Cloning and expression of the predicted α/β hydrolase enzyme from *Variovorax* sp. EA-ED0101

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ABSTRACT *Ralstonia solanacearum* is a gram-negative pathogen bacteria responsible for Moko disease in many crops of economic importance such as potato, tomato, begonia, heliconia, anthurium and mainly plantain and banana especially in Colombia, due their genetic variability makes it less resistance for a specific treatment, it becomes difficult and hard to handle the pathogenicity of *R. solanacearum* which Quorum Sensing is governed by 3OH-PAME molecule identified as the responsible of virulence factors and production of AHLs. Biological control is a tool with a promising way of treatment due it would not generate resistance in strains by the Quorum Quenching mechanisms discovered in many organisms after screening several places to identify an organism and enzyme responsible for driving this response over pathogens. Preliminary studies isolated endophytic microorganisms from Musaceae crops in Colombia and identified *Variovorax* sp. EA-ED0101 as potential strains to degrade 3OH-PAME, the bioinformatic analysis found α/β hydrolase gene responsible for the predicted enzyme. This work focuses on the molecular biology approach to corroborate bioinformatic predictions, so the gene is isolated, purified and cloned into the pET 101/D-TOPO vector expression system into *E. coli* TOP10, extracted using Miniprep techniques and check predicted hydrolase gene presence into the vector using insert specific primers and T7 specific primers present in the vector. Furthermore, with the transformation process into *E. coli* BL21 to drive the expression process after adding IPTG to the medium and collected a sample from the hour 0 of the addition until the hour 5 of the process and checked expression in SDS-PAGE electrophoresis corroborating partially previous bioinformatic analysis.

KEYWORDS Protein expression, *Ralstonia solanacearum*, Quorum Quenching, molecular biology...

Introduction

Bacteria were for a long time believed to exist as individual cells, yet, the discovery of communication between them made possible the understanding of how they coordinate particular behaviors together to get better nutrients, proliferate and form biofilms that provide an ability to be more resistant to certain harsh environments (De Kievit and Iglewski 2000). The language they use to communicate is based on molecules generated by themselves, called self-inducing molecules, with which bacteria manage to behave in a certain way according to their population density (De Kievit and Iglewski 2000). Communication between bacteria is known as Quorum Sensing (QS) which works in such a way that when a bacterium releases a self-inducing molecule into the environment it is not detected by another bacterium because the concentration is minimal, although, when there is enough population density to reach a concentration that achieves the required threshold it is when they can activate or repress certain genes together, depending on the ecology of the microorganism to regulate different processes such as sporulation, synthesis of antimicrobial peptides, biofilm formation, the plasmid transfer through conjugation, virulence, antibiotic resistance, swarming motility, and secondary metabolite production (De Kievit and Iglewski 2000; San José and Orgaz 2010; Zhou *et al.* 2016). The interactions between these microorganisms impact their physiology due to the alteration of the chemistry of the environment and shape microbial diversity (Zhou *et al.* 2016).

The identification of the QS molecules in many microorganisms is diverse, for example, in Gram-positive bacteria, QS signalling molecules mainly relies on small cyclic or linear peptides, which are detected by a membrane-bound sensor kinase and

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some Gram-negative bacteria respond to lipid signals such as the diffusible signal factor (DSF) or 3-hydroxypalmitic acid methyl ester (3OH-PAME), involved in QS (Fetzner 2015). In the case of pathogenic bacteria that use QS as a communication language to express virulence factors in plants as phytopathogens, results in a global problem because bacteria that present this type of behavior mostly affect crops of interest for human consumption (Nazzaro, Frantianni and Coppola 2013). Due to this problem, the use of numerous treatments has been tried for the diseased plants within the healthy crop, eradicating the source of infection, however, the transmission of the disease is a difficult factor to control because these bacteria have developed strategies to be increasingly versatile and increment its spread to more healthy individuals plants, for which the treatments are not fully efficient (Eljounadi, Lee and Bae 2016).

Bacterial wilt pathogen *Ralstonia solanacearum* is a gram-negative bacterium and a significant constraint on the production of bananas and plantains due to the limited options for its control, easy dispersion and devastating effects on a broad host range including economically important crops such as potato, tomato, plantain, banana, begonia, heliconia, anthurium and peanut for a large number of countries, especially in Colombia (Ramírez *et al.* 2020). The principal QS molecule of this pathogen is 3-hydroxy palmitic acid methyl ester (3OH-PAME; *Fig. 1.*), a volatile molecule capable of governing the expression of virulence factors in *R. solanacearum*, This pathogen enters roots through wounds or natural openings and migrates to the developing vascular bundles, reaching the xylem vessels (Lowe-Power, Khokhani and Allen 2018). After entering the xylem network, *R. solanacearum* spreads systemically through its host. These cells eventually grow into aggregates in a biofilm matrix that can fill entire vessels and potentially obstruct water flow (Lowe-Power, Khokhani and Allen 2018). Bacterial wilt virulence depends on a large consortium of plant cell-wall-degrading enzymes, extracellular polymeric substances (EPS), and the expression of a complex regulatory cascade dominated by the Phc quorum-sensing system which 3OH-PAME is the molecule required to activate this system (Lowe-Power, Khokhani and Allen 2018). However, interestingly the 3OH-PAME based QS system also regulates the expression of acyl-homoserine lactone based system, which has no contribution to virulence in *R. solanacearum* (Fetzner 2015).

The degradation of QS molecules is widely reported as Quorum Quenching (QQ), and this mechanism has been used as an alternate approach for biocontrol of phytopathogens by many researchers because it leads to observe less pathogenic effects on the infected plant (Achari and Ramesh 2015). There are so many compounds that are effective in showing selective interference with the QS of pathogenic bacteria, to target bacterial virulence, and to develop new anti-infective therapies (Fetzner 2015). Thus, the QQ is a mechanism used to inhibit the biosynthesis of virulence factors by different mechanisms of quorum quenching as (1) inhibition of synthesis of quorum-sensing signal, (2) inhibition of sensing of quorum-sensing signal, (3) degradation of quorum-sensing molecules, and other mechanisms (Achari and Ramesh 2019). The QQ is considered then, as a good strategy that is even better than bactericidal or bacteriostatic treatments because it is less likely to produce resistance in the phytopathogen (Fetzner 2015).

Ralstonia solanacearum has a broad spectrum of affecting various crops of human interest due to its geographical distribution, genetic diversity, pathogenicity, epidemiological relationships, and physiological properties (Alvarez *et al.* 2013). Bacterial communities have developed several sophisticated ways of interacting and associating with the environment they inhabit, and microorganisms can change by altering their phenotype so their metabolism and other activities can be successful in the new environment (Nazzaro, Frantianni and Coppola 2013). The evolution acting over pathogen *Ralstonia solanacearum* can be problematic due to the complex of strains they can form having clusters of strains with phenotypic differences (Fegan and Prior 2006). As a consequence of the extent variation found in its host range, pathogenicity and geographical distribution, *R. solanacearum* is recognized as a species complex which is *R. solanacearum* (American strains), *R. pseudosolanacearum* (Asian and African strains) and *R. syzygii* (Indonesian, Japanese and Australian strains) (Fegan and Prior 2006; Ramírez *et al.* 2020). Additional research has found some association between phylotypes and the ecological characteristics of their geographic distribution (Ramírez *et al.* 2020). The 3OH-PAME molecule in QS is a conserved communication system and one of the mechanisms by which *R. solanacearum* express their virulence factors to cause bacterial wilt in the host and later its death (Bassler 1999; Kumar *et al.* 2016). Once 3OH-PAME through which *R. solanacearum* communicates reached the quorum, it would be interesting to explore which biomolecule available can have a degradative effect on this compound, as a strategy of QQ and evaluate *R. solanacearum* response to this change over the molecule that governs its pathogenicity.

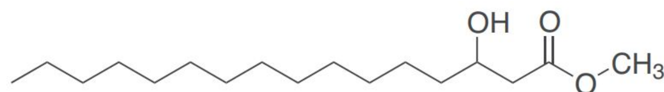

Figure 1 Chemical structure of 3-Hydroxypalmitic acid methyl ester (3OH-PAME), representative quorum sensing signal molecule of some Gram-negative bacteria.

Preliminary studies from EAFIT University (Villegas-Escobar and Londoño 2020) have allowed an approach to these biological control techniques against phytopathogen *Ralstonia solanacearum*, which performed the isolation of endophytic microorganisms from Musaceae crops and identified *Burkholderia* sp. EA-ED0047, *Microbacterium* sp. EA-ED0055 and *Variovorax* sp. EA-ED0101 as potential strains to degrade 3OH-PAME being the only source of carbon in the medium, those three strains showed a degradation halo against *R. solanacearum* after 48 hours of 5.5 +/- 0.8 mm, 3.1 +/- 0.4 mm, and 8.6 +/- 1.0 mm, respectively, choosing *Variovorax* sp. EA-ED0101 for its high degradation capacity on the compound, building a genomic library by fragmenting DNA and storing it into different cosmids generating various clones to observe which part of the entire genome of *Variovorax* contained the gene responsible of the high degradative capacity. Finally, choosing the clone 306 (C306) among the 500 clones generated to be the clone with this major activity, after sequencing this segment of the genome (cosmid of the clone 306), it was possible to predict putative genes using computational biology methods. Finding homologies with protein sequences to be the probable function protein (Data unpublished) revealed that it would be a hydrolase (*Fig.2.*).

Through BLAST-P, multiple sequence alignment at the NCBI revealed between 82.3% and 69.6% identity of the hypothetical protein with proteins defined as α/β hydrolase from *Variovorax*

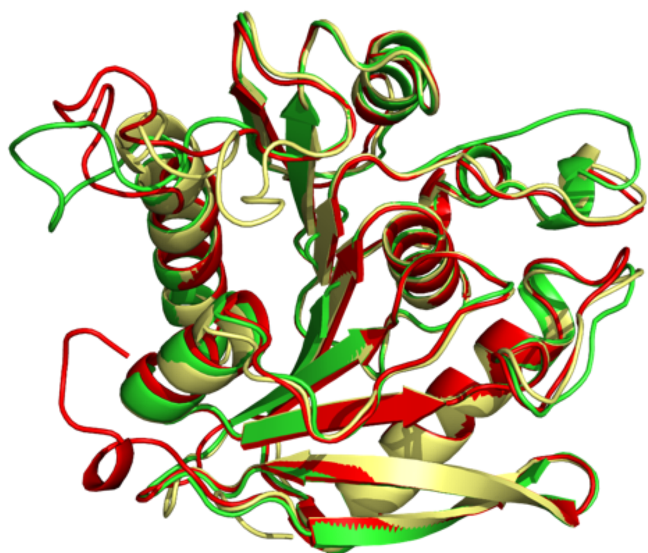

Figure 2 Predicted α/β hydrolase 3D structure from *Variovorax* sp. EA-ED0101, after identifying one consensus motif through bioinformatics tools (From data unpublished).

sp. EA-ED0101 Besides, using UniProt showed similar alignments with α/β hydrolase of several organisms. Additionally, one consensus motif was identified, which is conserved among serine esterases (Shinohara, Nakajima and Uehara 2007). Finally, 3D models of two other α/β hydrolase with the query obtained from *Variovorax* sp. EA-ED0101 showed more than 90% accuracy with good alignment. Due to all this work from (Villegas-Escobar and Londoño 2020), it helped to not only predict the probable gene responsible for producing the biomolecule enzyme that can perform QQ technique against phytopathogen *Ralstonia solanacearum* degrading the 3OH-PAME but also to find a possible function of the protein synthesized by the candidate gene responsible through bioinformatic analyses and that could be the protein structure (*Fig.2.*). The goal in this study is to not only successfully clone this gene in a heterologous organism but also to corroborate the functionality of this protein using a molecular biology approach expressing the candidate gene. It can be revealing because finding a molecule that can degrade 3OH-PAME could lead to future biological control techniques against *R. solanacearum* the pathogen that affects many human crops of economic importance. Molecular biology can evaluate the veracity of those bioinformatic analyses as a supporting tool with which can perform further *in vitro* assays once the potential protein is isolated from *Variovorax* sp. EA-ED0101 elucidating the degradative capacity.

Materials and Methods

Cosmid extraction from E. coli Clone 306

The *E. coli* TOP 10 clone 306 contained the cosmid, this clone showed a higher level of degrading activity against 3OH-PAME as the only source of carbon to quench *Ralstonia solanacearum*. The cosmid has ampicillin resistance gene so to reactivate that clone it was cultured overnight adding 1 mL in 100 mL of LB medium (10 gr/L Tryptone, 10 gr/L NaCl, and 5 gr/L Yeast extract) with 100 μg/mL of ampicillin, at the other day the sample was treated with alkaline lysis method for maxiprep and also was treated with QIAprep Spin Miniprep Kit (QIAGEN) following manufacturer's instructions with the modification of the last step where the elution of the DNA from the column is using EB buffer preheated at 70 ºC (QIAprep Spin Miniprep Kit Handbook Appendix C) in order to obtain higher yield recommended for large plasmids (>10 kb), where the cosmid by sequencing result showed a size of 36 kb. Once the cosmid was extracted with the kit we proceeded to visualize it through an electrophoresis gel at 1.2% of agarose using TBE 1X, GeneRuler 100 bp Plus DNA Ladder, 6X Loading Dye, SYBR (Thermo Fisher Scientific, CA).

Primer design

In order to isolate the gene of interest some special modifications were made in the sequences to use Directional TOPO cloning technology pHidroF (5'-CACCTTGACGAACCCCGAGGCG-3') and pHidroR (5'-GCCGATGCGCCAGGCCTG-3') were designed with primer BLAST at NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and also with Genome Compiler software (http://www.genomecompiler.com/), and synthesized at LGC Biosearch Technologies, California. We include a '5-CACC overhang to the forward primer before the start codon, while in the reverse primer we eliminated the stop codon in order to ligate the gene of interest to the vector (*Fig 3.*).

Gene amplification from Cosmid extraction

First to test primers specificity we used Taq DNA Polymerase (Thermo Fisher Scientific, CA) and pHidro primers looking for amplification of the target gene in the cosmid DNA as the template. This PCR was standardized using the next concentration of all the component in the master mix: Buffer Taq 1X; $MgCl_2$ 1,5 mM; dNTPs 0,3 mM; primer forward 0,2 μM; primer reverse 0,2 μM; Taq DNA Polymerase 1 U; DNA template 200 ng; DMSO 5%. For PCR conditions we used a gradient to visualize what temperature works better for the amplification of the gene of interest avoiding non-specifities, using: Initial denaturation at 95 ºC x 3 minutes; denaturation at 95 ºC x 30 seconds; annealing 60-70ºC x 30 seconds; extension at 72 ºC x 43 seconds; Repeat cycle from denaturation 34X; final extension 72 ºC x 5 minutes; 12 ºC x Infinite hold. All this procedure using C1000 Touch Thermal Cycler (Bio-Rad, USA).

Once we corroborated the correct size of the amplicon, we used Platinum™ SuperFi™ DNA Polymerase (from Invitrogen, USA) for final amplification and then ligation into the vector. PCR reaction was following the next concentrations: SuperFi™ Buffer 1X; dNTPs 0,2 mM; pHidroF 0,5 μM; pHidroR 0,5 μM; SuperFi™ GC Enhancer 1X; Platinum™ SuperFi™ DNA Polymerase 0,02 U/μL; DNA template 10-100 ng. Following the next PCR conditions: Initial denaturation at 98 ºC x 30 seconds; denaturation at 98 ºC x 10 seconds; annealing at 60-70 ºC x 10 seconds; extension at 72 ºC x 15 seconds; Repeat cycle from denaturation 34X; final extension 72 ºC x 5 minutes; 4 ºC x Infinite hold. We visualized the result of the gradient of temperature in an electrophoresis gel at 1.2% of agarose using TBE 1X, GeneRuler 100 bp Plus DNA Ladder, 6X Loading Dye, SYBR (Thermo Fisher Scientific, CA).

DNA purification before ligation in the vector

When we visualized the band in agarose gel with the UV light on we had to expose the gel little time to localize where was the exact spot of our gene of interest to perform the gel extraction, using sterile scalpel the gel was cut and placed into a 1.5 mL pre-weighted tube. Then we used the GeneJET Gel Extraction Kit

(Thermo Scientific, CA) following the manufacturer's instructions and quantified it on NanoDrop 2000 (Thermo Scientific, CA).

Sequencing the gene of interest

We sent two of our amplicons using the Platinum™ SuperFi™ DNA Polymerase to verify what we were going to ligate into the vector. We sent two reaction tubes with 50 μL containing 468,86 ng/μL and 455,06 ng/μL of our gene of interest, also sent 20 μL with 3,2 μM for each tube of primer (pHidroF and pHidroR), the samples were sent to MCLAB (Molecular Cloning Laboratories, South San Francisco, CA) using Sanger Sequencing method.

Trimming ends and NCBI Nucleotide BLAST

We used ABI files for trimming ends using a free pipeline made by Sam Kim from Duke University in Biopython and available in GitHub (https://github.com/sam-k/seq-quality-trimming). Multiple sequences can be trimmed at the same time by its quality and directly perform Nucleotide BLAST at NCBI. Sequences without good quality to merge were eliminated, once we merged the sequences with higher quality those were trimmed perfectly and immediately performed a homology search by the pipeline code with the Basic Local Alignment Search Tool (BLAST) software (http://blast.ncbi.nlm.nih. gov) algorithm at National Center for Biotechnology Information (NCBI). Additionally, we work with the trimmed sequences and upload it at the NCBI to align it and get a percentage of identity and a distance tree results.

E. coli TOP10 chemocompetent cells with CCMB80

The strain *E. coli* TOP10 (Invitrogen) is needed to perform the first step of transformation, which first we have to make our stock of chemocompetent cells. The cells were stored at -80 ºC and added to 100 mL LB medium (10 gr/L Tryptone, 10 gr/L NaCl, and 5 gr/L Yeast extract) and let them growth at 37 ºC until their Optical Density value 0,9 was reached, then we took 1 mL from that culture and transferred it to a new culture with the same volume and composition at 37 ºC too, being aware of the Optical Density (OD) do not go further 0,5 - 0,7 range. Once that OD is achieved we took 25 mL in 50 mL falcon tubes and proceeded with the CCMB80 protocol, we eliminated culture by precipitating cells, then we kept the cells cold chain during all the process, and add 50 mL of CCMB80 cold buffer (1M Potassium Acetate, pH 7.0, 10 mL; Calcium Chloride dihydrate, 11,8 gr; Manganese Chloride tetrahydrate, 4,0 gr; Magnesium Chloride hexahydrate, 2,0 gr; and Glycerol, 100 mL), resuspend cells, centrifugate, and repeat this process around 3-4 times mixing falcon tubes content after every step of centrifugation with resuspended cells. Then we store the cells with 10% of CCMB80 at -80 ºC.

Ligation into pET 101/D-TOPO cloning vector

After corroborating through sequencing which gene we were going to clone into the vector we proceeded to ligate this gene into the vector according to the manufacturer's instructions doing some modifications. We used Champion™ pET101 Directional TOPO™ Expression Kit (from Invitrogen) and to perform the TOPO cloning reaction to ligate the insert into the vector you have to add: Fresh PCR product (0.5 - 4 μL) depending on concentration; Salt Solution 1 μL; TOPO vector 1 μL and sterile water to a final volume of 6 μL and let this reaction for 5 minutes after mixing it gently. We standardized the TOPO Cloning reaction and performed the reaction in the following amounts: Fresh PCR product 2 μL to add 14,86 ng to the reaction; Salt Solution 2 μL and TOPO vector 2 μL and let the reaction proceeded during 10 minutes after mixing it gently. Then took 3 μL to perform the transformation into *E. coli* TOP10 chemocompetent cells.

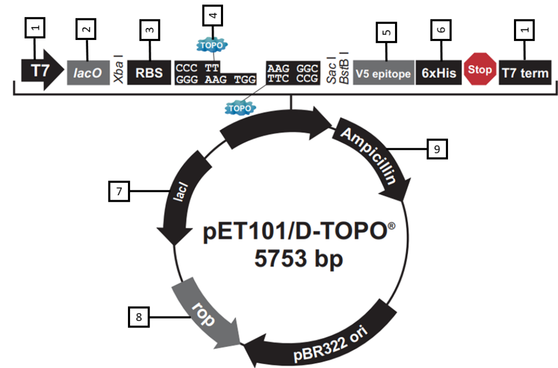

Figure 3 pET 101/D-TOPO vector used for expression of the gene with predicted α/β hydrolase activity, the components of the plasmid are (1) T7 Promoter and termination region, (2) Operon *lac*(*lacUV5*), (3) RBS (Ribosome Binding Site), (4) Topoisomerase, (5) V5 epitope region, (6) Polyhistidine (6xHis) region, (7) *lacI*, lac repressor, (8) *ROP* ORF, and (9) Ampicillin as marker of selection (Invitrogen, USA).

Transformation of E. coli TOP10 chemocompetent cells We thawed the cells on the ice around 15-30 minutes which were stored at -80 ºC, we proceeded to perform the transformation taking 3 μL from the TOPO cloning reaction and added it gently to the chemocompetent cells mixing it without pipetting up and down 15 times and let them on the ice during 30 minutes and then heat shock at 42 ºC during 90 seconds and immediately thawed them on the ice during 3 minutes and added 800 μL of prewarmed SOC medium (2% bacto tryptone; 0.5% yeast extract; 10 mM NaCl; 2.5 mM KCl; 10 mM $MgSO_4$; 10 mM $MgCl_2$; 20 mM glucose) at 37ºC and let them recover during 1 hour sealing the lid of the tube with fixomull at 37 ºC with 420 rpm. When the time of recovering is completed we plated 100 μL in prewarmed (37ºC) LB agar medium with 100 μg/mL of ampicillin, centrifuged the other volume at 8000 rpm during 2 minutes, decanted the supernatant and resuspended the cells in a reduced volume to plate in another prewarmed LB agar medium with the antibiotic of selection. Finally, let them grow in a range of 12-20 hours at 37 ºC.

Screening with colony PCR

We performed the PCR in the same concentration as when we isolated the gene which the master mix is: Buffer Taq 1X; $MgCl_2$ 1,5 mM; dNTPs 0,3 mM; primer forward 0,2 μM; primer reverse 0,2 μM; Taq DNA Polymerase 1 U; DMSO 5%; and here our DNA template is half of the biomass from the colony due to their size and went directly into the tube of reaction. With the same PCR conditions changing initial denaturation and annealing: Initial denaturation at 95 ºC x 8 minutes; denaturation at 95 ºC x 30 seconds; annealing at 64.5 ºC x 30 seconds; extension at 72 ºC x 43 seconds; Repeat cycle from denaturation 34X; final extension 72 ºC x 5 minutes; 12 ºC x Infinite hold. All this procedure using C1000 Touch Thermal Cycler (Bio-Rad, USA). The result was

visualized in an electrophoresis gel at 1.2% of agarose using TBE 1X, GeneRuler 100 bp Plus DNA Ladder, 6X Loading Dye, SYBR (Thermo Fisher Scientific, CA), to determine which clone was positive getting a vector with the desired insert.

Isolation of positive colony and Miniprep procedure

We took the positive clone and the negative clone to a new fresh medium (LB liquid medium with 100 μg/mL of ampicillin) and let them grow overnight during 12 hours exactly. When the 12 hours are finished we took a portion of the positive clone culture to preserve at -80 ºC adding a new fresh medium (LB liquid medium with 20% of glycerol). Once the cryopreservation is done, we proceeded with the miniprep protocol, here is relevant to achieve good integrity, quality, purity and quantity of extraction because the plasmid extracted is going to be used in the following step of cloning which is into *E. coli* BL21 (DE3). To achieve those objectives we used QIAprep Spin Miniprep Kit (QIAGEN) following the manufacturer's instructions to compare the results between positive and negative colonies. After the miniprep extraction, we visualized it in an electrophoresis gel at 1.2% of agarose using TBE 1X, GeneRuler 100 bp Plus DNA Ladder, 6X Loading Dye, SYBR (Thermo Fisher Scientific, CA).

PCR to reconfirmate gene insertion

After getting a positive and negative clone and performing plasmid extraction to both, we proceeded with another PCR using it as a DNA template, not the biomass from the colony but the plasmid from the extraction. We made a dilution 1:4 from the miniprep extraction, and we repeated it x4 times to use every dilution as a DNA template using 1 μL per tube of reaction. The master mix composition and the thermal cycler conditions we used is the same as the amplification of the gene of interest we isolated before. And the results were visualized in an electrophoresis gel at 1.2% of agarose using TBE 1X, GeneRuler 100 bp Plus DNA Ladder, 6X Loading Dye, SYBR (Thermo Fisher Scientific, CA). Also, we tested other primers that amplify from the T7 promoter priming site to the T7 reverse priming site, which primers were available in the kit provided by Invitrogen (Champion™ pET101 Directional TOPO™) T7 (5´-TAATACGACTCACTATAGGG-3´) and T7 reverse (5´-TAGTTATTGCTCAGCGGTGG-3´). The PCR concentrations in the master mix are: Buffer Taq 1X; $MgCl_2$ 1,5 mM; dNTPs 0,3 mM; primer forward 0,016 μM; primer reverse 0,016 μM; Taq DNA Polymerase 1 U; DMSO 5%; with 1 μL from the Miniprep extraction and another tube with 2 μL from the same tube of extraction. With the same PCR conditions changing annealing temperature calculated by its sequence with Thermo Scientific calculator: Initial denaturation at 95 ºC x 3 minutes; denaturation at 95 ºC x 30 seconds; annealing at 49 ºC x 30 seconds; extension at 72 ºC x 60 seconds; Repeat cycle from denaturation 34X; final extension 72 ºC x 5 minutes; 12 ºC x Infinite hold.

E. coli BL21 (DE3) electrocompetent cells with glycerol and chemocompetent cells with CCMB80

The strain *E. coli* BL21 (DE3) is needed to perform the second step of transformation to express our protein of interest. First, we have to make our stock of electrocompetent cells and chemocompetent cells, which were processed in the same way as *E. coli* TOP10 with the CCMB80 with the same conditions for chemocompetent cells. Meanwhile, for the electrocompetent cells, the BL21 (DE3) cells were stored at -80 ºC and added to 100 mL LB medium (10 gr/L Tryptone, 10 gr/L NaCl, and 5 gr/L Yeast extract) and grown at 37 ºC until their Optical Density = 0,9 were reached, then we took 1 mL from that culture to a new fresh culture with the same volume and composition at 37 ºC, being aware of the Optical Density do not go further 0,5 - 0,7 range. Once that OD is obtained we took 25 mL in 50 mL falcon tubes and proceeded with the glycerol protocol. We kept everything on ice or frozen, tips, glycerol, falcons, centrifuge, etc. The glycerol is crucial to use in a pretty clean material because detergents can affect the procedure later in transformation affecting its efficiency, so we prepared it with Type I Purified water sterilized and diluted the glycerol to 10% and sterilized it again in the autoclave and 0,45 μm filter too. Proceeding with the protocol we eliminated culture by precipitating cells, then, we kept the cells in a cold chain during all the process, and added 50 mL of cold 10% glycerol (100 mL glycerol, and 900 mL of Type I Purified water), resuspended cells gently, centrifuged, and repeated this process almost 3-4 times mixing tubes after every step of centrifugation with resuspended cells. Then we stored the cells with 10% of glycerol at -80 ºC, every tube with 40 μL of electrocompetent cells.

Transformation of E. coli BL21 electrocompetent and chemocompetent cells

The key to obtaining high efficiency of transformation in this process consists of keeping the cells on ice, even the cuvettes. We thawed them on the ice, wait 15-20 minutes, and added around 30 nanograms to the cells (without pipetting up or down) and incubated it on the ice for 30 minutes. Once incubation time is ready we use the cuvette previously chilled on -20 ºC fridge, and transfer the mixing reaction, i.e., the electrocompetent cells with the plasmid to it, carefully without generating any bubbles, tap the cuvette and settle the bacteria to the bottom of the cuvette. We use a 0.2 cm cuvette (Bio-Rad, USA) and MicroPulser™ Electroporation Apparatus (Bio-Rad, USA). And quickly introduce the cuvette into the arm of the electroporator and choose the pre-existent protocols, in this case, we used "Ec2" and "Ec3" which both gives one pulse, but "Ec2" gives a voltage of 2.5 kV meanwhile "Ec3" gives 3.0 kV. Once we selected a protocol we gave the electroporation pulsing "PLS" and heard a slight sound, then we added 900 uL of prewarmed SOC medium at 37 ºC into the cuvette once it is out of the electroporator, and let them recover during 1 hour sealing the lid of the tube with fixomull for better aeration and incubated it at 37 ºC with 420 rpm. When the time of recover is ready, we plated 100 μL in prewarmed (37ºC) LB agar medium with 100 μg/mL of ampicillin, centrifuged the other volume at 8000 rpm during 2 minutes, decanted the supernatant and resuspended the cells in a reduced volume of medium to plate in another prewarmed LB agar medium with the antibiotic of selection. Finally, grown in a range of 12-20 hours at 37 ºC.

The transformation of chemocompetent cells is the same as proceeded with *E. coli* TOP10 which was incubated the cells with the plasmid during 30 minutes on the ice, heat shock at 42 ºC during 90 seconds and immediately transferred it to the ice and incubated during 3 minutes, and repeated the same process of recovery with the same cultures. And, grown in a range of 12-20 hours at 37 ºC.

Induction of gene expression in E. coli BL21

From the positive clones obtained we took it to a 100 mL new fresh LB medium (10 gr/L Tryptone, 10 gr/L NaCl, and 5 gr/L Yeast extract) with 100 μg/mL of ampicillin and grown

overnight at 37 ºC, then we inoculated 100 mL of a new fresh LB medium with the antibiotic of selection with 5 mL of the overnight culture. And this new culture must achieve an OD range of 0.5-0.8 at 37 ºC in two-three hours. Once we had the needed OD range, we split the culture in two, letting a culture-induced and the other uninduced. For the induced culture the IPTG (Isopropyl β- d-1-thiogalactopyranoside, from Invitrogen) is added to a final concentration of 1 mM and took an aliquot of 500 μL every hour during 4-6 hours incubation at 37 ºC. For the uninduced culture, we took an aliquot of 500 μL every hour during 4-6 hours incubation at 37 ºC.

SDS-PAGE to visualize an increment in protein production

The samples collected previously (500 μL) are centrifuged (Rotina 380r, HETTICH) at 8000 rpm during 30 seconds to decant fully the supernatant and resuspend the cell pellet directly in 80 μL of 1X SDS-PAGE sample buffer (0.5 M Tris-HCl, pH 6.8, 1.25 mL; Glycerol (100%), 1.0 mL; β-mercaptoethanol, 0.2 mL; Bromophenol Blue, 0.01 gr; SDS, 0.2 gr; and bring to a final volume of 10 mL with sterile water), boil during 10 minutes at 85-90 ºC and centrifuge at maximum speed during 10 minutes. Finally, load 5-10 μl of the superficial part of the solution because the sample can become viscous and difficult to load on the SDS-PAGE gel. The resolving gel was made to 30 mL approximately at 7,5% with 6,25 mL of Acrylamide mix of 40% (29:1; from AMRESCO), 6,25 mL of Tris-HCl 1,5M pH 8,8, 0,25 mL of 10% SDS, 0,61875 mL of a mix Ammonium Persulfate (APS) and Tetramethylethylenediamine (TEMED), and 12 mL of deionized sterile water. The stacking gel was made to 10 mL approximately at 5% with 1,65 mL of Acrylamide mix of 40% (29:1; from AMRESCO), 2,5 mL of Tris-HCl 1M pH 6,8, 0,1 mL of 10% SDS, 0,25025 mL of a mix APS/TEMED, and 5,8 mL of deionized sterile water; in both gels added the mix of APS/TEMED at the end to allow polymerization. Added the running buffer (3 gr of Tris, 14,4 gr of glycine, and 1 gr of SDS), the conditions of electrophoresis were 60V until the samples reach the resolving gel (approximately 30 minutes) and voltage is changed for 100 V. After the running is finished (approximately 2 hours) the SDS-PAGE gel is stained with Staining solution (Blue coomassie 0,1%, glacial acetic acid 10%, and methanol 10%) during 2 hours in the gel shaker (BioBase, SK-D3309-Pro) when the time is completed the staining solution is retired and the Destain solution (glacial acetic acid 10%, and methanol 10%) is added overnight in the gel shaker. The next day, the Destain solution is replaced every 2-3 hours until the bands are visible.

Results

DNA cosmid extraction from E. coli Clone 306

The cosmid extraction was proceeded for the alkaline lysis with two replicas of extraction with this method, meanwhile with QIAprep Spin Miniprep Kit was extracted with four replicas to store more quantify of this cosmid. *Fig.4.* shows how the integrity of these two methods of extraction works.

The cosmid size is around 36 kb, and that was why it is relevant to modify the protocol using the kit according to the manufacturer's instructions (QIAprep Spin Miniprep Kit Handbook Appendix C). With the alkaline lysis, for the first sample, we got a yield of 10198,45 ng/μL with 260/280 relation of 1,95 and for the second sample, we obtained a yield of 16272,45 ng/μL with 260/280 relation of 1,97. With the kit we obtained for the first sample a yield of 170 ng/μL with 260/280 relation of 1,92 and 260/230 of 2,3; for the second sample a yield of 204 ng/μL with

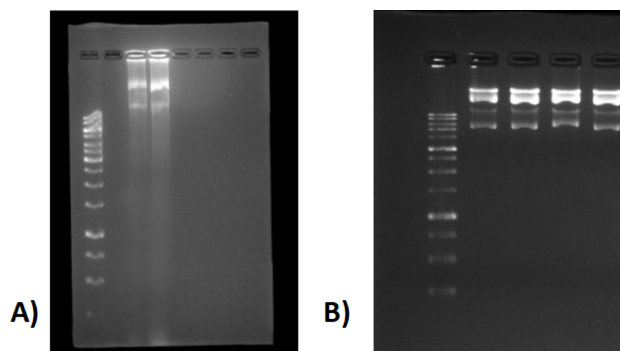

Figure 4 Maxiprep extraction through two different methodologies (A) Alkaline lysis adapted from Sambrook, (B) QIAprep Spin Miniprep Kit (QIAGEN); both using 1kb ladder (Thermo Scientific, CA). All lanes from each extraction method are replicates from the same activated culture.

260/280 relation of 1,91 and 260/230 of 2,3; for the third sample a yield of 159 ng/μL with 260/280 relation of 1,91 and 260/230 of 2,25; and for the fourth sample a yield of 213 ng/μL with a 260/280 relation of 1,91 and 260/230 of 2,2. We realized that both methods of extractions work perfectly for cosmid extraction, but, it is better to have an isolated and more integrous cosmid DNA to perform the gene isolation without any gDNA interference.

Gene amplification from Cosmid extraction

The gene isolation is programmed when designing the primers to avoid non-specificities and wrong annealing to other places in the cosmid and finally trying to get an amplification of the desired gene of interest, the amplification was performed first with a normal Taq DNA Polymerase (Thermo Fisher Scientific, CA) to corroborate the specificity of the primers on the gene we were trying amplify and to standardize a protocol for further screenings, which resulted in high specificity (*Fig. 5. A*), that result allowed us to use the Platinum SuperFi Taq Polymerase (*Fig. 5. B*) to isolate the gene in a way more specific than using the normal Taq DNA Polymerase, and to directly clone it into the vector. Both of these results were performed in a thermal gradient to evaluate the specificity of the primers in the annealing.

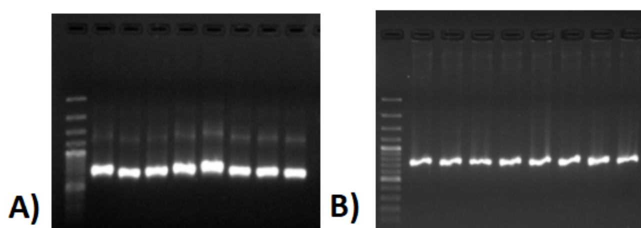

Figure 5 PCR from cosmid extraction to isolate the desired gene in a thermal gradient using (A) Taq DNA Polymerase and (B) Platinum™ SuperFi™ Taq for PCR and pHidroF/pHidroR primers. Ladder 1 Kb (Thermo Fisher Scientific, CA).

Once these results were ready the quantification is made, to perform the next step in cloning which is ligation we needed purification of the gene of interest from the agarose gel to ligate avoiding non-specificities, being aware of the fragment that is

going be insert into the pET 101/D-TOPO vector. To cut the DNA in a careful way we exposed the Agarose gel to UV a little time revealing the bands and immediately shouting it off, then, marked the place with a Kim wipe and cut the agarose gel with a sterile scalpel blade with UV light off, later revealing the cube of agarose cut to visualize the presence of the desired band. Then DNA gel band extraction is performed with GeneJET Gel Extraction Kit (Thermo Scientific, CA) getting a low yield of 7,43 ng/μL which is good because the ligation requires a low quantity of DNA to work.

Sequencing the gene of interest

Samples from the PCR with Platinum SuperFi Taq Polymerase were sequenced by Sanger Sequencing Method using the pHidroF and pHidroR complete gene (700 bp), and the results were processed and analyzed using a bioinformatic free pipeline at GitHub (https://github.com/sam-k/seq-quality-trimming). The results showed: GenBank accession: MN207134.1; which is *Variovorax* sp. EA-ED0101 alpha/beta hydrolase (qqrs101) gene. Corresponding to the desired gene, we are going to insert it into the vector with 100% of identity.

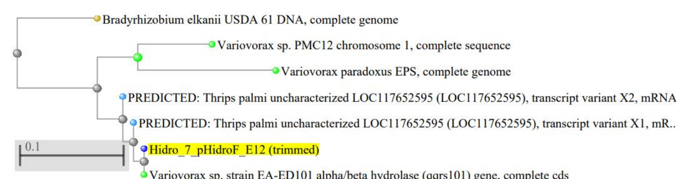

Figure 6 Distance Tree results from nBLAST - NCBI, showing the percentage of identity between the sample sequenced (i.e., the gene isolated from cosmid of the clone 306 with Platinum™ SuperFi™ Taq PCR) compared to the sequences stored at the database (NCBI) that share a close percentage of identity with our gene.

Variovorax sp. strain EA-ED101 alpha/beta hydrolase (qqrs101) gene, complete cds
Sequence ID: MN207134.1 Length: 702 Number of Matches: 1

Range 1: 89 to 471 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
708 bits(383)	0.0	383/383(100%)	0/383(0%)	Plus/Plus

```
Query  1    CGCCGCCTTCGCCCGAGGCCTGCTATGTGCTGGCGCACGGCGCCGGTGCCGGCATGACGC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  89   CGCCGCCTTCGCCCGAGGCCTGCTATGTGCTGGCGCACGGCGCCGGTGCCGGCATGACGC  148

Query  61   ATCCGTTCTTGGAGGCGGTGGCCGAGGGGCTCGCGCAGCGGCGCATCGCCACGCTGCGCT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  149  ATCCGTTCTTGGAGGCGGTGGCCGAGGGGCTCGCGCAGCGGCGCATCGCCACGCTGCGCT  208

Query  121  ACCAGTTTCCCTACATGGAGCGAGGCAGCAAGCGCACCGACGCGCCGGCCCTCGCGCATG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  209  ACCAGTTTCCCTACATGGAGCGAGGCAGCAAGCGCACCGACGCGCCGGCCCTCGCGCATG  268

Query  181  CCACGGTGCGCGCGGCGGTCGACTGCGCGGCGGCGCACTTCGGTGAGCTGCGCCTCTTTG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  269  CCACGGTGCGCGCGGCGGTCGACTGCGCGGCGGCGCACTTCGGTGAGCTGCGCCTCTTTG  328

Query  241  CCGGTGGCAAATCTTTCGGTGGCCGCATGACCTCGCAGGCCCAGGCGCTGCAGCCGCTGC  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  329  CCGGTGGCAAATCTTTCGGTGGCCGCATGACCTCGCAGGCCCAGGCGCTGCAGCCGCTGC  388

Query  301  CGGGTGTCGAAGGGCTGATCTTTCTCGGCTTTCCGCTGCACCTCTCGGGCGCGCCATCGA  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  389  CGGGTGTCGAAGGGCTGATCTTTCTCGGCTTTCCGCTGCACCTCTCGGGCGCGCCATCGA  448

Query  361  CCACGTGCGCCGAGCATTTGCAC  383
            |||||||||||||||||||||||
Sbjct  449  CCACGTGCGCCGAGCATTTGCAC  471
```

Figure 7 Alignment obtained at nBLAST - NCBI, showing an identity of 100% with *Variovorax* sp. EA-ED0101 α/β hydrolase (qqrs101) gene; GenBank accession: MN207134.1.

Transformation of E. coli TOP10 and colony PCR

To perform the TOPO Cloning reaction and transform *E. coli* TOP 10, after incubation time the PCR was immediately made, to select a positive clone from all the transformed colonies, many which a little could be correctly transformed, i.e., with the plasmid correctly ligated the desired gene properly. *Fig.8.B.* shows perfectly how the colony PCR works, in that gel, there are two positive controls in the down wells at the end the lasts two wells followed by the negative control. The gel shows slight bands of the desired insert in the first clones, and the same thing in the down wells, but only one clone is the perfect one that showed a suitable efficiency to ligate the gene into the pET 101/D-TOPO vector to express the protein, that clone, is taken to a new fresh medium LB with 100 μg/mL of ampicillin to further cryopreserve with a new fresh medium LB with 20% sterile glycerol.

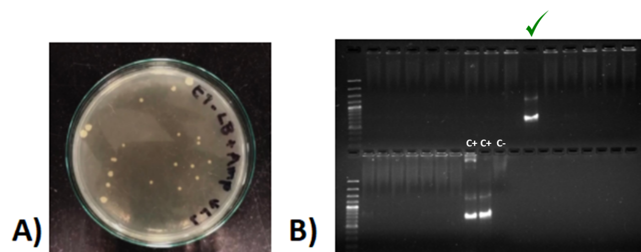

Figure 8 Transformation of *E. coli* TOP10 and Colony PCR (A) Colonies transformed with pET 101/D-TOPO + hydrolase candidate gene; (B) Colony PCR checking positive transformants with the desired size insert (700 bp).

The idea of choosing this clone is to identify which transformant has our gene of interest into the pET 101/D-TOPO vector.

Miniprep, PCR to reconfirm ligation and transformation of E. coli BL21 chemocompetent and electrocompetent cells

The cells were prepared after getting colonies in the selective medium, took both positive colony and negative colony to visualize any change in the miniprep extraction and further analyses. The transformed cells from the TOP10 result were useful to observe the miniprep differently as mentioned before because the miniprep extraction shows something interesting when you watch this process through an electrophoresis agarose gel, which is what shows *Fig. 9.*, a pattern where you can see the difference between the plasmid who were negative for the ligation of the desired insertion of the gene, where there are four bands present, meanwhile for the positive clone you can see one bold and strong band and another slight band. Nonetheless, to confirm successful gene insertion we proceeded with the PCR using gene-specific primers (pHidroF/pHidroR) and additionally, we used T7 primers to evaluate the promoter and termination T7 region and also the difference of size between an empty vector and a ligated vector with the gene of interest.

After miniprep extraction from the positive clone, it was important to visualize the presence of the desired gene insertion into the plasmid, so the strategy used was to part from the original stock of miniprep around 30 ng/μL, and make serial dilutions 4 times 1:4 and use 1 μL from every dilution including the original one to visualize how ligation worked into this positive colony of *E. coli* TOP10. Also, to corroborate ligation using the primers corresponding to T7 promoter and termination region, including the plasmid from the miniprep negative for the desired insert. *Fig. 10.* shows the result of this amplification.

The results show a great amplification for the gene target which it is 700 bp, and the T7 amplification is a relevant experi-

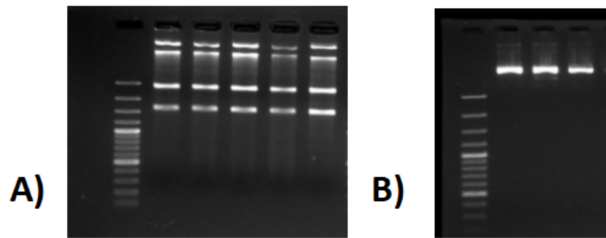

Figure 9 Miniprep extraction from transformed *E. coli* TOP10 (A) Miniprep of the negative colony, four replicates; (8) Miniprep of the positive colony, three replicates; both gels have the same ladder 100bp plus (from Thermo Fisher Scientific).

ment and conclusive essay because it shows that the primer is annealed at a promoter site until a terminus site and amplifies whatever it is inside, the results from that amplification show that every tube of miniprep extraction has two replicas in this amplification, one using 1 μL of plasmid DNA and another using 2 μL of plasmid DNA, after those six wells it shows a negative control for ligation (i.e., the plasmid extracted from the negative colonies in the colony PCR) exposing a band of different fragment size (300-400 bp), and the other well is a positive control that the kit brings, followed by a negative control for the PCR to visualize possible primer dimers. Finally, this result is pretty conclusive because it allows us to transform *E. coli* BL21 due to the presence of the desired gene into the expression vector.

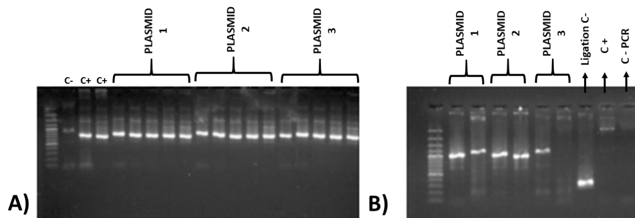

Figure 10 PCR from the miniprep extraction amplifying the gene of interest that must be ligated and T7 promoter and termination region (A) Result of amplification using specific gene primers and serial dilutions of template, 5 replicates per sample; (B) Amplification of T7 region with 2 replicas per sample, adding negative control for ligation which is the miniprep negative for the desired insert, control positive amplifying *lacZ* gene (Plasmid DNA included in the kit as positive control for T7 PCR, size around 3000 bp) and the negative control for PCR.

BL21 is known to have a lower transformation efficiency if we compare it with other *E. coli* strains as DH5 alpha, TOP10, etc. (Liu *et al.* 2014). So these two processes in parallel would determine if they could have a difference during this transformation step. Transforming the BL21 competent cells through thermal shock and electroporation. The two methods were performed just to realize which one was better than the other. Electroporation is an easy and quick method by its efficiency shown in diverse techniques to transform many organisms (Kotnik *et al.* 2015). And there is a difference using either “Ec2” or “Ec3” as electroporation protocol, using 0.2 cm cuvettes it is appropriate to use these two protocols, but, the evidence shows that “Ec3”

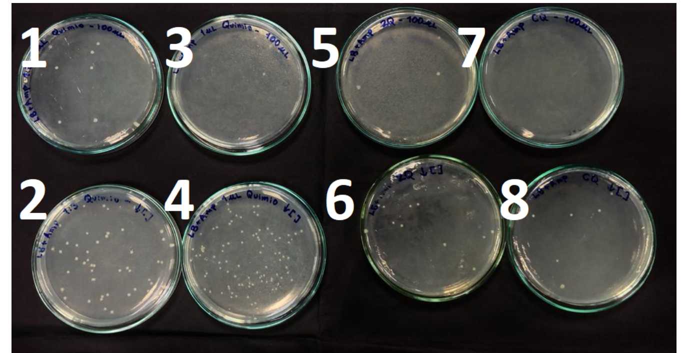

Figure 11 *E. coli* BL21 Chemocompetent cells transformed. Top (1) 10 ng of plasmid and 100 μL plated, and (2) pellet plated, (3) 30 ng of plasmid and 100 μL plated, and (4) pellet plated, down (5) 60 ng of plasmid and 100 μL plated, and (6) pellet plated, (7) 1 μL of plasmid control provided by the kit and 100 μL plated, and (8) pellet plated. Negative control, viability and transformation process are not shown.

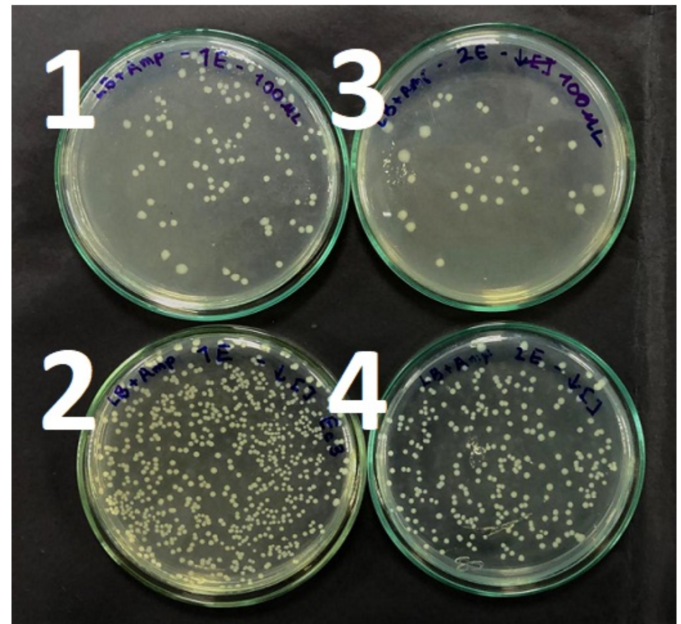

Figure 12 *E. coli* BL21 Electrocompetent cells transformed. (1) 30 ng of plasmid and 100 μL plated, and (2) pellet plated, (3) 60 ng of plasmid and 100 μL plated, and (4) pellet plated. (1) and (2) are the result of transforming with “Ec3” protocol, meanwhile (3) and (4) are the result of transforming with “Ec2”. Negative control, viability and transformation process are not shown.

has higher efficiency than "Ec2", however, both of this protocols works perfectly to obtain a high transformation efficiency. Meanwhile, the chemocompetent cells with buffer CCMB80 show a low transformation efficiency, but it works, and just one colony is needed to perform the induction of expression once the colony is moved to a new fresh medium with the antibiotic of selection.

SDS-PAGE to corroborate gene expression

This electrophoresis was the most important thing of all the process because it determines if all the cloning steps and decisions made through the experiments were accurate due the theory behind the technology and hoping to corroborate partially if the bioinformatic analyses were right, partially because it determines only weight, not biological function *Fig. 13.* There is a band getting strong through the time which the arrow points, finding a 27 kDa protein result of the induction with IPTG, this has sense with the predicted protein target.

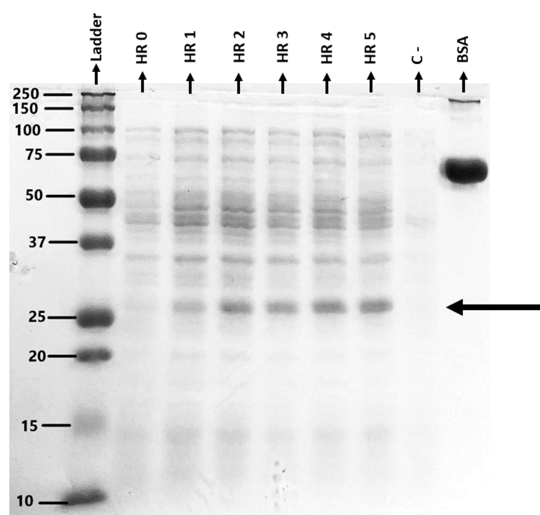

Figure 13 SDS-PAGE to visualise protein synthesis of predicted hydrolase, the wells indicate: (1) Ladder Precision Plus Protein All Blue Prestained Protein Standards (Bio-Rad, 1610373) ranging from 10–250 kD; (2) hour 0 of induction with IPTG; (3) hour 1; (4) hour 2; (5) hour 3; (6) hour 4; (7) hour 5; (8) negative control E. coli BL21 without any plasmid; and (9) positive control for electrophoresis, BSA (Bovine Serum Albumin). Arrow pointing the expected protein.

Discussion

The identification of the responsible gene was made through bioinformatic analysis, finding homologies in the sequences and their possible translation to protein, in this case, the isolation of this gene from a cosmid was made to ligate this sequence into an expression vector to produce the predicted α/β hydrolase and go for further analysis with the biochemical functions of the enzyme, mainly evaluating the activity against *Ralstonia solanacearum* and 3OH-PAME (Chen *et al.* 2013). It needs to be understood because the physiological function of most QQ enzymes is not consistently clear, and the biodiversity of exploring how much microorganisms can produce such enzymes with potential use in the biotechnological field, now with major importance by the pathogens that show resistance against the antibiotics, so this study can help as an alternative of such recent problematic because it becomes antimicrobial agents in controlling the microbial disease by interfering with the QS system, which relays on the degradations of compounds used by the pathogens and blocks microbial attack (Chen *et al.* 2013). This is a promising method of controlling microbial disease in this case of *R. solanacearum* kind of controlled by the strain Variovorax sp. EA-ED0101 which the genre *Variovorax* is known for the ability to grow on many acyl-homoserine lactones producing acylases to incorporate the molecule into its metabolism, specifically *Variovorax paradoxus*, and as we have mentioned acyl-homoserine lactones serve as cell-to-cell signalling molecules in many species of the class Proteobacteria (Leadbetter and Greenberg 2000). The phylogenetic relationship of this genre can lead us and tell us about how much this strain can be involved in this kind of ability to degrade a molecule used as QS of a pathogen bacteria, so that is why it is important to study more about the production of such enzymes especially if it belongs to a strain of this genre known for many studies of degrading many QS molecules, this would not be an exception and with then the isolation for its study would mind having a complete vision of its capacity (Jose *et al.* 2019; Dong and Zhang 2005). Also, it has been demonstrated that more bacteria can degrade the 3OH-PAME, the molecule which *R. solanacearum* uses involved in regulation of virulence, *Ideonella sp.*, *Acinetobacter sp.*, *Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa* and *Rhodococcus corynebacterioides* too (Achari and Ramesh 2015; Shinohara, Nakajima and Uehara 2007).

pET Directional TOPO technology

In this work, we have expressed in *E. coli* BL21 (DE3) a protein derived from strain *Variovorax* sp. EA-ED0101, and corroborated the bioinformatic analysis. The conditions for isolation of the gene of interest from the cosmid was successful and optimized, it generated a strong band of the desired gene at 700 bp and also others non-specificity, at the moment of purifying the DNA from the band, cloning into the vector and perform colony PCR is when that non-specificity have a sense from where it comes, which is from the own gene because once it was purified it would not have any other non-specificity. However, that is not a problem because the fragment of the desired gene (700 bp) was isolated from that non-specificity before performing the TOPO Cloning reaction. Furthermore, the Platinum SuperFi Taq DNA Polymerase is highly important in the workflow because the high fidelity it has to polymerase dNTPs, so it gives as a final product one amplicon with blunt ends and the overhang designed in the forward primer fully complementary to the "CACC", which allow us to proceed with directional TOPO and is what we aim to achieve for proceeding with the ligation. The taq is an excellent tool for cloning benefiting from supreme sequence accuracy.

The steps during the transformation between different strains of *Escherichia coli* (TOP10 and BL21) are a relevant factor during all the development of this research due to their each genotype characteristics, *E. coli* TOP10 is known to be specific for cloning purposes and plasmid propagation by the stability of this strain at the moment of replication so it fits well at the moment of propagating the expression vector that we will use as an expression of the predicted protein of interest in the other strain; meanwhile, *E. coli* BL21 is special for the expression under the T7 promoter contained in the plasmid because the gene expression system is based on bacteriophage T7 RNA polymerase (Studier and Moffatt 1986). The genotype of this strain is characteristic due to the integrated gene (by an integrase) that produces the T7 RNA

polymerase which is highly selective for its promoters contained in the vector of expression we are using (pET 101/D-TOPO), a phenomenon that does not occur naturally in *E. coli* but it does in strain *E. coli* BL21 (DE3), producing mRNA rapidly due to the T7 RNA polymerase that can saturate the translation machinery of *E. coli*, the rate of protein synthesis will depend on the efficiency of its translation and also T7 RNA polymerase can reach levels comparable with those for ribosomal RNAs in a normal cell (Studier and Moffatt 1986). When the mRNA is translated efficiently, a target recombinant protein can accumulate to greater than 50% of the total cell protein in three hours or less (Studier and Moffatt 1986).

The T7 RNA polymerase initiates the process of transcription to RNA chains very efficiently and elongates them faster than does *E. coli* RNA polymerase (Chamberlin and Ring 1973). And that is why it is so important to work with *E. coli* BL21 (DE3), naturally, this microorganism would not be able to produce an amount of mRNA and protein in that efficient way due to the work of the T7 RNA polymerase does, furthermore, *E. coli* BL21 (DE3) is genotypical designed to work with T7 RNA polymerase since this strain has basal expression (Pan and Malcolm 2000). This vector pET 101/D-TOPO includes the T7 promoter and T7 termination region, that means cloning into strain *E. coli* BL21 (DE3) would directly produce the amount of desired protein we aim to express, i.e., the predicted α/β hydrolase, also this vector and all the other T7 polymerase-based expression vectors have expressed successfully thousands of homologous and heterologous proteins to very high levels in *E. coli* strain BL21 (DE3) (Pan and Malcolm 2000). Nonetheless, it is important to work with both strains due to their genotypic information and advantages, strain *E. coli* BL21 (DE3) cannot manage some plasmids and are relatively unstable in this strain, that is why *E. coli* TOP10 is fundamental for plasmid propagation and maintenance, then *E. coli* BL21 (DE3) is relevant during all the process of expression and to carry this on in presence of the IPTG, which is fundamentally needed to trigger the expression process and has been demonstrated to trigger this process through RNA accumulation after induction with IPTG (Studier and Moffatt 1986).

Now that the T7 RNA Polymerase role is explained, the transcriptional process is carried out by the construct in the pET 101/D-TOPO vector that has the T7 promoter and the T7 termination region included and will improve mRNA transcription specifically of this candidate gene. It is relevant to understand the other components of the construct present in the plasmid as the lac repressor (*lacI*) and lac operon (*lacO*/*lacUV5*), which are used by the metabolism of *E. coli* BL21 and drive all this expression process. The concentration of the regulated enzymes, in turn, controls the flux through a given pathway, *E. coli* like plenty of organisms can supply their energy demands by altering enzyme concentrations to take full advantage of the fluctuating food supplies in their environment, and when glucose levels are low *E. coli* can take up and metabolize alternative sugars as lactose [25]. In the operon, the genes are regulated depending on the metabolic needs of the cell and to regulate a gene the operon requires a master switch which is a repressor molecule and binds to the operator negatively blocking the expression of structural genes of the operon. In the vector, the lac operon, *lacUV5* specifically, is a mutated sequence which that mutation gives the operon the ability to require IPTG to unbind the repressor from the operon, the traditional lacO requires more activators to unbind the repressor (Lewis 2005). So the expression can not be made if there is not IPTG (lactose metabolite, mimics allolactose) available in the medium, and that is related with the T7 RNA Polymerase, which cannot synthesize mRNA due steric hindrance by the protein produced due to the lac repressor and will not express the desired protein in high amounts until the lac repressor is removed from the lac operon which can be achieved through adding IPTG at some specific range of concentration. Besides, there is RBS (ribosome binding site) present in the sequence of the vector which allows once the mRNA is synthesized immediately goes to the translation process to the ribosome, thanks to the RBS sequence that drives this process efficiently.

The strategy of expression has more genes involved as in the case of the V5 epitope, which is a tag for the expressed protein with the 14 aa V5 epitope derived from parainfluenza virus 5 (Brennan, Li and Elliott 2011). This is a tool with so much potential at Western blot to identify our protein of interest expressed in a gel, besides, that is for identification, in the primer design we did not include the stop codon in the reverse primer because the polymerase continues to synthesize the information to include V5 epitope, 6xHis and finally the stop codon which the vector includes. So the 6xHis tag contains a sequence that when translated to protein becomes a chain of six histidines which makes the purification of the protein of interest an easy process due to the interaction with molecular charges, the recovery of the protein is succeeded by the affinity of the histidines for the charged resin, being a highly selective interaction that allows purification process modifying pH charges to avoid other proteins (Crowe, Masone and Ribbe 1995). Another fundamental compound in this vector is the TOPO enzyme isolated from *Vaccinia virus* (DNA Topoisomerase I), which recognizes the sequence ’5-CCCTT-3’ and after the last thymine cleaves the DNA strand forming a phosphotyrosine covalent bound, and this Directional TOPO technology allows the ligation when the overhang of the vector (GTGG, *Fig. 3.*) invades the 5′-CACC from the PCR product we will to insert into this vector and performs a nucleophilic reaction with the phosphotyrosine covalent bound to ligate itself into the vector and release the TOPO enzyme. This vector commercially comes linearized, so one end comes with the GTGG overhang and TOPO ligated after of recognizing the sequence ’5-CCCTT-3’ and in the other end comes with a blunt end and the sequence of recognizing with the ligated TOPO too. Also, there is included the *rop* gene, the repressor of primer which ensures a low copy number of plasmid replication and this gene has so much sense to be included in the vector because the pET vectors are used for expression of a specific protein, a high copy number of plasmid would lead to high production of the desired protein but as mentioned before the T7 RNA Polymerase gives a lot of appreciable amount of RNA waiting to be translated into protein, so a high copy number of plasmid plus induction through IPTG would shut down *E. coli* cellular machinery as mentioned before by (Studier and Moffatt 1986) a target protein can accumulate to greater than 50% of the total cell protein in three hours or less, so a low copy number caused by *rop* gene is the better strategy to conserve *E. coli* BL21 (DE3) viability and go for the expression of the desired protein, commonly, the replication process occurs by RNAI and RNAII transcription which RNase H elongates for the DNA polymerase extension, with the rop protein present the RNAI and RNAII form a complex that modulates the initiation of transcription of the primer RNA precursor (Cesareni, Muesing and Polisky 1982). These components are fundamental in the vector of expression due to their relevance taking care of *E. coli* BL21 (DE3) viability or any other organism desired to be

transformed under the T7 promoter. So, the expression vectors have the appropriate composition of genes thinking in every possible situation for the protein production (obviously avoiding the explanation of the *ori* and the gene of selection marker), here, we are focused on the expression vector components, and finally, allowing us to have a direct workflow in obtaining homologous and heterologous proteins according to our goals, it became an excellent tool to obtain diverse proteins of interest and develop many analyses based on the interest of isolating proteins for future application processes.

Ligation process into pET 101/D-TOPO

The TOPO Cloning Reaction was a little bit complicated by the low efficiency of ligation by the TOPO enzyme, we obtained other negative assays of transformation with colony PCR negatives (results are not shown), and this could be due to the low activity of the TOPO enzyme, we changed the concentration of the TOPO Cloning Reaction and at least we got one positive clone (*Fig.8.B.*) in the assay, changing the volumes and concentrations according to manufacturer's instructions, suggesting this might be due to the old storage of the kit and would be interesting to evaluate this result according to the efficiency of the enzyme through the time to reveal how much time of storage can affect the enzyme activity. We modified the ligation process (1) using a higher concentration of the Salt solution, (2) using a higher concentration of insert, (3) using a higher concentration of pET 101/D-TOPO vector, and (4) incubating the reaction 5 more minutes (10 minutes in total), to raise the concentration of the components in this TOPO Cloning reaction, i.e., obtaining a higher probability of each compound to interact with more molecules of the TOPO Cloning reaction and let them react more time to achieve the ligation process. Would be interesting to evaluate how the storage can modify enzymatic kinetics to establish a limit of useful life due to many laboratories cannot afford different kits and immediately finish them.

Transformation process in chemocompetent E. coli BL21, E. coli TOP10, and in electrocompetent E. coli BL21 cells

The result of transforming is important due to the probability, is higher getting a positive clone with a great number of colonies, i.e., a great efficiency of transformation. As the results showed (*Fig.8.A*) in *E. coli* TOP10 the number of colonies is not high, but it is sufficient to obtain a positive clone, could be by the low amount of plasmid given. When transforming *E. coli* BL21 (*Fig.11.* and *Fig.12.*) is visualized better with two different methods of transformation (heat shock and electroporation), where plating 100 μL of the tube of transformation from the heat shock procedure obtained 8 colonies per plate, with 31,43 ng of plasmid, meanwhile when plating 100 μL of the tube of transformation from the electroporation procedure obtained 91 colonies per plate with 31,43 ng of the plasmid. So the electroporation process was way more efficient in getting a high number of colonies than heat shock even for plasmids using an expression technology.

SDS-PAGE to visualize the predicted enzyme

According to the previous study about this enzyme, it would have a size of 24 kDa, but with this technology of Directional TOPO cloning step adds a 6xHis tag and V5 epitope, it increases the size of the protein to 27 kDa. The intention of visualizing different times of inducting IPTG is to watch any differential change in the protein production, which is needed to corroborate the usage of the expression vector, SDS-PAGE will help at visualizing this topic. Furthermore, the band will be notorious by increasing over time. Which is notorious in the samples from *Fig. 13.* Visualizing the desired band at 27 kDa with Precision Plus Protein™ ranging from 10–250 kD. The results are concise due to the thickness of the expected band through the time and visualizing the negative control (BL21 without any plasmid and the same OD) which has no presence of this band, so the cellular machinery has the responsibility of producing all this protein synthesis thanks mainly to the T7 promoter functioning by the addition of IPTG that released the lac repressor protein over the lac operon letting no steric hindrance to proceed with the transcription process, that is why it is so necessary the understanding of the technology behind this methods, due to all of this leads to the acknowledge how the biological process succeded. Finally, the presence of the band in the expected size gives us a hint about the presence of the predicted protein, other steps of recognition must be required as the use of V5 epitope for example, and the purification step to evaluate the predicted hydrolase phenomenon over *Ralstonia solanacearum* that will be tested in future assays.

Conclusions

pET D-TOPO vectors are suitable to work on the expression of proteins of interest knowing their sequence to obtain as a final product the desired translational process and modifications if it is needed. Also, the genotypic information about the organism we are working helps a lot to strategize so many procedures due to the diverse of genotypes that exist, and boost many others possible applications, in this case, strain *E. coli* TOP10 was helpful to propagate the pET 101/D-TOPO vector with the sequence of predicted hydrolase gene, meanwhile *E. coli* BL21 (DE3) was helpful to induce an appropriate response according to the sensing of a molecule responsible for trigger the expression by inactivating the lac repressor protein allowing the T7 RNA Polymerase transcribe the gene of interest. Many factors are involved through all this process, and being conscious of it can boost our improvement during all the procedure. The SDS-PAGE cell pellet was directly charged into the SDS-PAGE Buffer visualizing all the proteins contained inside the *E. coli* BL21 (DE3) cells, finally getting a bold 27 kDa band of protein through the time, and the previous bioinformatic analyses were right according to the molecular weight in protein size, the next step will involve many biochemical tests to evaluate previous predictions and corroborate its predicted α/β hydrolase activity. It would be interesting to evaluate the structure of this novel predicted enzyme because it does not come from any other *Variovorax* sp. organism that has been found in the literature with the same activity against the 3OH-PAME (Shinohara, Nakajima and Uehara 2007; Achari and Ramesh 2015).

A future perspective could be able to scale those QQ mechanisms, as transgenic plants expressing quorum quenching molecules, that already exists as an experiment in transgenic tobacco and potato plants expressing AHL lactonase, which these transgenic plants quenched QS signalling and showed reduced tissue maceration and enhanced resistance to pathogen infection (Achari and Ramesh 2019). The QQ is a useful strategy alternative to antibiotics, emerging as a new antivirulence to prevent the incidence of plant diseases even more important in those of economical impact. There are reports on the existence of new QS molecules with unknown structures indicating a need for exhaustive future research to understand this complexity regulating bacteria virulence on plants. And there is never enough,

new niches, extreme environments and even extreme organisms are potential resources for isolation of novel QQ bacteria enzymes and would be interesting to explore even more, how that biodiversity brings us a powerful tool emerging as a new technique to reduce the virulence occasioned by pathogen bacteria.

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