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ReViTA: A novel *in vitro* transcription system to study gene regulation

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ABSTRACT

ReViTA (**Re**verse *in* ***Vi****tro* **T**ranscription **A**ssay) is a novel *in vitro* transcription-based method to study gene expression under the regulation of specific transcription factors. The ReViTA system uses a plasmid with a control sequence, the promoter region of the studied gene, the transcription factor of interest, and an RNA polymerase saturated with σ^{70}. The main objective of this study was to evaluate the method; thus, as a proof of concept, two different transcription factors were used, a transcriptional inducer, AlgR, and a repressor, LexA, from *Pseudomonas aeruginosa*. After the promoters were incubated with the transcription factors, the plasmid was transcribed into RNA and reverse transcribed to cDNA. Gene expression was measured using qRT—PCR. Using the ReViTA plasmid, transcription induction of 55% was observed when AlgR protein was added and a 27% transcription reduction with the repressor LexA, compared with the samples without transcription factors. The results demonstrated the correct functioning of ReViTA as a novel method to study transcription factors and gene expression. Thus, ReViTA could be a rapid and accessible *in vitro* method to evaluate genes and regulators of various species.

Introduction

Gene transcription *in vivo* starts when RNA polymerase (RNAP) binds to DNA and catalyzes RNA production from the DNA template. Bacterial RNAP is an enzyme composed of five different subunits ($\alpha_2\beta\beta'\omega$) that additionally binds to a dissociable sigma (σ) factor in initiating the transcription process [1]. The σ factors bind to specific promoters to facilitate promoter recognition by RNAP and to activate the expression of a particular group of genes [2].

In vitro transcription (IVT) is a well-known technique that allows the synthesis of RNA. and which has been used to study the function of gene promoters, RNAP, σ factors, ribozyme biochemistry studies, antisense RNA and RNAi experiments, microarray analysis, *in vitro* translation, RNA vaccines [3], transcription inhibitors [4], and transcription factors [5], among others. In addition, it is a widespread methodology being used to study different organisms [6–9].

Over the years, IVT assays have evolved from using radiolabeled nucleotides [8] to developing nonradioactive PCR-based methods [9]. Additionally, in traditional IVT assays, the RNAP of the organism under study had to be purified and free of σ factors, which can be a limitation, especially when large amounts of pathogenic microorganisms need to be grown to obtain the RNAP [6–8,10,11]. This problem is easily solved by using the commercially available *Escherichia coli* RNAP. The RNAP holoenzyme from *E. coli* is saturated with the σ^{70} factor and thus can recognize only promoters with σ^{70} binding sites; however, it is an excellent solution to tackle the study of transcription regulators. In addition to the RNAP and σ factors, many other regulators are involved in the modulation of gene expression during transcription.

Transcription factors (TFs) are proteins with DNA-binding domains that recognize specific DNA sequences and regulate the expression of genes under specific conditions. TFs are essential to modulate genetic transcription. By binding to their promoter region, they can transcriptionally activate or repress gene expression [12,13]. Several TFs can regulate a specific gene, and one TF can regulate more than one gene.

Abbreviations: ReViTA, Reverse *in Vitro* Transcription Assay; RNAP, RNA polymerase; qRT—PCR, quantitative Real Time-Polymerase Chain Reaction; IVT, *in vitro* transcription; TF, Transcription Factor; RNR, ribonucleotide reductase; IMAC, immobilized metal affinity chromatography; EMSA, Electrophoretic mobility shift assay; sspDNA, salmon sperm DNA; BSA, bovine serum albumin; D3-PA, D3-phosphoramidite.

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Thus, it is crucial to consider TFs when studying transcription procedures [12–14]. Other investigators have developed IVT systems to detect regulons modulated by a specific transcription regulator [11]. In this study, an IVT-based system called ReViTA (**Re**verse *in* ***Vi****tro* **T**ranscription **A**ssay) is described, the aim of which is to understand and study how specific TFs affect RNAP activity over certain promoters to modulate the overall transcription process. This approach represents a previously unexplored application of IVT assays. The ReViTA system uses RNAP from *E. coli* saturated with σ^{70} and a plasmid (pReViTA) that includes two gene promoter regions. One of them is used to study the promoter of interest to which the TF binds, and the other is used as a control gene to which the TF does not bind and is specifically used to standardize the methodology. As a proof of concept, in this study the ReViTA system is used to measure the expression of two genes of *Pseudomonas aeruginosa* PAO1 after incubation with specific TFs. The work involves *nrdA*, which encodes class Ia ribonucleotide reductase (RNR) and its transcriptional inducer AlgR [15], and *dinB*, a type IV polymerase transcriptionally repressed in the presence of the protein LexA [16,17]. Using ReViTA, it was determined that specific concentrations of AlgR and LexA transcriptionally induce and repress the expression of *nrdA* and *dinB*, respectively. In addition, it was confirmed that the ReViTA system produced comparable results to those obtained using traditional linear DNA fragments, making it a novel system to use in the study of *in vitro* gene regulation.

Materials and methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used are listed in suppl. Table S1. The different *Pseudomonas aeruginosa* and *Escherichia coli* strains were routinely grown in Luria-Bertani (LB; Scharlab, Barcelona, Spain) medium at 37 °C. Liquid cultures were shaken at 200 rpm. Antibiotics were added to the culture medium, when necessary, at the following concentrations: 50 μg/mL ampicillin, 10 μg/mL gentamicin, 50 μg/mL kanamycin, and 34 μg/mL chloramphenicol for *E. coli*, and 100 μg/mL gentamicin for *P. aeruginosa*.

ReViTA cassette design

The ReViTA cassette (see Fig. 1A) was synthesized as a GeneArt gene synthesis product cloned into the pMK-RQ vector (Thermo Fisher Scientific, Waltham, MA, USA), generating pMK-RQ::ReViTA (pETS251). The cassette was designed as a sequence of random DNA with 65% GC (ReViTA-INSERTION) and a sequence of DNA corresponding to the internal region of *gyrB* (*E. coli* K12 MG1655) with single nucleotide modifications to reduce the chance of internal promoters (ReViTA-TRIAL). Two double terminators flank ReViTA-INSERTION and ReViTA-TRIAL sequences, the terminator rrnBT1-T7TE forward (Registry of Standard Biological Parts #BBa_B0015) upstream, and the bidirectional T7TE-LuxIA double terminator (Registry of Standard Biological Parts #BBa_B0014) downstream.

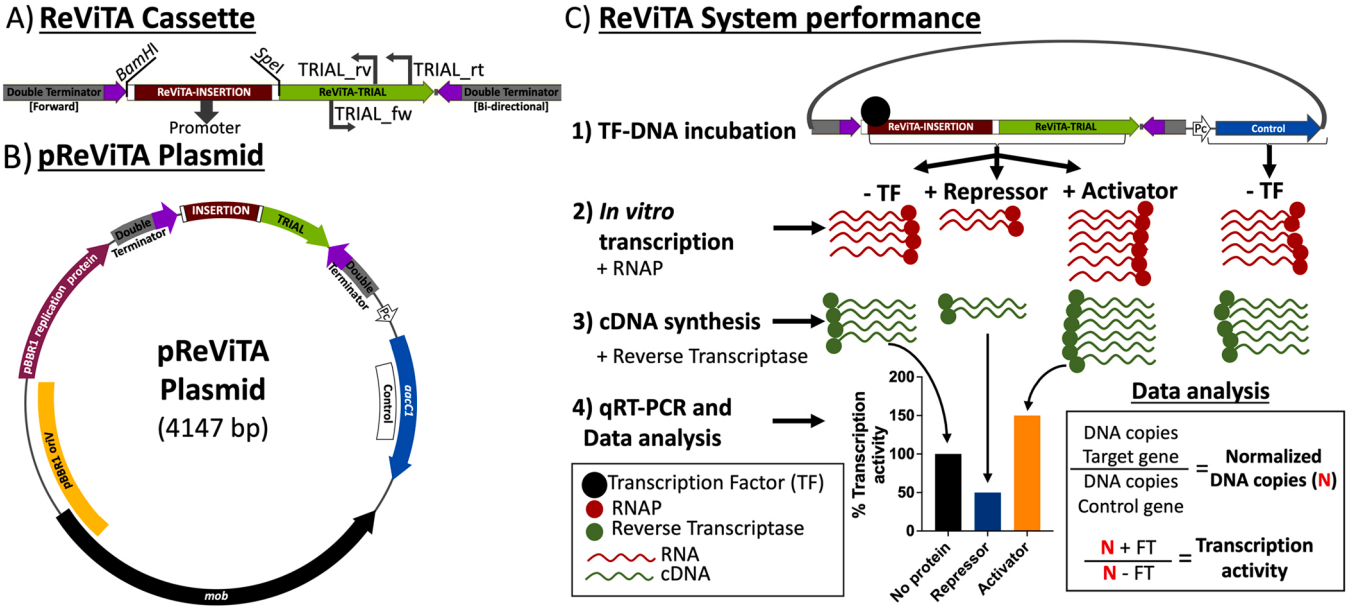

Fig. 1. Overview of the ReViTA system. A) Schematic representation of the ReViTA cassette. The cassette includes a random DNA sequence where the promoter to be studied would be inserted (ReViTA-INSERTION) and a specific sequence to measure gene expression (ReViTA-TRIAL). Double terminators flank the sequences ReViTA-INSERTION and ReViTA-TRIAL; one of them stops transcription in one direction (Forward), and the other in both directions (Bidirectional). The primers to reverse transcribe the RNA from the promoter into cDNA (TRIAL_rt) and to perform qRT—PCR (TRIAL_fw and TRIAL_rv) bind to the ReViTA-TRIAL sequence. B) Map of the pReViTA plasmid, which includes the ReViTA cassette inserted into the pBBR1MSC5 backbone vector, the *aacC1* gene, which confers resistance to gentamicin and was used as a control sequence for the *in vitro* transcription (IVT) assay, the *mob* gene for mobilization functions, pBBR1 *oriV* as a replication origin (used for broad-host-replication range), and finally the pBBR1 replication protein[19]. C) pReViTA system performance. The ReViTA system is based on the IVT methodology to measure gene expression. The study transcription factor (TF), which can be a transcriptional repressor or an activator, binds to the promoter region of the target gene and does not bind to the promoter of the control sequence. Once the plasmid is incubated with the TF, the RNA polymerase (RNAP) binds to the promoters and transcribes the genes. Depending on the presence or absence of the TF, the transcription levels of the genes are different. Afterward, the reverse transcriptase catalyzes the conversion of RNA into cDNA. The cDNA levels of each sample were measured using qRT—PCR. The data obtained in the qRT—PCR were analyzed using the formulas found in the bottom right square of the image to obtain the transcription activity of each sample. If the TF is a repressor, the samples with protein will show less transcription activity than samples with no protein, and if the TF is an activator, the samples with protein will show higher transcription levels than the no-protein sample.

DNA manipulation and plasmid construction

Recombinant DNA manipulations were performed using standard protocols [18]. The manufacturer's instructions were followed in using the molecular biology kits and enzymes. DNA amplifications were performed with the primers listed in suppl. Table S2 and using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) or DreamTaq Green PCR MasterMix (Thermo Fisher Scientific). DNA fragments were isolated from agarose gels using the GeneJet Gel Extraction Kit (Thermo Fisher Scientific). The plasmids constructed throughout the work were extracted from *E. coli* DH5α using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific). The constructs obtained were verified *via* DNA sequencing by Eurofins Genomics (Ebersberg, Germany). All the enzymes used in this study were purchased from Thermo Fisher Scientific except those indicated otherwise.

To construct the pReViTA (pETS252) plasmid, the backbone of pBBR1MSC5 was amplified with PCR using primers 1 and 2 listed in suppl. Table S2 [19]. The fragment was gel purified, cloned into pJET1.2b, and transformed into *E. coli* DH5α to obtain pJET1.2b::[pETS130]bb (pETS250). Then, the plasmids pMK-RQ::ReViTA (pETS251) and pJET1.2b::[pETS130]bb (pETS250) were digested with *Xba*I-*Aat*II. The fragments obtained were gel purified, ligated using the T4 ligase enzyme, and transformed into *E. coli* DH5α. The sequence of the plasmid pReViTA has the GenBank accession no. OP909926.

The AlgR overproducer plasmid (pETS28a-AlgR) was as constructed previously and transformed into the *E. coli* Rosetta (DE3) strain [15]. The LexA overproducer plasmid was constructed by cloning the *lexA* gene from *P. aeruginosa* PAO1 (PA3007) into the pET28a overexpression system (Sigma-Aldrich, St. Louis, MO, USA). The primer pairs 3/4 listed in suppl. Table S2 were used to amplify a PCR band of 615 bp.Thereafter the amplified PCR band and the plasmid pETS were digested using the restriction enzymes *Nco*I and *Xho*I. The bands were gel purified, ligated using the T4 ligase enzyme, and transformed into *E. coli* DH5α, generating the plasmid pET28a-LexA (pETS255). The constructed plasmid was subsequently transformed into the *E. coli* Rosetta (DE3) strain.

Overexpression and purification of transcription factors (TFs)

The protein AlgR-His was overproduced and purified, as described previously [15]. The protein LexA-His in the C-terminal end of the protein was overproduced in the Rosetta (DE3) strain and induced with IPTG 1.0 mM for 4 h at 37 °C. The cells were centrifuged, and the pellet was resuspended in LexA buffer lysis buffer (20 mM Tris-HCl, 300 mM NaCl, 5 mM imidazole, and 1 mM DTT) to prepare the crude protein extract. The suspension was sonicated, and the crude extract was obtained by centrifuging the sonicated suspension for 30 min at 15,000*g* at 4 °C.

An FPLC system (BioLogic DuoFlow System, Bio-Rad, Hercules, CA, USA) was used to purify the LexA protein with a 5 mL His-Trap™ HP column (GE Healthcare, Chicago, IL, USA) by immobilized metal affinity chromatography (IMAC). Different volumes of buffer A (20 mM Tris-HCl, 300 mM NaCl, 5 mM imidazole, and 1 mM DTT) and buffer B (20 mM Tris-HCl, 300 mM NaCl, 500 mM imidazole, and 1 mM DTT) were used to generate specific imidazole concentrations. The purified protein was visualized with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS—PAGE, 12% acrylamide protein gel, Bio-Rad) and stored at − 80 °C. Bradford reagent (Bio-Rad) was used to determine the protein concentration using bovine serum albumin (BSA, Bio-Rad) as a standard.

Electrophoretic mobility shift assay (EMSA)

The promoter regions of the genes *nrdA* and *dinB* and the control were used in this assay to check TF binding. To produce the DNA probes used in EMSA, the primer pairs 5/6 and 8/9 listed in suppl. Table S2 were used to amplify the promoter regions of *nrdA* (491 bp) and *dinB* (242 bp), respectively, from the genomic DNA of *P. aeruginosa* PAO1. Primers 1 and 10 listed in suppl. Table S2 were used to amplify the promoter of the control sequence using the plasmid pReViTA (pETS252) as the template (264 bp). The primer M13 added the arbitrary sequence 5′-CTGGGCGTCGTTTTAC-3′ at the 3′ end of every probe. The bands obtained in the first PCR were used as templates for a second PCR that used the WellRED dye-labeled oligo (Sigma-Aldrich) coupled to the near-infrared fluorophore D3-phosphoramidite (D3-PA) to obtain the EMSA probes.

The promoters of *nrdA* and the control sequence were used at 50 fmol per reaction. The purified AlgR protein was added at 0, 0.5, and 1 pmol in each binding reaction. The DNA bands and the AlgR protein were mixed with binding buffer containing 20 mM Tris-HCl (pH 7.8 at 25 °C), 120 mM KCl, 2 mM $MgCl_2$, 2 mM DTT, 10% glycerol, 50 ng/µl sspDNA (salmon sperm DNA), and 12.5 ng/µl BSA. AlgR-*nrdA* reactions were incubated for 20 min at RT before gel electrophoresis. The purified LexA protein was used in binding reactions at 0, 0.125, or 0.5 pmol per reaction. The DNA bands, *dinB* and control, were used at 50 fmol per reaction. The binding reactions also contained 50 ng/µl BSA, 50 ng/µl sspDNA, 20 mM Tris-HCl (pH 8.0 at 25 °C), 50 mM KCl, 2 mM MgCl2, 1 mM EDTA, 2 mM DTT, and 5% glycerol. LexA-*dinB* reactions were incubated for 30 min at 37 °C before gel electrophoresis.

EMSA was performed in 4% acrylamide gels using 37.5:1 acrylamide:bis-acrylamide (Sigma-Aldrich), 5% triethylene glycol (Sigma-Aldrich) and 2 mg/mL ammonium persulfate (Sigma-Aldrich). The running buffer was 40 mM TAE (pH 7.8 or 8.0 at 25 °C) for the proteins AlgR and LexA, respectively. Images were obtained by scanning the gels in the 700-nm channel of the Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE, USA).

Construction of promoter carrying ReViTA for in vitro transcription

The plasmids pReViTA-P*nrdA* (pETS253) and pReViTA-P*dinB* (pETS254) were constructed as follows. First, the promoter regions of *nrdA* (P*nrdA*, 786 bp) and *dinB* (P*dinB*, 504 bp) were amplified by PCR using the primer pairs 5/7 and 14/15 listed in suppl. Table S2, and the genomic DNA from *P. aeruginosa* PAO1 was used as the template. The fragments were gel purified, ligated into the vector pJET1.2b with the enzyme T4 ligase, and transformed into *E. coli* DH5α. The resulting plasmids and the plasmid pReViTA were digested with *Bam*HI/*Spe*I, and the fragments were gel purified and ligated with the enzyme T4 ligase, generating pReViTA-P*nrdA* and pReViTA-P*dinB*. The primers ReViTA-Test-fw and ReVita-Test-rv were used to verify the insertion of P*nrdA* and P*dinB* into the ReViTA cassette through PCR and sequencing.

Construction of promoter-carrying PCR bands for in vitro transcription

The genes used in this study were amplified *via* PCR using primers 12/13 to amplify *nrdA* (P*nrdA*-PCR-IVT, 1045 bp) and 14/16 to amplify *dinB* from genomic DNA of *P. aeruginosa* PAO1, obtaining a PCR band of 954 bp (P*dinB*-PCR-IVT). Simultaneously, the promoter region of the control sequence was amplified using primers 1/17 listed in suppl. Table S2 using the plasmid pReViTA as the DNA template, obtaining an amplified band of 869 bp (CTRL-PCR-IVT). The *in vitro* transcription fragments were extracted using the Gel Extraction Kit.

In vitro transcription assay

The RNAP used in the *in vitro* transcription assay was commercially available *E. coli* RNA polymerase (New England Biolabs, Ipswich, MA, USA). This holoenzyme is saturated with the σ^{70} factor, which recognizes specific σ^{70} promoters to initiate RNA transcription. The *E. coli* RNAP Reaction Buffer 5 X (New England Biolabs) was used in the experiment.

First, the promoter templates (ReViTA-P*nrdA*, ReViTA-P*dinB*, and their linear PCR transcription fragment counterparts mixed with the

control sequence) were incubated with the transcription factor and the reaction buffer (New England Biolabs) of RNAP for 30 min at RT using AlgR and 37 °C using LexA. In each reaction, 25 fmol of the template (25 nM) per reaction was used. A specific protein concentration was tested in each reaction.

The *in vitro* transcription buffer was composed of 1X reaction buffer, 0.5 mM NTP mix (ATP, UTP, CTP, and GTP, Sigma-Aldrich), 1 mM spermidine (Sigma-Aldrich), 0.06 U pyrophosphatase (Sigma-Aldrich), and 20 U Ribolock RI (Thermo Fisher Scientific). The *in vitro* transcription buffer was mixed with the protein—DNA complex and incubated at RT or 37 °C for 30 min. Subsequently, 0.5 U of *E. coli* RNAP was added to each reaction. The *in vitro* transcription reaction was performed at 37 °C for 30 min. All the incubations were carried out using the T100 Thermal Cycler (Bio-Rad).

The samples were diluted 1:2 with Milli-Q water and mixed with 1X Turbo DNase I buffer and Turbo DNase I (Thermo Fisher Scientific) to remove the DNA template. DNase treatment was performed at 37 °C for 1 h with gentle shaking. The DNase inactivation reagent (Thermo Fisher Scientific) at 1X was added to each sample, and the mix was incubated for 5 min at room temperature with occasional mixing. After centrifuging the samples for 90 s at 10,000*g*, the supernatant was recovered. A PCR was performed using specific primers as DNA absence test to ensure that the samples were DNA free. The control sequence was used as the positive control.

Reverse transcription, qPCR, and data analysis

The mRNA produced from the *in vitro* transcription reaction was reverse transcribed using 200 U Maxima Reverse Transcriptase (Thermo Fisher Scientific), 0.5 mM dNTPs mix (dATP, dTTP, dCTP, and dGTP, Sigma, Spain), 1 X Thermo RT buffer (Thermo Fisher Scientific), 20 U Ribolock RI, and gene-specific primers. The primers ReViTA_TRIAL_rt and ReViTA_ctrl_rt were used to reverse transcribe the plasmid pReViTA; the primers qRTgreen_IVT_PAO-nrdA_rv, IVT_dinB-PAO1_rt and ReViTA_ctrl_rt were used to reverse transcribe the PCR band templates of *nrdA*, *dinB*, and the control, respectively. The mixture was incubated following the manufacturer's instructions. The cDNA obtained was quantified by qRT—PCR with the StepOne-Plus 96-well real-time PCR system (Thermo Fisher Scientific). Samples were mixed with 1X PowerUp SYBER green Master Mix (Thermo Fisher Scientific) and 200 nM specific amplification primers. The primers used are specified in suppl. Table S2. Additionally, calibration curves of the tested amplicons were prepared. These standards were used to determine the number of copies of DNA in each reaction and to calculate the percentage of transcription activity in each sample.

Promoter prediction

The software phiSITE (with predetermined matrices) was used to predict σ^{70} promoters in the studied genes (http://www.phisite.org/main/index.php?nav=tools&nav_sel=hunter) [20]. The promoter regions that were cloned into the plasmid pReViTA (P*nrdA*, 786 bp and P*dinB*, 504 bp) were submitted *in silico* to the software and analyzed.

Statistical analysis

GraphPad Prism 9.0 (GraphPad Software, Boston, MA, USA) was used to perform statistical analyses. Single comparisons were performed with unpaired Student's *t* tests. The data values are expressed as the mean and standard deviation.

Results

ReViTA system: characterization and functioning

The ReViTA system was developed to address the need to study transcription factors and their specific roles and functions *in vitro*. The ReViTA cassette is a synthetic sequence explicitly designed to facilitate the study of regulators and their regulons using the widely known *in vitro* transcription technique (Fig. 1). Fig. 1A shows a representative scheme of the ReViTA cassette. It is composed of a random DNA sequence (ReViTA-INSERTION) where the promoter of interest will be cloned using the *Spe*I-*Bam*HI restriction sites. Downstream of the INSERTION site, the ReViTA-TRIAL sequence corresponds to an internal region of *gyrB* (*E. coli* K12 MG1655). This sequence is used to measure and quantify the expression of the promoter to be studied, and the primers used in the assay are designed to bind specifically to it. Double terminators flank the ReViTA-INSERTION and ReViTA-TRIAL sequences to stop transcription in both directions, with the expression measurements being gene specific. Finally, the ReViTA cassette also includes a control sequence that corresponds to the *aacC1* gene to standardize the reaction, as further described.

The ReViTA cassette was integrated into a pBBR1MSC5 plasmid backbone, as shown in the representative scheme in Fig. 1B [19]. The pBBR1MSC5 backbone sequence was amplified by PCR and cloned into pJET1.2b. The plasmid pJET1.2b::[pETS130]bb and the ReViTA cassette were digested with *Aat*II-*Xba*I and ligated, generating the pReViTA (pETS252) vector (see Materials and Methods). In this plasmid, the gene *aacC1* encodes a gentamicin 3-N-acetyltransferase, which confers resistance to gentamicin. Part of the *aacC1* sequence is used in the IVT assay as the control sequence. *aacC1* has its promoter (Pc), a class 1 integron promoter, and should not be recognized by the studied TF. In addition, pReViTA includes a *mob* gene for mobilization functions and the pBBR1 oriV and pBBR1 replication protein used in various microorganisms for plasmid maintenance and replication.

Any promoter of interest can be cloned into the ReViTA-INSERTION site, and the IVT assay can be performed to evaluate the function of the studied TF. An experiment with the ReViTA system will encompass five different steps: 1) DNA template preparation, 2) *in vitro* transcription, 3) cDNA synthesis, 4) qRT—PCR, and 5) data analysis (Fig. 1C).

DNA template preparation

This step includes the cloning of the promoter of interest into pReViTA. The final plasmid concentration was set to 25 nM to have 25 fmol of plasmid in each IVT transcription reaction. To ensure that the DNA template was high quality, the DNA was extracted from *E. coli* DH5α (see Materials and Methods).

In vitro transcription (IVT)

The commercially available RNAP holoenzyme from *E. coli* was used to generate the RNA from the DNA templates used. To carry out IVT, the TF of interest was first incubated with the DNA template (plasmid or free DNA fragment). The time and temperature for DNA-transcription factor incubation may vary depending on the TF used.

The buffer composition for the TF-DNA incubation and the IVT assay was determined empirically by trial and error. When choosing a buffer, it is crucial to bear in mind the salt concentration present in the buffer, such as NaCl, $MgCl_2$, or KCl, which can dramatically alter the transcription reaction. Different buffers were assessed, and the best was the buffer of RNAP itself mixed with additives such as spermidine, pyrophosphatase, and Ribolock RI (see Materials and Methods).

The amount of RNA transcribed from the DNA template will depend on the strength of the promoter and TF binding. It is hypothesized that when testing a transcriptional repressor, the amount of RNA transcribed from the sample incubated with the protein will be less than that from the sample without protein. Simultaneously, it is believed that if the TF is an activator, the amount of RNA transcribed from the DNA template incubated with the protein will be higher than that in the sample without protein. In all cases, the amount of RNA obtained with the transcription of the control sequence should not vary, as the TF would

not bind to this DNA region. After transcription, the DNA template was eliminated using Turbo DNase I for 1 h at 37 °C. A DNA absence test using the control sequence as the positive control was performed to ensure that the DNA template was eliminated.

cDNA synthesis

The mRNA obtained from the tested promotor and the control sequence, independent of whether they were incubated with the TF, was reverse transcribed to cDNA using specific transcription primers (listed in suppl. Table S2) and Maxima Reverse Transcriptase. The primer used to reverse transcribe the RNA from the tested promoter binds to the TRIAL sequence. We assumed that for each RNA molecule, a cDNA molecule was obtained.

qRT—PCR

qRT—PCR was used to measure cDNA amplification. Specific primers (suppl. Table S2) and the PowerUP SYBR Green Master Mix (1 X) were used to amplify DNA copies. With the ReViTA system, primers used to measure transcription of the promotor of interest bind to the TRIAL sequence; thus, they are independent of the tested promotor cloned into the plasmid. The amplicons from the ReViTA-TRIAL and the control sequence were designed to have similar lengths to obtain the same PCR efficiencies and comparable results.

Data analysis

As the signal intensity in qRT—PCR is proportional to the number of amplified DNA copies, a calibration curve of each amplicon was used to calculate the number of DNA copies in each reaction. Assuming that every RNA molecule was reverse transcribed into a DNA molecule, the original number of RNA copies in each sample and the percentage of transcription activity in each reaction were calculated. To determine whether a TF is a transcriptional repressor or an activator, it is necessary to compare the transcriptional activity of the pReViTA incubated with the protein and the plasmid with no TF. The transcription values of the control sequence were used to normalize the data for analysis, facilitating the comparison between samples.

Use of the ReViTA system to measure transcription of PnrdA and PdinB P. aeruginosa promoters after specific TF binding

As proof of concept, the ReViTA system was used to measure the expression of the well-known *P. aeruginosa* genes *nrdA* with the transcriptional activator AlgR [15] and *dinB* in the presence of the transcriptional repressor LexA [16,17]. *nrdA* is part of the operon *nrdAB,* which encodes the class Ia ribonucleotide reductase constitutively active in *P. aeruginosa.* Different TFs tightly regulate the transcription of this promoter. One of them is AlgR, which transcriptionally induces the expression of *nrdA* through an AlgR binding box in its promoter [15]. The other gene analyzed in this work is *dinB*, which encodes a DNA polymerase IV with no proofreading activity that is transcriptionally repressed by LexA and it is part of the SOS-response system [21]. The promoter region of *dinB* (P*dinB*) has one LexA-binding sequence.

The genes used in the IVT assay require a σ^{70}-dependent promoter to be recognized by the RNAP used in the assay. The transcription of *dinB* has been thoroughly studied in *E. coli*, and it was determined that *dinB* presents a single promoter that can be transcribed using σ^{70} (RpoD, constitutive) or σ^{38} (RpoS, stationary phase, and stress) [22]. However, there were no data available for its counterpart in *Pseudomonas*. In the case of *P. aeruginosa* PAO1, there was no evidence that σ^{70} bound to the promoter regions of *nrdA* and *dinB*. After bioinformatic prediction, several putative σ^{70} binding regions were detected in the promoter sequences of *nrdA* and *dinB* (suppl. Fig. S1). In the *nrdA* promoter region, three putative σ^{70} binding sites were found, two of which were downstream of the AlgR binding box, overlapping with the TF NrdR binding sites [23]. In the *dinB* promoter region, the experimentally demonstrated LexA box was found to overlap one of the σ^{70} binding sites; two more were found upstream (and thus could still be controlled by LexA), and the last two were found downstream of the LexA box (suppl. Fig. S1). Therefore, it was hypothesized that *nrdA* and *dinB* would have a σ^{70}-dependent promoter.

The TFs used in this work are shown on a 12% SDS—PAGE gel (Fig. 2A-B), which corresponded to a His-tagged AlgR protein (27 kDa) and a His-tagged LexA protein (23 kDa). When purifying AlgR, some nonspecific bands were observed; however, AlgR was found mainly as a monomer (signaled in the gel with a m). LexA was found as both a monomer (m, 23 kDa) and a dimer (indicated in the gel with a d, 46 kDa). The purified proteins were used in electrophoretic mobility shift assay (EMSA) to confirm specific AlgR-P*nrdA* [15] and LexA-P*dinB* binding [21] and verify that the AlgR-Control and LexA-Control complexes did not form (Fig. 2C-D). It was observed that the highest concentration of TF increased the amount of shifted P*nrdA* and P*dinB*, with the highest shift observed when using 1 and 0.5 pmol of protein, respectively. The control region used in these assays was not bound to the protein AlgR (Fig. 2C). However, it was bound lightly to LexA when using 1 pmol of protein (Fig. 2D), indicating that there may be some competitivity among *dinB* and the control when using LexA.

AlgR transcriptionally induces nrdA expression, and LexA transcriptionally represses dinB expression via in vitro transcription

To evaluate the performance of the ReViTA system and as a proof of concept, an IVT assay was conducted using the ReViTA plasmid or linear

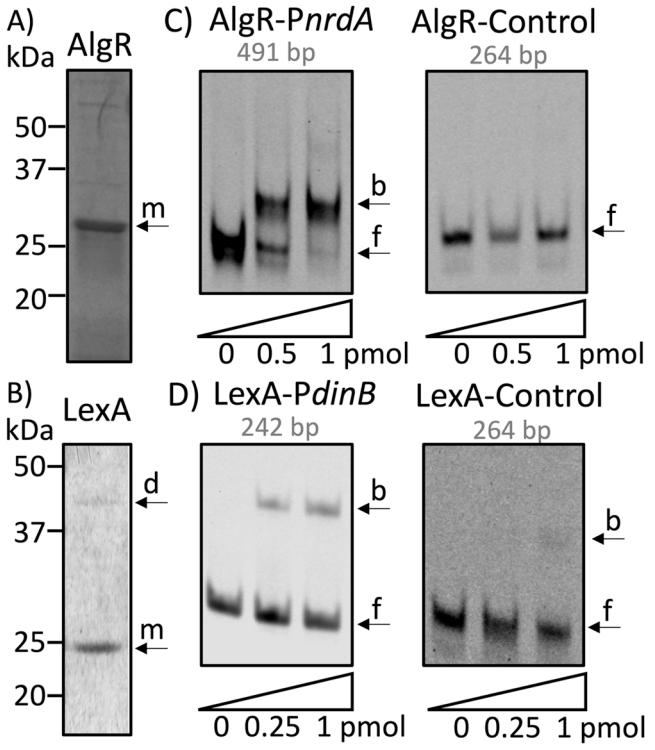

Fig. 2. Study of TF binding to promoter regions. SDS—PAGE (12%) gel of *P. aeruginosa* PAO1 A) purified AlgR-His (27 kDa), and B) LexA-His (23 kDa). The bands indicated with arrows on the gel correspond to the protein monomer (m) and dimer (d). C) EMSA experiments using the promoter region of *nrdA* (P*nrdA*, 491 bp) from *P. aeruginosa* and the promoter control sequence of the ReViTA plasmid (264 bp) and the AlgR protein. D) EMSA using the promoter of *dinB* (P*dinB*, 242 bp) from *P. aeruginosa* and the control sequence (264 bp). Fifty femtomoles of each probe was used per reaction. The number of picomoles of AlgR-His or LexA-His used is labeled. f, free DNA; b, bound DNA.

DNA fragments as DNA templates (see Materials and Methods) and Fig. 3. The promoter region of *nrdA* and *dinB* were cloned in the ReViTA-INSERTION sequence of the ReViTA plasmid (pReViTA-P*nrdA* and pReViTA-P*dinB,* Fig. 3A and C). The linear DNA fragments were obtained by PCR amplification of P*nrdA,* P*dinB* and the control sequence, which were 1045 bp, 954 bp, and 869 bp, respectively (Fig. 3B and D).

Fig. 3A and B show that *nrdA* expression increased by 29% and 55% after adding 0.25 and 0.5 pmol of AlgR, respectively, when using the ReViTA system and by 17% and 40% when using the PCR bands. The increase in both cases was similar and consistent with the higher *nrdA* expression as the protein concentration increased. The raw data of a representative experiment using the ReViTA system and the PCR bands can be found in suppl. Fig. S2A.

Fig. 3C and D show that the expression of *dinB* decreased by 27% when using the plasmid pReViTA and by 30% with the PCR bands after incubation with 0.125 pmol of LexA. However, when the protein concentration was increased to 0.5 pmol, P*dinB* expression decreased only by 10% and 11% in the ReViTA system and PCR bands, respectively although the tendency is clear but not statistically significant. Suppl. Fig. S2B shows the raw data of a representative experiment when the FT LexA is used with the ReViTA plasmid and with PCR bands.

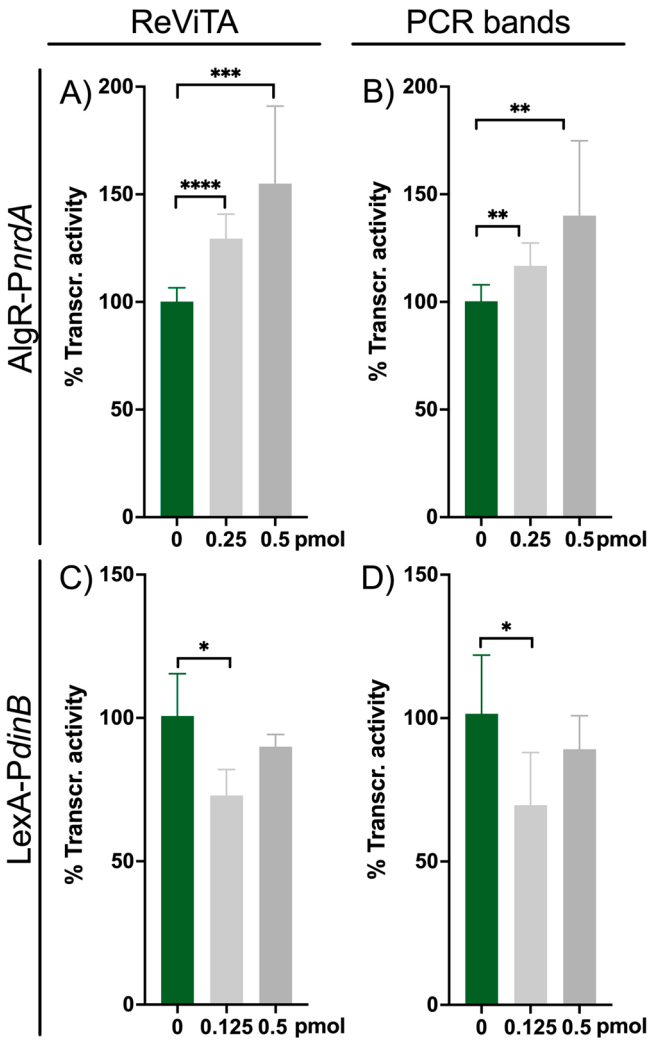

Fig. 3. *In vitro* transcription (IVT) assays. IVT assay using 25 fmol of A) the plasmid pReViTA-P*nrdA* with the promoter region of *nrdA*, B) the PCR bands of P*nrdA* (1045 bp) and the control sequence (P*aacC1-aacC1*, 869 bp), C) the plasmid pReViTA-P*dinB*, with P*dinB*, and D) the PCR bands of P*dinB* (954 bp) and control gene (P*aacC1-aacC1*, 869 bp) promoter regions. The Y-axis of the graphs represents the percentage of transcription of the template. The numbers below the X-axis represent the pmol of AlgR (A and B) or LexA (C and D) used in the assays. The data are expressed as the mean ± standard deviation. Student's unpaired *t* test was used to determine significant differences between samples with protein (0.125, 0.5, and 2.5 pmol) and the sample without protein (0 pmol) (*, p value < 0.05; **, p value < 0.01; ***, p value < 0.001; ****, p value < 0.0001).

Discussion

In vitro transcription assays are a widely used technique to produce RNA from DNA templates in the laboratory. Here, a novel IVT system, ReViTA, was designed to measure gene expression after a TF binds to a promoter of broad-spectrum organisms. The ReViTA system uses the plasmid pReViTA to transcribe the promoter of interest and the control gene into RNA in the presence of a given TF. The main goal of ReViTA is to evaluate TF activity by measuring gene expression under the binding of a specific TF. Different TFs can be studied and quantifying RNA expression changes can reveal whether these TFs inhibit or activate gene expression (Fig. 1).

The use of ReViTA offers several advantages over traditional IVT techniques. First, there is no need to genetically modify the host organism, which is highly useful in organisms whose genome is difficult to manipulate and when studying genes that cannot be mutated. Secondly, when studying a TF *in vitro*, the specific outcome from the regulator-regulon binding is obtained. *In vivo*, however, due to the complexity of the system, other unknown factors, proteins, or sRNA may modulate gene expression in an unknown way. Thus, the gene expression measured *in vivo* may not be as clear as the specific gene expression measured *in vitro.* Thirdly, no radiolabeling is needed, as the ReViTA system method is based on quantifying the RNA produced in the reactions by qRT—PCR, which prevents health concerns [9]. Finally, the DNA template is protected in a plasmid, preventing degradation by 5′-exonucleases. It is known that using supercoiled plasmids as DNA templates offer advantages compared to linear DNA fragments [24]. The signals from the transcripts of promoters in supercoiled plasmids are higher than those from transcripts of linear templates, where the signal is weaker and can lead to misinterpretations. Even though it is easier for linear fragments to form the RNAP-DNA complex, these complexes tend to be less stable than specific promoter-RNAP complexes [25].

However, the use of ReViTA also presents some disadvantages. First, the RNAP used in the assay is the commercial *E. coli* RNAP saturated with σ^{70}; thus, only promoters recognized by the σ^{70} factor can be used. Therefore, the methodology would be limited when studying promoters with alternative sigma factors or promoters from other species if the promoter cannot be recognized by heterologous RNAP [8]. However, this problem can be solved by using the specific σ, if available, or with a combination of purified RNAP and σ factor complexes [11,26]. Secondly, RNAP-promoter complex formation is affected by several parameters, such as salt concentration, temperature, template topology, and RNAP concentration. These IVT conditions depend on the TF and promoter used and must be optimized empirically [24]. Finally, to perform IVT, the TF needs to be purified, with associated challenges. Using a TF whose *in vitro* conditions are very different from the RNAP conditions may limit the use of ReViTA.

Considering the above points, it was evaluated whether the ReViTA system is a good option for studying gene expression after TF binding. The *nrdA* and *dinB* promoter regions from *P. aeruginosa* were cloned into pReViTA and their expression measured after incubation with the transcriptional inducer AlgR and transcriptional repressor LexA, respectively. AlgR and LexA from *P. aeruginosa* were purified using *E. coli* Rosetta (DE3) as overproducing cells (Fig. 2A-B). The AlgR protein was purified as a monomer, and the remaining bands in the gel may correspond to nonspecific proteins, as there is no evidence of AlgR being active as a dimer. LexA was found to be present as both a monomer and a dimer. Some studies claim that LexA is active in its dimeric form in *E. coli*, *Bacillus subtilis*, and *Mycobacterium tuberculosis* [27–29]. When this dimer is cleaved by RecA, LexA loses its transcription repression

function. As only one LexA-binding box has been experimentally found in the promoter region of *dinB* [21], the presence of a protein dimer may indicate that in *P. aeruginosa,* LexA needs to form a dimer to be active; however, more experiments should be performed to confirm this hypothesis. The protein AlgR, on the other hand, was found to be a monomer, which may indicate that it is the active form of the TF (Fig. 2A).

When studying AlgR and LexA binding to P*nrdA* and P*dinB*, respectively, and the control sequence, it was demonstrated experimentally that small concentrations of the TF, 0.5 pmol of AlgR, and 0.25 pmol of LexA bound to P*nrdA* and P*dinB*, respectively, shifting the bands in the EMSA gel (Fig. 2C-D). When studying the binding of the TF to the control sequence, band shifting was not observed when using AlgR (Fig. 2C), but a slight shift was seen when using 1 pmol of LexA (Fig. 2D), which indicates that there may be some promoter competition when using the transcriptional repressor LexA.

To evaluate whether the ReViTA system is suitable for studying gene regulation, IVT assays were conducted using the plasmid pReViTA and linear PCR fragments with the promoter region of *nrdA* or *dinB* and the control sequence (Fig. 3). It was observed that in both types of experiments, the incubation of the TF with its specific promoter changed the transcriptional activity. The use of 0.25 or 0.5 pmol of AlgR increased the transcription of *nrdA* by 29% and 55% when using the ReViTA system and by 17% and 40% when using the PCR bands. These results are consistent, as an increased protein concentration led to higher *nrdA* expression, as can be observed in the raw data shown in suppl. Fig. S2A.

Additionally, using 0.125 pmol of LexA decreased the transcription of *dinB* by 27% and 30% when using the ReViTA plasmid and the linear PCR bands, respectively. However, a higher concentration of LexA (0.5 pmol) did not result in lower expression; in contrast, the expression of *dinB* seemed to decrease by only 10% and 11% when using the ReViTA plasmid or the PCR bands, respectively. Although the tendency is clear, it did not show statistical significance. It is believed that the nonspecific binding of LexA to the control region observed in Fig. 2D, and thus the competition among promoters, may be the reason for not finding a higher repression of *dinB* when using an increased protein concentration of LexA. The reasoning behind this hypothesis is shown in the raw data of suppl. Fig. S2B. The Ct values of the control sample without FT show a slight increase when adding 0.5 pmol of the protein LexA (for example in the PCR bands samples, the control Ct changes from 25.34 to 26.57), which may indicate that its transcription is been slightly repressed. While this outcome may suggest that performing an EMSA before using the ReViTA system is necessary, we are confident that, due to the high sensibility of ReViTA, it is not completely essential to carry out the EMSA. Nevertheless it is helpful to test whether the TF to be studied is a good candidate or be used in a high throughput method to determine binding to a specific promoter region.

Moreover, as the data in suppl. Fig. S2 shows, there is some variability among experiments. Thus, it is important to normalize the data before comparing experiments. To normalize the data, first, the %Act (percentage of activity) of the DNA sequences with protein was compared with the %Act of the DNA sequences without protein. Thereafter, the test was compared with the control samples obtaining the %Act nor (percentage of activity normalized) with corresponds to the transcriptional activity of the IVT experiment. Although, it seems that the unspecific TF-promoter binding could be avoided by performing the experiment using the test sequence with and without the TF, it is believed to be important to run the control along with the TF to ensure that the transcription activity obtained in the experiment is due to the specific binding between the promoter and the protein and not due to non-specific DNA interactions. Nonetheless, the results obtained with pReViTA and linear PCR bands are similar, which may indicate the correct functioning of the plasmid pReViTA. It was also observed that this system is specific enough to differentiate between a transcriptional activator and a repressor; thus, it could be used to study the specific unknown function of a TF.

Thus, the ReViTA system is a novel technique to measure gene expression and study TF regulation, whose benefits outweigh its disadvantages. In addition, the results obtained from the experiments conducted with pReViTA and the linear PCR fragments as DNA templates are very similar, which confirms its specificity and functionality.

Conclusion

ReViTA is a novel system to study transcriptional regulation of genes from broad-spectrum organisms as it consists of a plasmid to study transcription factors *in vitro*. Its simplicity and easy functioning offer many advantages over traditional techniques, overcoming its disadvantages. The transcription factors used as proof of concept were a transcription activator and repressor. The transcription activator increased gene expression and the transcription repressor decreased gene expression after comparing with the samples with no protein, showing the right functioning of the system ReViTA.

CRediT authorship contribution statement

ARC and ET wrote the manuscript. ARC and LP designed the plasmid and performed the biological assays. ET directed the research and revised the experimental data. All authors have approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare no financial or commercial conflicts of interest.

Data availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2023.04.005.

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