by PCR. This linear PCR fragment was transformed directly into the *S. flexneri* strain with only the helper plasmid and the *S. flexneri* with both the helper plasmid and the covering plasmid. Cells in which *ssrA* was replaced by the kanamycin resistance gene were selected on LB plates containing kanamycin. In principle, using this method *ssrA* can be deleted in both strains. But in practice, results from colony PCR showed that *ssrA::kan* colonies can only be obtained from *S. flexneri* with the covering plasmid (Fig.3). The knockout was further confirmed by southern blot (Fig.4). This result suggests that *ssrA* is essential in *S. flexneri* 2a 2457T.

Co-transduction experiment also showed *ssrA* is essential in *S. flexneri* 2a 2457T.

To further confirm that *ssrA* is essential in *S. flexneri*, a co-transduction experiment was also performed. If *ssrA* is truly essential in *S. flexneri*, the *ssrA::kan* marker cannot be co-transduced into wild type *S. flexneri* with an adjacent marker unless a covering plasmid is
present. To perform this experiment, a tetracycline resistance gene was inserted just upstream of the ssrA locus in the ssrA::kan S. flexneri strain by phage transduction (Singer et al., 1989). The locus of the tet marker is 58.97 min. and the locus of the ssrA::kan marker is 59.35 min. on the chromosome. The distance between the two markers is d=59.35-58.97=0.38min. Since a phage particle can package as much as 2.1 min of DNA, the two markers are close enough to be co-transduced. According to the equation of co-transduction frequency:

$$F = [1-(d/2.1)]^3 = [1-(0.38/2.1)]^3 = 54.9\%.$$  

This number suggests that when P1 phage is prepared from this strain, if one marker (e.g. the TetR marker) is packaged into a phage particle, there is 54.9% chance that the other marker (e.g. the KanR marker) is also packaged into the same particle. The phage was used to infect both S. flexneri strains with and without the covering plasmid. Tetracycline resistance colonies were first selected on LB plates with tetracycline and the percentage of these TetR colonies that are also KanR was determined by patching the tetR colonies onto LB plates with kanamycin. It was found that when transducing into S. flexneri with the covering plasmid, 59% of the tetR colonies were also kanR; when transducing into S. flexneri without the covering plasmid, none of the tetR colonies were also kanR. This result showed that the ssrA::kan marker can not be co-transduced with the tet marker unless a covering plasmid is present, which further confirms that ssrA is essential in S. flexneri 2a 2457T.

**S. flexneri 2a 2457T colonies that do not require ssrA were identified after conjugation with an E. coli Hfr strain**

As previously mentioned, S. flexneri and E. coli are closely related. But unlike in S. flexneri, ssrA is not essential in E. coli. In order to find what is responsible for this difference in phenotypes, S. flexneri cells were conjugated with a set of seven E. coli Hfr strains that cover the whole E. coli genome (Fig. 5). Following the conjugation, cells that do not require
tmRNA were selected by knocking out *ssrA* in all the transconjugants using the dual-marker (Tet<sup>R</sup>, *ssrA::Kan*) phage transduction. The dual-marker phage instead of the single marker *ssrA::kan* phage was used because spontaneous Kan<sup>R</sup> mutations were prone to occur when kanamycin was the only antibiotic. The addition of the tetracycline can eliminate the spontaneous mutants.

In the experiment, after conjugating *S. flexneri* strain with the *E. coli* No.4 Hfr strain, *S. flexneri* colonies that did not require tmRNA were identified. The loci of the oriT and selected marker on the No.4 Hfr chromosome are 45min. and 25min (Fig. 5). This result suggested that either some DNA element(s) in the 25min.-45min. region on the *E. coli* chromosome is responsible for making *ssrA* non-essential in *E. coli*, or some *S. flexneri* chromosomal DNA element(s) in the corresponding region is responsible for making *ssrA* essential in *S. flexneri*. In addition, since colonies that can bypass the phenotype were not found after No.3 or No.5 Hfr conjugation, the part that contains the responsible element(s) can even be narrowed down to the transfer region in No.4 that does not overlap with No.3 and No.5 Hfr (35min~42min).

![Wild type control ssrA::kan](image1.png)

**Fig. 3** Colony PCR showed the *ssrA::kan* replacement. This replacement can only be obtained from *S. flexneri* with covering plasmid but not from wild type *S. flexneri*.

![Wild type control ssrA::kan](image2.png)

**Fig. 4** The *ssrA::kan* replacement was further confirmed by southern blot.
Fig. 5 Hfr mapping set. Arrowheads indicate the origin and direction of transfer for each Hfr strain; bars indicate the positions of the selective markers on the transfer region.
Discussion

The data here demonstrated that \textit{ssrA} is essential in \textit{S. flexneri} but not in \textit{E. coli}. More importantly, some DNA element(s) can bypass the \textit{ssrA}-essential phenotype in \textit{S. flexneri} after the conjugation. So the immediate plan for the downstream research would be to map and identify the DNA element(s) that is responsible for bypassing the phenotype. After mapping the responsible DNA element(s), depending on which species the responsible DNA element belongs to, two distinct models can be proposed.

\textbf{If the responsible DNA element belongs to \textit{E. coli}}

If some \textit{E. coli} specific gene(s) is found to be recombined into the \textit{S. flexneri} genome through Hfr conjugation, it is possible that this gene complements the \textit{ssrA} deletion phenotype in \textit{S. flexneri} through a gain-of-function manner. So the possible model can be that the product of this responsible gene has similar function as tmRNA and therefore constitutes a backup system for tmRNA.

\textbf{If the responsible DNA element belongs to \textit{S. flexneri}}

If some \textit{S. flexneri} specific gene(s) is lost or truncated from the Hfr conjugation, it is possible that the inactivation of this gene bypasses the \textit{ssrA} deletion phenotype in \textit{S. flexneri} through a loss-of-function manner. So a possible model can be that some protein produced from this responsible gene is toxic when their level in the cell is too high. tmRNA may directly or indirectly regulate the level of these proteins in the cell through tagging and degradation so the amount of the proteins is controlled at a certain level. When the responsible genes are inactivated, the toxic proteins will not be produced so tmRNA is no longer required to control the level of the proteins.

tmRNA is important for bacterial pathogenesis. It is indispensable for full virulence in
Salmonella typhimurium and Yersinia pseudotuberculosis. (Julio, 2000; Okan, et al., 2006). It is also essential in Shigella flexneri and Neisseria gonorrhoeae. (Huang, et al., 2000). Therefore it has the potential to become the target for developing new antibiotics. Since no tmRNA has been found in eukaryotes, antibiotics against tmRNA can be very specific with a broad target range but has little side effect on human beings or animals.
Materials and methods

Bacterial Strains

The wild-type *S. flexneri* strain used in this study is *Shigella flexneri* 2a 2457T (From ATCC center). The wild-type *E. coli* strain used in this study is mg1655. Both *S. flexneri* and *E. coli* strains were grown at 37°C in Luria-Bertani broth (Sambrook et al.1989) supplemented with 100 µg/ml ampicillin, 30 µg/ml chloramphenicol, or 30µg/ml kanamycin as necessary, and monitored by optical density at 600 nm.

Transformation

*S. flexneri* was grown at 37°C in Luria-Bertani broth to OD of ~0.3 at 600nm. 5ml of bacterial culture was harvested by centrifugation and the supernatant was disregarded. The cell pellet was washed with 1.5ml ice-cold ddH2O three times and resuspended in 50ul ice-cold ddH2O. Plasmid was added into the solution and kept on ice for at least 20 minutes. The mixture was then electroporated and resuspended in 0.5ml LB broth immediately. The culture was shaked at 37°C for at least 2 hours. 50ul of the culture will then be spreaded onto LB plates with appropriated antibiotics and kept at 37°C over night.

Gene knock out using the Wanner method

The helper plasmid used in this study is pkd46 (Kirill, 2000). The help plasmid is pJS14 inserted with *E. coli ssrA* driven by its endogenous promoter. The primers used to amplify the linear kanamycin knock out fragment from pKD4 is Shi ssra del-F (5’-CACAAATGTTGCC ATCCCATTTGCTTAATCGAATTTGAGCGATTGTGTAGGCTGGAGCTGCTTC-3’) and Shi ssra del-R (5’-TCGGATGACTCTGGTAATCACCGATGGAGAATTTTGATGGGAATTCGGAGCAGCTGCTTC-3’)
**P1 Transduction**

The recipient strain is grown in LB broth over night. 1ml culture is harvested by centrifugation and the supernatant is disregarded. The cell pellet is resuspended in 0.5 ml solution of 10mM MgSO$_4$, 5mM CaCl$_2$. The cell suspension and the P1 phage is mixed according to table 1.

**Table 1 Phage transduction table**

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell suspension</th>
<th>P1 phage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100ul</td>
<td>0ul</td>
</tr>
<tr>
<td>2</td>
<td>100ul</td>
<td>10ul</td>
</tr>
<tr>
<td>3</td>
<td>100ul</td>
<td>50ul</td>
</tr>
<tr>
<td>4</td>
<td>100ul</td>
<td>100ul</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>100ul</td>
</tr>
</tbody>
</table>

The mixture from each group is incubated at 37°C for 30 min with the cap of the tube open. Then 1ml of fresh LB broth is added and the mixture is incubated at 37°C for another 1 hour before spreading onto LB plates with appropriate antibiotics (Miller, 1972).

**Southern Blot**

Equal amount of genomic DNA (~2mg) was digested with appropriate Restriction enzyme over night at 37 °C and loaded onto 0.7% agarose gel. After 6 hour electrophoresis at ~50V, the DNA fragments were transferred to membrane, hybridized with [32P]-labeled DNA probes at 65 °C and visualized by using a PhosphorImager. The primers used to amplify the probe from the *S. flexneri* genomic DNA are shi ssra south F (5' - cgcgaatatctttcacc-3') and shi ssra south R (5' - tttctgcataagcttgctg -3').
Hfr Conjugation

Hfr donor and recipient strains were grown at 37°C over night. 0.5ml of each strain was mixed and harvested by centrifugation at 13,000 rpm for 1 minute. The cell pellet was washed with ddH$_2$O twice. The mixture was then kept at 37°C for ~ 30 minutes with the cap of the tube open. The pellet was resuspended in 1ml LB broth. The solution was then vortex for at least 2 minutes to break the pili between the donor and recipient strains. The mixture was finally spreaded onto LB plates with appropriate antibiotics and kept at 37°C over night. The donor and recipient strains were killed by the antibiotics and only the transconjugants can survive (Singer et al., 1989).


CHAPTER 3

IDENTIFICATION OF MUTANTS THAT BYPASS THE PLASMID MAINTENANCE DEFECT IN ΔSSRA C. CRESCENTUS
Abstract

*ssrA* is a universally conserved gene found in all bacteria. Its product, tmRNA, plays an important role in ribosome rescue and protein quality control. *ssrA* deletion in *Caulobacter crescentus* causes two distinct phenotypes: 1) a specific delay in DNA replication initiation 2) defect in maintaining pBBR1 plasmids. Through genome wide random mutagenesis and selection in Δ*ssrA* C. crescentus strain, three mutants that are able to bypass the plasmid maintenance defect were identified. It is further discovered that the three mutations only bypasses the plasmid maintenance defect but not the DNA replication initiation delay, which suggests that the two phenotypes are controlled in two different pathways.
Introduction

*Caulobacter crescentus* is a gram-negative aquatic bacterium with two different cell morphologies: swarmer cell and stalker cell (Poindexter et al., 1981). *C. crescentus* starts its cell cycle from a swarmer cell, which has a single flagellum and therefore motile (Huguenel et al., 1982). The swarmer cell cannot initiate DNA replication until it differentiates into an immotile stalk cell. During this process the flagellum is removed and a stalk is constructed at the same pole of the flagellum. The stalk cell is able to initiate DNA replication and will asymmetrically divide into a swarmer cell and a stalk cell. The progeny stalk cell will start DNA replication immediately while the progeny swarmer cell will need to differentiate into a stalk cell before it can start DNA replication. The morphology change between swarmer cell and stalk cell coincides with the G1 to S phase transition in cell cycle, which makes *C. crescentus* a great model system to study cell cycle regulation and bacterial development (Ryan et al., 2003) (Fig 6).

There is a distinctive difference between tmRNA from *C. crescentus* and most other bacteria. tmRNA in *C. crescentus* and other α-proteobacteria, cyanobacteria consists of two RNA chains while tmRNA in most other bacteria such as *E. coli*, consist of one RNA chain. This is because there is a circular permutation in the *ssrA* gene of these α-proteobacteria. The one-piece transcript from *ssrA* gene must be processed into two RNA chains to form a mature tmRNA molecule. The two-piece tmRNA carries similar function as those one-piece tmRNA since it can tag proteins from a non-stop mRNA for degradation (Fig 7, Keiler et al., 2000).

When *ssrA* is deleted in *C. crescentus*, two phenotypes have been noticed. The first phenotype is there is a 40 minutes delay in swarmer-cell to stalk-cell transition in Δ*ssrA C. crescentus* comparing with wild type *C. crescentus*, which results in a much longer doubling time for the deletion strains. Since the morphology change coincident with the G1 to S phase
transition in \textit{C. crescentus}, this specific delay suggest that tmRNA is required for the correct timing of DNA replication initiation in \textit{C. crescentus} (Keiler et al., 2003).

The second phenotype is that \textit{\Delta ssrA C. crescentus} is not able to maintain a set of pBBR1 family plasmids, which are readily maintained in wild type \textit{C. crescentus} (Hong SJ dissertation). However, the factors which are responsible for this plasmid maintenance defect are still unknown. Identification of these factors is the focus of this chapter.

![Fig. 6 The cell cycle of C. crescentus (Kelly, 1998)](image)

![Fig 7. Processing tmRNA in C. crescentus (Keiler et al., 2000)](image)
Results

Discovery of Six mutants that bypass the plasmid maintenance defect in $\Delta ssrA$ C. crescentus

To study the factors that are responsible for the plasmid maintenance defect in $\Delta ssrA$ C. crescentus, $\Delta ssrA$ C. crescentus cells were mutagenized and selected for the mutants that can maintain pBBR1 plasmids. The random mutations were introduced by transposon mutagenesis (EZ-Tn5<TM> <R6Kgori/KAN-2>Tnp Transposome™ Kit). The transposon carries a Kanamycin resistance gene so the mutants were first selected on LB plate with Kanamycin. All the mutants on the plates were scraped off and collected in one test tube. This mixed culture was subsequently made competent by ice-cold H$_2$O wash and transformed with pJS14, a pBBR1 family plasmid that carries a chloramphenicol resistance gene. After the transformation, all the transformants were spreaded onto LB plates with chloramphenicol. Six mutants were able to grow on the plates. Plasmid extraction showed that they all harbored pJS14, which means the mutation does bypass the plasmid maintenance defect.

Identification of the mutants

The insertion loci of the transposon in all the six mutants were mapped and it turned out that they located in three different genes, CC0153, CC1008 and CC2264. To confirm if inactivation of these genes does bypass the phenotype, single knock out of each of the three genes were generated in $\Delta ssrA$ C. crescentus. All the knockouts were confirmed by southern blots (Fig 8). Further transformation of pJS14 into these three knockouts showed that they were capable of maintaining plasmid, which suggests that the inactivation of any of the three genes is responsible for bypassing the defect. However, we also noticed that although all the three knockouts were able to maintain pJS14, their transformation efficiency were 100 to
1000 fold lower than transformation of pJS14 into wild type *C. crescentus*. The reason for this difference is still unknown.

**nactivation of the three genes does not bypass the DNA replication initiation delay in ∆ssrA C. crescentus**

In order to test if the inactivation of the three genes can also bypass the DNA replication initiation delay in ∆ssrA *C. crescentus*, growth curve experiment of the three knock outs were performed. Our data showed that they all grew slower than wild type *C. crescentus* (Fig 9, Table 2). This data suggests that the mutation only bypass the plasmid maintenance defect in ∆ssrA *C. crescentus*.

![Southern Blot results confirm the knockouts of the three target genes](image)
Fig 9. The growth curve of the three mutations
Table 2. The doubling time of all three mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Doubling time in PYE (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt C. crescentus</td>
<td>94.34</td>
</tr>
<tr>
<td>tmRNA deletion</td>
<td>114.52</td>
</tr>
<tr>
<td>CC1008 deletion</td>
<td>110.44</td>
</tr>
<tr>
<td>CC2264 deletion</td>
<td>118.27</td>
</tr>
<tr>
<td>CC0153 deletion</td>
<td>113.63</td>
</tr>
</tbody>
</table>
Discussion

The data presented here demonstrated that inactivation of CC0153, CC1008 or CC2264 in \( \Delta ssrA \) *C. crescentus* bypasses the plasmid maintenance defect. CC0153 encodes a protein called HrcA, which is a heat shock protein transcription repressor (Michelle et al., 2004). CC1008 encodes a protein called RsaD, which presumably is a component of the type I secretion system (Michael et al., 2004; Peter et al., 1998). CC2264 encodes a phosphomannomutase, which catalyses the transition between \( \alpha\)-D-glucose 1 phosphate and \( \alpha\)-D-glucose 6 phosphate (Matthew et al., 2007). Although the three genes seem unrelated and do not form a very good model why they can bypass the plasmid maintenance defect, they do give some hints regarding future research. HrcA is a heat shock protein transcription repressor, which indicates that the tmRNA activity is again linked to stress response. Also there are several LPS genes located just downstream of CC1008, which means the transposon insertion in CC1008 may affect the expression of these downstream genes. Meanwhile the product of CC2264, the phosphomannomutase is involved in LPS synthesis. Therefore the relationship between LPS synthesis, tmRNA activity as well as plasmid maintenance can be investigated.

Furthermore, although the inactivation of the three genes can bypass the plasmid maintenance defect to some extend, the low transformation efficiency comparing with the wild type suggests that there are other factors that are also involved in the plasmid maintenance mechanism. More mutagenesis and selection could be done to identify the other unknown factors.

Last but not least, the inactivation of the three genes only bypasses the plasmid maintenance defect but not the DNA replication initiation delay, which suggests that the two phenotypes are controlled in two different pathways. Unfortunately the delay in DNA replication initiation is not a selectable phenotype and is therefore more difficult to study than
the plasmid maintenance defect. To identify mutants that can bypass the DNA replication initiation delay, one can start with looking for mutants that bypass the two phenotypes at the same time. To carry out this plan, more mutagenesis and selection can be done and the “bigger” colonies on plates will be isolated and more closely studied. They are the fast growers and possibly bypass the DNA replication initiation delay.
Materials and methods

Bacterial Strains

The wild-type *C. crescentus* strain used in this study is CB15N (Evinger et al., 1977). CB15N were grown at 30°C in PYE or M2G broth (Ely, 1991) supplemented with 5 mg/ml kanamycin, 1 mg/ml chloramphenicol as necessary, and monitored by optical density at 660 nm.

Transposon mutagenesis and mutation mapping

The transposon mutagenesis was performed by electroporate the Tn5 transposon into Δ*ssrA* *C. crescentus* and selected on LB plates with kanamycin. To map the mutation, the genomic DNA of the identified mutants was extracted and digested over night with a chosen restriction enzyme. All the pieces of the genomic DNA were self ligated into circular DNA fragments and transformed into *E. coli*. Since the transposon has an origin in its sequence, the circular DNA fragment that contains the transposon becomes a plasmid and therefore could replicate in *E. coli* cells. All the other circular DNA fragments that do not contain the transposon insertion will be lost and those cells cannot grow on media. The plasmid with the transposon is extracted and sequenced using primers anneal to a fragment in the transposon. The sequencing data will reveal the exact insertion site of the transposon on the genome (Fig 10).
Specific Gene knockout

The upstream and downstream 1kb fragments of the ORF of the target gene are cloned into pNPTS138 (Keiler, 2003) and transformed into \( \Delta ssrA C. crescentus \). The target gene is then knocked out using the two-step recombination method (Gay et al., 1985) and verified by southern blot analysis.

Southern Blot

Equal amount of genomic DNA (~2mg) was digested with appropriate Restriction enzyme over night and loaded onto 0.7% agarose gel. After 6 hour electrophoresis at ~50V, the DNA fragments were transferred to membrane hybridized with [32P]-labeled DNA probes at 65 °C and visualized by using a PhosphorImager. The probes used in this chapter are always the upstream 1kb fragments of the ORF of the target genes. The primers used to generate the knockout construct are listed in table 3.
Table 3. Primers for specific gene knockout.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequences</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0153 Up F</td>
<td>GGACTAGTCGGTCAGGTGAAA</td>
<td>amplify cc0153 upstream 1kb</td>
</tr>
<tr>
<td></td>
<td>TCACCTG</td>
<td></td>
</tr>
<tr>
<td>0153 Up R</td>
<td>CGCGGATCCGTCCTGCCGCTC</td>
<td>amplify cc0153 upstream 1kb</td>
</tr>
<tr>
<td></td>
<td>CGATCAAG</td>
<td></td>
</tr>
<tr>
<td>0153 Down F</td>
<td>CGCGGATCCGAGACTTTACCATA</td>
<td>amplify cc0153 downstream 1kb</td>
</tr>
<tr>
<td></td>
<td>ATGACCGA</td>
<td></td>
</tr>
<tr>
<td>0153 Down R</td>
<td>CTAGCTAGCGGGTTCAGCCTG</td>
<td>amplify cc0153 downstream 1kb</td>
</tr>
<tr>
<td></td>
<td>CGCGGGG</td>
<td></td>
</tr>
<tr>
<td>2264 TA upF</td>
<td>GGACTAGTGATGAAGGCGCAA</td>
<td>amplify cc2264 upstream 1kb</td>
</tr>
<tr>
<td></td>
<td>GATTCAGC</td>
<td></td>
</tr>
<tr>
<td>2264 TA upR</td>
<td>CCCAAGCGTTTGAAGGTCGCT</td>
<td>amplify cc2264 upstream 1kb</td>
</tr>
<tr>
<td></td>
<td>TCAGTTGA</td>
<td></td>
</tr>
<tr>
<td>2264 downF</td>
<td>TACCCAAAGCTTGAGCTGAGAT</td>
<td>amplify cc2264 downstream 1kb</td>
</tr>
<tr>
<td></td>
<td>GCTCACCA</td>
<td></td>
</tr>
<tr>
<td>2264 downR</td>
<td>TACTAGCTAGCGGGGAAGATCAT</td>
<td>amplify cc2264 downstream 1kb</td>
</tr>
<tr>
<td></td>
<td>GCCGTAGT</td>
<td></td>
</tr>
<tr>
<td>1008 upF 1.1</td>
<td>GGAGAGAGCGAAGCTGACGAA</td>
<td>amplify cc1008 upstream 1kb</td>
</tr>
<tr>
<td></td>
<td>GGGACTGCGAGCTCCTGGTA</td>
<td></td>
</tr>
<tr>
<td>1008 upR anneal 1.1</td>
<td>GCCGCGCGGA</td>
<td>amplify cc1008 upstream 1kb</td>
</tr>
<tr>
<td></td>
<td>GGGAGGCGAAGCTGACGAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GGGCGCTGAGCTCCTCCAGGAC</td>
<td></td>
</tr>
<tr>
<td>1008 downF anneal 1.1</td>
<td>TCGCGCGGCGTCTTGAAGGCT</td>
<td>amplify cc1008 downstream 1kb</td>
</tr>
<tr>
<td></td>
<td>CACAGCGCGCTCCTGAGCTC</td>
<td></td>
</tr>
<tr>
<td>1008 downR 1.1</td>
<td>CTAGCTAGCGGGCCTCCGTTG</td>
<td>amplify cc1008 downstream 1kb</td>
</tr>
<tr>
<td></td>
<td>AAGAAGCGCGAGG</td>
<td></td>
</tr>
</tbody>
</table>

**Growth curve**

Cultures of strain CB15N, tmRNA deletion strain, knockouts of cc1008, cc2264, cc0153 respectively in ΔssrA CB15N were grown in PYE medium to an OD660 of 0.5-0.6. Cells were then diluted into fresh PYE at an OD660 of 0.05. Cultures were grown at 30°C to an OD660 of 0.5-0.6 and samples were taken periodically to monitor the changes in optical density at 660 nm. The absorbance values were fit to an exponential function to calculate the doubling time of each strain.
Reference


Keiler, K. C., Shapiro, L., and Williams, K. P. (2000). tmRNAs that encode proteolysisinducing tags are found in all known bacterial genomes: A two-piece tmRNA functions in Caulobacter. Proc Natl Acad Sci USA 97, 7778-7783.


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