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Application of Double-stranded RNA (dsRNA) Produced by *E. coli* HT115 (DE3) and Vector L4440 in Reverse Genetics Studies in Insects

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ABSTRACT

RNA interference is a cellular process for regulating gene expression by double-stranded RNA (dsRNA). In the past two decades, this cellular process has been used as a tool for the temporary knockdown of gene expression to study gene function in reverse genetics studies. In this regard, double-stranded RNA has been made in various ways and used to knock down the corresponding gene. In the past decade, the potential of the technique for insect pest management has become clear although the costs associated with the production of dsRNA are not reasonable and affordable for such use. Even on the laboratory scale, making the dsRNA for RNAi experiments using dsRNA production kits is not affordable for most researchers and laboratories. Therefore, researchers are focused on ways to make the production of dsRNA more affordable. The conventional method of carrying out RNAi experiments uses a vector called pL4440 and a host strain of *E. coli* called HT115 (DE3) to make dsRNA. This method which is called bacterium-mediated RNAi (bmRNAi) has been used successfully for the knockdown of many genes in *Caenorhabditis elegans*. However, the number of studies that used this technique so far in insects is limited to a few major insect orders, namely Coleoptera, Lepidoptera, Diptera, and Hymenoptera. In this review, the bmRNAi technique is discussed in detail and the studies successfully conducted using this technique are introduced.

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Introduction

In 1998, Andrew Fire and Craig Mello announced that double-stranded RNAs (dsRNA) cause Post-Transcriptional Gene Silencing (PTGS) in the nematode *Caenorhabditis elegans*, and called this phenomenon RNA interference or RNAi for short (Fire *et al*., 1998). This phenomenon explained confusing observations of gene silencing in plants and fungi and started a revolution in biology leading to the conclusion that non-coding RNAs are the main regulators of gene expression in Eukaryotes (Setten *et al*., 2019). Since the bacteria are the dominant diet of *C. elegans* in the laboratory, engineered bacteria to produce dsRNA have been used to provide dsRNA to them (Timmons and Fire, 1998). A cloning vector L4440 (pPD129.36), containing two convergent T7 polymerase promoters in opposite directions separated by a multiple cloning site (MCS), has been constructed (Fig. 1) (Timmons and Fire, 1998). The vector has been used in an *E. coli* strain (BL21/DE3; (Studier and Moffatt, 1986)) expressing bacteriophage lambda T7 polymerase gene from an inducible promoter (Lac) (Timmons and Fire, 1998). Despite the observation of specific interference in the studies conducted with dsRNA-producing bacteria, which indicated the suitability of ingested dsRNA, limited phenotypes were observed in terms of penetration and expression. Therefore, increasing the efficiency of this technique in

various ways has been the subject of subsequent studies. These studies showed that a strain of bacterium lacking specific dsRNA endonuclease, known as RNaseIII (rnc-), can be cultivated to produce high amounts of specific dsRNA. Also, these bacteria can effectively induce strong and target gene-specific phenotypic responses during feeding assays in *C. elegans* (Timmons *et al*., 2001). This effective strain was HT115 (DE3), constructed by deleting the *rnc* gene from the W3110 strain with T7 RNA polymerase under an IPTG-inducible promoter derived from bacteriophage λDE3 (Studier and Moffatt, 1986) and tetracycline resistance (Tn10) (genotype: HT115: W3110, rnc14::ΔTn10) (Dasgupta *et al*., 1998; Takiff *et al*., 1989). After introducing the combination of vector L4440 and host strain HT115 (DE3) as the most effective combination in the production of dsRNA in the study of RNAi in *C. elegans* (Timmons *et al*., 2001), this method became a conventional method in such studies. In this review, we will briefly introduce the process of RNAi in general, and in particular, will focus on bacteria-mediated RNAi (bmRNAi) and the studies conducted using this technique on insects.

Environmental RNA interference process

The process of RNA interference can be divided into four steps: (1) cleavage of long double-stranded RNA by Dicer, (2) formation of the RNA Induced Silencing Complex (RISC), (3) unwinding of the double-stranded siRNA and activation of the silencing complex, as well as (4) cutting and destroying target mRNA (Kim, 2003; Szweykowska-Kulińska *et al*., 2003). The first step of the environmental RNAi process begins with the delivery of long exogenous dsRNA whose sequence is completely homologous to the target RNA sequence. The dicer enzyme recognizes dsRNA and typically cuts it into 21-25 nucleotide fragments (Hannon, 2002) of double-stranded siRNA, depending upon the species, in a reaction dependent on ATP energy. In the second step, Dicer-cleaved siRNAs are incorporated into the inactive RNA-Induced Silencing Complex (RISC), a multicomponent nuclease complex. In the third step, in an ATP-dependent process, the enzyme helicase opens up the two strands of siRNA and activates the RISC. In the final step the RISC, coupled with an antisense strand of siRNA, tracks down the target mRNA and cuts it into 22 nucleotides long fragments (Fig. 2). After the completion of cleavage, the RISC-siRNA complex is dissociated and the released siRNA can repeat the mRNA recognition and cleavage cycle (Ali *et al*., 2010; Hammond *et al*., 2001; Hannon, 2002).

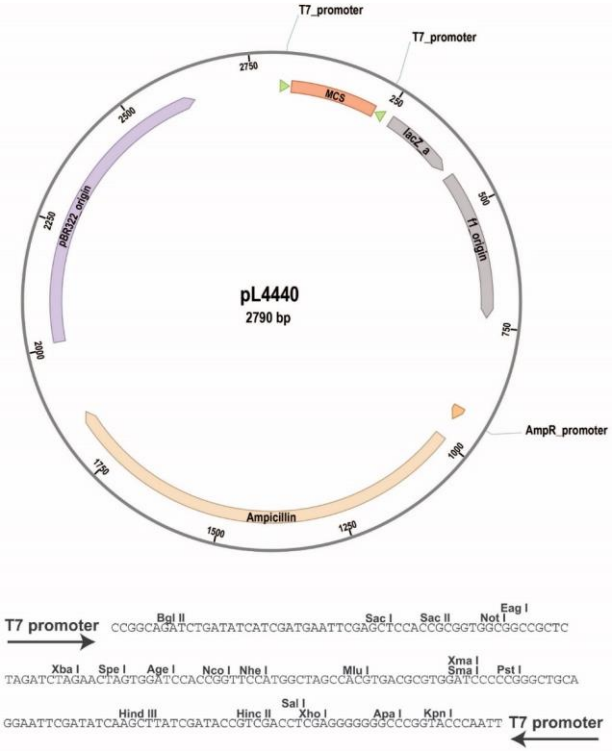

Fig. 1. Map of L4440. The empty RNAi vector (L4440) is a modified version of pBluescript with convergent T7 promoters on each side of a 185 base pairs (bp) Multiple Cloning Site (MCS) (bottom sequence of the fig.) that directs the transcription of RNA from both DNA strands (Andersen *et al*., 2008). It is worth mentioning that a newer version of this vector has been made with T7 terminators upstream of the promoters (Sturm *et al*., 2018).

Studies conducted on insects using bmRNAi

E. coli strain HT115 (DE3) was first transformed with the L4440 vector in 2001 and since then, it has become a useful tool for RNA interference experiments in invertebrates (Timmons *et al*., 2001). The studies conducted using this method in insects and the methods of providing dsRNA to different insects are summarized in Table 1, showing the effectiveness of this inexpensive dsRNA production and delivery method in the four major orders of insects, namely Coleoptera

(beetles and weevils), Lepidoptera (moths and butterflies), Diptera (true flies) and Hymenoptera (wasps, bees and ants). Currently, the production of dsRNA by fermentation is considered the cheapest method. The lack of data on the effectiveness of this inexpensive dsRNA production and delivery method and the importance of the remaining insect orders from different perspectives, including pest control and gene function analysis, emphasizes the necessity of conducting such studies. The potential pathogenicity of *E. coli* for several insect species shows that if this method is used in pest control, beneficial insects can also be negatively affected by the use of this delivery system (Vogel *et al*., 2019). Considering that in some of the reviewed studies, the dsRNA-producing bacteria added to the diet has shown better efficiency, and in others purified dsRNA, the delivery method may be different depending on the insect species.

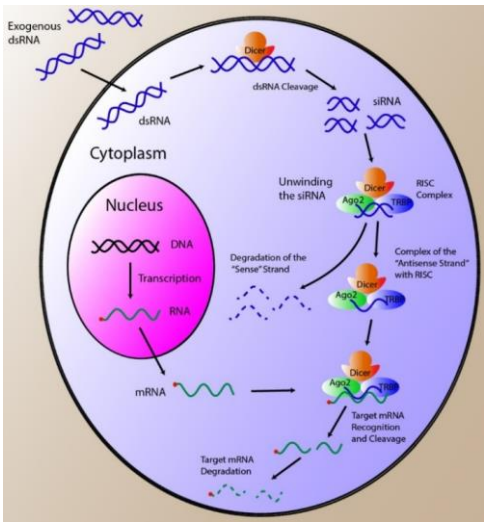

Fig. 2. Mechanism of environmental RNA interference. In this phenomenon, foreign long dsRNA enters the cell and typically is converted into smaller pieces of 21-25 nucleotides by the Dicer enzyme, which is called siRNA. The siRNA molecules are then placed in an RNA-Induced Silencing Complex (RISC) and the two strands are separated. The complementary strand of the target mRNA is kept as a guide in the complex and the non-complementary strand is left and degraded. Then, by finding the target mRNA and matching the sequence, the complex proceeds to cut the target mRNA. The cleaved mRNA is then targeted and degraded by the nucleases of the cell.

Step-by-step process of bmRNAi

The step-by-step process of conducting an RNA interference experiment with bacterial dsRNA is briefly mentioned in the following.

1- Designing the best possible double-stranded RNA specific to the target gene and organism in order to increase the knockdown efficiency: E-RNAi website is one of the online tools for dsRNA design. By providing the sequence or Accession Number of the desired gene, it suggests the best part of the sequence for making dsRNA and provides relevant primers (Horn and Boutros, 2010).

2- Selecting restriction enzymes according to the designed sequence and the multiple cloning sites (MCS) on the L4440 vector (Fig. 1) and adding the restriction site of these enzymes along with 3 additional A nucleotides at the 5′ end of the primers from step 1 to create enough binding space for the restriction enzymes (Ahn *et al*., 2019). The selected restriction enzymes should not have cut sites on the designed sequence. It is worth mentioning that it is better to choose restriction enzymes whose cut site in the vector map has the shortest distance from the T7 promoter (the target sequence must lack these cut sites). In this way, a large part of the MCS will be removed in the cloning process and fewer extra sequences will be built into the dsRNA.

3- Extracting RNA from the studied insect, synthesizing cDNA, and performing PCR using the primers designed in step 2 and doing PCR product clean-up.

Table 1. Studies conducted on insects by bmRNAi approach using *E. coli* strain HT115 (DE3) and vector L4440.

Insect orders	Insect species	Target gene	dsRNA delivery method	References
Lepidoptera	*Spodoptera exigua*	*Chitin synthase A*	coated food with a suspension of live bacteria	(Tian *et al*., 2009)
	Sesamia nonagrioides	*Juvenile hormone esterase*	coated food with a suspension of live bacteria	(Kontogiannatos *et al*., 2013)
	Helicoverpa armigera	*cytochrome P450 CYP6B6*	dsRNA extracted from bacteria	(Zhang *et al*., 2013)
	Helicoverpa armigera	*Ultraspiracle protein*	coated food with a suspension of bacteria, dsRNA extracted from bacteria	(Yang and Han, 2014)
	Spodoptera exigua	*Integrin β1*	coated food with a suspension of live bacteria, heat-killed bacteria, and damaged bacteria by sonication	(Kim *et al*., 2015)
	Chilo suppressalis	*ptth, torso, spook and nm-g*	dsRNA extracted from bacteria	(Zhu *et al*., 2016)
	Mythimna separata	*chitinase*	coated food with a suspension of live bacteria	(Ganbaatar *et al*., 2017)
	Spodoptera exigua	*Chymotrypsin 2*	coated food with a suspension of heat-killed bacteria and damaged bacteria by sonication	(Vatanparast and Kim, 2017)
	Spodoptera littoralis	*Sl 102*	coated food with a suspension of killed bacteria by sonication using an ultrasound homogenizer	(Caccia *et al*., 2020)
	Tuta absoluta	*Juvenile hormone inducible protein, Chintin synthase A, Carboxylesterase, Arginine kinase*	coated food with a suspension of live bacteria	(Bento *et al*., 2020)
	Spodoptera frugiperda	*Chitinase, Chitin synthase B, Sugar transporter SWEET1, and Hemolin*	coated food with a suspension of live bacteria	(Wan *et al*., 2021)
	Spodoptera litura	*Natalisin*	dsRNA extracted from bacteria	(Wang *et al*., 2021)
	Helicoverpa armigera	*Dicer2*	dsRNA extracted from bacteria with various methods	(Verdonckt and Vanden Broeck, 2022)
Coleoptera	*Leptinotarsa decemlineata*	*b-actin, Protein transport protein sec23, Coatomer subunit beta*	dsRNA extracted from bacteria	(Zhu *et al*., 2011)
	Helicoverpa armigera	*ecdysone receptor*	dsRNA extracted from bacteria	(Zhu *et al*., 2012)
	Plagiodera versicolora	*actin, signal recognition particle protein 54k, heat shock protein 70, shibire, cactus, soluble N-ethylmaleimide-sensitive fusion attachment proteins*	coated food with a suspension of bacteria	(Zhang *et al*., 2019)
	Leptinotarsa decemlineata	*cuticular protein, cytochrome P450 monoxygenases, glutathione synthetase*	coated food with a suspension of live bacteria	(Naqqash *et al*., 2020)
	Agrilus planipennis	*shibire, heat shock protein-70kDA*	coated food with a suspension of live bacteria	(Leelesh and Rieske, 2020)
	Harmonia axyridis	*vestigial*	dsRNA extracted from bacteria	(Ma *et al*., 2020)
Diptera	*Aedes aegypti*	*doublesex*	coated food with a suspension of live and heat-killed bacteria	(Whyard *et al*., 2015)
	Drosophila suzukii	*pyrokinin*	dsRNA extracted from pretreated bacteria with sonication or heat	(Ahn *et al*., 2019)
Hymenoptera	*Camponotus floridanus*	*peptidoglycan recognition proteins, PGRP-LB and PGRP-SC2*	dsRNA extracted from bacteria	(Ratzka *et al*., 2013)

4- Enzymatic digestion of L4440 vector and the pure PCR product and performing agarose gel electrophoresis followed by purification of the fragments. Ligation of the purified linearized L4440 vector and the PCR product to create a recombinant vector.

5- Making chemically competent *E. coli* strain HT115 (DE3) (Green and Rogers, 2013; Swords, 2003) and transforming it by recombinant L4440 vector using heat shock treatment of 42 °C for 40 seconds. Culturing the transformed *E. coli* in super optimal broth with catabolite repression

(SOC) medium (Lessard, 2013) for 1 hour and 30 minutes. Finally, transferring 150 ul of culture into an LB medium plate containing tetracycline (12.5 μg/ml) and ampicillin (50 μg/ml) and incubating at 37 degrees overnight.

6- Selecting individual colonies and doing direct colony PCR using primers pL4440F (ACCTGGCTTATCGAA) and pL4440R (TAAAACGACGGCCAGT) (Whyard *et al*., 2015) followed by agarose gel electrophoresis to confirm the success of the process (by knowing the product size). Sequencing the fragment for complete assurance.

7- Liquid culture of the successful colony in selective LB medium containing tetracycline (12.5 μg/ml) and ampicillin (50 μg/ml) at 37 degrees overnight.

8- Transferring 4 milliliters of the cells to 400 milliliters of fresh selective LB Amp-Tet medium and incubating for 4 hours at 37 °C with shaking. When the Optical Density (OD) reaches higher than 0.4, bacterial cells can be induced with sterile filtered isopropyl β-d-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and then incubated for another 5 hours under the same conditions. At this stage, the bacteria contain dsRNA and are ready to be delivered to insects in different ways.

9- If it is necessary to purify dsRNA from bacteria, the cells are pelleted by centrifugation (4000 relative centrifugal force (rcf) for 10 minutes at 4 °C) and suspended in 1 milliliter of distilled water and transferred to a 15 milliliters Falcon tube. To destroy the cells and release dsRNA, they are treated with various enzymatic, heat or sonication methods. Purification of dsRNA from bacteria is carried out using regular RNA or dsRNA extraction methods (Chomczynski and Sacchi, 1987; Diaz-Ruiz and Kaper, 1978) followed by enzymatic digestion of DNA and single-stranded RNA; or selective precipitation of dsRNA (Verdonckt and Vanden Broeck, 2022).

10- Delivering the dsRNA to the studied insect in various ways, including overlaying the insect's food in a suspension of live or killed bacteria, extracting and purifying dsRNA by enzymatic or selective precipitation methods. Then evaluating effects of dsRNA on insect by phenotypic assay and/or real-time PCR assay.

Conclusion

The RNA interference technique is considered an efficient, cheap, and quick method in gene function analysis studies. This method is especially important in organisms that are not considered as model organism since the genetic tools for gene function analysis are not available in these organisms. After the introduction of this phenomenon and using it, in a short period of time, the potential of this technique for insect control became clear and studies were directed in that direction. The bottleneck of using the RNA interference technique in insect control and management is the high cost of dsRNA production on a large scale; therefore, efforts are underway to make dsRNA production cheaper on this scale. The bmRNAi is considered the cheapest method of RNAi. Considering that the bmRNAi studies conducted are limited to only four large orders of insects, it is necessary to prove the effectiveness of this technique and delivery method in other insect orders as well.

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