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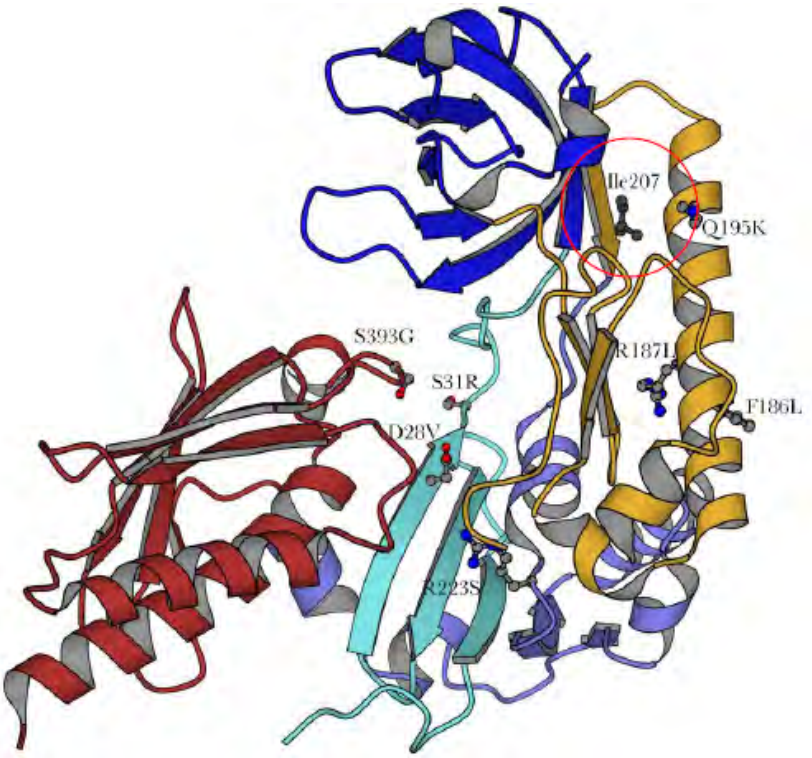
Ribonuclease E

Reversion of a temperature-sensitive phenotype by external suppressors in *Salmonella typhimurium*



Eva Garmendia Espinosa

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Biology Education Centre and Department of Cell and Molecular Biology, Uppsala University
Supervisors: Diarmaid Hughes and Jessica Bergman

SUMMARY

The ribonuclease E (RNase E) plays an essential role in the metabolism of messenger, transfer and ribosomal RNAs. The RNase E polypeptide consists of two domains: a catalytic one where the actual ribonucleolytic activity is located and a scaffolding one with which several other proteins interact to form the multienzyme complex known as the degradosome.

In *Salmonella typhimurium* several temperature-sensitive (ts) mutants in RNase E gene (*rne*) were isolated. These mutations render the enzyme non-functional at high temperatures (43 ºC) causing lack of growth. During the study of these conditional mutations a number of suppressors of the ts phenotype were found. Most of the suppressors were second-site mutations within *rne* gene.

Further studies identified additional suppressors of the ts phenotype, which mapped in other genes: so-called external suppressors. Two external suppressors were mapped prior to the beginning of this project (Disa Hammarlöf, unpublished data). In the course of this project the map positions for these two suppressors were confirmed and, in addition, the map positions of four other external suppressors were confirmed. The basic approach used for the mapping was transduction of each of the suppressors into the genetic background of the original ts strain: so-called backcrossing.

Based on the mapping data I suggest two different and contrasting models to explain the ts phenotype of the original RNase E mutants as well as its suppression by the external suppressors. These models are based on the known characteristics of the six external suppressors mapped in three different genes. The models explain also why RNase E is essential for growth and why mutations in the ‘suppressor’ genes make the RNase E enzyme no longer essential.

INTRODUCTION

RNase E: features and activity

The ribonuclease E (RNase E) is an essential endoribonuclease involved in the turnover of a large number of mRNAs, in the maturation of many tRNA precursors and in the processing of 5S rRNA (reviewed by Cohen and McDowall, 1997). The structure of the enzyme can be divided in an N-terminal half which contains the catalytic domain and a C-terminal half that forms the scaffold for the formation of the RNA processing multienzyme called the degradosome (Miczak *et al.*, 1996) (**Figure 1**). In the degradosome, RNase E works together with a phosphorylase (PNPase), an RNA helicase (RhlB) and an enolase (reviewed by Carpousis, 2007). Recently it has been shown that RNase E is a membrane binding protein and that its localization to the inner cytoplasmic membrane is crucial for normal cell growth (Khemici *et al.*, 2008).

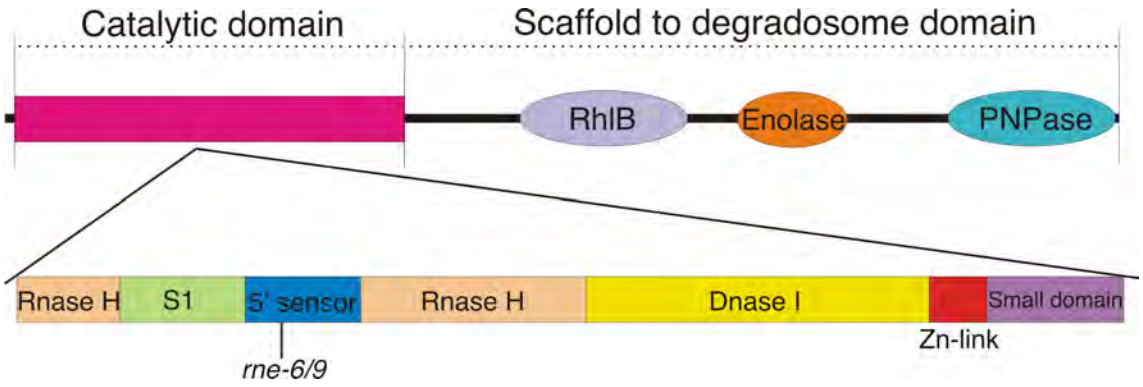

Figure 1. Structure of the RNase E polypeptide. Different motifs within the catalytic domain and the regions of attachment of the different enzymes on the non-catalytic domain in the degradosome are shown. In the insert, the location of the *rne-6* and *rne-9* alleles is shown. The picture was adapted from Perwez *et al*, 2008.

RNase E is encoded by the *rne* gene (Miczak and Apirion, 1993) which is essential in the model Gram-negative bacterium *Escherichia coli* (McDowall and Cohen, 1996). Regulation of the *rne* gene expression occurs mostly at mRNA level and RNase E is able to autoregulate its own synthesis by cleaving the *rne* mRNA (Mudd and Higgins, 1993; Jain and Belasco, 1995).

The S1 RNA binding motif of the catalytic domain of RNase E is essential for RNase E activity during RNA degradation. Here, a pocket formed by the S1 motif interacts specifically with the terminus of 5'-monophosphorylated RNA. It also permits access to single-stranded RNA (reviewed by Carpousis, 2007). The catalytic action of RNase E consists in cutting RNA substrates at internal sites in A+U rich regions, generating RNA fragments that are degraded afterwards by several other RNase enzymes (McDowall *et al.*, 1994).

Temperature-sensitive mutants

The temperature-sensitive (ts) RNase E mutants were first isolated from an RNase III-deleted strain of *E. coli*. It was observed that the cleavage between the 23 S and the 5 S cistrons did not occur at elevated temperatures (43º C), presumably causing the observed lack of growth (Apirion and Lassar, 1978). The best-studied ts mutants in *E. coli* are *rne-1* and *rne-3071*. Both mutations are associated with inability to process 9S rRNA to 5S, and with reduced rates of mRNA turnover (McDowall *et al*., 1993).

In *Salmonella enterica* serovar Typhimurium (*S. typhimurium)* several *rne* mutations were isolated as suppressors of a mutation in the gene that codes for the translation factor EF-Tu (*tufA499* Gln125Arg) (Hammarlöf and Hughes, 2008). These *rne* mutations reverted the slow-growth phenotype caused by the substitution in the *tufA* gene. The genetic analysis mapped these rne mutations in or close to the N-terminal end of the RNase E containing the catalytic domain. The *rne* mutations were then studied in more detail and some of them were shown to have a ts phenotype, similar to the phenotype of the *E. coli rne-3071*, although not as defective in the 9S processing (Hammarlöf and Hughes, 2008).

During the study of the *rne* mutations, many strains with a suppressed ts phenotype were isolated. The study of those strains revealed that many of the suppressors were second-site mutations within *rne*, but some appeared to map in different genes (Disa Hammarlöf, unpublished data). Mapping of the suppressed strains confirmed locations for two external suppressors. The first suppressor is a nonsense mutation in the gene for the 30S ribosomal protein S1 (*rpsA*) and the second is a deletion in the gene coding for the ribonuclease R (*vacB*) (Disa Hammarlöf, unpublished data).

Aim

The aim of this project was to confirm map positions for several more of the external suppressors found for both the *rne-6* and *rne-9* ts alleles, and to begin the identification of a completely unknown external suppressor. The aim was to use knowledge of the functions of the mapped external suppressors to rationalize how each suppressor reverses the ts phenotype. Based on that rationalization the greater aim was to propose a reasonable hypothesis to explain why RNase E is essential for growth.

RESULTS

STM1551-1 as external suppressor of ts *rne*

The strain TH7620 carries the temperature-sensitive (ts) mutation *rne-6* but is no longer ts due to the presence of an unknown external suppressor. In order to study this suppressor, TH7620 was transduced with the strain TH8018, which previously had been constructed to carry the wild type sequence for this suppressor linked to an EZ-Tn5 kanamycin resistant marker. For this transduction a P22 phage lysate was made on TH8018 (donor) in order to move the marker and link it to the suppressor found in TH7620 (recipient) (**Figure 2**).

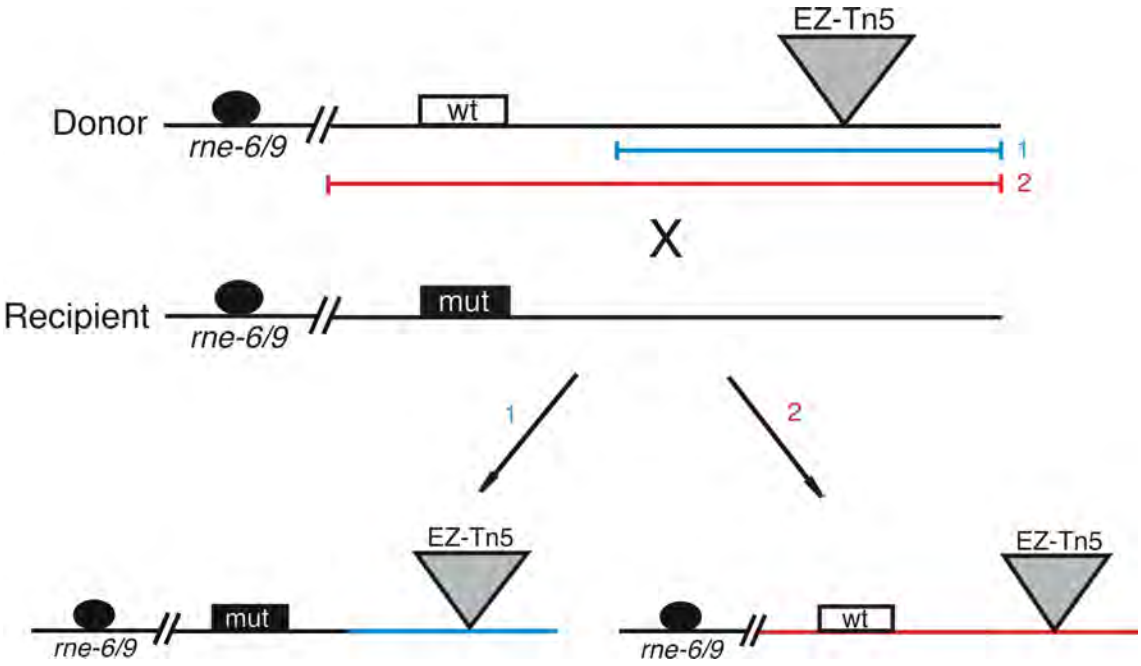

Figure 2. Transduction to link the marker EZ-Tn5 to the suppressor. The donor strain has the wild-type allele for the suppressor (white box) with the EZ-Tn5 linked and it is crossed with the strain that has the suppressor (black box). The selection for kanamycin resistance gives two classes of transductants: 1) the ones with the suppressor mutation linked to the EZ-Tn5 and 2) the ones with the wild-type allele linked. The class 1 transductants could grow at 43 ºC in the screening while the class 2 could not. One transductant of the class 1 was stored as a new strain. Note that the strains always carry one of the two original ts mutations in the *rne* gene.

To test whether the transductants were ts or not and determine the linkage between the EZ-Tn5 and the candidate suppressor, a screening was done by streaking each transductant on LA plates with incubation at both 43 ºC and 30 ºC (the latter used as control of growth). This gave 97.5 % of linkage. A control plate with the two parental strains was used during the screening to test if the phenotype of the transductants growing at 43 ºC was the same as the suppressed strain TH7620.

To test the hypothesis that the suppression of the ts phenotype is caused by a mutation in the *STM1551* gene (as previous experiments indicated) I PCR amplified and sequenced this gene from 20 suppressed transductants and from 20 that retained the ts phenotype.

If the hypothesis is correct, all of the suppressed ones should carry the same mutation in the gene while the ts ones should lack it.

Analyzing the sequences, I found that the substitution mutation Val45Met in the *STM1551* was present in the 20 suppressed transductants but absent in the 20 ts transductants, strongly suggesting that the point mutation is source of suppression (data not shown). The same gene was also amplified in both TH8018 and TH7620 as negative and positive controls respectively, and the wild-type sequence (LT2) of the gene was taken from the NCBI database as a reference. Since the suppressed transductants had the EZ-Tn5 linked to the *STM1551-1* suppressor one of them was stored as the new strain TH8122.

The mutations *rne-6* and *rne-9* affect the same amino acid. I therefore decided to do the control backcross of the suppressor into the original ts strain (*rne-6* for the *STM1551-1* suppressor) as well as into the *rne-9* strain TH7589. This was done to test whether the suppressor mutation in *STM1551* suppressed each of the different ts mutations in *rne* (**Figure 3**).

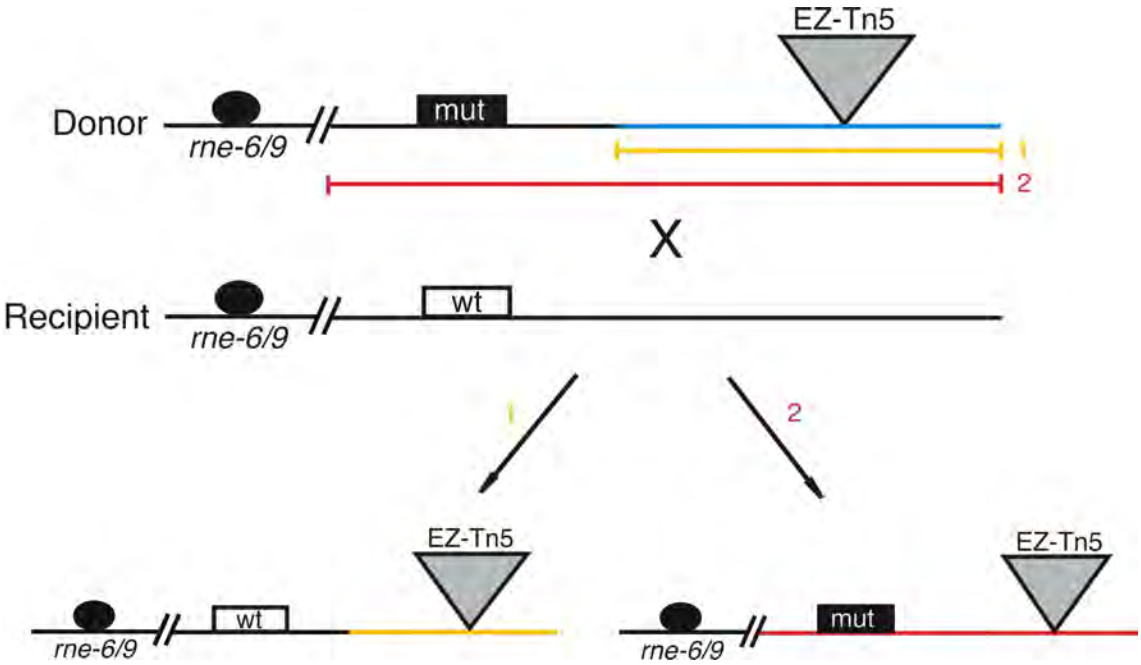

Figure 3. Backcross transduction. Here the suppressor is moved into the genetic background of the original ts strains. As figure 2, the selection gives two classes of transductants during the screening: 1) no growth at 43 ºC because of the wild-type allele and 2) growth at 43 ºC because it carries the suppressor.

To this end, I made a P22 lysate on the TH8122 strain (donor) and transduced the strains TH7180 and TH7589 (recipients). The transductants were screened at both 43 and 30 ºC and 10 transductants of each class (ts and suppressed) were picked. The *STM1551* gene from the 20 isolated transductants was PCR amplified, purified and sequenced. The same gene from parental strains was also PCR amplified and sequenced as a control. The analysis of the sequences showed that, indeed, all the suppressed transductants carried the *STM1551-1* suppressor while the ts strains did not (**Figure 4**).

Figure 4. Complete *STM1551* alignment. V: Valine. M: Methionine. *: Stop codon. TH7180 and TH7589 represent the genotype of the both recipient strains and TH8122 the amino acid sequence of the donor strain. The ts and suppressed sequences represent the amino acid sequence of the two classes of transductants obtained.

vacB-2, vacB-3 and *vacB-4* as external suppressors of ts *rne*

The alleles named *vacB-2*, *vacB-3* and *vacB-4* have previously been found to suppress the ts *rne* phenotype of the strains TH7618, TH7622 and TH7625 respectively. Preliminary studies showed them to map in the *vacB* gene but this result had to be confirmed. To test if they suppressed the ts phenotype of original *rne-6* and *rne-9* strains, as with the *STM1551-1* suppressor, I first needed to link the EZ-Tn5 to the suppressor, transducing the mentioned strains (recipients) with the TH8035 one (donor) (**Figure 2**). One suppressed transductant from each cross was stored as the new strains TH8123, TH8124 and TH8125.

Each of the suppressors was then backcrossed into both the originals *rne-6* and *rne-9* ts strains (**Figure 3**). 10 transductants of each class (growing and not growing at 43 ºC) in each transduction were picked and the region of interest in the *vacB* gene was PCR amplified. These PCR products along with the PCR products obtained for the parental and LT2 strains were purified and sent for sequencing.

The *vacB-2* allele had a deletion of 13 amino acids in the *vacB* gene from amino acid 524 to 535. This mutation was present in the 10 transductants growing at 43 ºC, while the ones lacking growth had the wild-type *vacB* allele (**Figure 5**). Thus, the results confirmed the *vacB-2* allele as the suppressor of the studied ts phenotype.

Figure 5. Alignment of a fragment of *vacB* gene (between amino acid 498 and 573). The deletion is from one X to the other. TH7180 and TH7589 represent the amino acid sequence of the both recipient strains and TH8123 the amino acid sequence of the donor strain. The ts and suppressed sequences represent the amino acid sequence of the two classes of transductants obtained.

The *vacB-3* allele was found to have an Arg446Pro substitution within the *vacB* gene and the sequencing results showed that the mutation was present in the 10 transductants with the suppressed phenotype and absent in the 10 transductants showing the ts phenotype (**Figure 6**). The vacB-3 is therefore another mutation in the *vacB* gene that restores the normal growth at high temperatures.

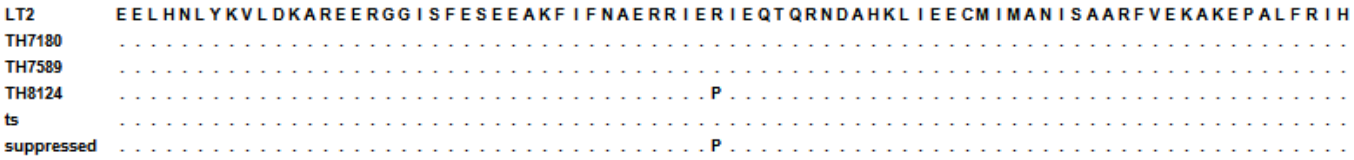

Figure 6. Alignment of a fragment of *vacB* gene (between amino acid 408 and 486). R: Arginine. P: Proline. TH7180 and TH7589 represent the amino acid sequence of the both recipient strains and TH8124 the amino acid sequence of the donor strain. The ts and suppressed sequences represent the amino acid sequence of the two classes of transductants obtained.

The *vacB-4* allele had the substitution Ile444Ser in the *vacB* gene. The sequences showed that the allele was present in the transductants with suppressed phenotype but it was absent in the ones with ts phenotype (**Figure 7**). These results confirmed that *vacB-4* is another independent mutation in the *vacB* gene, capable of suppressing the ts phenotype due to the *rne-6/9* mutations in the *rne* gene.

Figure 7. Alignment of a fragment of *vacB* gene (between amino acid 408 and 486). I: Isoleucine. S: Serine. TH7180 and TH7589 represent the amino acid sequence of the both recipient strains and TH8124 the amino acid sequence of the donor strain. The ts and suppressed sequences represent the amino acid sequence of the two classes of transductants obtained.

None of the suppressors affect the growth by themselves

It is possible that a mutation allows the cell to live while acting as a suppressor but at the same time affect the growth if the RNase E is working properly. To address that question I incubated the plates containing the suppressed strains at 30 ºC, where the suppression is not needed because the RNase E is active.

Normal growth for all of the strains was observed, strongly suggesting that each one of the mutations allow the cell growth. Indeed, the growth phenotype of these colonies was the same as in LT2 (data not shown).

Identification a completely unknown suppressor

Before the start of this project, nothing was known about the external suppressor of temperature-sensitive RNase E in the strain TH7630. To map this suppressor I transduced the strain with an EZ-Tn5 hop pool grown in the LT2 strain in order to insert the marker in random positions throughout the genome. The hope was to find at least one transductant during the screening process that showed the loss of suppression, meaning that the piece of DNA inserted had the EZ-Tn5 linked to the region that contained the wild-type allele of the suppressor gene. During the screening I found two transductants that did not grow at 43 ºC, which was confirmed by a second streak.

The next step was to rescue the EZ-Tn5 from these two ts transductants. For that, I extracted the genomic DNA from the clones and digested it with the restriction enzyme EcoRI. The resulting small fragments were then ligated with the T4 ligase to construct plasmids. The ligation products were purified and transformed into the electrocompetents *E. coli pir*$^{+}$ cells using electroporation and then plated onto LA-Kan plates for selection.

The EZ-Tn5 contains an origin of replication, therefore only the plasmids with it will be replicated and only the cells with the plasmid that contains the EZ-Tn5 will be able to grow on the selective plates. The clones from the LA-Kan plates were used to extract the plasmid DNA. The samples were sequenced in both directions out of the EZ-Tn5 to determine the genome location of the EZ-Tn5 (**Figure 8**).

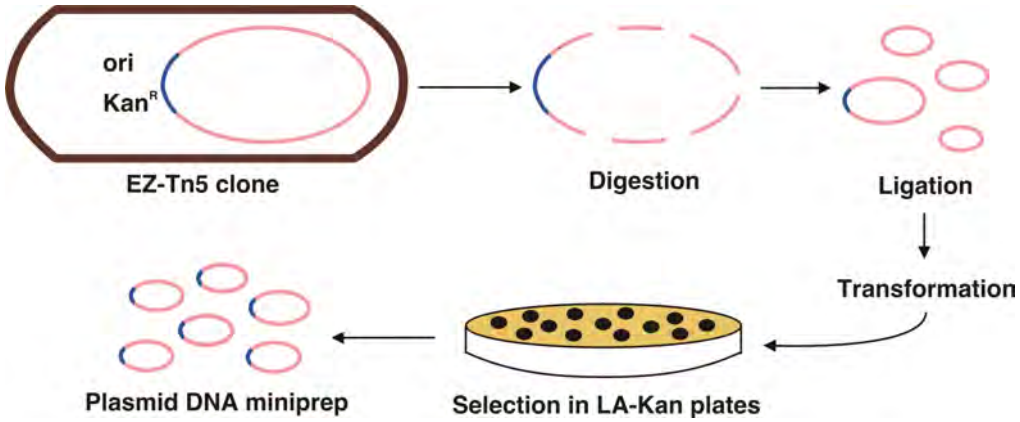

Figure 8. Steps for the localization of the EZ-Tn5 insertion. Picture adapted from Epicentre Biotechnologies protocol.

The sequences showed that the EZ-Tn5 was inserted in the same place as in the strain TH8018 (data not shown), suggesting that the suppressor in the strain TH7630 was the same as in the TH7620. To see if the suppression was due to the same *STM1551-1* allele I tried to PCR amplify the *STM1551* gene from the TH7630 strain and the two transductants that lost the suppression during the screening. I observed no amplification at all in the TH7630 but a normal amplification for the two transductants (**Figure 9)**, suggesting a deletion or another mutation in the TH7630 genome resulting a mismatch in the primer annealing sequence as the reason for the suppressed phenotype.

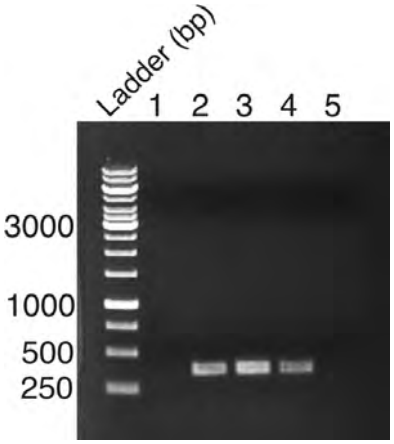

Figure 9.Agarose gel electrophoresis of *STM1551* PCR fragments. Well 5 shows the negative control without DNA on the reaction. PCR amplification was performed on a colony from the strain TH7630 (well 1), and from the two ts transductants from the screening (wells 2 and 3). A positive control was done by amplifying DNA from a LT2 colony (well 4).

To confirm that the suppressor in the TH7630 is indeed in the region where the *STM1551* lies I made a transduction with the P22 phage lysate grown on the TH8018 strain (donor) into the TH7630 (recipient) (**Figure 2**). If the suppressor is in the region, due to the high linkage of the Tn5, all of the transductants screened should lack growth at 43 ºC because of the introduction of a wild-type piece of DNA. The results confirmed what I expected, since there was no growth at high temperatures. This strongly suggests that the suppression of the TH7630 strain is due to some mutation in or very close to *STM1551* that also affects the annealing of my primers designed to amplify the *STM1551* gene.

DISCUSSION

In microbial genetics, one of the most useful tools are conditional lethal mutations and specifically temperature-sensitive mutations. The common basis for a temperature-sensitive (ts) phenotype is that the mutation changes an amino acid required for protein stability and the protein is no longer functional at high temperatures due to an unstable structure (Snyder and Champness, 2003). Because of this conditional phenotype it is really easy to isolate the mutations that causes it using a simple screening based on 43º and 30 ºC incubations. Here I have worked with two ts strains which had two different amino acid substitutions in the *rne* gene for the RNase E. Moreover, structural studies have revealed that these substitutions affect the proper folding of RNase E at high temperatures (unpublished data).

The study of the ts strains yielded several suppressors of the ts phenotype that mapped in external genes. Previous work has confirmed one mutation in the *rpsA*, gene that codes for the ribosomal protein S1, and another in the *vacB*, gene coding for the ribonuclease R, as suppressors of the ts RNA E phenotype. During this project, I confirmed three additional and different mutations in the *vacB* gene and one in a new gene, *STM1551*, which product is homologous to the *E. coli* antitoxin RelB. Furthermore, the results obtained in an attempt to identify a novel suppressor suggested that it is likely that a mutation in the *STM1551* flanking regions can restore growth at 43 ºC (**Table 1**).

Table 1. Summary of the suppressors found for the temperature-sensitive RNase E.

Allele	Gene	Product	Mutation
rpsA-1	*rpsA*	Ribosomal protein S1	Glu438Stop
vacB-1	*vacB*	Ribonuclease R	Δaa 435-438
vacB-2	"	"	Δaa 524-535
vacB-3	"	"	Arg446Pro
vacB-4	"	"	Ile444Ser
STM1551-1	*STM1551*	Putative antitoxin protein	Val45Met
STM1551-2?*	To determine	---	Not confirmed

***** This suppressor was still in process to be found by the end of this project.

To confirm each one of the suppressors as such, the final and crucial step is to move the suppressor into the genetic background of the ts strain where it arose, to see if the growth is restored at 43 ºC. Then one can sequence the suppressor to find the suppressor mutation. The most powerful example of this approach is, as Kock postulated back on 1884 (Madigan *et al.,* 2000), that only a pathogen can be confirmed as cause of a certain disease, if after isolating it from a sick patient the inoculation into a health individual

can reproduce the disease and it can be isolated again. Here I did the same with every suppressor.

A mutation in the S1 ribosomal protein gene restores the growth at high temperature

During previous work on this project, the nonsense mutation Glu438Stop in the *rpsA* gene was confirmed as suppressor of the ts phenotype rendered by either the *rne-6* or *rne-9* alleles (**Table 1**). The *rpsA* gene codes for the ribosomal protein S1, located in 30S small subunit of the 70S prokaryotic ribosome (Schnier *et al*, 1981). The S1 protein is essential during the translation initiation, where it is involved in the recognition and binding of the majority of mRNAs to the 30S subunit (Subramanian, 1983).

Several mutations in the ribonuclease R gene restores the growth at high temperature

In this project, I confirmed three independent mutations in the *vacB* gene (also known as *rnr*) acting as suppressors of the ts RNase E. In addition, another different suppressor was found in previous studies to map in the same gene. The nature of these suppressors varies, with two of them being deletions in different parts of the gene and the other two substitutions in two amino acids locate close to each other (**Table 1**).

The product of the *vacB* gene is the exoribonuclease known as ribonuclease R (RNase R). The activity of the RNase R consists in the processive degradation of RNA molecules from the 3' to the 5' end, which generates di-nucleotides as final products. Besides, RNase R has a special specificity in degrading structured RNA molecules (reviewed by Andrade *et al.*, 2009).

A mutation in a putative antitoxin gene restores the growth at high temperature

A substitution in the *STM1551* gene has been confirmed here as the suppressor of the ts phenotype (**Table 1**). The *STM1551* is listed in the NCBI database as a gene for a putative cytoplasmatic protein in the *Salmonella typhimurium* LT2 genome, which gave me no information about its possible role on the cell. Doing blast with the amino acid sequence I found identical similarity with several putative stability proteins StbD in different *Salmonella* strains, and furthermore, a high similarity with a known antitoxin with the same name in some *E. coli* strains.

The toxin-antitoxin (TA) systems are found on mobile genetic elements and in bacterial chromosomes. Generally, the activity of the toxin is blocked by the interaction with the antitoxin, either at the protein level or with antisense RNA. StbD is the antitoxin of StbE toxin and their *stbDE* genes are found organized in an operon, with the upstream gene encoding the antitoxin and the toxin gene downstream and overlapping (Hayes, 1998). These characteristics are also found in the *Salmonella typhimurium* LT2 genes *STM1551* and *STM1550*, the latter being the putative toxin on the system. This genetic

organization is representative of the TA system type II (Hayes, 1998). In addition, this new *STM1550/1551* system shows similarity with the well-known TA type II system *relEB* suggesting that the mode of their action can also be similar. The type II toxins work as translation inhibitors. More specifically, RelE cleaves the mRNA codon in the A site of the ribosome at its second position (Christensen and Gerdes, 2003).

Model proposals

Model I. Essential-gene model

In this model, I propose that RNase E is essential for growth because it is needed to processes a polycistronic messenger RNA to allow the translation of an essential downstream gene (**Figure 10**).

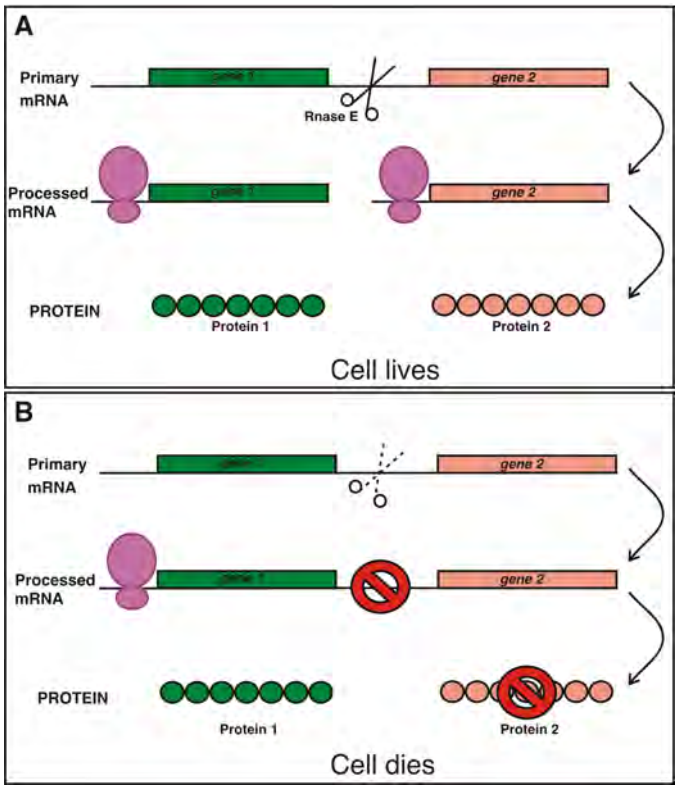

Figure 10. Activity of the RNase E according to the essential-gene model. Panel A shows how the RNase E cuts a primary mRNA inside the cistron and that allows the ribosome to go in and bind to the ribosome binding region of the gene 2, which product is essential for growth. In B RNase E is not active, i.e. at high temperatures in the case of temperature-sensitive mutations. Here the absence of cleavage prevents the ribosome to bind to the gene 2 transcript, causing lack of protein 2, and therefore the cell death.

Suppression by mutated S1 ribosomal protein

Within this model, I suggest that the mutated ribosomal protein S1 allows the ribosome to bind the essential gene transcript even in absence of cleavage by RNase E. The mutation found as suppressor is a nonsense mutation affecting to the amino acid nº 438, close to the C-terminal end. Since the major ribosome-binding domain on the S1 is located near to the N-terminal end (Schnier *et al.*, 1982), the protein could still be part of the ribosome but with the activity somehow affected.

Suppression by mutations on ribonuclease R

In this model, a mutated ribonuclease R (four different mutations in the gene have been found as suppressors) could replace RNase E in cutting the poly-cistronic mRNA and therefore the ribosome could bind to the essential gene transcript.

Suppression by mutation on a putative antitoxin

As is explained early in the discussion, the STM1551 antitoxin protein in a normal situation would bind to the putative STM1550 toxin, preventing the toxin to act in the cell. Here I propose that when the antitoxin is mutated, its interaction with the toxin is compromised, allowing the toxin to act in the cell. The toxin, if being of the type II as I suggested before, would cut the mRNA when it is being translated by the ribosome. It is known that toxins, such as RelE, cut stop codons with some specificity (Perdersen *et al.*, 2003). I suggested that the toxin would cut the mRNA at the stop codon of the gene 1 (**Figure 10**) allowing then the ribosome to bind to the gene 2 transcript.

Model II. Toxin model

This model is completely opposite to the previous one. Here RNase E would be essential because the enzyme in required to cut and degrade the mRNA of some product toxic for the cell (**Figure 11**).

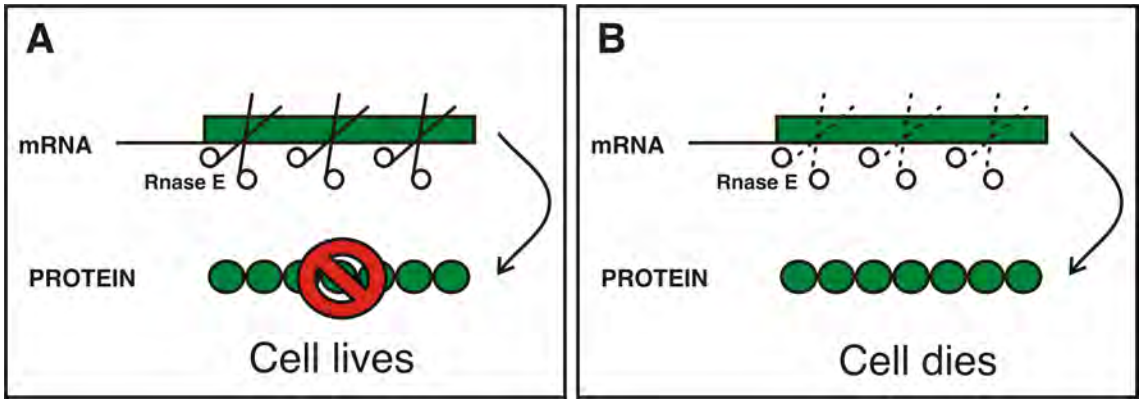

Figure 11. Activity of the RNase E according to the toxin model. Panel A shows how RNase E, in normal situation would cut the messenger RNA of a possible toxin compound, preventing the cell to die. In B the opposite situation is illustrated, when RNase E is non functional. In this case the mRNA of the toxin product is not degraded, going into translation and the cell is exposed to the toxic compound.

Suppression by mutated S1 ribosomal protein

In this model, the mutated S1 protein, instead of allowing the ribosome to bind to certain mRNA would prevent this from happening and, therefore, although the mRNA is not degraded because the RNase E is not functional, the mRNA would not be translated and the cell would not be exposed to the toxic compound.

Suppression by mutations on ribonuclease R

To fit in this “toxin” model, the mutated RNase R would replace RNase E in degrading the mRNA of the toxin, preventing the cell to die.

Suppression by mutation on a putative antitoxin

Within the “toxin” model, the mutation in the putative antitoxin acting as suppressor can be explained in two different ways. The first way is, as I explained in the previous model, that the mutated antitoxin could no longer bind correctly to the toxin and the toxin cuts the mRNA on the ribosome. It is known that when a toxin cuts a transcript while is still being transcribed the tmRNA helps to rescue the ribosome (reviewed by Moore and Sauer, 2007) and the immature product is degraded by several enzymes because the tmRNA tags it. This way the toxin product would be degraded after the toxin cuts the mRNA and the tmRNA is tagged the toxic polypeptide.

The other way of suppression can be that the mutated antitoxin can now bind, in addition to its natural toxin, also to this toxin compound, preventing the toxicity and the cell death.

Future perspectives

First of all, it is important to identify the suppressor in the strain TH7630. To this end a new pair of primers annealing outside of the current ones should be designed and used to amplify and sequence from that strain. If any mutation is found then the EZ-Tn5 maker should be linked to that mutation in order to move it into the originals *rne-6* and *rne-9* strains and confirm it as suppressor.

To test the hypothesis of the essential-gene model, the first step could be to find an essential gene inside a polycistronic mRNA. Once a candidate gene is identified, one way to test if the RNase E cuts that transcript could be a northern blot using a probe for that mRNA to see if there is a difference in the length of mRNA between the ts mutant and the wild type. At high temperatures in a test with a wild type strain several products should be obtain while in the ts strain only one big product is expected. This will confirm the cutting activity of the RNase E at that mRNA.

MATERIAL AND METHODS

Media and growth conditions

Liquid and solid media used for bacterial growth were Luria Bertani (LB) culture media (10 g NaCl, 5g yeast extract, 10 g tryptone, filled up to 1 liter with di stilled water and autoclaved, pH 7.2-7.4) and LA plates (LB supplemented with 1.5 % agar). Strains were always grown at 30 ºC and liquid cultures, when needed, were incubated with shaking (200 r.p.m). The antibiotic kanamycin (Kan) was used at final concentration 50 μg/ml both in liquid media and plates.

Bacterial strains

All *S. typhimurium* strains that I used were derived from the sequenced wild-type strain known as LT2 (McClelland, M. et al 2001). A list of the strains used in these experiments is shown in **Table 2** and all of them were lab stocks.

Table 2. Bacterial strains

Strain	Genotype	Comments
TH4527	Wild type (wt)	*Salmonella enterica* serovar Typhimurium, LT2
TH7180	*rne-6*	Ile207Ser, ts at 43 ºC
TH7589	*rne-9*	Ile207Asn, ts 43 ºC
TH7620	*rne-6, STM1551-1*[1]	Val45Met, growth at 43 ºC
TH7618	*rne-6, vacB-2*[1]	Δaa 524-535, growth at 43 ºC
TH7622	*rne-9, vacB-3*[1]	Arg446Pro, growth at 43 ºC
TH7625	*rne-9, vacB-4*[1]	Ile444Ser, growth at 43 ºC
TH7630	*rne-9*, unidentified suppressor	
TH8018	*rne-6, STM1551-1* (wt) linked to EZ-Tn5	97% linkage, Kan^R
TH8035	*rne-9 vacB* (wt) linked to EZ-Tn5	36% linkage, Kan^R
TH8122[2]	*rne-6, STM1551-1* linked to EZ-Tn5	97% linkage, Kan^R
TH8123[2]	*rne-6, vacB-2* linked to EZ-Tn5	36% linkage, Kan^R
TH8124[2]	*rne-9, vacB-3* linked to EZ-Tn5	36% linkage, Kan^R
TH8125[2]	*rne-9, vacB-4* linked to EZ-Tn5	36% linkage, Kan^R

1. Suppressor studied in these experiments. **2.** Constructed during this work.

Transductions

All transductions were made using phage P22 (HT105 *int*) (Schmieger and Backhaus, 1973). All donor strains were previously constructed transducing the original suppressed strain with EZ-Tn5 hop pool generated in TH4527, selecting for Kan^R and screening for ts phenotype at 42 ºC.

A P22 lysate grown on donor strains was made mixing 1 ml of overday culture with 100 µl of P22 lysate grown on wild type LT2. 4 ml of soft agar, TTA-LB (7g agar, 10 g tryptone, 8 g NaCl, 1 g glucose, filled up to 1 liter with distilled water, autoclaved and supplemented with 45 % LB) were added to the mixture and poured right away on LA plates. Then, after one night at 30 ºC, the soft agar from the several plates was collected in a Falcon tube with 4.5 mL of LB and vortex until an even slurry was obtain. Finally, the tubes were centrifuged for 10 minutes (3000 x g) and the supernatant was filtered using a 0.2 µm filter, obtaining the P22-donor strain lysate (pfu/ml was approximately 4.2×10^{11}).

This lysate was used to transduce the different recipient strains by mixing 1 ml of overnight culture with 15 µl and 50 µl of the lysate leaving them for 1 hour incubation at 30 ºC. 100 µl of each mixture were plated onto LA + Kan plates, selecting there for Kan^R. Plates were incubated for one night at 30 ºC. The day after, the transductants were re-streaked on the same kind of selective plates for cleaning from phage.

To test the phenotype of each transductant, they were screened on LA plates both at 43 ºC and at 30 ºC.

PCR amplification and DNA sequencing

The genome sequence of *S. typhimurium* available on the NCBI database (accession number NC_003197) was used to design oligos for the PCR amplification of the STM1551 and *vacB* genes. The design oligos were ordered from Sigma-Aldrich (sequences in **Table 3**).

Table 3. Primer sequences

Primer	Sequence 5' → 3'	PCR settings
STM1551 fw	TTTAAACTGTTCCTGTATGG	Annealing temperature: 60 ºC
STM1551 rv	CCTTCGTGAAGGATGCTTTT	Time of extension: 1 minute
vacB-2 fw	GTTGACAGGCTGTGTATGGT	Annealing temperature: 54 ºC
vacB-2 rv	TGCGTGGATGCCGTAAATCA	Time of extension: 1.5 minutes
KAN-2 FP1	ACCTACAACAAAGCTCTCATCAACC	
R6KAN-2 RP1	CTACCCTGTGGAACACCTACATCT	

DNA samples used in the PCR amplification were obtained by the dilution of fresh colonies in 100 µl of sterile water, and then boiled for 5 minutes. The PCR reaction was carried out using PuReTaq Ready-To-Go PCR beads (GE Healthcare, Uppsala) following the manufacturer protocol. The final volume of each reaction was 25 µl with 0.4 nM forward and reverse primers and 2 µl of DNA sample.

The PCR program used was the following one: first denaturation at 95 ºC for 5 minutes, and then a series of 30 cycles of 95 ºC for 30 seconds, 60 or 54 ºC for 30 seconds and 72 ºC for 1 or 1.5 minute(s), depending on the pair of primers. The final extension was done at 72º for 7 more minutes.

PCR products were purified before sending them to sequencing. I used the QIAquick PCR purification kit (Qiagen, VWR International AB, Stockholm) following the instructions provided by the manufacturer and eluting in 30 µl EB buffer. To be sure that the samples were good enough for sequencing, the DNA concentration was determined using a Nanodrop NO-1000 spectrophotometer (Nanodrop, Wilmington). Each sample had around 20 - 80ng of DNA per microliter. The actual sequencing was performed at the Macrogen sequencing facilities (Macrogen, Seoul, South Korea) using aABI3730XL machine.

The sequencing files were edited and studied with BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and alignments were done with Clustal W alignment application implemented in BioEdit.

Gel electrophoresis

Amplification products were visualized using 1 % agarose gel electrophoresis with 1 %Tris-acetate-EDTA buffer (40 mM Tris acetate and 2 mM $Na_2EDTA{\cdot}2H_2O$, pH 8.5). The gel was stained with ethidium bromide (EtBr) to determine that the amplification was correct.

The samples were diluted with 6x loading dye (Fermentas) to a final concentration of 1x before loading them on the gel. The marker used for size determination was the 1kb DNA Ladder (Fermentas, Thermo Fisher Scientific).

Extraction of genomic DNA

To extract DNA from a given strain, 0.5 mL of overnight culture were prepped using the Epicentre Masterpure^{TM} DNA purification Kit (Epicentre® Biotechnologies), following the general instructions and eluting in 30 µl of EB buffer.

The final DNA concentration was measured in the Nanodrop NO-1000 spectrophotometer (Nanodrop, Wilmington).

Enzyme digestion of genomic DNA

To chop the genomic DNA, I used the EcoRI fast digest (Fermentas, Thermo Fisher Scientific). The reaction had the following components: 5µl of 10x fast digest buffer, 5 µg/µl of DNA and 5µl of enzyme, filling out to a final volume of 50 µl. The mixture was put at 37 ºC for 10 minutes for digestion and right after that at 80 ºC for 5 minutes for inactivation.

Ligation of DNA fragments

The ligation reaction was carried out with the T4 DNA Ligase (Fermentas, Thermo Fisher Scientific) in a reaction with 1 µl of buffer T4, 5 µl of digested DNA and 2 µl (10u) of T4 ligase in a total of 10 µl. The incubation was for 1 hour at room temperature and the inactivation of the enzyme at 70 ºC for 5 minutes.

The ligation product was purified in order to remove all the salts with the QIAquick PCR purification kit (Qiagen, VWR International AB, Stockholm) in the same way as in the PCR product's purification. The DNA concentrations of the products were measured with the Nanodrop NO-1000 spectrophotometer (Nanodrop, Wilmington).

Transformation of plasmids

Transformation was done adding 2 µl of plasmid DNA (between 20 - 30 ng) to a 25 µl of electrocompetents TransforMax^{TM} EC100D pir^{+} cells (Epicentre® Biotechnologies) and filling with 25 µl of 10% glycerol. The mixture was place in a cold 0.1 cm cuvette (Cell Projects, UK) and the electroporation was performed in a Bio-Rad Gene Pulser with a Pulse Controller (settings: 1.8 kV, 25µF, 200 Ω). After each pulse, 1 mL of LB was added and incubated shaking at 37 ºC for 1 hour. The cells were plated out onto LA-Kan plates to rescue the Kan^{R} clones.

Plasmid purification and determination of Tn5 position

To extract the plasmid from the cells I used the QIAprep Spin Miniprep Kit (Qiagen, VWR International AB, Stockholm) and a 4 mL of overnight culture following the given instructions and eluting in 30 µl of buffer EB. The final concentration was measured in Nanodrop NO-1000 spectrophotometer (Nanodrop, Wilmington).

To determine the position of insertion of the Tn5 I sent off the plasmid DNA for sequencing at Macrogen sequencing facilities (Macrogen, Seoul, South Korea) with the primers (**Table 3**) from the EZ-Tn5™ <R6K*γori*/KAN-2> Tnp Transposome™ Kit (Epicentre® Biotechnologies). Sequences were blasted on the NCBI webpage (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to find the position.

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