CENTRO DE INVESTIGACIÓN Y DE ESTUDIOS AVANZADOS DEL INSTITUTO POLITÉCNICO NACIONAL

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DEPARTAMENTO DE GENÉTICA Y BIOLOGÍA MOLECULAR

"Análisis de la interacción *in vivo* de Ribonucleasa II, Ribonucleasa PH, y Degradosoma de RNA en *Escherichia coli*"

Tesis que presenta:

Biol. NOÉ ARROYO VÉLEZ

Para obtener el grado de

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En la especialidad de

Genética y Biología Molecular

Director de Tesis

Dr. Jaime García Mena

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ABBREVIATIONS.

RNAD	RNA Degradosome
FRET	Förster resonance energy transfer
CFP	Cyan fluorescent protein
YFP	Yellow fluorescent protein
RNase PH	Ribonuclease PH
RNase II	Ribonuclease II
PNPase	Polynucleotidephosphorylase
RNase R	Ribonuclease R
RhlB	ATP-dependent DEAD-box RNA helicase B
RNase E	Ribonuclease E
Eno	Enolase
mRNA	Messenger RNA
tRNA	Transfer RNA
rRNA	Ribosomal RNA
E. coli	*Escherichia coli*

RESUMEN

En *Eschericha coli* el degradosoma de RNA (RNAD) lleva a cabo la mayor parte de la degradación regulada del RNA Aunque el RNAD presenta componentes canónicos (RNasa E, Helicasa Rhlb, PNPasa y Enolasa), también es capaz de interaccionar con proteínas alternativas, principalmente con otras ribonucleasas como RNasa PH y RNasa II modificando de esta forma su estructura, función y especificidad por los RNAs. La interacción entre componentes canónicos (RNasa E) y no canónicos (RNasa II y RNasa PH) del RNAD ha sido comprobada previamente mediante experimentos *in vitro* por lo que resulta importante comprobar tales interacciones *in vivo*. En este trabajo se trataron de evaluar las interacciones entre la Ribonucleasa E y las Ribonucleasas II y PH *in vivo* mediante análisis FRET. Para llevar a cabo este objetivo se construyeron cepas de *E. coli* que contienen fusiones cromosomales de las proteínas RNasa PH o RNasa II, fusionadas a la proteína fluorescente phi-Yellow (phi-YFP) que funciona como aceptor de fluorescencia y de la proteína RNasa E o su versión trunca en el extremo C-terminal incapaz de ensamblar el RNAD (701), fusionadas a una proteína fluorescente Cyan (CFP) que funciona como donadora de fluorescencia por medio de recombinación homóloga. Las cepas construidas fueron comprobadas mediante PCR y fluorimetria. Finalmente se evaluó la transferencia de energía entre las moléculas fluorescentes fusionadas a las ribonucleasas en cuestión mediante fluorimetría de punto final y se calculó la eficiencia aparente de FRET (FRET app) sin encontrarse resultados concluyentes ni replicables que comprobaran las interacciones *in vivo* entre la ribonucleasa E y las ribonucleasas II y PH. Estos resultados fueron adversos como consecuencia de los bajos niveles de fluorescencia detectados para el fluoroforo phi-YFP, esto podría deberse a que las ribonucleasas fusionadas a la proteína phi-YFP (RNasa II y RNasa PH) presenten bajos niveles de expresión o a otros fenómenos que impiden la cuantificación de la fluorescencia de la proteína phi-YFP. El alcance de este proyecto impide profundizar más a este respecto sin embargo resulta fundamental la realización de más pruebas para identificar y corregir los problemas que impiden el análisis FRET y por lo tanto la caracterización de las interacciones entre las ribonucleasas de interés.

ABSTRACT

In *Eschericha coli* the RNA degradosome (RNAD) accomplish most of the regulated degradation of RNA. Even though RNAD presents canonical components (RNase E, Helicase RhlB, PNPase y Enolase), is also capable to interact with alternative proteins, mainly with other ribonucleases like RNase PH and RNase II thus modifying its structure, function and specificity for certain RNAs. Interactions between canonical (RNase E) and non-canonical (RNase II and RNase PH) components of RNAD have been proved previously *in vitro* so it is very important to corroborate such interactions *in vivo*. In this work we tried to evaluate the interactions between Ribonuclease E and Ribonucleases II and PH *in vivo* by FRET analysis. To achieve this goal we constructed *E. coli* strains carrying chromosomal fusions of native RNase PH or RNase II, fused to the fluorescent protein phi-Yellow (phi-YFP) which functioned as fluorescence acceptor and RNase E protein or its C-*terminal* truncated version which cannot be assembled in a functional RNAD (701), fused to the Cyan Fluorescent Protein (CFP) that functioned as a donor of fluorescence by recombineering method. Constructed strains were corroborated by PCR of recombined genes and Fluorometric assay. Finally it was evaluated the energy transfer between fluorescent proteins fused to the ribonucleases by end-point Fluorometric assay. Furthermore it was calculated the apparent FRET efficiency (FRET app) without obtaining conclusive or replicable results that corroborate interactions *in vivo* between ribonuclease E and ribonuclease II and PH. These results were adverse as a consequence of the low levels of fluorescence detected for the phi-YFP fluorophore, this could be due to the ribonucleases fused to the phi-YFP protein (RNase II and RNase PH) present low levels of expression or due to other phenomena that prevent the quantification of the fluorescence of the protein phi-YFP. The scope of this project impedes further exploration in this regard, however, it is essential to carry out more tests to identify and correct the problems that impede the FRET analysis and therefore the characterization of the interactions between the ribonucleases of interest.

I. INTRODUCTION

In bacterial cells, protein synthesis can be regulated at any of three phases: translation, transcription, and mRNA degradation. RNA stability has a key role in genetic expression regulation. The continuous degradation and synthesis of mRNAs not only give rise to metabolic variations required as cells grow and reproduces, but also rapid adaptation to changing environmental conditions (Arraiano *et al.,* 2010a). From fragile messenger RNA to the more stable noncoding RNAs, all molecules are eventually degraded. Elimination of unnecessary transcripts involves RNAs whose expression is no longer required, and the removal of defective RNAs. Moreover, it contributes to recycling of nucleotides in the cell (Andrade *et al.,* 2009). Processing, degradation, decay and quality control of RNA is controlled by ribonucleases (RNases), and requires the combined action of endoribonucleases (endo-RNases) and exoribonucleases (exo-RNases) (Carpousis A. J., 2007).

Most of mRNA decay starts with internal cleavage by endonucleases leading 5' fragments, which no longer have a protective stem-loop at their 3'end and are therefore susceptible to rapid 3'-exonucleolytic degradation. The 3'-fragments generated by internal cleavage regularly possess a protective stem-loop at its 3'-end which difficult processive 3'-exonucleolytic degradation and so, successive rounds of endonucleolytic cleavage are required until resulting fragments length allows its degradation by oligoribonucleases (Bandyra *et al., 2013*; Baker and Mackie, 2003). To complement the activity of endonucleases, bacteria have a set of 3' exoribonucleases which function by one of two mechanisms. These mechanisms are either hydrolytically and irreversibly to yield nucleoside monophosphate products or phosphorolytically to produce nucleoside diphosphates in a reversible reaction. Polynucleotide phosphorylase (PNPase) and Ribonuclease PH (RNase PH) are prototypical representatives of the former group; in the second group principal enzyme are members of the RNR superfamily and in *E. coli* are represented by Ribonuclease II (RNase II) and Ribonuclease R (RNase R) (Hui *et al.,* 2014).

On regard to endoribonucleases, Ribonuclease E (RNase E) is a primary enzyme involved in decay and degradation of nearly every transcript as well as messenger (mRNA), transfer (tRNA), and ribosomal (rRNA) RNAs processing (Hammarlöf *et al.,*

2015). *Escherichia coli* RNase E makes most internal cleavages of RNA in both 5'-end sensing and 5'-bypass pathways, this process is the first step in mRNA degradation process (Bandyra *et al*., 2013). In some works trying to isolate and purify RNase E, this protein was found included in a multiprotein complex that was called RNA degradosome (RNAD) (Carpousis *et al.,* 1994, Py B *et al.,* 1994, Causton *et al.,* 1994). In *E.coli* RNase E contains in its N-terminal end a hydrolytic endoribonuclease function domain, an S1 RNA binding domain that forms a pocket that specifically interacts with the terminus of 5´ monophosphorylated RNA and a channel that only permits access to the catalytic site of single-stranded RNA conferring specificity for single-stranded chains (Carpouis A. J., 2007). In its C-terminal end RNase E presents RNA binding domains (RBD and AR2), a membrane targeting sequence and binding sites to the phosphorolytic exoribonuclease PNPase, the ATP-dependent DEAD-box RNA helicase (RhlB) and the glycolytic enzyme enolase (Eno) (Khemici *et al.,* 2005). Union between these proteins and their corresponding binding sites forms the canonical RNA-degradosome in its minimal functional composition, which accomplish messenger RNA degradation, and process and matures structured RNAs precursors into their active forms (Worral *et al.,* 2008).

The cooperation of components of the degradosome facilitates substrate turnover, their assembly can be highly flexible and entail intriguing functional consequences for substrate capture and processing (Bandyra *et al.,* 2013). Moreover the degradosome can form a stable complex with the 70S ribosome and polysomes, suppressing the catalytic activity of RNase E for 9S RNA processing, whereas there is little effect on single-stranded substrates cleavage, suggesting that the enzyme maintains its ability to cleave unstructured RNAs when associated with the ribosome (Tsai *et al.,* 2012).

Additionally the RNAD is reported to present other minor proteins as GroEL and DnaK (Miczak *et al.,* 1996) and interact with trans-acting proteins like Hfq, RraA and RraB. All these interactions could induce changes in RNA degradosome structure and composition, modulating its operation and causing alterations in RNA decay patterns (Gao *et al.,* 2006). It has been shown that RraA can bind to the RNA helicase RhlB and the two RNA-binding sites in the degradosome-scaffolding domain of RNase E, affecting multiple components of the RNA degradosome (Gorna *et al.,* 2010). In other cases RNA degradosome remodeling can be induced by the

expression of components related with conditions of stress, suggesting that this remodeling might be driven by increasing the concentration of a non-canonical component (Aït-Bara *et al.,* 2015).

In addition to PNPase and RNase E, *E. coli* cells possess other ribonucleases with important roles in RNA processing and degradation that can associate with RNA-degradosome. Co-immunoprecipitation and gel affinity studies show that hydrolytic exoribonuclease RNase II can be associated with proteins that form the canonical RNA-degradosome, indicating that RNase II may be associated and be part of RNA-degradosome structure. (Lu and Taghbalout, 2014).

RNase II was described as transcribed from the 1794 bp *rnb* gene (Spahr P. F., 1964). This gene encodes a protein of 598 amino acid residues, with a calculated molecular mass of 67,583 (Zilhão *et al.,* 1993). RNase II is a membrane-associated protein that binds the cytoplasmic membrane via an amino-terminal amphipathic α-helix, fluorescence localization studies showed it is present within the cell as a membrane-associated ordered structure (Lu and Taghbalout, 2013). *E. coli* RNase II is the prototype of the RNase II-family of enzymes that processively degrade RNA in the 3' to 5' direction with a hydrolytic activity releasing 5'-nucleotide monophosphates. It is the major hydrolytic enzyme, responsible for 90% of the exoribonucleolytic activity in crude extracts, its activity is sequence independent but it is sensitive to secondary structures (Arraiano *et al.,* 2010b). Besides, RNase II has four domains: two N-terminal cold shock domains (CSD1 and CSD2) involved in RNA binding, one central RNB catalytic domain, and one C-terminal S1 domain responsible for the binding to the substrate (Amblar *et al*., 2006).

In *E. coli* cell extracts most RNA degradation is performed by the hydrolytic pathway (Deutscher *et al.,* 1991), suggesting a hydrolytic ribonuclease as part of the RNA-degradosome, this supports studies pointing to RNase II as part of multiprotein complex RNA-degradosome. Deletion studies indicate that although RNase II is an important enzyme for survival and regulation, cells require the combined action of multiple ribonucleases for accurate function. Cells lacking both PNPase and RNase II are related to an *in vivo* accumulation of RNA species 100-1500 nucleotides long and to an increase in the half-life of total mRNA (Donovan and Kushner, 1986).

Additionally transcriptomic analysis showed deletion of RNase II, RNase R and PNPase changed the expression of genes related with flagellum assembly, motility and biofilm formation, many of such changed transcripts were down-regulated in the exoribonuclease mutants when compared to the wild-type control (Pobre and Arraino, 2015). All above suggest ribonucleases work as an intricate network whose interactions determine their function, this emphasizes the importance of knowing how many and how they interact within *E. coli* cells *in vivo*.

On the other hand, it has been found evidence suggesting the interaction of a second phosphorolytic ribonuclease called RNase PH. *In vitro* and *in vivo* studies confined this interaction to an 80 amino acids basic region in RNase PH and an 16 amino acids acidic region (1021-1036) in C-terminal domain of RNase E (Figure 1), overlapping with PNPase union site (Perez-Medina *et al.,* 2014, Duran-Figueroa *et al.,* 2006). RNase PH was first discovered as a 45-50 kd phosphate-requiring exonuclease that catalyzes the final trimming of 3' terminus of tRNA precursors (Deutscher *et al.,* 1988). Subsequent studies demonstrated it is an essential enzyme for the processing of tRNA precursors (Kelly *et al.,* 1992) and play a key role that affects ribosome metabolism in such way that this function cannot be taken over by any of the hydrolytic exoribonucleases present in the cell (Zouh and Deutscher, 1997). For 16S rRNA, degradation during starvation begins with shortening of its 3′-end in a reaction catalyzed by RNase PH. In the absence of this RNase, there is no 3′ end trimming of 16S rRNA and total RNA degradation is greatly reduced (Basturea *et al.,* 2011) in addition this enzyme digests through RNA duplexes of moderate stability and has been related with RNA fragments degradation that have short regions of secondary structure (Jain C., 2012). Phylogenetic analysis revealed PNPase (PNP1 and PNP2) domains share substantial sequence and structural homology with RNase PH and that PNP2, and RPH are sister taxa which arose through duplication of the ancestral PNP1 domain (Sokhi *et al.,* 2014). Further RNase PH enzyme presents redundant functions with PNPase and RNase R, this may indicate all these enzymes could participate in a common pathway (Jain C., 2012).

RNA-degradosome is a highly dynamic heterogeneous complex that can change by transient recruitment of its components in response to changes in environmental

conditions (Prud'homme-Généreux *et al.,* 2004; Marcaida *et al.,* 2006). It has also been demonstrated by studies testing *in vivo* RNAD assembling, that also found variations in RNAD proteins expression in response to changes in growth medium from rich LB to minimal M9 medium (Domínguez-Malfavón *et al*., 2013). Changes in structure composition of RNA-degradosome could modulate its function and alter RNA decay patterns (Gao *et al.,* 2006) modifying in this way gene expression profiles.

Among the different methods to study protein-protein interaction Förster resonance energy transfer (FRET) or fluorescence resonance energy transfer (FRET), assay is a convenient method to demonstrate close proximity of molecules *in vivo*. FRET is a physical phenomenon described by Thomas Förster (Förster T., 1948*)* that comprises the interplay between two fluorescent proteins in close proximity (10-100 Å). In such phenomenon a fluorophore in its exited state called donor, transfers energy in a non-radiative form to a second fluorophore called acceptor (Zheng J., 2006). Donor molecule can be exited with light pulses in a specific wave length. Absorption spectrum of acceptor overlaps with emission spectrum of donor. The efficiency of energy transfer decreases with the sixth power of the inverse distance (Helms V., 2008) hence FRET results an excellent reporter of close proximity between molecules. Several *in vivo* studies in *E. coli* have successfully used FRET analysis to characterize interactions between molecules coupled to fluorophores. Examples of these are the discovery of the interaction between monofunctional transpeptidases PBP2 and PBP3 when both are present at midcell (Van-der-Ploeg *et al*., 2013), the study of selective interaction of lactose permease (LacY) enzyme with its surrounding phospholipids Pyr-PE and Pyr-PG (Suárez-Germà *et al*., 2012) and the validation of *in vivo* assemble of RNAD through the analysis of interactions among RNase E, PNPase Enolase and RhlB (Domínguez-Malfavón *et al.,* 2013). All this point FRET analysis could be used as a correct tool to characterize interactions among RNAD canonical (RNase E) and non-canonical (RNase PH or RNase II) proteins.

II. SCOPE

We study the interaction *in vivo* among RNAD's protein RNase E, RNase II and RNase PH of *E. coli* using FRET analysis with spectrofluorometric and microscopy approaches. Based on measured distances it will be set a model of RNAD structure when associated to RNase PH and RNase II.

III. RATIONALE

Regulation processes of genetic expression are essentials for cell survival and development, allowing synthesis of necessary compounds and repression of compounds useless in specific situations. All this yield an efficient and suitable distribution of resources that confers adaptive capacity to cell. RNAD participates in decay, degradation and maturation of RNA playing a key role in posttranscriptional regulation processes. Although there is a well-defined canonical RNAD, this complex is a dynamic structure whose composition may vary depending on environmental conditions. It has been proven the interaction between canonical and non-canonical (RNase PH and RNase II) ribonucleases based on *in vitro* studies therefore, it results very important to test the existence of such interactions *in situ* since variations in RNA-degradosome components may modify its function. The discovery of RNA-degradosome structure and its components may provide us a useful tool in understanding its function and relevance in cell survival and development.

IV. HYPOTHESIS

In *Escherichia coli*, RNase II and RNase PH interact and stay in proximity to RNase E *in vivo*, assembling in an alternative RNA-degradosome.

V. GENERAL AIM

To demonstrate that Ribonucleases RNase II and RNase PH interact and stay in proximity with the canonical RNAD *in vivo* and may be part of the multi- complex RNA-degradosome structure in *Escherichia coli*.

VI. SPECIFIC AIMS

Specific Aim 1. To re-evaluate and characterize each of the chromosomal fusions previously generated for evidence of proximity studies in the RNAD (LEC, LE701C and LEBNY-Km).

Specific Aim 2. To transform the strains LEC and LE701C with plasmid pCP20 and induce protein expression of flippase in order to suppress the Km resistance cassette by FRT sites recombination.

Specific Aim 3. To generate recombining cassettes containing phi-YFP from previously generated plasmid pL05CtY

Specific Aim 4. To construct chromosomal fusions of ribonucleases RNase II and RNase PH with phi-Yellow Fluorescent Protein (phi-YFP) at their C-ends for FRET analysis.

Specific Aim 5. To analyze *in vivo* the interaction between RNase E-Cyan Fluorescent Protein (CFP) and RNase II, and RNase PH by FRET assay.

VII. METHODS

1. Bacterial strains and media

E. coli strains, plasmids and oligonucleotides required in this work are listed in Tables 1, 2 and 3 respectively. Media were prepared as described [Sambrook and Russell, 2001]. Incubation was done at 32° C, with the exception of DH5-α grown at 37º C. When necessary, the media were supplemented with 50 µg/ml kanamycin, 50 µg/ml of ampicillin or 12.5 µg/ml chloramphenicol.

2. Experimental *in silico* design

The nucleotide DNA sequences of target genes encoding RNase PH and RNase II were obtained from the GenBank database (Gi: 15829254 and 556503834). The DNA sequences encoding the phi-yellow fluorescent protein (phi-YFP), were obtained from the on line database of commercial supplier (Evrogen). Fusions between RNases resident enzymes and selected Föster Resonance Energy Transfer proteins (FRETP) were designed *in silico* using the Vector NTI v.6.0 software

(Invitrogen). Codon analysis of the fluorescent protein coding sequences to ensure proper protein expression in *E. coli*, were done employing translate tool (http://web.expasy.org/translate/).

3. Plasmid DNA isolation by Alkaline Lysis protocol (MINIPREP) [Domínguez-Malfavón L., 2013]

A single bacterial colony was transferred to 5 mL of culture media with the proper antibiotic, and was grown at 32º C overnight. The culture was then pelleted in a 1.5 mL Eppendorf tube. The pellet was resuspended in 200 µL of cold Solution I (per liter: Glucose 9.0 g, 25.0 mL Tris 1M pH 8.0, 2.0 mL of EDTA 0.5 M pH 8.0). Cells were lysed with 400 µL of fresh made Solution II (NaOH 0.2 N, SDS 1%). The solution was mixed by inverting the tubes and was stored in ice for 4 minutes. Neutralization was done by adding 300 µL of cold Solution III (for 100 mL; 60.0 mL of potassium acetate 5 M, 11.5 mL of acetic acid and 28.5 of distilled water) and mixed gently by inversion. Tubes were centrifuged at maximum speed during 5 minutes and supernatant were transferred to a new tube. DNA precipitation was done with 480 µL of isopropanol during 15 minutes at room temperature. DNA was by centrifugation at maximum speed during 5 minutes. DNA pellet was washed with 1.0 mL of 70 % ethanol and air dried during 5 minutes. DNA was resuspended in 50 µL of sterile deionized water.

4. Phenol DNA extraction [Adapted from Domínguez-Malfavón L., 2013]

5 mL of culture media were grown at 32º C overnight. Then the culture was pelleted in a 1.5 mL Eppendorf tube. The pellet was resuspended in 500 µl of distilled water. A volume of phenol were then added and mixed vigorously with vortex and centrifuged at 6000 rpm during 5 minutes. Aqueous phase was recovered and transferred to a new tube. 500 µl of chloroform were added, mixed vigorously and centrifuged for 5 minutes at 6000 rpm. Aqueous phase were recovered and transferred to a new tube and DNA was precipitated with 1/10 of 3 M sodium acetate and 2 volumes of absolute ethanol during 1 hour at -20ºC. DNA was recovered by centrifugation at maximum speed during 5 minutes and the pellet was washed with 1.0 mL of 70% ethanol. DNA was resuspended in 50 µL of deionized water.

5. PCR amplifications

All PCR reactions were carried out using the Taq DNA Polymerase system (Thermo scientific). Each reaction were set to a volume of 50 µL and contained 1X Buffer, 2 mM MgCl, 0.2 mM dNTPs, 0.025 u/µl of Taq polymerase, 0.2 µM of oligonucleotide forward, 0.2 µM of oligonucleotide reverse and 10-50 ng of DNA template. Using the appropriate annealing temperature for each pair of primers calculated using the Vector NTI v.6.0 software (Invitrogen). 30 amplification cycles were applied to all reactions. Thermal cycling conditions are described in Table 4.

6. Gel electrophoretic assays

For visualization and separation of genomic DNA, plasmid DNA and PCR products, electrophoresis assays were carried out in 0.8% agarose gels added with 0.8µl of Midori Green Nucleic Acid Staining Solution (Bulldog Bio) which were placed in an electrophoretic chamber with TBE 1X buffer (89mM of Tris, 89mM of borate and 2mM EDTA at pH 8.2). Gene Craft 1kb ladder were used as gen ruler when needed. Pictures for analysis were taken with Molecular Imager Gel Doc XR System (BioRad) and images processed with Image Lab software (BioRad).

7. Restriction enzymes assays

DNA was cut using the conditions recommended by the correspondent manufacturer. All restrictions assays were carried out employing 5 U of restriction enzyme for each µg of DNA.

8. Competent Cells [Adapted from Sambrook and Russell, 2001]

A single bacterial colony was grown in 5 mL of LB medium at 32º C with agitation overnight. The next day 1 mL of this culture were used to inoculate 50 mL of LB medium in a 500 mL Erlenmeyer flask and were grown until an OD_{600} of 0.4. The culture was chilled on ice and centrifuged in an Eppendorf (5804 R) centrifuge stabilized to 4º C (5 minutes, 7000 rpm). Cells were resuspended in 5 mL of chilled

sterile $CaCl_2$ (100 mM) and incubated in ice for 10 minutes. Then cells were pelleted in an Eppendorf (5804 R) centrifuge stabilized to 4º C (5 minutes, 7000 rpm). Cells were resuspended in 1 mL of chilled sterile $CaCl_2$ (100 mM) and incubated in ice for 30 minutes.

9. Transformation with plasmid DNA [Adapted from Domínguez-Malfavón L., 2013]

Approximately 70 ng of plasmid DNA were added to 200 µL of competent cells, mixed by pipetting and incubated in ice-water bath for 30 minutes, then cells were submitted to a thermal shock (60 seconds, 42º C) and incubated in ice again for 2 minutes. Cells were recovered in 0.8 mL of LB medium pre-warmed to 32° C and incubated at 32° C for 1 hour and spread in LB plates added with correspondent antibiotic

10. Induction of Flippase [Adapted from Domínguez-Malfavón L., 2013].

A single colony were added to 5 mL of LB medium supplemented with ampicillin (50 µg/mL), and incubated overnight 32° C under agitation. The overnight culture was diluted 1:10 in 5 mL of LB (Ap, 50 µg/mL) and incubated for 8 hours at 32° C under agitation. Then the culture was incubated at 42º C during 15 more minutes. From this second incubation, serial dilutions were made and 100 µL of each dilution were spread in LB plates added with ampicillin (50 µg/mL).

11. Antibiotic resistance selection

After being transformed and induced, colonies grown on the plates were taken with a toothpick and seeded in gridded plates, each colony was taken and seeded in one square of a gridded plate of LB medium later the same toothpick was used to seed the same colony in the corresponding square of a gridded plate of LB medium added with kanamycin. Plates were incubated overnight at 32º C. Later search for the antibiotic sensitive or resistant colonies were done.

12. Purification of recombining cassettes [Adapted from Domínguez-Malfavón L., 2013].

All recombining cassettes were amplified as mentioned above (PCR conditions). Using the appropriate annealing temperature for each pair of primers calculated using the Vector NTI v.6.0 software (Invitrogen).

After amplification, PCR products were purified using Wizard SV Gel and PCR Clean-Up System (Promega) as manufacturer instructions and resuspended in nuclease free water. DNA samples were cut with 5 units of Dpn I restriction enzyme (New England Bio Labs Inc.) per microgram of DNA as manufacturer instructions for one hour at 37° C.

DNA from samples was purified as follows, a volume of phenol-chloroform-iso amylic alcohol (25:24:1) was added to the samples, mixed vigorously with vortex and centrifuged at 6000 rpm during 5 minutes. Aqueous phase was recovered and transferred to a new tube. A volume of chloroform-isoamyl alcohol (24:1) was added, mixed vigorously and centrifuged for 5 minutes at 6000 rpm. Aqueous phase was recovered and transferred to a new tube. For precipitation 1 µL of glucogen (1µg/µL) was added for each microgram of DNA in the samples and mixed by pipetting several times, then 2 volumes of absolute ethanol were added and mixed by inversion. Tubes were placed at -20ºC overnight and DNA was recovered by centrifugation at maximum speed during 5 minutes. The resulting pellets were washed with 1.0 mL of 70% ethanol. DNA was resuspended in 50 µL of deionized water.

13. Induction of the RED recombination system and preparation of recombinant cells [Domínguez-Malfavón L., 2013]

A single bacterial colony was grown in 5 mL of LB medium at 32º C with agitation overnight. The next day 1 mL of this culture was used to inoculate 50 mL of LB medium in a 500 mL Erlenmeyer flask and was grown until an OD600 of 0.5. 10 mL of this culture were transferred to a 125 mL Erlenmeyer flask, and the RED system was induced for expression at 42º C during 15 minutes. Immediately after induction, the flask was quickly chilled in an ice bath. 10 mL of non-induced cells were also

chilled in ice as a control. Induced and non-induced cells were centrifuged in an Eppendorf (5804 R) centrifuge stabilized to 4º C (8 minutes, 7000 rpm). Cells were resuspended in 1 mL of chilled sterile deionized water and transferred to a previously chilled Eppendorf tube. Cells were washed 3 times with chilled deionized water (centrifuging and resuspending). After the final wash bacteria were resuspended in 100 mL of chilled deionized water and kept in ice.

14. Electroporation [Adapted from Domínguez-Malfavón L., 2013]

300 ng of recombining cassette DNA were added in a previously chilled electroporation 0.1 cm cuvette (BioRad). Then 50 µL of electrocompetent cells were added to the cuvette and immediately electroporated with a “Micropulser” with voltage of 1.8 kV and 200 ohms for a constant time longer than 5 mseconds. Immediately after, bacteria were recovered in 1 mL of LB medium and incubated at 32º C for 1 hour. Selection of recombinant bacteria was made in LB medium plates added with chloramphenicol (30 µg/ml) and incubated at 32º C, 48 hours.

15. Spectrofluorimetric assays [Domínguez-Malfavón L., 2013]

Bacteria were grown overnight in LB medium (32° C). Cultures were washed and resuspended in 1X PBS, normalizing to OD600 = 5. Scans were done in a Synergy™ H4 Hybrid Microplate Reader emission scans were done from 440 to 650 nm with excitation wavelength of 433 nm and excitation scans were done from 300 to 490 nm with emission wavelength of 505 nm for cyan fluorescence assays. For yellow fluorescence assays emission scans were done from 530 to 700 nm with excitation wavelength of 525 nm and excitation scans were done from 400 to 550 nm with emission wavelength of 537 nm. For FRET measurements of whole cultures, bacteria were grown overnight in LB medium. FRET assays were performed by endpoint− method Cultures were washed 2 times with 800 µL and resuspended in 600 µL 1X PBS pH 7.4. Samples of 200 µL were read in the donor channel (458/505 nm excitation/emission), acceptor channel (514/537 nm excitation/emission) and the FRET channel (458/537 nm excitation /emission) under agitation at room temperature.

16. FRET calculations

Calculations for FRET efficiency were done following two strategies, in the first, calculations were made as described by Dominguez-Malfavon (Domínguez-Malfavón L., 2013) as follows: $FRET_{app} = A\text{-}A_o$, where A_o is given by the ratio of the signal B1 in the FRET channel and the signal A1 in the acceptor channel of strains containing only the acceptor fusions. A_o= B1/A1, and A is the ratio of the subtraction of the crosstalk C to the signal B2 in the FRET channel and the signal B2 in the acceptor channel of strains containing both acceptor and donor fusions A= (B2-C)/A2. The crosstalk C of the donor is calculated by the ratio of the signal C1 in the FRET channel and the signal C2 in the donor channel of strains containing only the donor fusions. This ratio C1/C2 will be then multiplied to the signal C3 in the donor channel of strains containing both acceptor and donor fusions. C = (C1/C2)*C3. So that: $FRET_{app}$ =[(B2 – C)/A2]-B1/A1].

VIII. Results

This work was accomplished in the Laboratorio de Referencia y Apoyo para la Caracterización de Genomas, Transcriptomas y Microbiomas del Departamento de Genética y Biología Molecular within Cinvestav facilities over a period of 13 months.

1. Experimental Design

In order to provide a description of the interactions among RNase II, RNase PH, and RNAD's RNase E, it were designed a FRET analysis with a single pair of fluorophores, where CFP fused to RNase E wild type or its C-terminus truncated version (701), function as FRET donor and phi-YFP fused to C-terminus of RNase PH or RNase II function as FRET acceptor (Figure 2).

FRET donors were planned to be obtained from two strains (LEC and LE701C) previously generated in our group (Domínguez-Malfavón *et al*., 2013). The first carries an RNase E gene tagged with a six glycine hinge followed by Cyan Fluorescent Protein (CFP) in its C-terminus. The second carry an RNase E gene

truncated in its C-terminal end that cannot assemble a canonical RNAD (Morita *et al*., 2004) tagged with a six glycine hinge and CFP in its C-terminus (Domínguez-Malfavón *et al*., 2013).

As FRET acceptor, phi-Yellow Fluorescent Protein (phi-YFP) were planned to be fused to the end of native RNase PH or RNase II in strains LEC and LE701C. Additionally it was planed the construction of two more strains necessary for FRET calculations that only harbor the FRET acceptor (phi-YFP). Strains LEC and LE701C are derivatives of strain DY329 and therefore express recombination system RED in a temperature dependent way. Final part of native RNases (RNase II and RNase PH) genes were planned to be fused to a genetic construct composed of a 18nt sequence which coding for a 6 glycine hinge followed by phi-YFP gene and a Kanamycin resistance cassette gene by recombineering method (Yu *et al*. 2000).It was planned to obtain the construct from strain LEBNY (Dominguez-Malfavón *et al* 2013) by PCR amplification, afterward electroporate such amplified sequences in cells of DY329, LEC and LE701C strains in combinatorial way (Figure 3) and induce recombination in their chromosomes (Figure 4), generating strains LHY, LBY, LECHY, LECBY, LEC701HY and LEC701BY.

2. Characterization of LEC and LE701C strains.

Genotype of previously generated strains LEC and LE701C (Figure 5) needed to be corroborated in order to ensure their correct function and suitability in this project. To achieve the above cultures of both strains were grown overnight LB medium, harvested and subjected to DNA Phenol extraction (Methods-4 Phenol DNA extraction) followed by PCR essays with oligonucleotides pair CMJKmR-LDM46F which hybridize with the medium parts of Kanamycin resistance gene and RNase E gene respectively (Figure 6), annealing temperature of reactions was set to 60.5° C and extension time set to 90 seconds. Amplified sequences were analyzed in 0.8% agarose gel electrophoresis, 5 μL of reaction were stained with loading buffer and loaded into the gel wells. Reaction carried with LE701C DNA as template showed an amplified sequence of 1600bp. Reaction carried with LEC DNA showed an amplified sequence of 2600bp, both amplified sequences agreed with sizes expected (Figure 7).

Additionally fluorescence of cultures of LEC, LE701C and DY329 strains were measured by spectrofluorimetry as mentioned in methods (15 Spectrofluorimetric assays) in order to test the functionality of fluorescent proteins, assays were done by triplicate. Samples were exited to 458nm and fluorescence measured at 505nm to trace CFP. Bacterial fluorescence was normalized to PBS medium fluorescence. LEC and LE701C strains presented higher levels of fluorescence (Figure 8) when compared to DY329 strain which lack of CFP. Together PCR and spectroflurometry tests confirmed the presence and correct function of CFP in LEC and LE701C strains.

3. Elimination of kanamycin resistance cassette

Genetic constructs intended to fuse in LEC and LE701C genomes contained a kanamycin resistance cassette as selection marker. Since the strains LEC and LE701C already contained a kanamycin resistance cassette attached to the CFP gene, it was necessary to suppress such kanamycin resistance gene from the bacterial chromosome in order to avoid unspecific homologous recombination, this goal was achieved with help of plasmid pCP20 (Cherepanov and Wackernagel, 1995) which encodes for flippase enzyme that recognized and recombined FLP sites present in the beginning and end of kanamycin resistance gene present in strains LEC and LE701C suppressing in this way kanamycin resistance gene and leaving a small FRT scar in the bacterial chromosome (Figure 9).

In order to probe the integrity of pCP20 plasmid sample used in this work, 2 µl of plasmid sample were proved in a .8 agarose gel electrophoresis (Figure 10) Cultures of LEC and LE701C strains were grown overnight in LB medium, 1 mL of this culture were used to inoculate 50 mL of LB medium then cells were made electrocompetents with $CaCl_2$ (Methods-8 Competent Cells) and transformed (Methods-9 Transformation with plasmid DNA) with 70ng of pCP20 plasmid. Transformed cells were recovered in 800 µL of pre-warmed LB medium during 1 hour at 32° C then spread in LB plates added with ampicillin (50 µg/mL). Growth is shown in Table 5. 38 hours later resultant colonies were isolated and a single colony of each transformed strain (LEC and LE701C) were added to 5 mL of LB medium supplemented with

ampicillin (50 µg/mL), and incubated overnight 32° C under agitation and placed 15 minutes at 42° C for induce for flippase expression as mentioned in methods (Methods-10. Induction of flippase) then cells were spread in LB plates. Plates were grown overnight and resultant colonies were seeded in gridded plates of LB medium and LB medium added with kanamycin (Methods-11 Antibiotic resistance selection) (Figure 11). Gridded plates were grown overnight, colonies that grew in LB medium but didn't in LB medium added with kanamycin were identified as sensitive to the antibiotic and preserved in LB plates. In order to corroborate the loss of resistance kanamycin cassette such sensitive colonies were DNA phenol extracted (Methods-4 Phenol DNA extraction) and proved by PCR reaction using oligonucleotides pair LDM42F-LDM46R which hybridize in the medium part of RNase E gene and in the region after the stop codon of RNase E gene respectively, PCR conditions were set to 57.6° C as annealing temperature and extension time set to 90 seconds. 5µL of resulting reactions were proved by 0.8% agarose gel electrophoresis, getting amplification of sequences of 1300 pb for LE701C and 2400pb for LEC (Figure 12) corresponding with the sizes expected. Given that flippase expression process is sufficient to eliminate pCP20 plasmid from bacteria (Dominguez-Malfavon 2013), so that resultant strains sensitive to kanamycin were seeded in LB medium added with ampicillin (50 µg/µl) in order to test for ampicillin sensitivity, all strains proved were unable to grow in medium added with ampicillin corroborating the loss of pCP20 plasmid (Figure 13).Finally plasmid were recovered from bacteria by plasmid isolation (Methods-3 Plasmid DNA isolation by Alkaline Lysis protocol (MINIPREP) for its preservation.

4. LEBNY Characterization

LEBNY strain was selected as template to amplify a genetic construct composed of a 6 glycine hinge, phi-YFP and a kanamycin resistance cassette. This made necessary to corroborate the presence and function of the genes of interest, to achieve this first LEBNY strain was stained with gram staining technique and observed in a Leica DM500 optical microscope with a Leica PLAN 100X/1.25 objective. Bacterial cells appeared as pink bacillus, this agreed with gram negative nature of *E. coli* (Figure 14). Additionally LEBNY strain was seeded in LB plates and

LB plates added with kanamycin (50 μg/μl) in triplicate. All plates seed presented bacterial growth showing that strain was resistant to kanamycin as expected (Figure 15). Finally bacteria were cultured, DNA phenol extracted (Methods-4 Phenol DNA extraction) and proved by PCR reaction with oligonucleotides pair LDM28F-CMJKmR which hybridize in the medium part of Enolase gene and in the medium part of Kanamycin resistance cassette respectively (gene map in figure 16), PCR conditions were set to 60.5° C as annealing temperature and extension time set to 90 seconds, resulting in the amplification of a sequence of 1600 pb approximately (Figure 17) as was expected. All proves mentioned above corroborate the desired genotype and phenotype of LEBNY strain.

5. Oligonucleotide synthesis

Two pairs of primers were designed for recombination assays using oligo 7 software (Molecular Biology insights Inc.) and proved in silico with Vector NTI v.6.0 program (Invitrogen Corporation). Oligonucleotides were designed with 50 nt recombination region in its 5' that is homologous (H1 for sense, and H2 for anti-sense primers) to the specific chromosome site were recombination were directed (end of RNase II or RNase PH), and with 25-30 nt region in its 3' for PCR priming to the sequence desired for amplification, sense primers also presented a sequence which codify for a 6 glycine hinge (18 nt) that separates recombination region from priming region (Figure 18). Forward primers were named NAV01F and NAV03F and their last 30 bp 3' were designed to hybridize with the start codon and 22 bp down river of phi-YFP gene present in LEBNY chromosome, in their 5' the firs 50nt were designed to hybridize with 50nt up river of stop codon of RNase PH and RNase II respectively. Reverse primers were named NAV02R and NAV04R, their last 25 bp in 3' side were designed to hybridize a few base pairs later of the last FRT site that followed the Kanamycin resistance gene present in LEBNY chromosome, in their 5' the firs 50nt were designed to hybridize with the stop codon and 50nt down river of RNase PH and RNase II genes respectively

Designed oligonucleotides were synthesized at 50 nmole scale and purified by PAGE (Sigma-Aldrich) and used in PCR at 10 mM concentration, oligonucleotides

were denatured in a boiling bath during 3 minutes, and fast-chilled on ice prior to use in the PCR assay.

6. Obtaining of recombination cassettes

LEBNY strain was selected as the source of phi-YFP protein, in its genomic context this protein was preceded by the enolase gene and followed by the kanamycin resistance gene flanked by FRT sequences. Oligonucleotides pairs were designed for hybridize to the beginning of phi-YFP sequence and some nucleotides down river from last FRT site. When these oligonucleotides were used in PCR reaction they were planned to add the homologous regions in the ends of the amplified sequences needed for the homologous recombination process in the end of RNase PH and RNase II genes.

In order to generate the recombining cassettes to electroporate in strains LEC and LE701C, cultures of LEBNY strain were grown overnight in LB medium, harvested and subjected to DNA Phenol extraction (Methods-4 Phenol DNA extraction) followed by PCR reaction with oligonucleotides pairs NAV01-NAV02 (For recombination in RNase PH end) (Figure 19) and NAV03-NAV04 (For recombination in RNase II end) (Figure 20) PCR conditions were set to 64.5° C and 66.3° C respectively as annealing temperature and extension time set to 2 minutes for both reactions. Reactions were analyzed by 0.8% agarose gel electrophoresis without obtaining any amplification (Figure 21); several attempts were done changing PCR reaction conditions including annealing temperature, annealing and extension times, salt concentrations and number of cycles without any improvement.

Given that difficulties with PCR reaction could be due to unspecific primers hybridization we were forced to try another strategy. This new strategy (Figure 22) consisted of amplifying a wider region that contained the sequence of interest (phi-YFP/KmR) with a 20nt pair of primers that hybridize several base pairs before and after of sense and antisense recombining primers respectively, the following steps of this new strategy consisted of purifying the amplified sequence and use it as template for PCR reactions with recombining primers NAV01-NAV02 and NAV03-NAV04 trying to increase in this way the probability of specific hybridization.

Following the above mentioned PCR reactions were carried out with oligonucleotides pair LDM28F-LDM43R which hybridize to medium and after regions of enolase gene respectively (Figure 23) PCR conditions were set to 58.3° C as annealing temperature and extension time set to 90 seconds. These reactions were analyzed in 0.8% agarose gel electrophoresis. Genomic DNA of LEBNY strain was used as template and genomic DNA of DY329 strain was used as positive control. In positive control 2 sequences were amplified one of 600 bp as expected and other faint in the gel of 2400 bp that wasn't expected. Reactions with LEBNY showed a single band of the same size of the faint band in DY329 reaction (Figure 24). The latter could indicate the amplified sequence was unspecific. Several attempts were done to increase the specificity of the reaction in order to eliminate the faint band in reactions with DY329. None of the attempts were successful; this let us to think there were troubles with LEBNY strain that impede hybridization of primers. The scope of this project didn't allow us to explore more with this regard so that it became necessary to design an alternative way to obtain phi-YFP protein.

7. Redesign of construction strategy

Due to troubles with original experimental design it was necessary to redesign the strategy of construction. In this new experimental design phi-YFP was obtained from previously generated plasmid pL05CtY (Dominguez-Malfavon 2013) that carries phi-YFP gene under *lac* promoter followed by a chloramphenicol resistance gene transcribed in the same open reading frame as is shown in figure 25. Because of this last it was decided to replace the phi-YFP-KmR cassette proposed in the first strategy of construction (Results-1 Experimental design) for a phi-YFP-CmR cassette in this new that would be obtained by PCR amplification from pL05CtY plasmid, using pairs of primers that add at the beginning and end of amplified cassettes sequences homologous to the desired recombination sites in bacterial chromosomes. Once the cassettes would be obtained they would be purified and electroporated in cells of strains DY329, LEC and LE701C. Finally cells that accepted and recombine the cassettes in their genomes would be selected with the proper antibiotic (Figure 26).

8. Recovery and transformation of plasmid pL05CtY

Cells of strain Top 10 (Invitrogen) were made competent with $CaCl_2$ (Methods-8 Competent Cells) and transformed (Methods-9 Transformation with plasmid DNA) with 1µl of plasmid pL05CtY (130 ng/ µl). Transformed cells were recovered in 1 ml of LB medium during 1 hour at 32° C in constant agitation, and then cells were spread in LB plates added with chloramphenicol (30 µg/ml). Plates were incubated overnight at 32° C The next day resistant colonies were isolated and seeded in plates of LB medium added with chloramphenicol (30 µg/ml) then 5 ml of LB medium were inoculated with a single colony of bacteria harboring pL05CtY plasmid and submitted to plasmid DNA isolation with the Gene Jet Plasmid miniprep Kit used as manufacturer's instructions and analyzed by 0.8 agarose gel electrophoresis in order to confirm that the plasmid was successfully integrated by the Top 10 cells. Once plasmid DNA was seen in agarose gel, sample was restricted with restriction enzymes Hind III and Xho I (New England Biolabs) with 5U of enzyme for µg of DNA sample and analyzed by 0.8% agarose gel electrophoresis (Figure 27).

9. Obtaining of recombining cassette

In order to obtain phi-YFP-Chloramphenicol resistance cassette two reverse 75 nt length oligonucleotides were designed as described before (Results-5 Oligonucleotide synthesis), their last 25 nt in 3' side were designed to hybridize several base pairs later of the stop codon of the chloramphenicol resistance gene in pL05CtY plasmid. In their final 5' parts one of such oligonucleotides (H2-RNase II) (Figure 28) presented 50 nt designed to hybridize with the stop codon and 47 nt down river of RNase II gene the other oligonucleotide (H2-RNase PH) (Figure 29)presented 50 nt designed to hybridize with the stop codon and 47 nt downstream of RNase PH gene respectively. In order to prove their integrity 2µl of the synthesized oligonucleotides were analyzed by 4% low melting point agarose gel electrophoresis (Figure 30). Both primers presented the desired size and no signs of degradation were seen in the gel.

As forward oligonucleotides (NAV01F, NAV03F) designed previously were able to hybridize with phi-YFP gene present in pL05CtY plasmid it was not necessary to

design new forward oligonucleotides so they were paired with the newly synthesized oligonucleotides H2-RNase II and H2-RNase PH.

For amplification of phi-YFP-Cm^R cassette that would be recombined in the end of RNase PH, 20 PCR reactions were carried out using oligonucleotides pair NAV01-H2 RNase PH. 10 ng of DNA of plasmid pL05CtY were used as template (Figure 31), PCR reactions were set to a volume of 50 µL, annealing temperature adjusted to 61.9° C, and extension time of 90 seconds. On the other hand for amplification of phi-YFP-CmR cassette that would be recombined in the end of RNase II, other 20 PCR reactions were carried out using oligonucleotides pair NAV03-H2 RNase II. 10 ng of plasmid pL05CtY were also used as template (Figure 32), in this case annealing temperature were set to 63.7° C, and extension time of 90 seconds. 2µL of resulting samples were analyzed by 0.8% agarose gel electrophoresis. All lanes loaded with PCR samples in the agarose gel presented amplified sequences corresponding to 1900 bp which was appropriate for the size expected for the phi-YFP-Chloramphenicol resistance cassette as is shown in (Figures 33 and 34).

The reactions carried out with the same oligonucleotides pair were mixed and purified using the Wizard SV Gel and PCR Clean-Up System (Promega) as manufacturer's instructions. After purification process, cassettes integrity were verified by 0.8% agarose gel electrophoresis assay, both cassettes appeared as clear and intense bands with a size corresponding to 1900 bp when visualized in the agarose gel.

Once purified, the samples were subjected to enzymatic digestion with restriction enzyme Dpn I (New England Biolabs) in order to eliminate the residues of plasmid pL05CtY that could enter into bacterial cells during subsequent electroporation steps. Restriction reactions were carried out using 5U of enzyme per microgram of DNA sample during 1 hour at 37° C. After restriction, cassettes integrity were verified by 0.8% agarose gel electrophoresis assay, both cassettes appeared as intense bands with size of 1900 bp and without signs of degradation when visualized in the agarose gel as is shown in Figures 35 and 37. Finally the resulting samples were purified using phenol-chloroform method avoiding using salts (Methods-4 Phenol DNA extraction) with the purpose of removing residues from the digestion reactions.

Purified samples were proved by 0.8% agarose gel electrophoresis assay resulting in clear bands of 1900 bp in size without signs of degradation (figures 36 and 38).

10. Construction of LHY, LECHY and LE701CHY strains

Cells of overnight cultures of strains DY329, LEC and LE701C were induced for expression of RED recombination system (Methods-13 Induction of the RED recombination system and preparation of recombinant cells) and electroporated as is described in methods (Methods-14 Electroporation), using the Gene Pulser Xcell Electroporation System (Bio Rad). 50 µL of competent cells were placed in previously chilled electroporation cuvette (Bio Rad) with 300 ng of the recombining cassette obtained as mentioned above with oligonucleotides pair NAV01-H2 RNase PH and pulsed. Then electroporated cells were recovered in 1 ml of LB medium for 1 hour and cultured in LB medium plates added with chloramphenicol (30 µg/ml). As control, cell cultures of all strains that did not go through the process of induction of RED system were subjected to electroporation without adding any DNA and then cells were cultured in LB plates and LB plates added with chloramphenicol (30 µg/ml) in order to prove antibiotic efficiency and cell viability after electrical shock. When DY329 cells (LHY) were electroporated, plates added with chloramphenicol seeded with induced cells presented 156 colonies and seeded with non-induced cells presented 8 colonies. With regard to LEC (LECHY) strain, plates added with chloramphenicol seeded with induced cells presented 3 colonies and seeded with non-induced cells did not present any colony. Finally when talking about LE701C (LE701CHY) strain plates added with chloramphenicol seeded with induced cells presented 12 colonies and seeded with non-induced cells did not present any colony. In all cases plates of LB medium seeded both with induced and non-induced cells of all strains presented massive growth which probes cell viability after the processes of induction and electroporation (Table 6). All colonies obtained with LEC and LE701C and 16 colonies obtained with DY329 strain were isolated in chloramphenicol plates for their preservation

11. Construction of LBY, LECBY and LE701CBY strains

Overnight cultures of strains DY329, LEC and LE701C were induced (Methods-13 Induction of the RED recombination system and preparation of recombinant cells) and electroporated (Methods-14 Electroporation) in the same way as above with 300ng of recombining cassette obtained with oligonucleotides pair NAV03-H2 RNase II. Then electroporated cells were cultured in plates of LB medium and LB medium added with chloramphenicol (30 µg/ml). Growth on plates was registered (Table 7). When DY329 (LBY) cells were electroporated, plates added with chloramphenicol seeded with induced cells presented 973 colonies and seeded with non-induced cells did not present any colony. With regard to LEC (LECBY) strain, plates added with chloramphenicol seeded with induced cells presented 352 colonies and seeded with non-induced cells did not present any colony. Finally when talking about LE701C (LE701CBY) strain plates added with chloramphenicol seeded with induced cells presented 2986 colonies and seeded with non-induced cells presented 267 colonies. In all cases plates of LB medium seeded both with induced and non-induced cells of all strains presented massive growth which probes cell viability after the processes of induction and electroporation. 16 colonies obtained of each strain were isolated in chloramphenicol plates for their preservation

12. Proving for mutant strains

Isolated colonies of each electroporated strain were cultured overnight in 5 ml of LB medium and genomic DNA was extracted (Methods-4 Phenol DNA extraction). Two oligonucleotides pairs were designed to hybridize with medium part and some nucleotides after stop codon of RNase II (NAV05F-NAV06R) (Figure 39) and RNase PH (NAV07F-NAV08R) (Figure 40) genes. In order to corroborate efficient recombination of phi-YFP-Chloramphenicol cassettes in desired sites of bacterial chromosome, PCR reactions were carried out using aforementioned genomic DNA as template and oligonucleotides pairs NAV05F-NAV06R when proving LBY, LECBY and LE701CBY strains, with annealing temperature of 59° C and NAV07F-NAV08R oligonucleotides pair when proving LHY, LECHY and LE701CHY with annealing temperature set to 57.9° C. Genomic DNA of strain DY329 was used as control.

When testing LHY strain 5 colonies resulted positives (Figure 41), when LBY strain 4 (Figure 42) colonies, when LECHY strain 2 colonies (Figure 43), when LECBY strain 6 colonies (Figure 44), when LE701CHY strain 3 colonies (Figure 45) and when LE701CBY strain 7 colonies (Figure 46).

Additionally colonies of all strains were verified by spectrofluorometric assays (Methods-15 SpectroFluorimetric assays), measures were done exciting all strains at 514nm and registering emission at 537 which corresponds to phi-YFP channel. DY329 was used as non-fluorophore labeled control and its fluorescence signal was subtracted from the fluorescence signals of other strains proved in order to suppress the natural fluorescence of bacteria, in this experiment n=4. In all cases samples presented increased fluorescence when compared with DY329 strain as was expected (Figure 47). Both tests together corroborated the successful recombination of phi-YFP fluorescent proteins in the desired sites of the bacterial chromosome.

13. FRET assays

All strains were analyzed as described in methods (Methods-15 Spectrofluorimetric assays) additionally previously generated strains LECPY and LE701CPY (Dominguez-Malfavon *et al* 2013) were also evaluated as experiment controls since these bacteria have been successfully used in FRET experiments and their properties are well established. Other strain that harbors plasmid pL07CtPYC was also used as control since such plasmid express CFP protein fused directly to phi-YFP protein and therefore the transfer efficiency of FRET is maximal for such fusion. Fluorescence measurements were registered and then auto fluorescence was suppressed subtracting DY329 fluorescence from fluorescence of samples. Then resulting values were used to calculate apparent FRET efficiency (FRETapp) as mentioned in methods (Methods-16 FRET calculations). Results are shown in figure 48. Control strain pL07CtPYC presented high values of fret efficiency corresponding to 66.5 as was expected. LECPY strain presented higher values (2.137) of FRET efficiency when compared to strain LE701CPY which presented negative values (-1.39), difference was tested with t-student, significance values $p<.001$. In regard to the other strains LECHY (-244), LE701CHY (-130), LECBY (-100) and LE701CBY (-113), 4 replicates of each stain were evaluated. All presented high negative values

with a lot of variance between measurements from the same strain. Differences between strains weren't significant (95 %) when compared with t-student test (Figure 48). These results hugely differ from those obtained for strains LECPY, LE701CPY and pL07CtPYC therefore don't let us to ascertain RNase E, RNase II and RNase PH interactions.

As RNase PH and RNase II activity increase when cells go through prolonged periods of stationary phase and starvation (Sulthana *et al* 2017), emission scans were made at 525 nm of excitation for phi-YFP signal detection. Strains LHY and LBY were measured at 16 h and at 40 h of culture. Cells grown for 40 h presented higher values of fluorescence when compared with LHY and LBY (Figure 49) suggesting that long periods of stationary phase increase LBY and LHY expression. In spite of the increase, fluorescence values of phi-YFP remain very low and are still insufficient to perform fret analysis.

IX. Discussion

In this work it was not possible to evaluate the interactions between RNase E and RNase II or RNase E and RNase PH by FRET assays. Values of FRET app obtained from strains LECHY, LE701CHY, LECBY and LE701CBY resulted very high (in the order of hundreds) and negative which do not correspond with the values that were expected taking into account that FRET app values of control strain pL07CtPYC that exhibit the highest FRET transfer rates are 66.5 in average moreover the FRET app values of control strains LECPY and LE701CPY are extremely larger than those of constructed strains. Altogether these let us to think that $FRET_{app}$ values of constructed strains are out of range and do not provide any reliable information in regard to RNA degradosome assembly. It is very important to discern if such fluorescence values result from the phenomena that occur naturally inside the bacteria or due to troubles generated during design and construction of strains or during methodology of FRET measurements. Data obtained in FRET measurements reveled low fluorescence of phi-YFP protein fused in RNases II and PH whereas fluorescence values for CFP remained normal. This low fluorescence of phi-YFP tagged proteins could be due to low expresion of RNase II and PH as was found by

Dominguez-Malfavon and coworkers for RhlB expression values when analyzing the interactions of RNA degradosome canonical components (Malfavon *et al* 2013), in this last FRET couldn't be evaluated due to the low levels of RhlB in bacterial cells. Taking into account this precedent, further studies must be carried out to quantify native expression values of both RNase II and PH to ascertain if such values correspond with the fluorescence detected in this work. Another possibility to consider is that in the conditions of measurements of FRET union sites to RNases II and RNase PH in RNase E would occupied by canonical RNAD canonical components specifically PNPase and RhlB which competing and impeding RNase PH and RNase II recruitment, to solve this problems it would be necessary to construct strains that do not express PNPase neither RhlB, or search for the optimal conditions where interaction between RNase E and RNase PH or RNase II are maximal and more stable. In other scenario low levels of fluorescence could be a consequence of the fusion of phi-YFP to the end of RNases II and PH causing a low transcription level of RNases, misassembling of the proteins or unspecific interactions between phi-YFP and sites important for RNases function and interactions, that would lead to the incorrect function of the RNases and probably avoid interaction with RNA degradosome components. Deletion or reduction of the function of RNases II or PH would bring about many effects. (Pobre V. *et al* 2015) found that deletion of RNase II significantly affect 187 transcripts genes related with flagellum assembly, motility and biofilm formation; moreover, Sulthana and coworkers (Sulthana *et al* 2017) proved that RNase II is required for cell survival during prolonged stationary phase and additionally upon starvation, both RNases PH and II are fundamental for cell development at low temperature (Awano N *et al* 2008). Thus for all above in case of low transcription, misassembling or malfunction of modify RNases in constructed strains bacterial growth and survival would be affected when cultured at low temperature or starving conditions evidencing troubles with RNases function. On the other hand phi-YFP low fluorescence could be due to artifacts produced by the nature of the fluorophore. RNase II is found as dimer in cells whereas RNase PH is found as trimer, inasmuch that tagged genes are in chromosomes all copies of these RNases are fluorophore labeled this could be a problem since fluorophores, when close enough, might exhibit self-quenching, which would reduce the total fluorescence intensity and change fluorescence anisotropy (Zheng). Besides protein interaction alone might produce fluorophore quenching.

Low fluorescence values of phi-YFP fused to the end of RNases II and PH could also be due to problems during strains construction. phi-YFP fusions were corroborated by PCR amplification of inserted sequences, all strains presented the desired size for amplified sequence although conventional end point PCR did not allow us to know the sequence amplified neither if such amplified is in the correct reading frame so that it is necessary to sequence the inserted genes to discard a wrong recombination in chromosomes of constructed strains.

All above indicates that further studies are needed to identify the cause of the low levels of phi-YFP fluorescence and if possible to correct them in order to obtain sufficient fluorescence values to perform FRET assays and characterize interactions between RNase E and RNases II and PH.

X. Conclusions

PCR and Fluorimetric analysis revealed that previously generated strains LEC, LE701C and LEBNY-Km presented the features of interest in this work wich consist of CFP protein fused to RNase E and truncated RNase E-701 and phi-YFP protein fused to enolase gene respectively.

Kanamycin resistance cassette was successfully eliminated from strains LEC and LE701C with help of plasmid pCP20 as was proved by PCR and antibiotic resistance assays.

Strain LEBNY-Km did not serve to obtain phi-YFP-Kanamycin genes by PCR amplification.

Recombining cassettes phi-YFP-Chloramphenicol were successfully obtained from previously generated plasmid pL05CtY by PCR reactions.

Generated recombining cassettes were sufficient to recombine phi-YFP protein in the end of ribonucleases RNase II or RNase PH genes in the chromosome of bacteria of LEC and LE701C by recombineering technique. Effective recombination

was corroborated by PCR amplification of modified genes finding the sizes expected for each construction.

Generated strains LHY, LECHY, LE701CHY, LBY, LECBY and LE701CBY presented high negative values of FRET app which resulted out of the range expected and don't allow us to analyze RNase E, RNase II and RNase PH interactions. Further experiments are needed to ascertain if such unexpected values are the result of RNases properties or artifacts of methodology or troubles during construction or expression of the genetic fusions.

XI. Perspective

To elucidate if low fluorescence values of phi-YFP are the result of RNases properties or artifacts of methodology or troubles during construction or expression of the genetic fusions.

To determine native expression values of RNase PH and RNase II.

To design a new FRET experiment that adjusts to the expression values of the RNase II and RNase PH.

TABLES (section)

Table 1: Strains required in this work		
Strain	**Relevant genotype**	**Source/Reference**
TOP 10α	*F– Φ80*lac*ZΔM15 Δ(*lac*ZYA*-arg*F) U169* rec*A1* end*A1* hsd*R17 (rK–, mK+)* pho*A* sup*E44 λ–* thi-*1* gyr*A96* rel*A1*	Invitrogen
DY329	W3110 Δ *lac*U169 *nadA*::*Tn10gal*490 *pgl*Δ8[λ *c*I857 Δ*cro bio* A)]	Yu *et al.*, 2000
LEC	DY329 *rne*::*rne-6xGly-cfp*	Domínguez-Malfavón L. 2013
LE701C	DY329 *rne*::*rne701-6xGly-cfp*	Domínguez-Malfavón L. 2013
LEBNY	DY329 *rne*::*rne701-6xGly-bfp, eno:eno-6xGly-phiyfp*	Domínguez-Malfavón L. 2013
LBY	DY329 *rnb*::*rnb-6xGly-phiyfp, rph-1*	This work
LHY	DY329 *rph*::*rph-6xGly-phiyfp, rnb*	This work
LECBY	DY329 *rne*::*rne-6xGly-cfp, rnb*::*rnb-6xGly-phiyfp, rph-1*	This work
LECHY	DY329 *rne*::*rne-6xGly-cfp, rph*::*rph-6xGly-phiyfp, rnb*	This work
LE701CHY	DY329 *rne*::*rne701-6xGly-cfp, rph*::*rph-6xGly-cfp, rnb*	This work
LE701CBY	DY329 *rne*::*rne701-6xGly-cfp, rnb*::*rnb-6xGly-cfp, rph-1*	This work

Table 2: Plasmids required in this work		
Plasmid	**Notes**	**References**
pCP20	Expresses Flp enzyme in thermo-inducible way.	Cherepanov and Wackernagel, 1995
pL05CtY	Carries phiYFP cistron upstream of the chloramphenicol gene.	Dominguez-Malfavón *et al* 2013.
pL07CtPYC	Carries the phiYFP-6xGly-CFP in frame fusion under the bacterial promoter T5 upstream of the chloramphenicol gene.	Dominguez-Malfavón *et al* 2013.
pL08CtPC	Carries the phiYFP under the bacterial promoter T5 upstream of the chloramphenicol gene.	Dominguez-Malfavón *et al* 2013.
pL09CtPY	Carries the CFP under the bacterial promoter T5 upstream of the chloramphenicol gene.	Dominguez-Malfavón *et al* 2013.

Table 3: Oligonucleotides required in this work. Last 5 primers were designed previously in the working group (Dominguez-Malfavon *et al* 2013)		
Primer	**Sequence**	**Notes**
NAV01F	Gagggggaatcgaatccattgtagcgacgcagaaggc ggcgctggcaaacggcggtggcggcggtggcatgaga ggatcgggatccagcggcgccctg	Forward; Amplifies phi -YFP sequence from pL05CtY plasmid. Adds 6 gly hinge and H1 region for recombination in *RNase PH* gene.
NAV02R	Cttacttttctacagacaaaaaaaaggcgactcatcagtc gccttaaaaatcccggcggatttgtcctactcagg	Reverse; Amplifies kanamycin cassette from pL05CtY.Adds H2 region for recombination in *RNase PH* gene.
H2-Rnase PH	Cttacttttctacagacaaaaaaaaggcgactcatcagtc gccttaaaaaggcaaaccagcaatagacataagcg	Reverse; Amplifies chloramphenicol cassette from pL05CtY.Adds H2 region for recombination in *RNase PH* gene.
NAV03F	Ttgccgaagtccgcatggaaacccgcagcattattgcgc gcccggtcgcgggcggtggcggcggtggcatgagagg atcgggatccagcggcgccctg	Forward; Amplifies phi -YFP sequence from pL05CtY plasmid. Adds 6 gly hinge and H1 region for recombination in *RNase II* gene.
NAV04R	Gagcggggaaataaacggcccatccatgaggaatgg gccgtgaaaggagatcccggcggatttgtcctactcagg	Reverse; Amplifies kanamycin cassette from pL05CtY.Adds H2 region for recombination in *RNase PH* gene.
H2-Rnase II	Gagcggggaaataaacggcccatccatgaggaatgg gccgtgaaaggagaggcaaaccagcaatagacataa gcg	Reverse; Amplifies chloramphenicol cassette from pL05CtY.Adds H2 region for recombination in *RNase II* gene.
NAV05F	Acgcgcccacaggatgaaat	Forward; Targets *rnase II* gene, 280 bp upstream the stop codon.
NAV06R	Acgaaggtagagcggggaaa	Reverse; Targets the region after *rnase II* gene
NAV07F	Gcgctgggtgagttcaccat	Forward; Targets *rnase PH* gene, 400 bp upstream the stop codon.
NAV08R	Tggtttcatgccttcgctcc	Reverse; Targets the region after *rnase PH* gene
LDM28F-	Cgttctggctggcgaaggca	Forward; Targets *eno* gene, 526 bp upstream the stop codon.
LDM42R	Cagggcttgattactttgagc	Reverse; Targets the region after *rne* gene
LDM43R	Ggctggcatttttaaatcag	Reverse; Targets the region after *eno* gene
CMJKmR-	Gcctacccgcttccattgct	Reverse; Targets medium part of Kanamycine resistance gene.
LDM46R	Ccaacgccagctgaacctgc	Forward; Targets ~500 pbs before *rne701* gene ends.

Table 4: PCR conditions. Reactions were carried during 30 cycles.

Period	**Initial denaturation**	**Denaturation**	**Annealing**	**Extension**	**Final extension**
Temperature/ Time	**94° C/ 5 min**	**94° C/ 30 sec**	**Variable**	**72° C / 30 sec**	**72° C/ 5 min**

Table 5. Growth of colonies after transformation of bacteria		
Transformation efficiency		
	LB S/A	**LB + Ap**
Control LEC	**massive growth**	**0**
LEC	**massive growth**	**10**
LE701C	**massive growth**	**0**
Control LE701C	**massive growth**	**4**

Table 6: Number of colonies grown when phi -YFP-Cloramphenicol cassette where recombined in RNase PH gene		
Strain	**LB**	**LB+Cm**
DY329 induced	**Massive Growth**	**8**
DY329 Non induced	**Massive Growth**	**156**
LEC Induced	**Massive Growth**	**3**
LEC Non induced	**Massive Growth**	**0**
LE701C Induced	**Massive Growth**	**12**
LE701C Non induced	**Massive Growth**	**0**

Table 7: Number of colonies grown when phi-YFP-Cloramphenicol cassette where recombined in RNase PH gene		
Strain	**LB**	**LB+Cm**
DY329 induced	**Massive Growth**	**973**
DY329 Non induced	**Massive Growth**	**0**
LEC Induced	**Massive Growth**	**352**
LEC Non induced	**Massive Growth**	**0**
LE701C Induced	**Massive Growth**	**2986**
LE701C Non induced	**Massive Growth**	**267**

FIGURES (section)

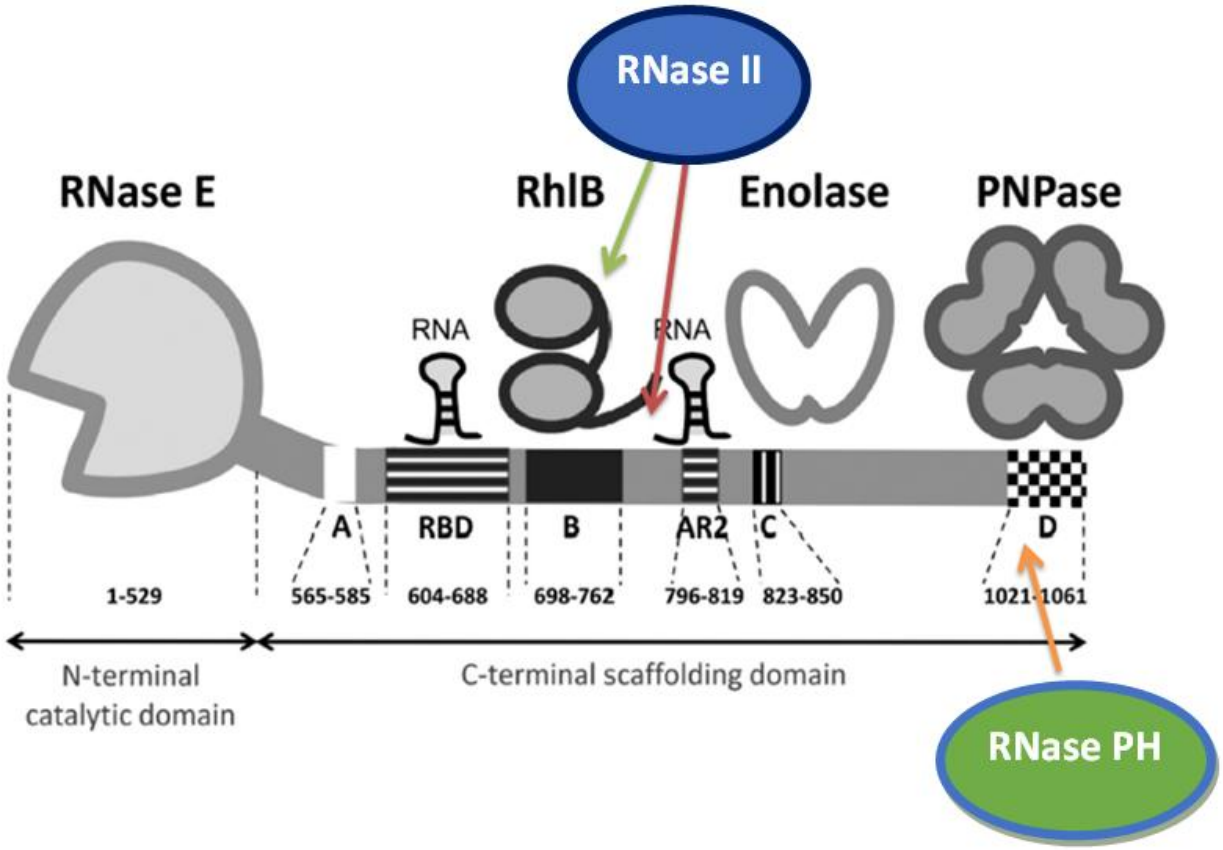


Figure 1. *RNase E binding sites to RNase II confined to amino acids 698-762 (Lu and Taghbalout 2014) and RNase PH confined to amino acids 1021-1036 (Pérez-Medina et al., 2014) in RNA-degradosome. Figure modified from Tsai et al., 2012.*

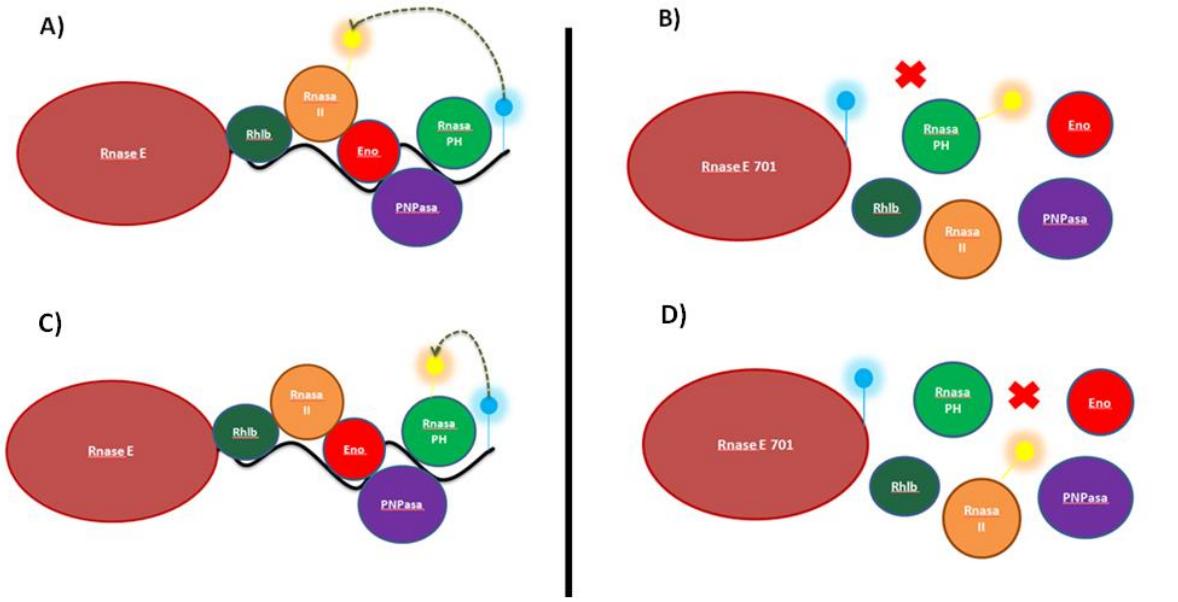


Figure 2. *FRET analysis with a single pair of fluorophores. Strains A) LECBY and B) LECHY RNase E-RNase II and RNase E-RNase PH respectively. Strains B) LE701CBY and D) LE701CHY were not expected to present evidence of proximity between fluorophores.*

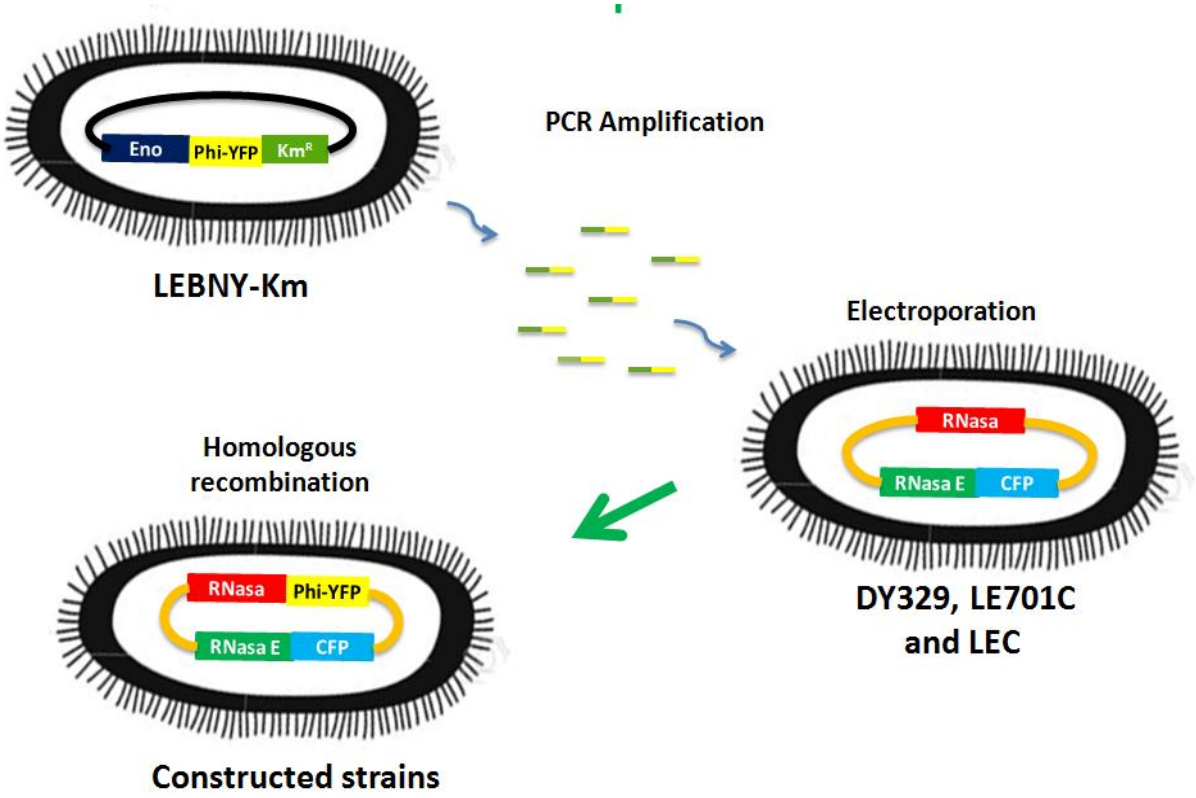


Figure 3. *Experimental design for strains construction.*

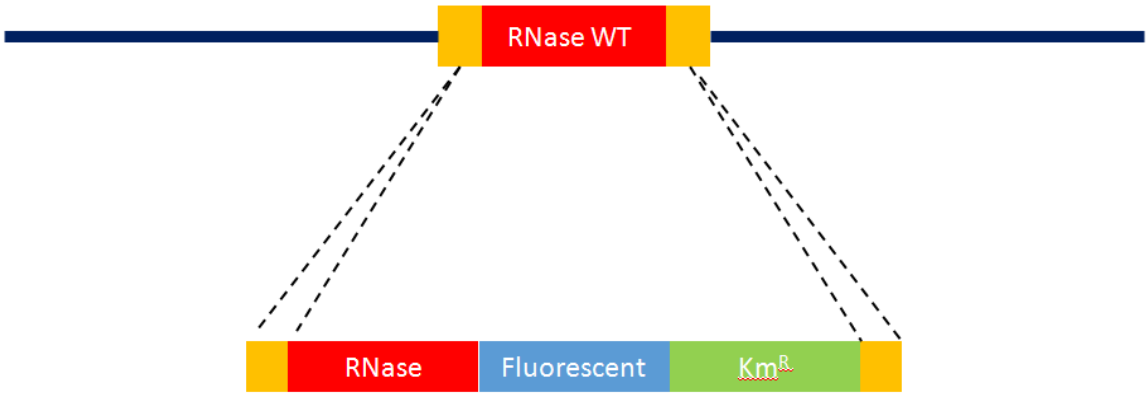


Figure 4. *Chromosomal recombination model. Recombinant cassette with 25-30 homologous regions in its ends to the specific chromosome site where recombination is directed.*

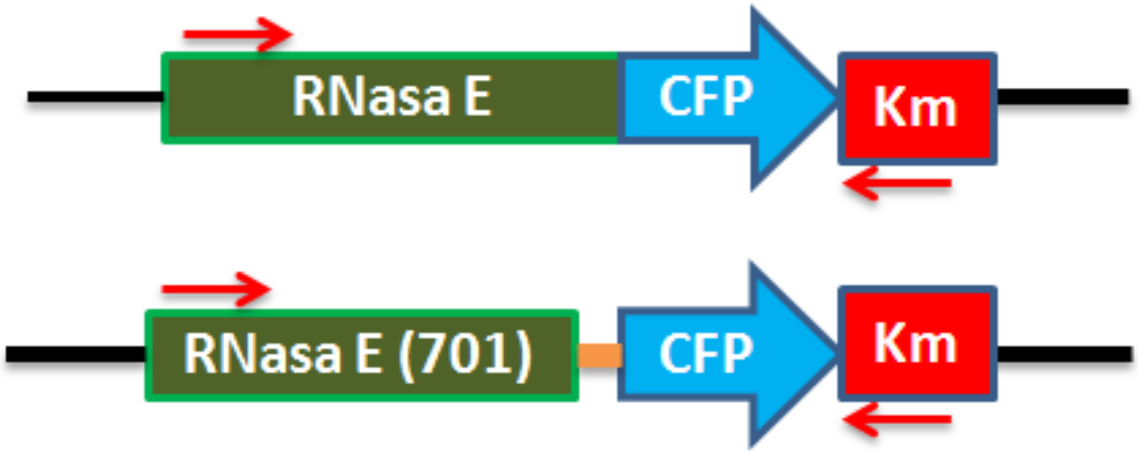


Figure 5. *Genotype of previously generated strains LEC and LE701C*

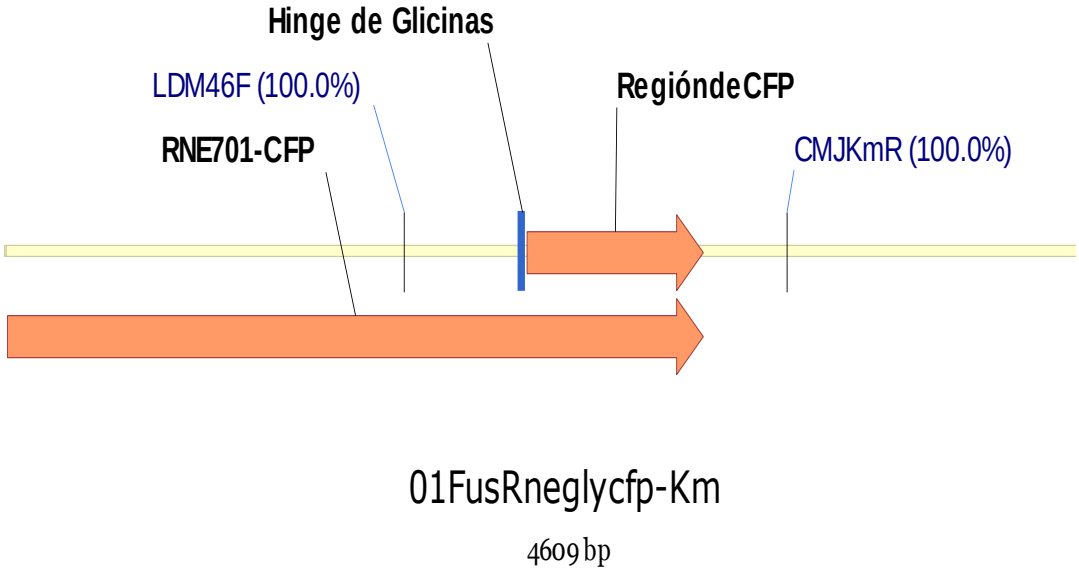


Figure 6. *Targeting sites of oligonucleotides pair CMJKmR-LDM46F in RNase E gene*

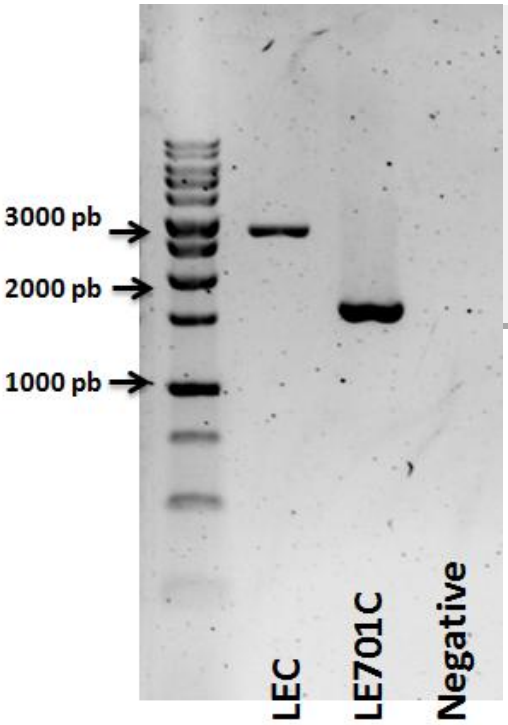


Figure 7. *0.8% Agarose gel of PCR amplification of RNase E with* oligonucleotides *pair CMJKmR-LDM46F. Amplification sequences correspond to 2600bp for LEC and1600 for LE701C*

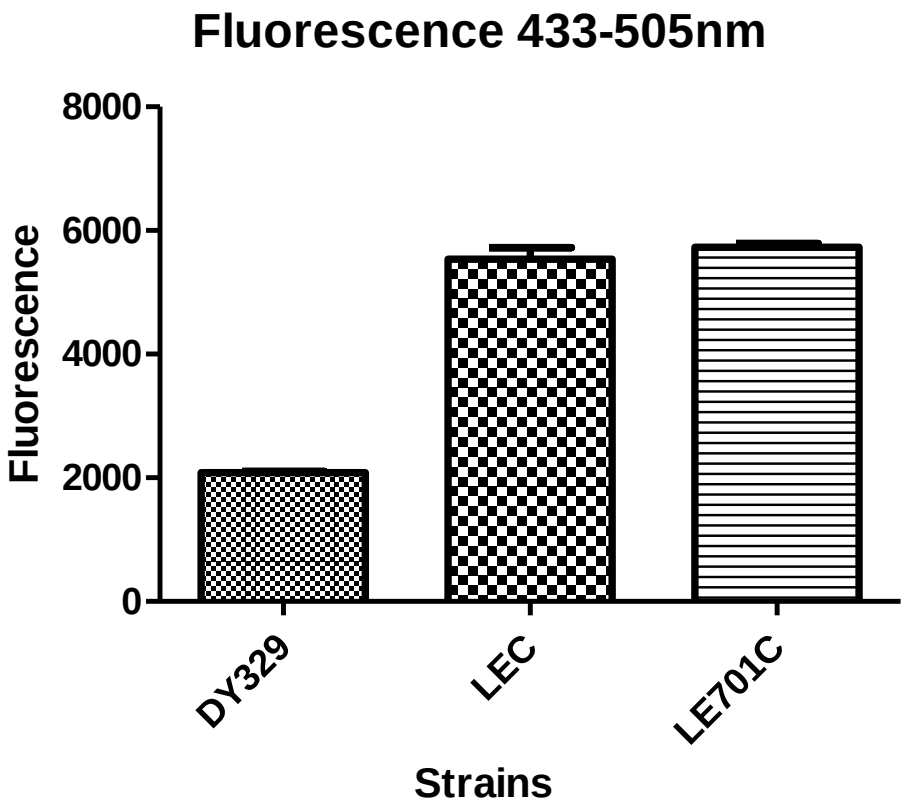


Figure 8. *.Fluorometric assay of strains LEC and LE701C after Kanamycin resistance loss. Bacteria were tested whit the channel corresponding to CFP with excitation at 433nm and measured emission at 505nm. Both strains presented higher values of fluorescence when compared to DY329 strain when analyzed whit t-student test, p<.001.*

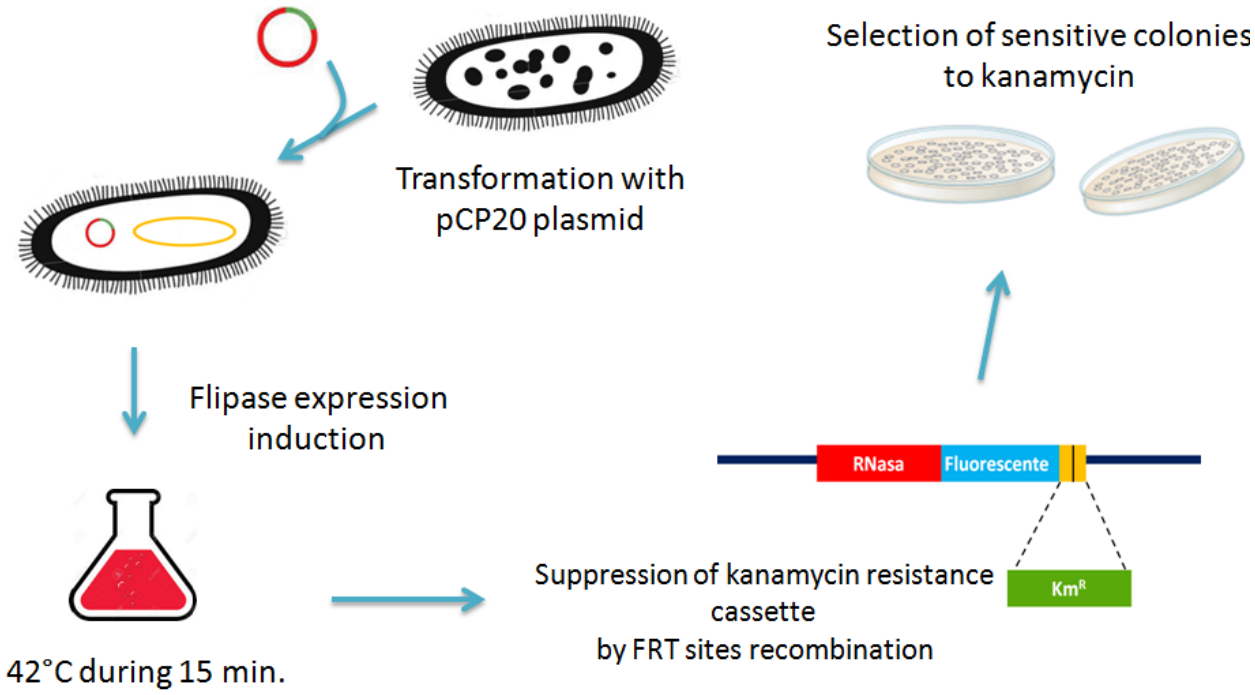


Figure 9. *Kanamycin resistance gene suppression by helper plasmid pCP20*

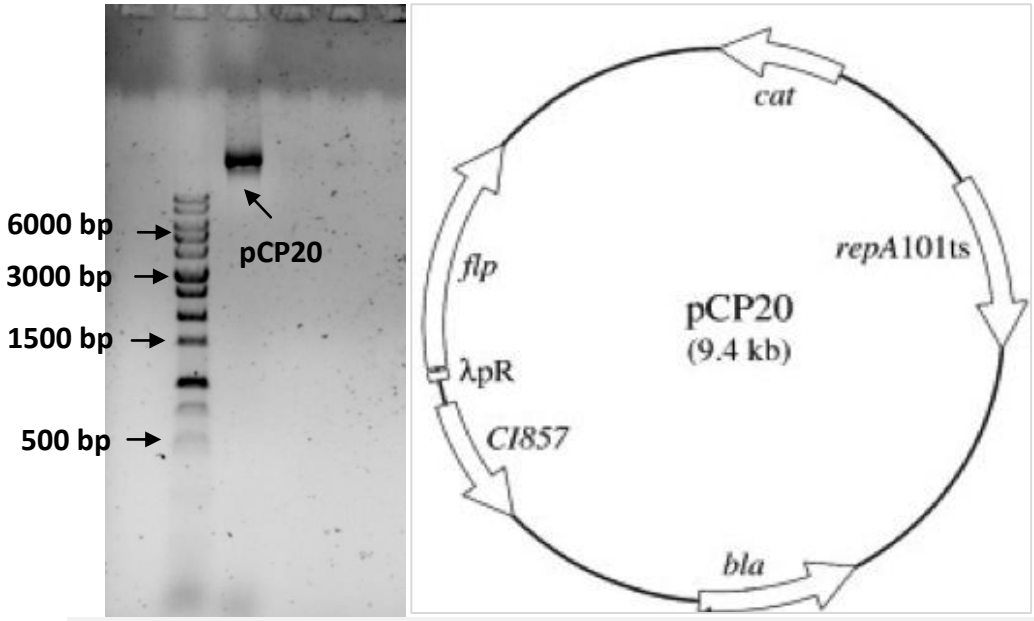


Figure 10. *.pCP20 plasmid. Left: 0.8% agarose gel with 2µl (70ng/ µl) of supercoiled pCP20 plasmid. Right Genetic map of Plasmid pCP20*

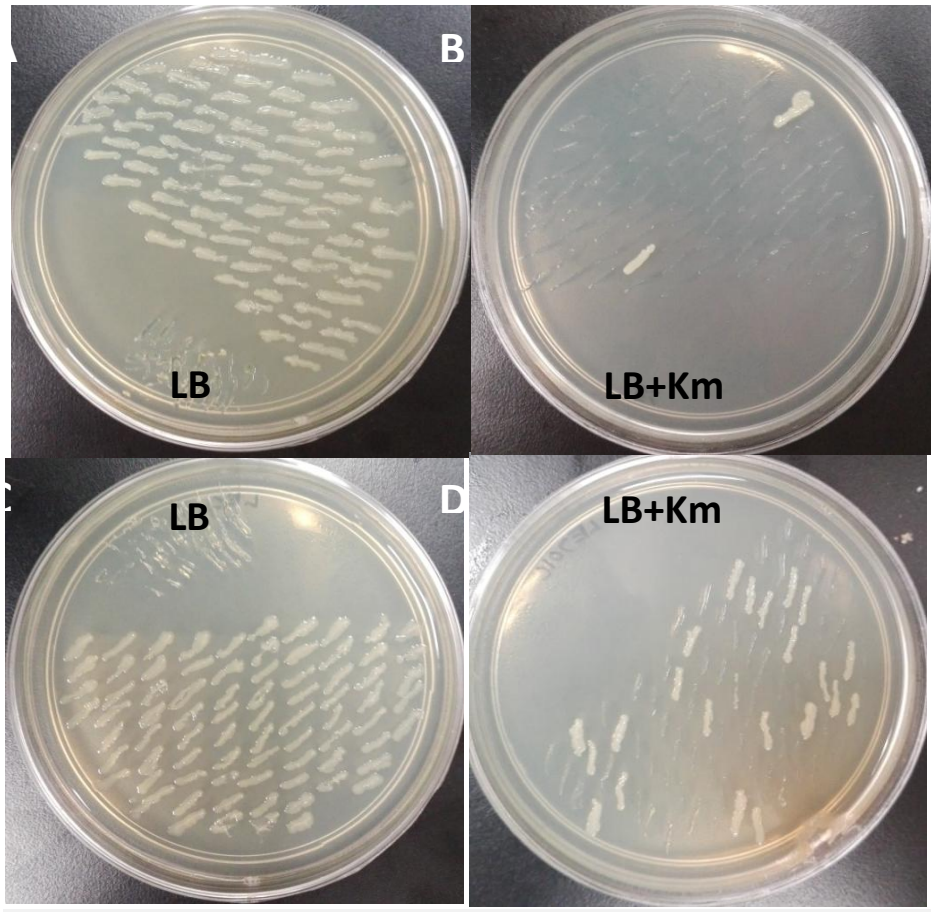
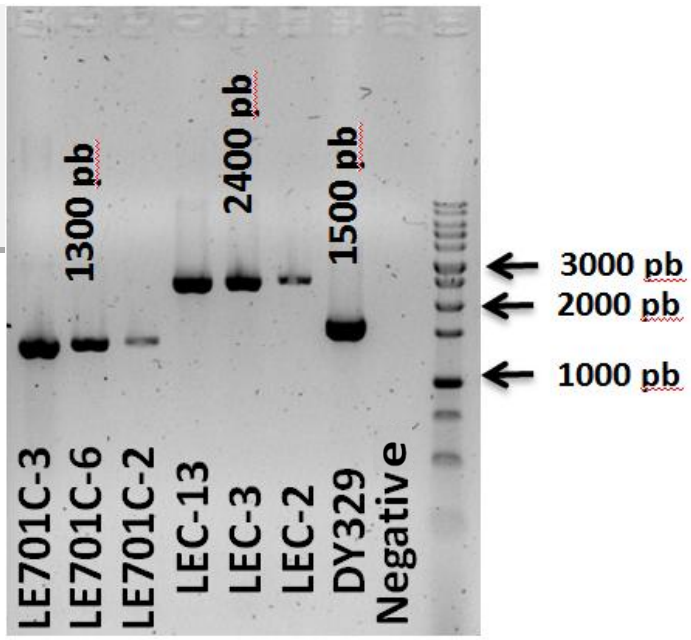


Figure 11. *Kanamycin sensitive colonies selection with gridded plated. Top plates seeded with LEC strain. Down plates seeded with LE701C strain.*

Figure 12. *PCR amplification whit* oligonucleotides *pair LDM42R-LDM46F 0.8% Agarose gel.*



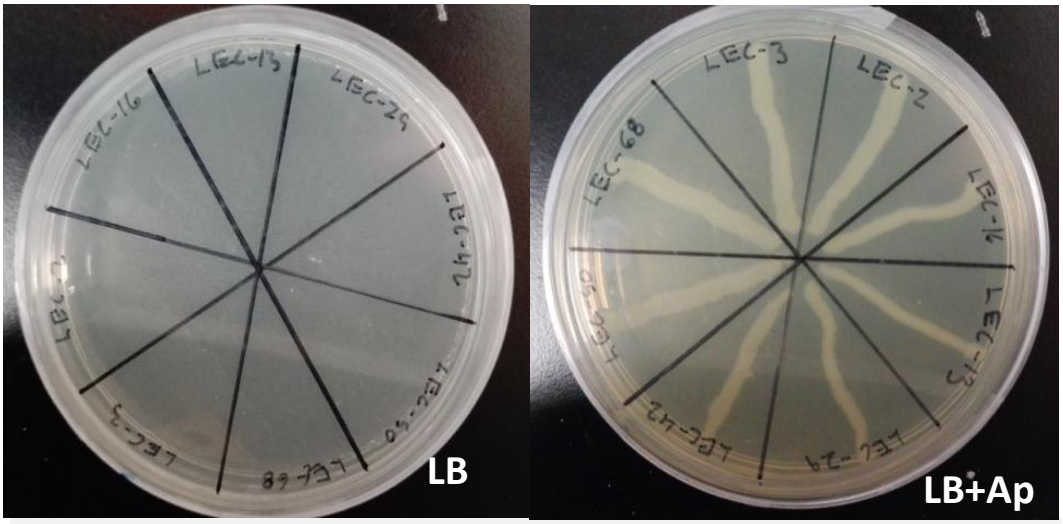


Figure 13. *Ampicillin sensitivity loss which indicate pCP20 plasmid lack.*

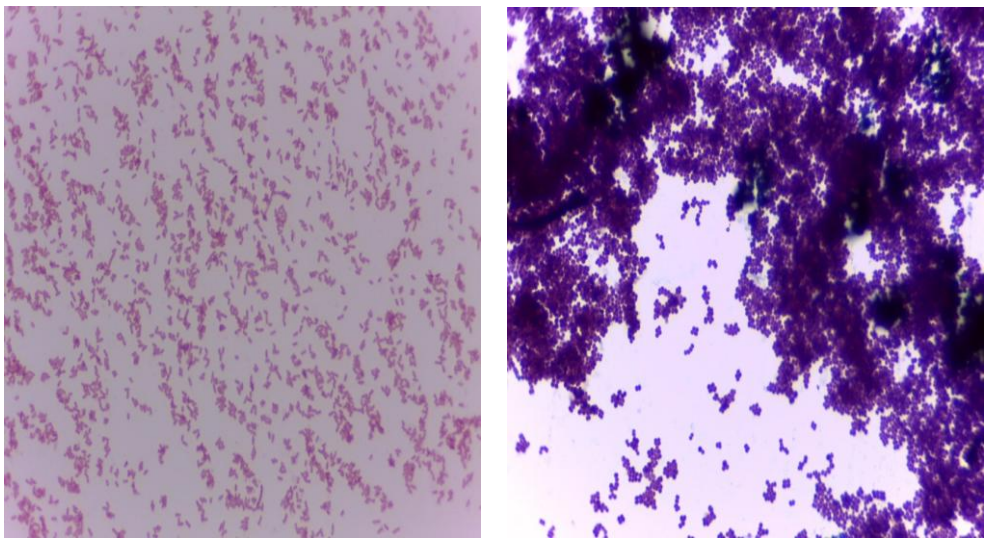


Figure 14. *LEBNY strain (Left). Control Gram positive bacteria of genus Kocuria (Right). Optical microscopy 40X*

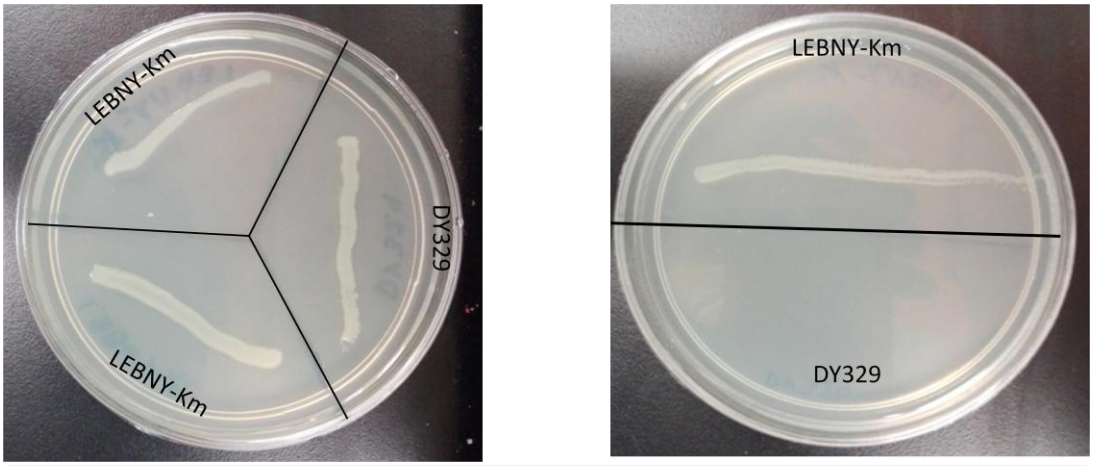


Figure 15. *Antibiotic resistance assay. LB plate (Left). LB+Km plate (Right)*

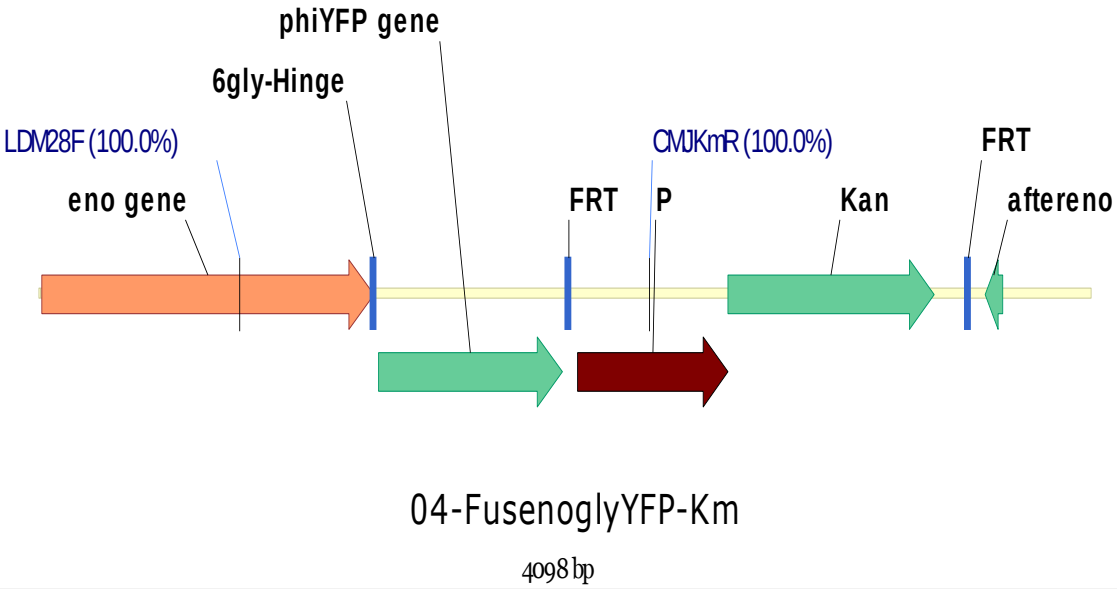
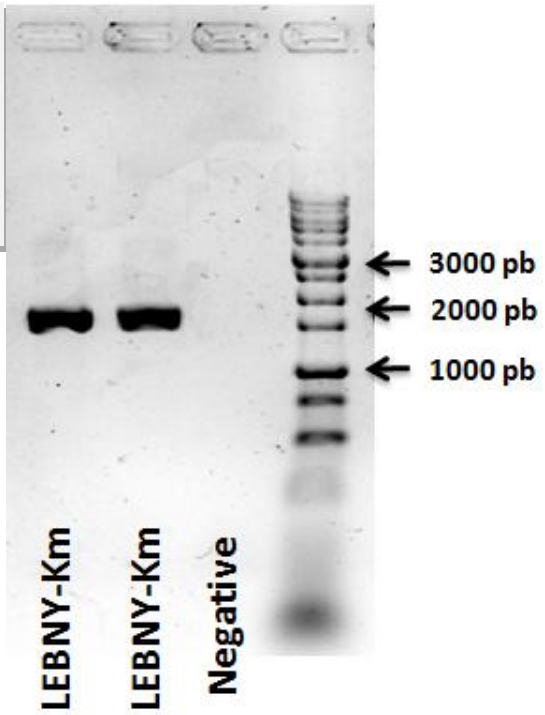


Figure 16. *Genetic map of enolase gene in LEBNY strain including Phi-YFP protein and Kanamycin resistance cassette.*

Figure 17. *PCR amplification with* oligonucleotides *pair LDM28F-CMJKmR. 0.8% agarose gel.*



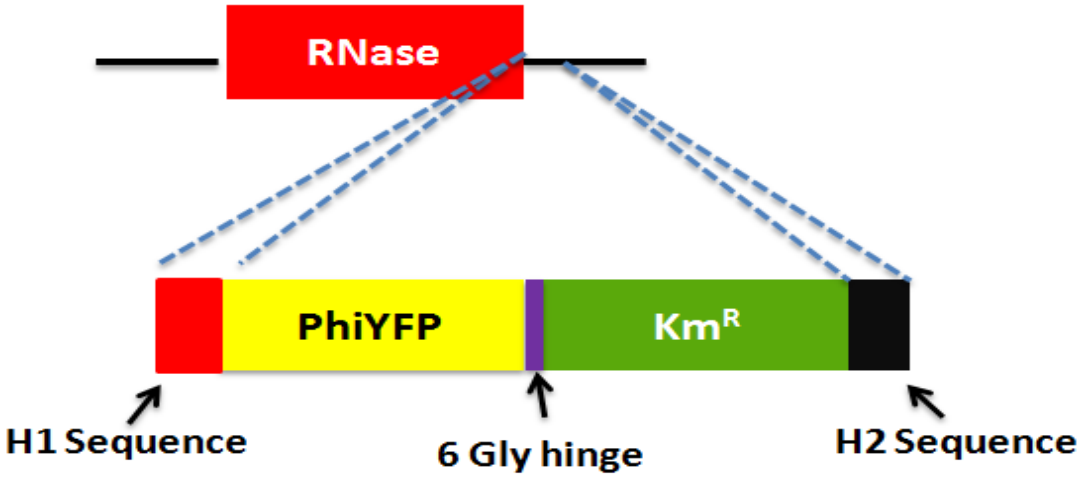


Figure 18. *Scheme of forward recombining oligonucleotides for homologous recombination in ribonucleases ends.*

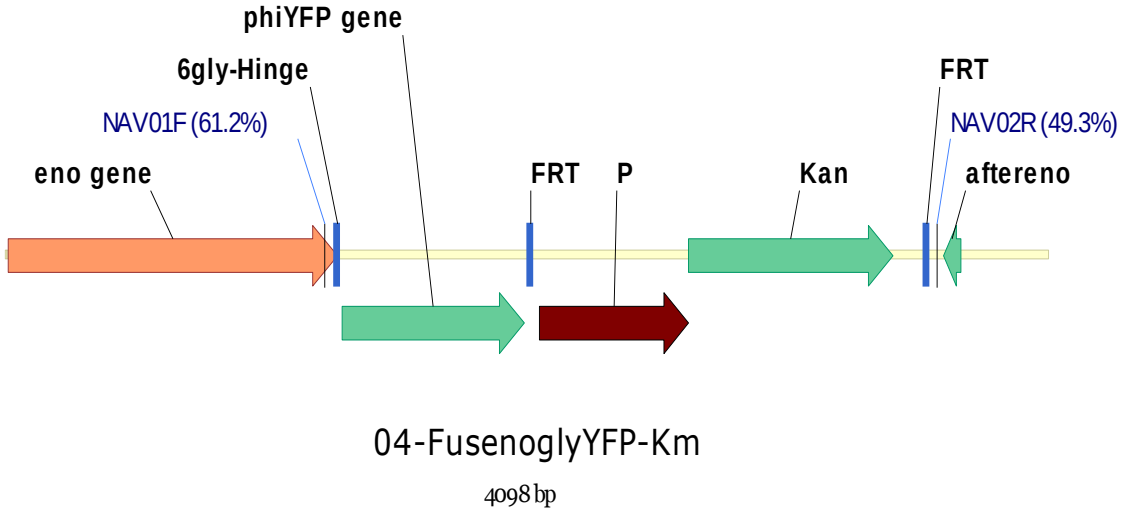


Figure 19. *Hybridization sites of NAV01 and NAV02 primers in enolase gene in LEBNY strain.*

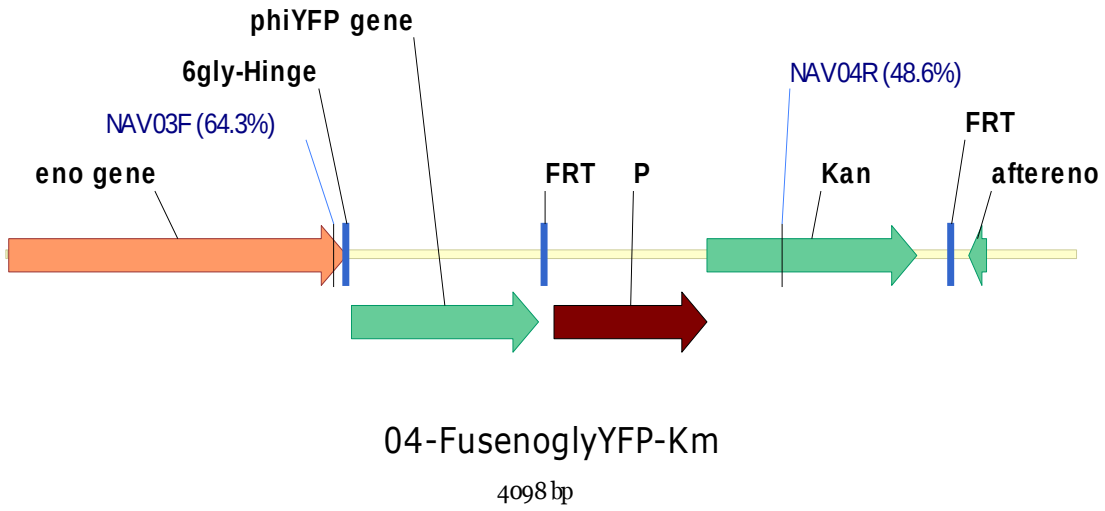


Figure 20. *Hybridization sites of NAV03 and NAV04 primers in enolase gene in LEBNY strain.*

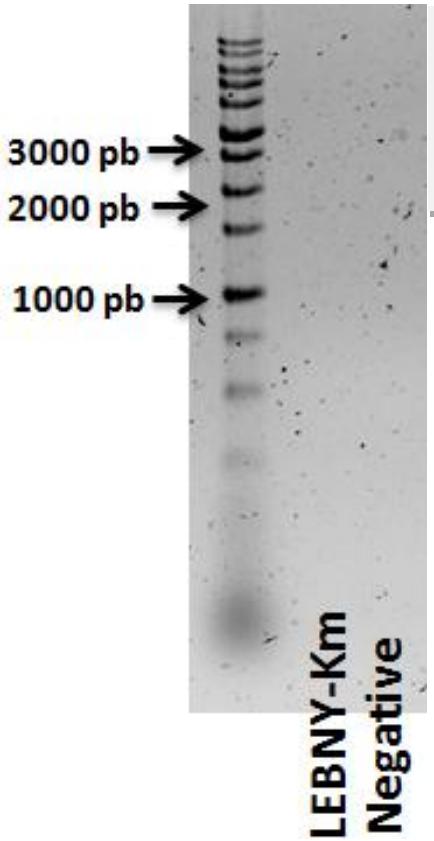


Figure 21. *Failed PCR reaction carried out with* oligonucleotides *pair NAV01F-NAV02R. 0.8% agarose gel.*

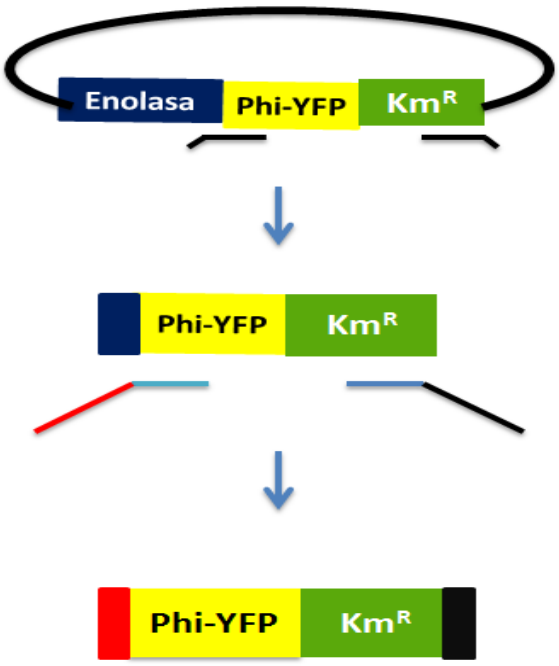


Figure 22. *Emerging strategy to obtain Phi-YFP Kanamycin resistance cassette*

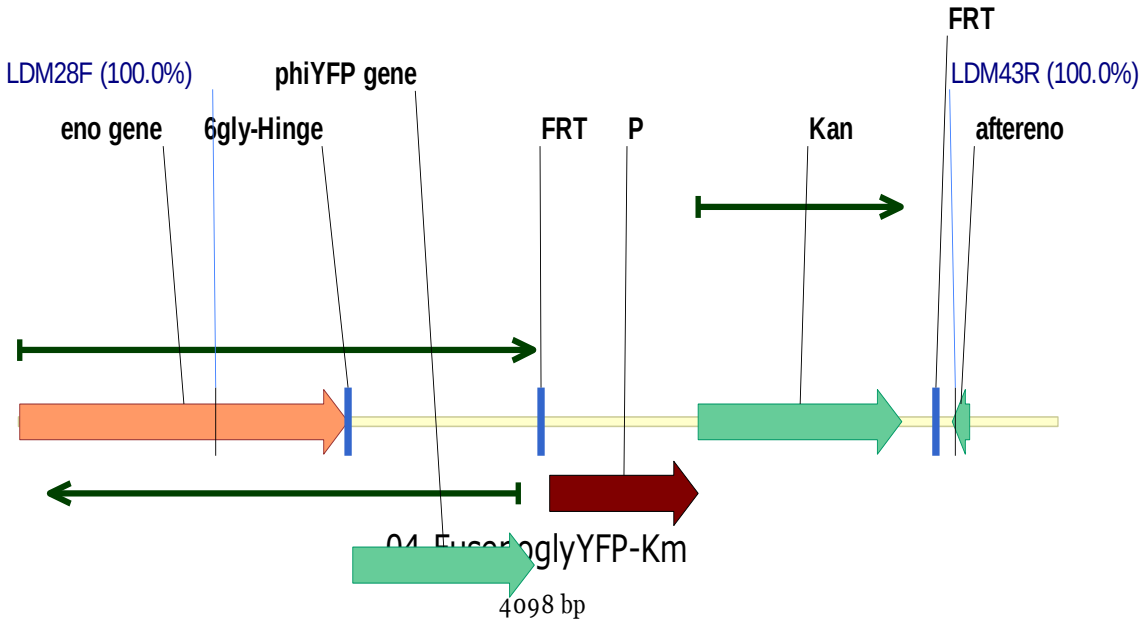


Figure 23. *Targeting sites of LDM28F and LDM43R primers in enolase gene in LEBNY strain.*

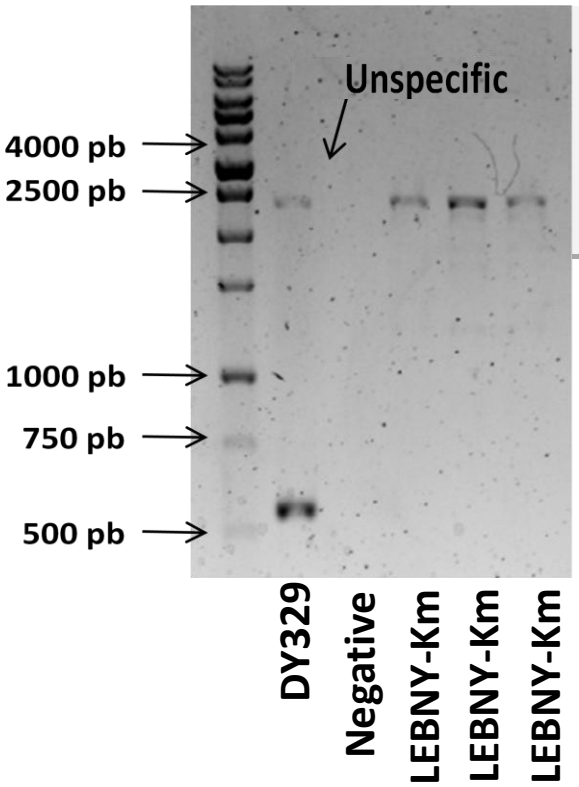


Figure 24. *PCR amplification with* oligonucleotides *pair LDM28F-LDM43R .8% Agarose gel*

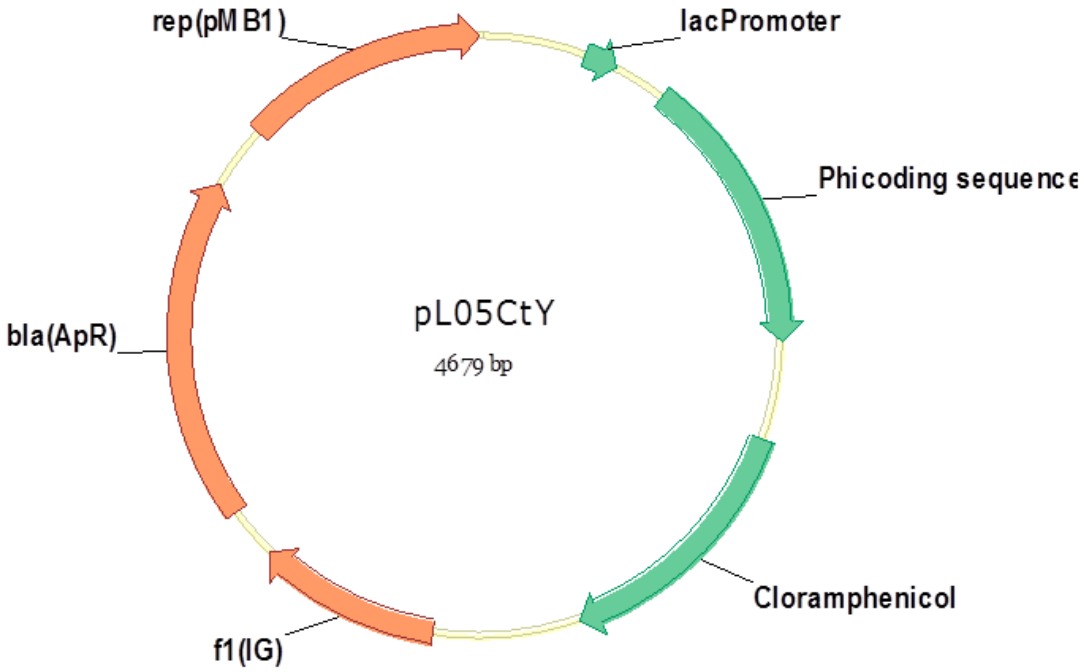


Figure 25. *Genetic map of plasmid pL05CtY (Dominguez-Malfavon et al., 2013)*

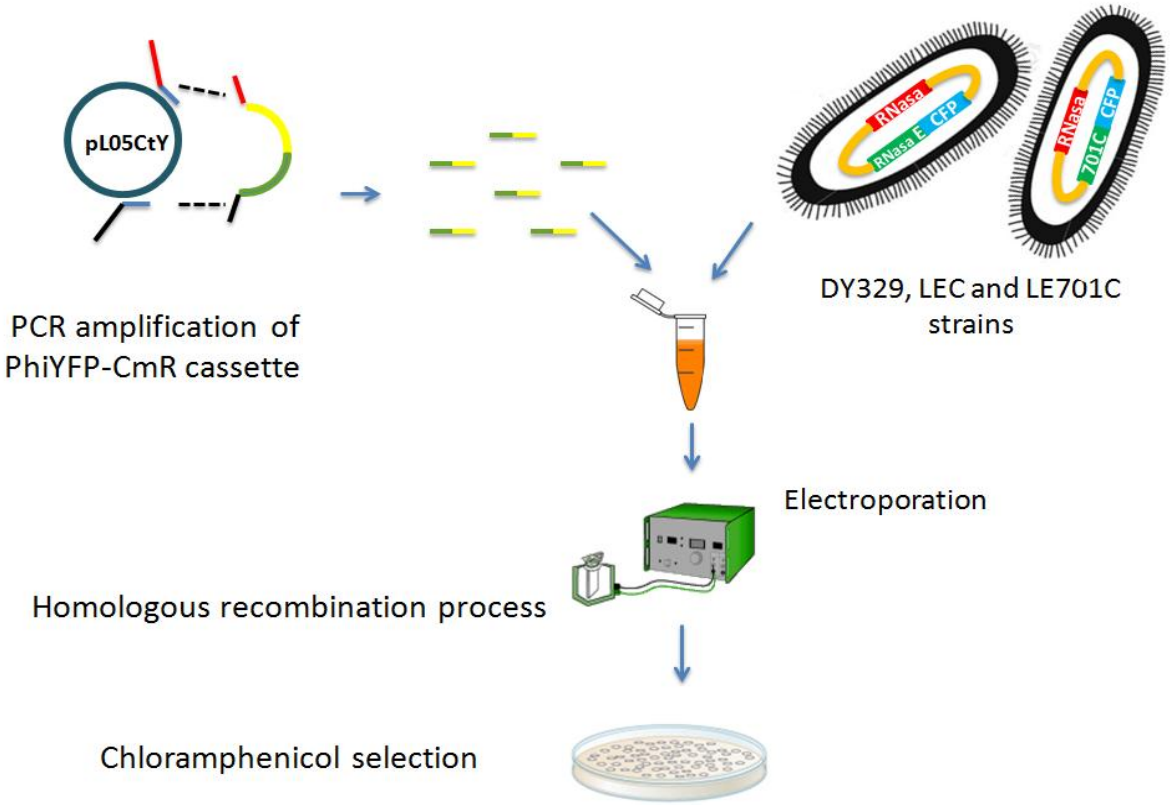


Figure 26. *Redesign of strategy of construction.*

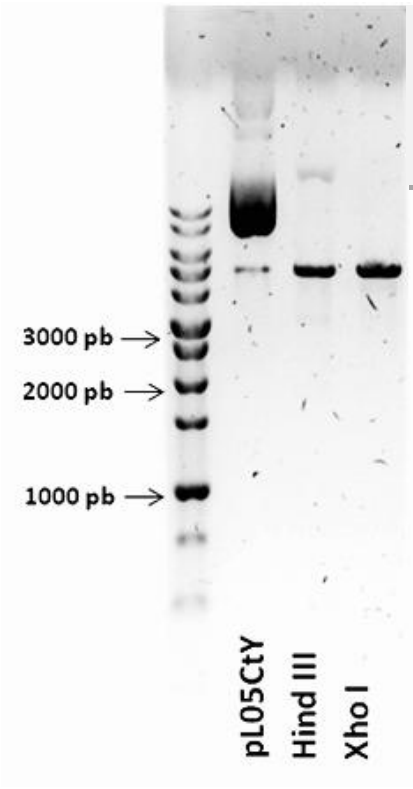


Figure 27. *pL05CtY plasmid linearized with restriction enzymes Hind III and Xho I. 0.8% agarose gel electrophoresis*

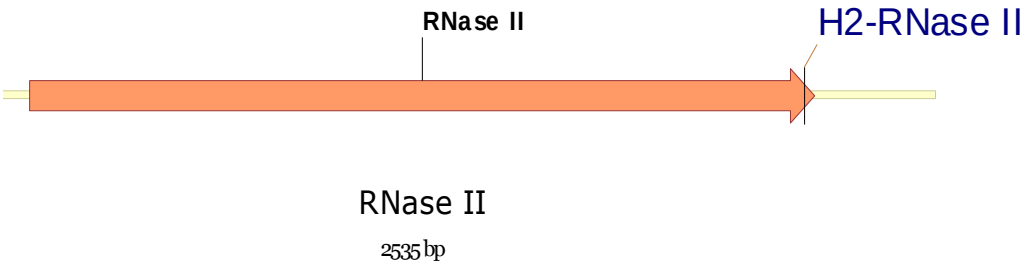


Figure 28. *Targeting sites of oligonucleotide H2-RNase II in RNase II gene.*

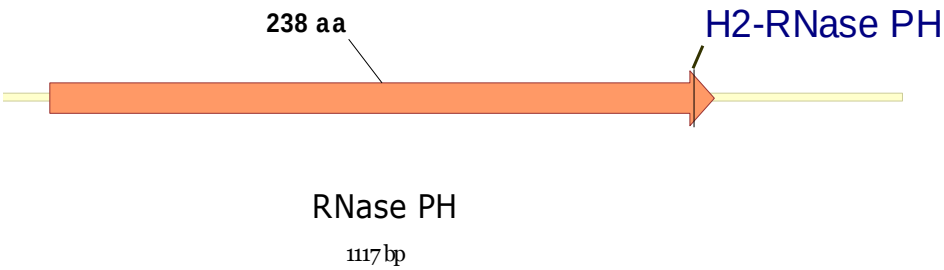


Figure 29. *Targeting sites of oligonucleotide H2-RNase PH in RNase PH gene.*

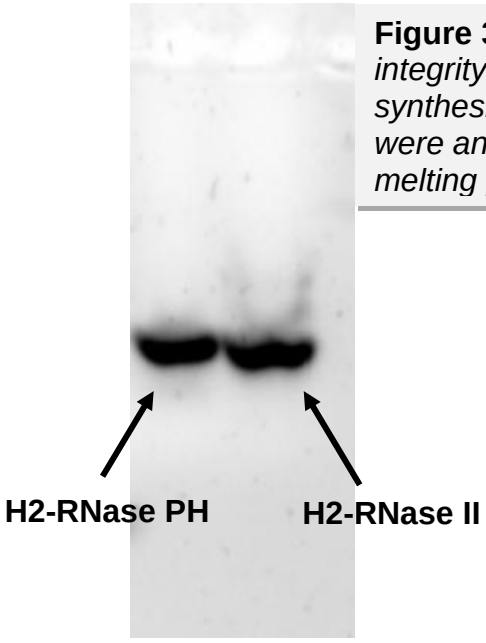


Figure 30. *Oligonucleotides integrity test. 2µl of the synthesized oligonucleotides were analyzed by 4% low melting point agarose gel*

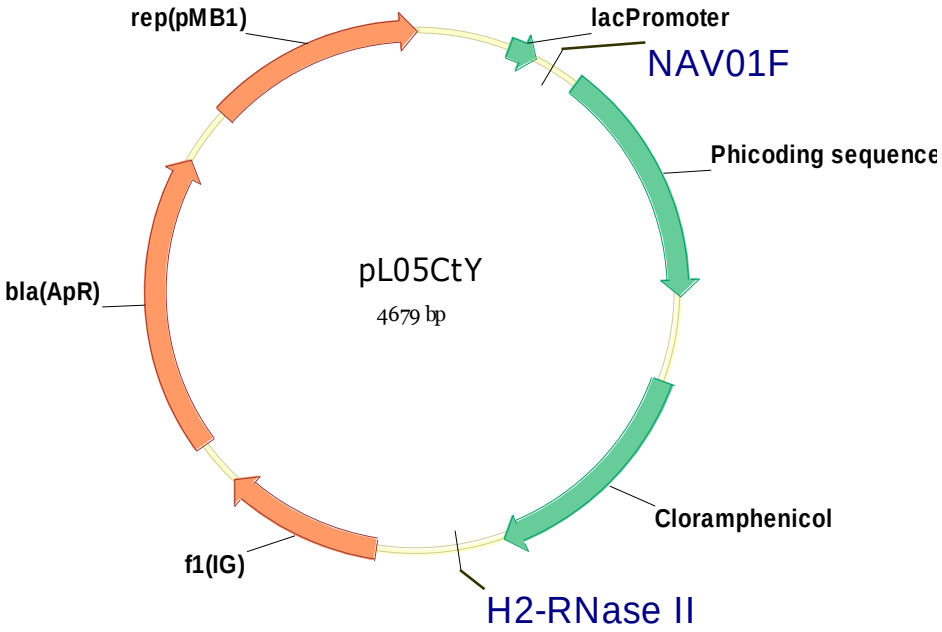


Figure 31. *Targeting sites of* oligonucleotide *H2-RNase II in RNase II gene.*

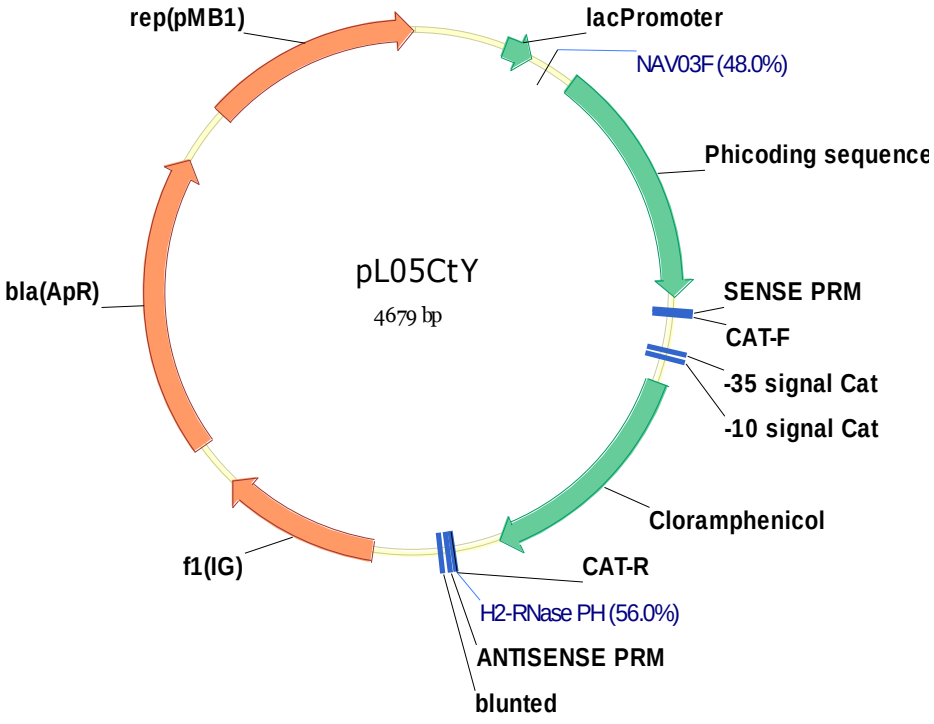


Figure 32. *Targeting sites of* oligonucleotides *H2-RNase PH in RNase PH gene.*

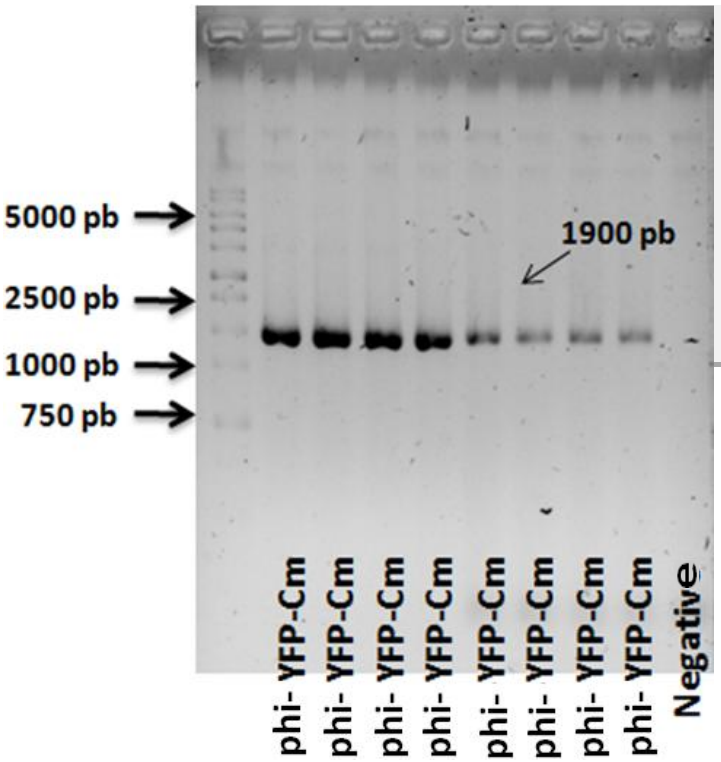


Figure 33. *PCR amplification of phi-YFP-Chloramphenicol cassette with* oligonucleotides *pair NAV01F-H2 RNase II. 2µl of reactions in 0.8% agarose gel* electrophoresis

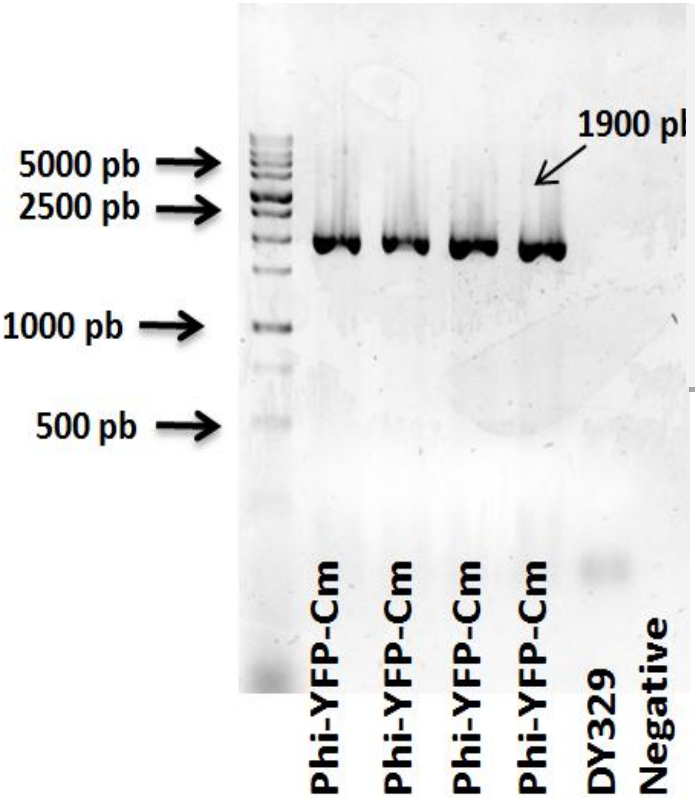


Figure 34. *PCR amplification of phi-YFP-Chloramphenicol cassette with* oligonucleotides *pair NAV03F-H2 RNase PH. 2µl of reactions in 0.8% agarose gel electrophoresis*

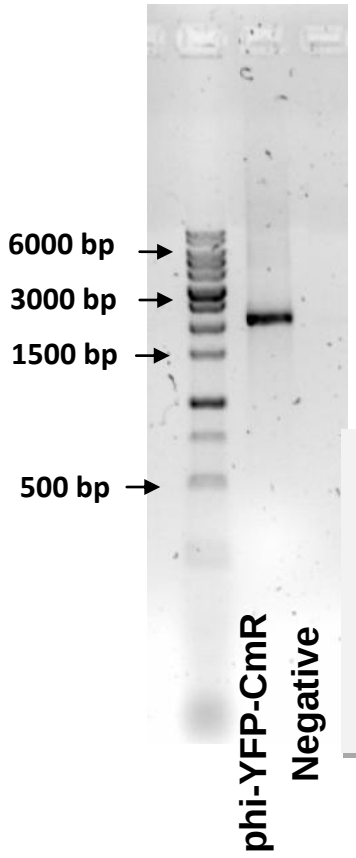


Figure 35. *phi-YFP-Chloramphenicol cassette for recombination in RNase PH gene after restriction with Dpn I enzyme 0.8% agarose gel electrophoresis*

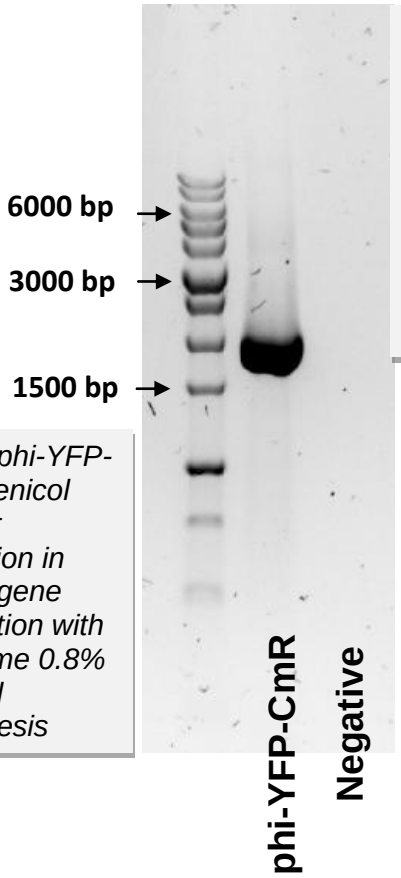


Figure 36. *phi-YFP-Chloramphenicol cassette for recombination in RNase PH gene after extraction with phenol-chloroform 0.8% agarose gel electrophoresis*

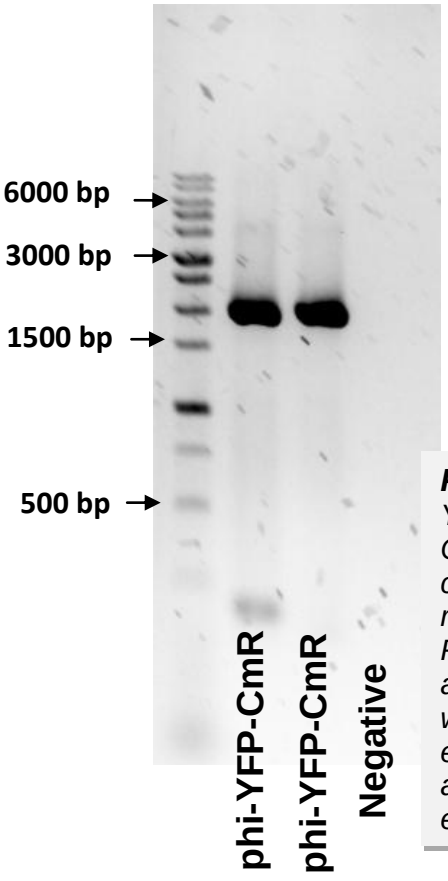


Figure 37. *Phi-YFP-Chloramphenicol cassette for recombination in RNase II gene after restriction with Dpn I enzyme 0.8% agarose gel electrophoresis*

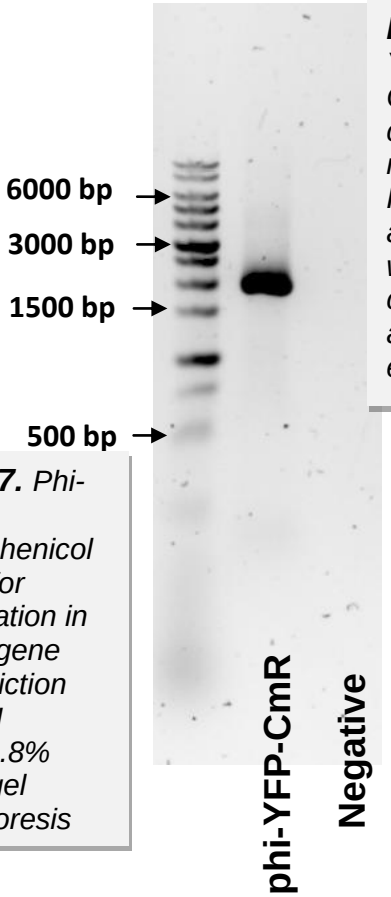


Figure 38. *Phi-YFP-Chloramphenicol cassette for recombination in RNase II gene after extraction with phenol-chloroform 0.8% agarose gel electrophoresis*

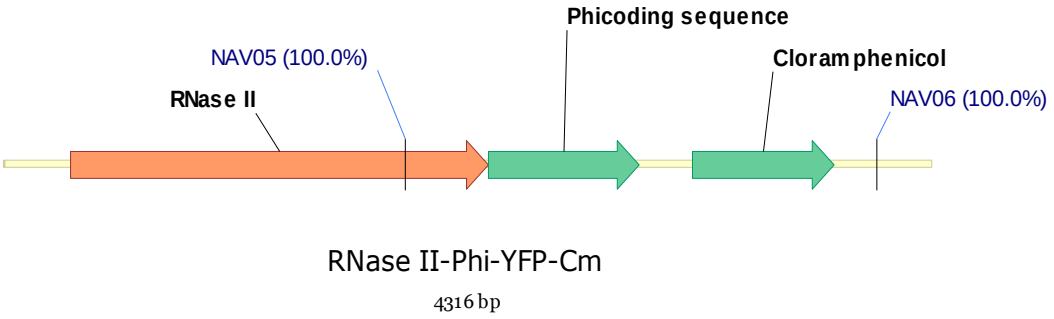


Figure 39. *Targeting sites of oligonucleotide NAV05F in NAV06R gene.*

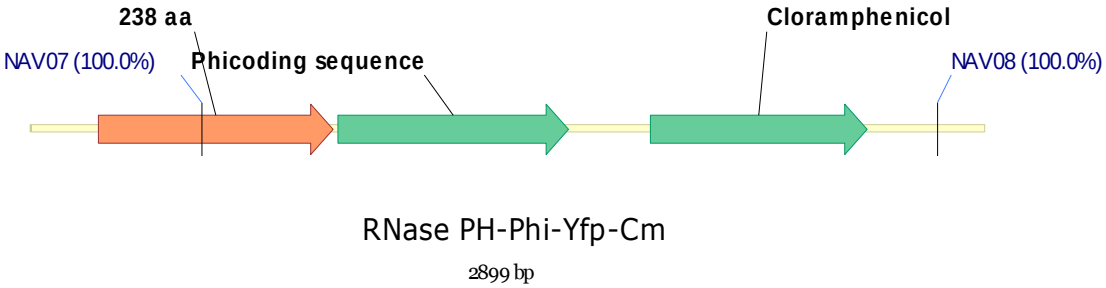


Figure 40. *Targeting sites of oligonucleotide NAV07 in NAV08.*

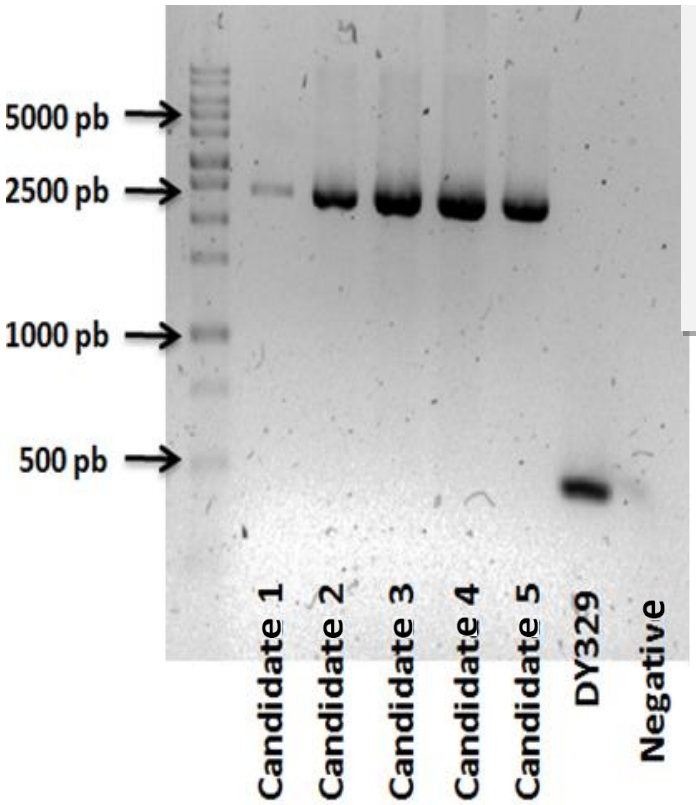


Figure 41. *PCR reactions for proving candidate colonies for LHY strain using NAV05F-NAV06R primers. All candidates resulted positive. Agarose gel at 0.8%.*

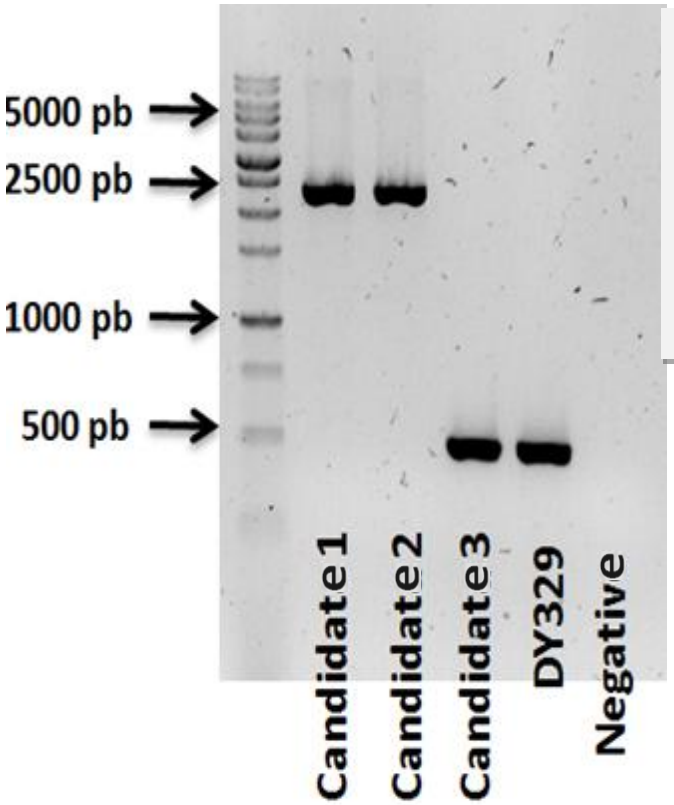


Figure 42. *PCR reactions for proving candidate colonies for LECHY strain using NAV05F-NAV06R primers. First two candidates resulted positive. Agarose gel at 0.8%.*

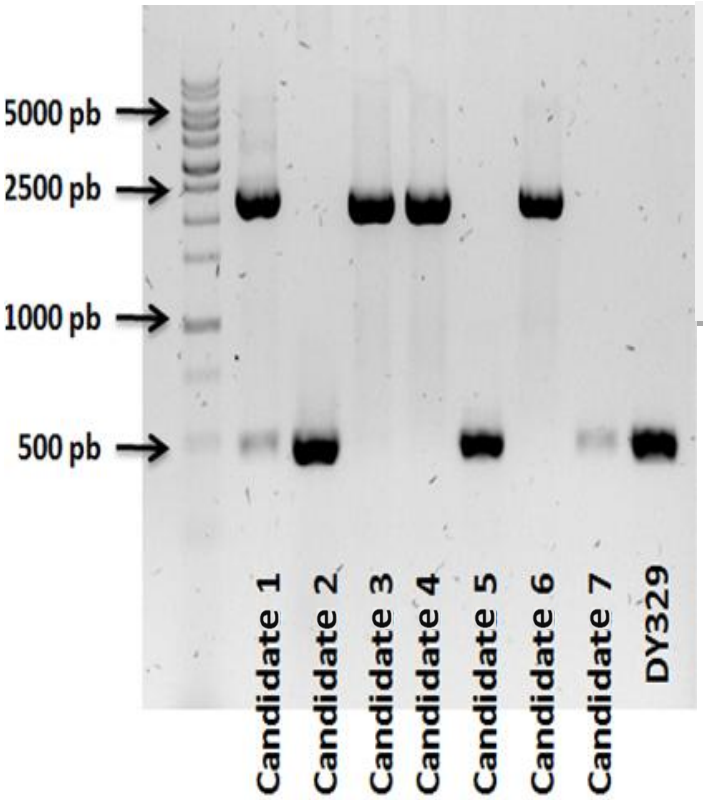


Figure 43. *PCR reactions for proving candidate colonies for LE701CHY strain using NAV05F-NAV06R primers. Candidates 3, 4 and 6 resulted positive. Agarose gel at 0.8%.*

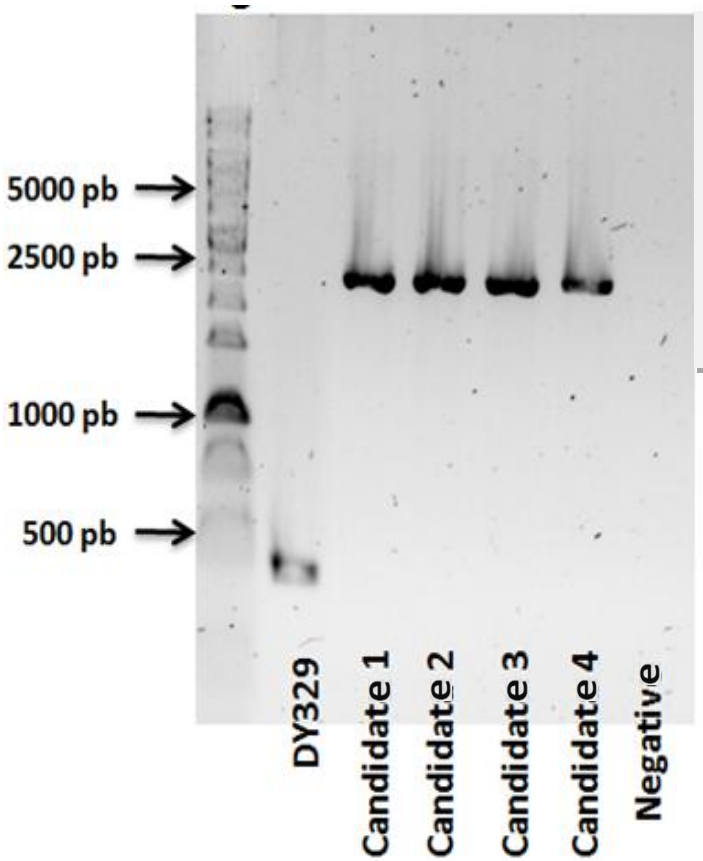


Figure 44. *PCR reactions for proving candidate colonies for LBY strain using NAV07F-NAV08R primers. All candidates resulted positives. Agarose gel at 0.8%.*

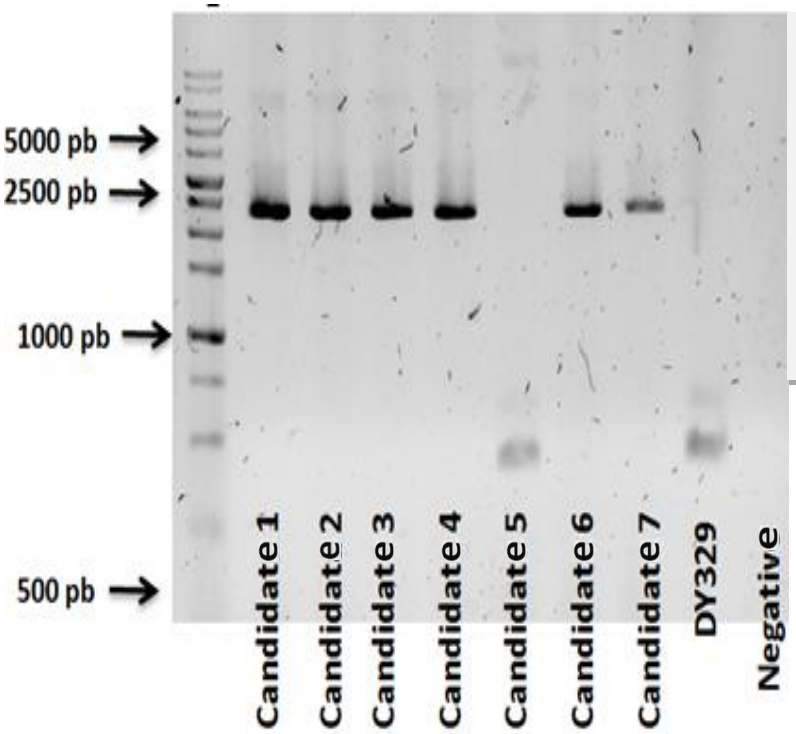


Figure 45. *PCR reactions for proving candidate colonies for LECBY strain using NAV07F-NAV08R primers. All candidates but 5 resulted positives. Agarose gel at 0.8%.*

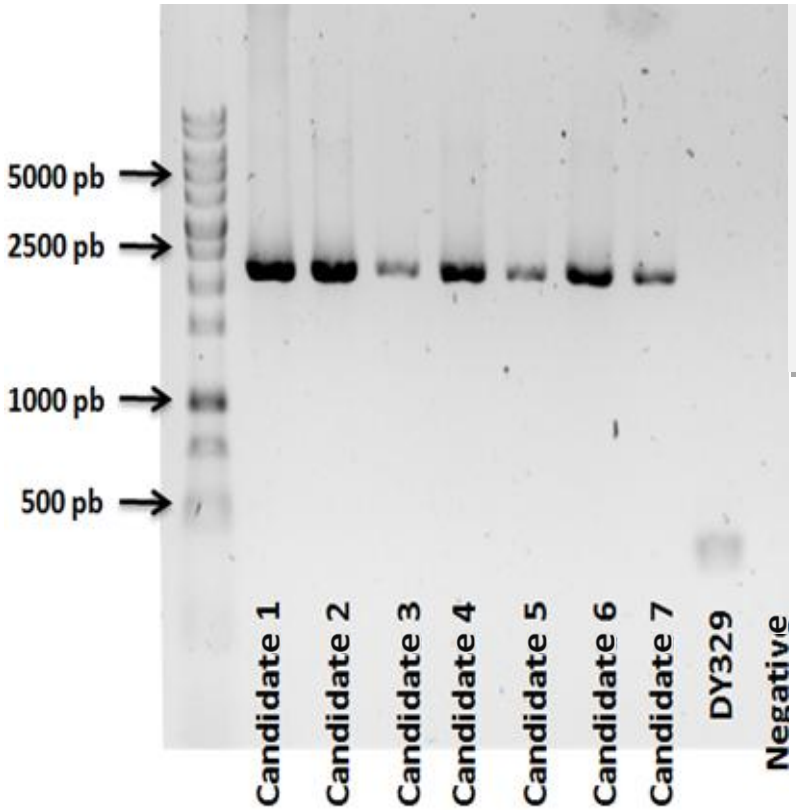


Figure 46. *PCR reactions for proving candidate colonies for LE701CBY strain using NAV07F-NAV08R primers. All candidates resulted positives. Agarose gel at 0.8%.*

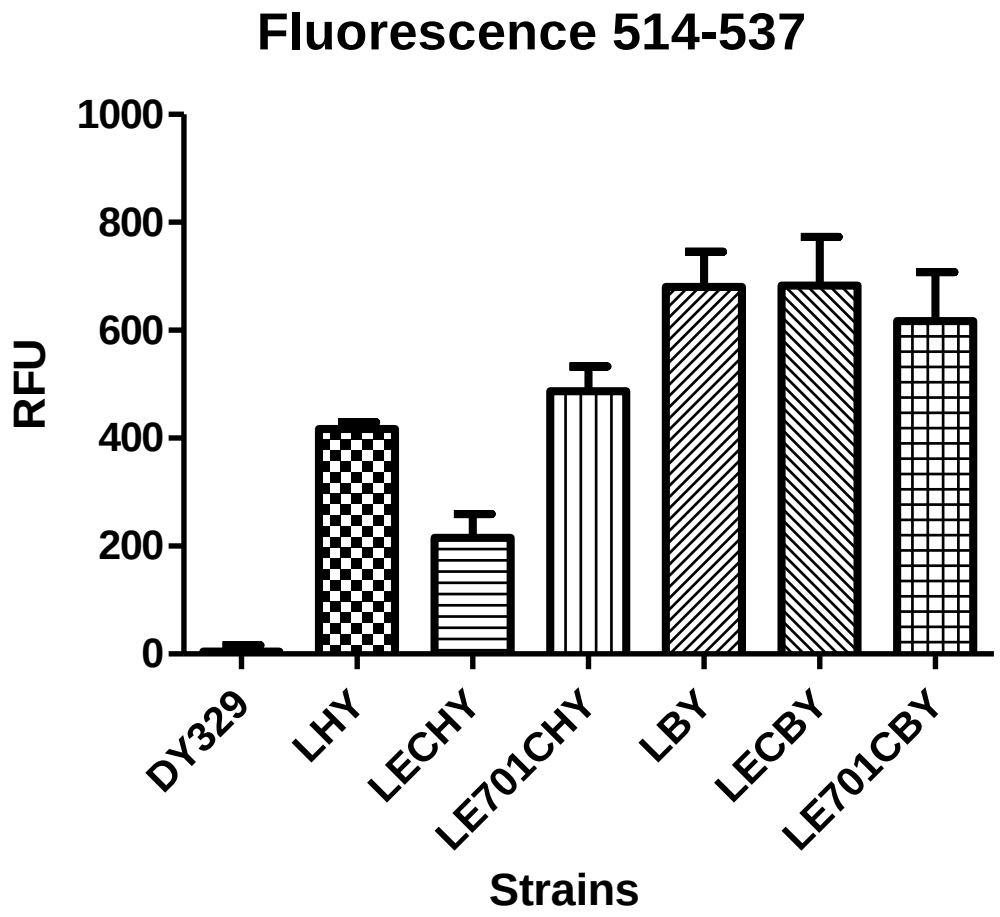


Figure 47. *Fluorimetric assay of all constructed strains. Bacteria were tested whit the channel corresponding to Phi-YFP whit excitation at 514nm and measured emission at 537nm. Fluorescence where normalized to fluorescence of wild type strain DY329. All strains presented higher values of fluorescence when compared to DY329 strain. n=6.*

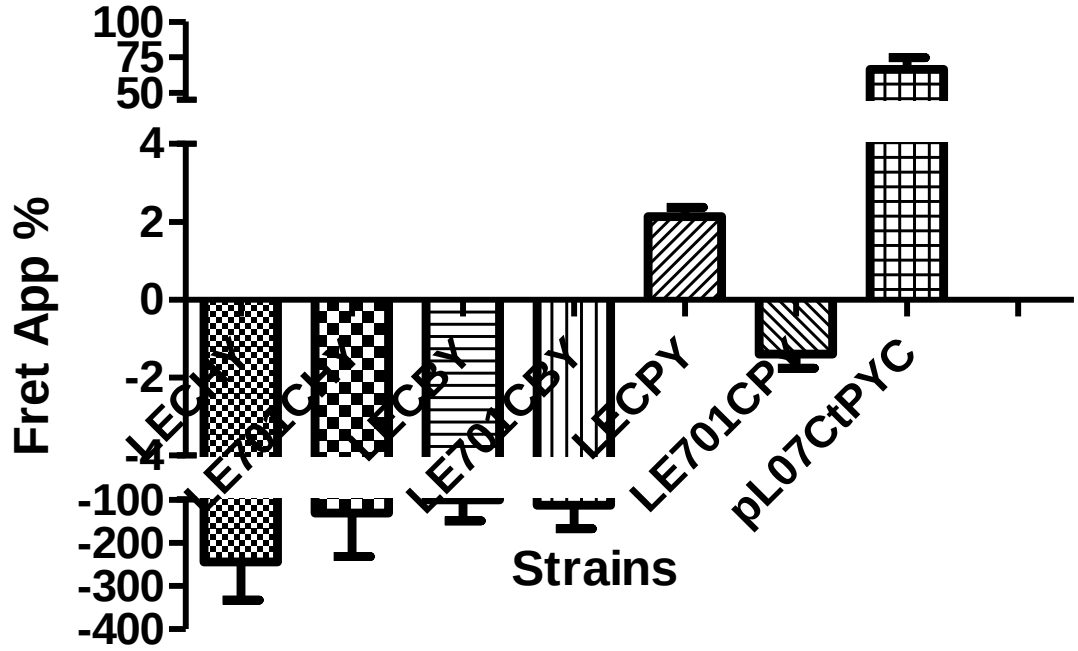


Figure 48. *FRET App of all strains. Control strain pL07CtPYC presented high values of fret efficiency (66.5).LECPY presented higher values (2.137) of FRET efficiency when compared (significance whit t-student p<.001) to strain LE701CPY which presented negative values (-1.39). LECHY(-244), LE701CHY(-130), LECBY(-100) and LE701CBY (-113) strains presented high negative values with a lot of variance and no significant differences (95 %) between strains when compared with t-student test. n=4.*

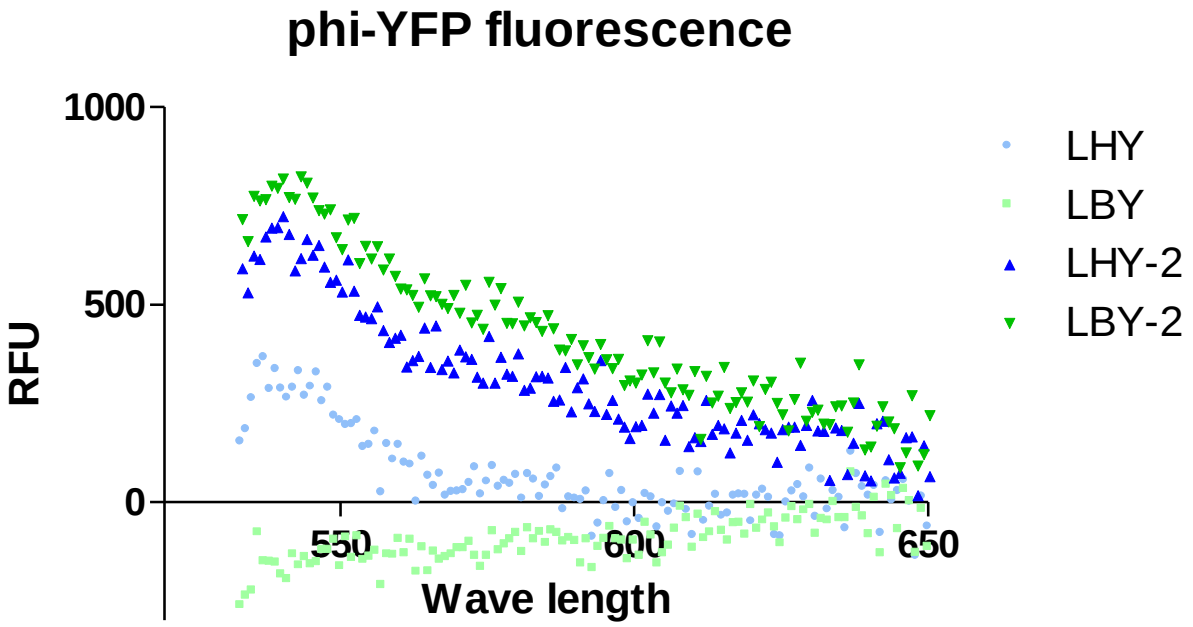


Figure 49. *Increase of phi-YFP fluorescence in stationary phase in strains LBY and LHY. Emission scans were made at 525 nm of excitation. Both strains were measured at 16 h (LHY and LBY) and at 40 h (LBY-2 and LHY-2) of culture. LBY-2 and LHY-2 presented higher values when compared with LHY and LBY suggesting that long periods of stationary phase increase LBY and LHY expression*

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