

Research Article



Construction of New Genetic Tools as Alternatives for Protein Overexpression in *Escherichia coli* and *Pseudomonas aeruginosa*

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Background: *Pseudomonas* protein expression in *E. coli* is known to be a setback due to significant genetic variation and absence of several genetic elements in *E. coli* for regulation and activation of *Pseudomonas* proteins. Modifications in promoter/repressor system and shuttle plasmid maintenance have made the expression of stable and active *Pseudomonas* protein possible in both *Pseudomonas* sp. and *E. coli*.

Objectives: Construction of shuttle expression vectors for regulation and overexpression of *Pseudomonas* proteins in *Pseudomonas* sp. and *E. coli*.

Materials and Methods: *Pseudomonas-Escherichia* shuttle expression vectors, pCon2(3), pCon2(3)-Kan and pCon2(3)-Zeo as well as *E. coli* expression vectors of pCon4 and pCon5 were constructed from pUCP19-, pSS213-, pSTBlue-1- and pPICZαA-based vectors. Protein overexpression was measured using elastase strain K as passenger enzyme in elastinolytic activity assay.

Results: The integration of two series of IPTG inducible expression cassettes in pCon2(3), pCon2(3)-Kan and pCon2(3)-Zeo, each carrying an *E. coli* lac-operon based promoter, P_{lac} , and a tightly regulated $T7_{(A1/O4/O3)}$ promoter/repressor system was performed to facilitate overexpression study of the organic solvent-tolerant elastase strain K. These constructs have demonstrated an elastinolytic fold of as high as 1464.4 % in comparison to other published constructs. pCon4 and pCon5, on the other hand, are series of pCon2(3)-derived vectors harboring expression cassettes controlled by $P_{T7(A1/O4/O3)}$ promoter, which conferred tight regulation and repression of basal expression due to existence of respective double operator sites, O_3 and O_4 , and $lacI^q$.

Conclusions: The constructs offered remarkable assistance for overexpression of heterogeneous genes in *Pseudomonas* sp. and *E. coli* for downstream applications such as in industries and structural biology study.

Keywords: Elastase strain K, $LacI^q$, Overexpression, Regulation, $T7_{(A1/O4/O3)}$

1. Background

Over the past years, shuttle expression vectors used in *Pseudomonas* sp. are derived from modification of reported constructs in which majority of the vectors are originated from RSR1010, RK2 and pRO1600-based plasmids (1). The application of *Pseudomonas* as host, particularly in expression of *Pseudomonas* proteins, has gained much attention in recent years as the routinely used *E. coli* is

unable to express these proteins at satisfactory levels due to (a) major difference of *Pseudomonas* promoter sequence to that of *E. coli* (2), (b) lacking of particular regulators and activators in *E. coli*, which are presumably essential for *Pseudomonas* genes (3), and (c) poor translation of *Pseudomonas* mRNA in view of its high G+C content and thus resulted in occurrence of unique codon that causes unwanted distribution of rare codons (4). Such nuisance

can be resolved by implementing genetic tools derived from *E. coli*. Further, such modifications can also be extended to *Pseudomonas* expression system. The transfer of *E. coli*-based technology to *Pseudomonas* would be beneficiary in terms of established manipulation protocols and commercially available reagents for the host (5). In our laboratory, vectors containing two different origins of replication have been constructed, in which *rep* (pMB1) (6) that is solely active in *E. coli* and stabilizing fragment (SF) (7) for plasmid replication in *Pseudomonas*. Other basic requirements include selection markers (kanamycin and zeocin) for screening of recombinant clones and a multiple cloning site (MCS) for insertion of foreign DNA.

2. Objective

The shuttle expression vectors, constructed in this study, are aimed to regulate and overexpress soluble and active *Pseudomonas* proteins for possible applications in industries and protein study.

3. Materials and Methods

3.1. Source of Bacteria and Plasmids

All bacteria and plasmids reported in this communication were obtained from Enzyme and Microbial Technology Laboratory, Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia. The constructed vectors were derived from pUCP19 (ATCC 87110), pSS213 (8), pUCP19/HindIII1500PstI (9), pPICZ α A (Invitrogen, USA) and pSTBlue-1 (Novagen, USA). Bacterial strains such as *P. aeruginosa* strain PAO1 (ATCC 47085) [lacI^q Δ (lacZ)M15⁺ tetA⁺ tetR⁺] and S5 (10) as well as *E. coli* strain TOP10 (Invitrogen, USA), KRX (Promega, USA) and TunerTM (DE3) pLacI (Novagen, USA) were among the expression hosts used in this study.

3.2. Construction of Vectors

Construction workflow of expression vectors overview is documented in Figure 1. Maps of vectors were

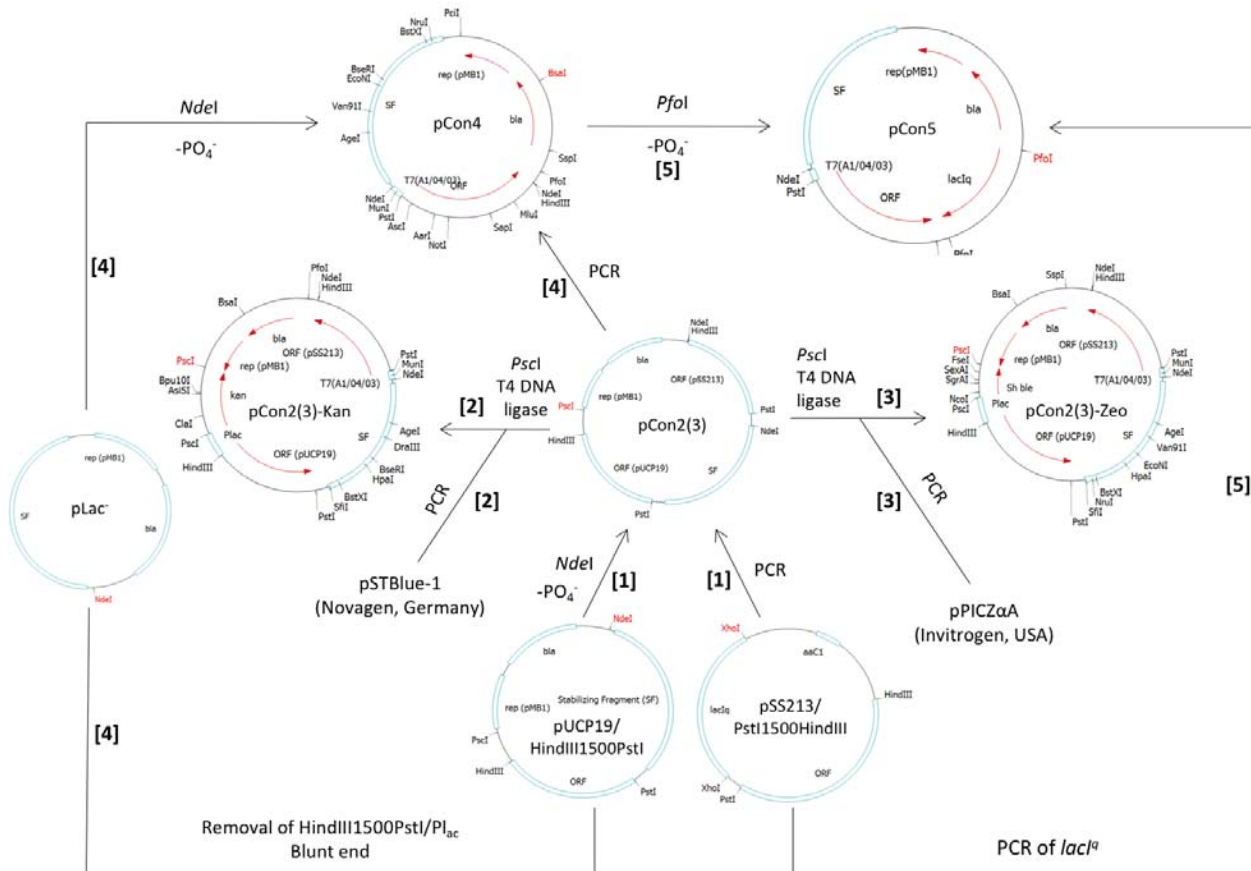


Figure 1. Workflow of vectors construction. Two shuttle vectors namely, pUCP19/HindIII1500PstI and pSS213/PstI1500HindIII, were acted as backbone for all vectors construction in this study. Pathways for construction of pCon2(3), pCon2(3)-Kan, pCon2(3)-Zeo, pCon4 and pCon5 are indicated by [1], [2], [3], [4] and [5], respectively. Arrows in red are indicating direction of protein expression. The maps were generated by Clone Manager 9 Basic Edition (Scientific and Educational Software, USA) and not drawn according to scale.

Table 1. Sequences of primers used in vector construction.

Primer	Sequence (5'-3') ^a	Tm (°C)	Amplicon size (bp)
ForC1 (<i>NdeI</i>)	GGCC <u>CATATG</u> AAGCTTTTTTACAAC	65.2	1,600
RevC1 (<i>NdeI</i>)	GCCG <u>CATATG</u> AAAATTTATCAAAAAGAG	64.3	
ForKan (<i>PciI</i>)	GCAT <u>ACATGT</u> CTGTGTCAGACCAAGTTTA	66.8	900
RevKan (<i>PciI</i>)	GAAT <u>ACATGTTT</u> AGAAAAACTCATCGAGCA	65.6	
ForZeo (<i>PciI</i>)	CTAA <u>ACATGT</u> GTTGTTGACAATTAATCATCGG	66.9	450
RevZeo (<i>PciI</i>)	GTCG <u>ACATGTT</u> CAGTCCTGCT	66.5	
ForlacIq (<i>PfoI</i>)	CTCT <u>TCCCGG</u> AAAATTTTCTCGA	65.1	1,200
RevlacIq (<i>PfoI</i>)	AGTAT <u>CCCGGG</u> AATATACTCCG	66.6	

^aRestriction endonuclease sites are indicated by underlined sequences.

generated by Clone Manager 9 Basic Edition (Scientific and Educational Software, USA) and primer sequences are summarized in Table 1.

3.2.1. Construction of pCon2(3)

A *NdeI* site at nucleotide 183 of the *Escherichia-Pseudomonas* shuttle vector, pUCP19/*HindIII*1500*PstI*, was chosen as location of insertion for a PCR generated fragment from pSS213/*PstI*1500*HindIII*. Briefly, the unique *NdeI* site of pUCP19/*HindIII*1500*PstI* was digested to form sticky ends and, subsequently, dephosphorylated with calf intestine alkaline phosphatase to avoid recirculization. *PstI*1500*HindIII*/T7_(A1/O4/O3), a PCR fragment encoding the ORF of organic solvent tolerant elastase from *P. aeruginosa* strain K under the control of promoter T7_(A1/O4/O3), was generated by ForC1 (*NdeI*) and RevC1 (*NdeI*) in a Corbett Research Gradient PCR CG1-96 for 30 PCR cycles, each with 1 min denaturation at 94 °C, 1 min annealing and 1 min extension at 55 and 72 °C. The final elongation step set at 72 °C for 7 min.

*PstI*1500*HindIII*/T7_(A1/O4/O3) was converted to a unique *NdeI* site by digesting with *NdeI*. Each of the process stated above was accompanied by DNA purification steps using QIAquick® Gel Extraction Kit (Qiagen, Germany) according to the manufacturer's instructions. Both of the DNA fragments were then ligated with T4 DNA ligase and transformed into *E. coli* TOP10. The resulting recombinant clones harboring pCon2(3) were selected based on bacterial growth on skimmed milk agar (SMA) supplemented with carbenicillin (100 µg.mL⁻¹) and gentamicin (10 µg.mL⁻¹).

3.2.2. Construction of pCon2(3)-Kan and pCon2(3)-Zeo

A *PciI* site at nucleotide 5980 of pCon2(3) was selected as a point of insertion for *kan/pro* coding sequence and

Sh ble/EM7, regions encoding kanamycin resistant gene from pSTBlue-1 and zeocin resistant gene from pPICZαA, respectively. pCon2(3) was initially linearized by *PciI* and dephosphorylated with calf intestine alkaline phosphatase to avoid recirculization. Primers ForKan (*PciI*) and RevKan (*PciI*) were employed to generate *kan/pro* at an annealing temperature of 55 °C.

Sh ble/EM7, on the other hand, was generated by ForZeo (*PciI*) and RevZeo (*PciI*) at 56 °C as the annealing temperature. Both of the PCR fragments were digested with *PciI*, purified and ligated to *PciI* digested-pCon2(3) to give rise to pCon2(3)-Kan and pCon2(3)-Zeo, which subsequently transformed into *E. coli* TOP10. Selection of positive transformants harbouring pCon2(3)-Kan and pCon2(3)-Zeo were conducted on carbenicillin (100 µg.mL⁻¹)/kanamycin (35 µg.mL⁻¹) and carbenicillin (100 µg.mL⁻¹)/zeocin (50 µg.mL⁻¹) supplemented on SMA plates.

3.2.3. Construction of pLac⁻

Restriction endonuclease sites, *PstI* and *PciI*, at nucleotide 2423 and 5980, respectively, of pUCP19/*HindIII*1500*PstI* were digested, thus releasing the ORF of elastase, multiple cloning sites (MCS) and promoter *P_{lac}* of pUCP19. The resulting construct, pLac⁻, was blunt ended with Quick Blunting Kit (NEB, USA), religated with T4 DNA ligase and transformed into *E. coli* TOP10. The pLac⁻ will serve as an intermediate vector for subsequent constructs, pCon4 and pCon5.

3.2.4. Construction of pCon4

In pCon4, an initial *P_{lac}*-controlled expression cassette of pUCP19/*HindIII*1500*PstI* was substituted by an expression system controlled by T7_(A1/O4/O3) from pSS213/*PstI*1500*HindIII*. Similar to pCon2(3), a *NdeI* site at nucleotide 2064 of pLac⁻ was digested and

dephosphorylated with *NdeI* and calf intestine alkaline phosphatase, respectively. PCR of *PstI*1500*HindIII*/*T7*_(A1/O4/O3) was carried out to amplify a region encoding the promoter of *T7*_(A1/O4/O3), ORF of elastase and MCS of pUC18. The *NdeI* digested-*PstI*1500*HindIII*/*T7*_(A1/O4/O3) was then ligated to *NdeI* digested- pLac⁻ to form pCon4. The resulting recombinant clones, *E. coli* TOP10/pCon4, were selected based on bacterial growth on SMA supplemented with carbenicillin (100 µg.mL⁻¹).

3.2.5. Construction of pCon5

A Lac repressor gene, *lacI^q*, is yet another essential element appeared in pCon5. *lacI^q* was amplified by ForlacIq (*PfoI*) and RevlacIq (*PfoI*) in 30 cycles, each with 1 min denaturation at 94 °C, 1 min annealing at 55 °C and 1 min extension at 72 °C. The final elongation step at 72 °C was for 7 min. The resulting PCR products were purified, digested with *PfoI* and ligated to *PfoI* digested-pCon4 to form pCon5. Recombinants of *E. coli* TOP10/pCon5 were screened on carbenicillin (100 µg.mL⁻¹) supplemented SMA plates.

3.3. DNA Sequences

Nucleotide sequences of pCon2(3), pCon2(3)-Kan, pCon2(3)-Zeo, pCon4 and pCon5 were deposited in GenBank®, with accession numbers JF276396, JF276397, JF276393, JF276394 and JF276395, respectively.

3.4. Expression of Elastase Strain K by the Constructed Vectors in *E. coli* and *P. aeruginosa*

All the constructs were transformed into chemically competent *E. coli* TOP10, KRX and Tuner™ (DE3) pLacI according to standard procedures, unless otherwise stated. Meanwhile, *P. aeruginosa* competent cells were prepared and electroporated as described in (11).

Cultures derived from colonies harboring empty vectors and recombinant plasmids were inoculated into 10 mL of LB (10 g.L⁻¹ tryptone, 5 g.L⁻¹ yeast extract, 1 g.L⁻¹ NaCl, pH 7.2) and carbenicillin (100 µg.mL⁻¹) for 16 h at 37 °C. Subsequently, the cell cultures (1 % v/v) were transferred into Schott Duran bottle (250 mL) containing LB on a rotary shaker (250 rpm) at 37 °C. Induction was carried out for 6 h by 1 mM of isopropyl-1-thio-β-D-galactopyranoside (IPTG) into culture with OD₆₀₀ of approximately 0.5. Cells were collected and the expression level was looked at.

Bacterial cells were centrifuged at 5000 ×g and 4 °C for 10 min. Cell pellet was washed and resuspended with physiological saline and immediately followed by ultrasonic disintegration (UD-200) at 60 W for 4

min intermittently on ice. Cell extract was obtained by centrifugation at 5000 ×g and 4 °C for 10 min. Both the culture supernatant and cell extract were subjected to elastinolytic assay (12) for detection of the recombinant elastase.

4. Results

4.1. Analysis of Constructed Vectors

Confirmation on the presence of genetic elements in constructs pCon2(3), pCon2(3)-Kan, pCon2(3)-Zeo, pCon4 and pCon5 was conducted *via* restriction endonuclease digestion and agarose gel electrophoresis, as shown in Supplementary material 1, Supplementary material 2 and Supplementary material 3. Circular conformation of pCon2(3), pCon2(3)-Zeo and pCon2(3)-Kan were extracted from *P. aeruginosa* strain PAO1 and S5 with the size of approximately 6-10 kb (Supplementary material 4). Comparable to pUCP19/*HindIII*1500*PstI*, the yield of the constructs replicated from PAO1 and S5 were qualitatively low compared to the ones found in strains of *E. coli*. This result had indicated that the copy number of pUCP19-derivatives was somehow remained unchanged, although multiple insertions of DNA fragments were carried out on the native plasmid during vector construction. The pCon4 and pCon5, however, were unable to replicate in both PAO1 and S5 as desired bands were undetected following plasmid extraction (Supplementary Material 4).

4.2. Expression Analysis of Constructed Vectors

All constructed shuttle vectors were transformed into various strains of *E. coli* and *P. aeruginosa* to determine the best elastinolytic expression level exhibited by the vectors in multiple hosts. In general, *E. coli* TOP10 was able to replicate and express the heterogeneous protein harbored by all constructed vectors (Table 2). Construct pUCP19/*HindIII*1500*PstI*, which carried single expression cassette controlled by *P_{lac}*, showed the highest protease activity of 4.24 A₄₉₅.h⁻¹.mL⁻¹ with an increase of 16.31 in TOP10 compared to control (pUCP19) (Fig. 2). Although exhibited high activity, constructs that harbored two expression cassettes such as pCon2(3), pCon2(3)-Kan and pCon2(3)-Zeo were unable to exhibit greater protease activity due to the fact that *E. coli* TOP10 is incapable of utilizing *T7*_(A1/O4/O3) promoter for protein expression. This was also evidenced in TOP10/pCon4 and TOP10/pCon5 that controlled only by *T7*_(A1/O4/O3) promoter, in which slight increase in activities was observed. Among the constructs with double expression cassettes in TOP10,

Table 2. Expression analysis of constructed vectors in various strains of *E. coli* and *P. aeruginosa*. Cell cultures were harvested by centrifugation following 6 h of induction by 1.0 mM IPTG.

Host	Elastinolytic activity ($A_{495}, \text{min}^{-1} \cdot \text{g}^{-1}$)*						
	pUCP19	pUCP19/ <i>HindIII</i> 1500 <i>PstI</i>	pCon2(3)	pCon2(3) -Kan	pCon2(3) -Zeo	pCon4	pCon5
TOP10 [#]	0.26±0.001	4.24±0.23	2.69±0.08	3.56±0.44	3.42±0.580	0.27±0.05	0.27±0.04
KRX [#]	0.03±0.01	1.39±0.07	2.77±0.04	2.68±0.03	2.76±0.03	0.26±0.09	0.14±0.02
Tuner TM (DE3) pLacI [#]	0.37±0.02	4.33±0.02	3.18±0.24	3.24±0.19	1.06±0.10	0.43±0.09	0.39±0.02
S5 [^]	4.28±0.07	5.31±0.11	4.24±0.09	4.39±0.06	4.85±0.06	ND	ND
PAO1 [^]	0.02±0.003	0.22±0.01	0.35±0.02	0.31±0.003	0.11±0.01	ND	ND

[#]Strains of *E. coli*. Elastinolytic activities of all constructs were detected in intracellular fraction of the hosts.

[^]Strains of *P. aeruginosa*. Elastinolytic activities of all constructs were detected in extracellular fraction of the hosts.

*Values are represented by mean value ± standard deviations (n = 3), which obtained after 6 h of induction with 1.0 mM IPTG.

ND denotes no elastase assay was carried out due to incapability of constructs to replicate in respective hosts.

pCon2(3)-Kan had demonstrated the best expression of $3.56 A_{495} \cdot \text{h}^{-1} \cdot \text{mL}^{-1}$, an increase of 13.69-fold against its control (Fig. 2).

Overexpression of elastase strain K was viewed on *E. coli* KRX/pCon2(3) (Table 2). It displayed greatest elastinolytic activity and fold difference of $2.77 A_{495} \cdot \text{h}^{-1}$.

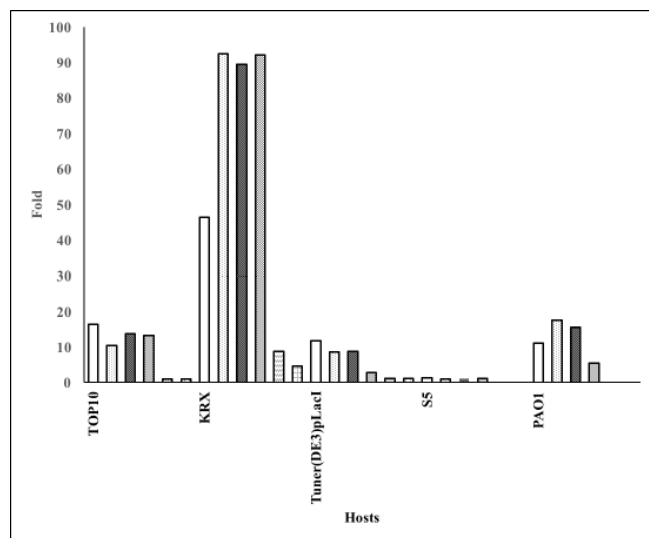


Figure 2. Expression profile of constructed vectors in tested hosts. Values were obtained by comparing elastinolytic activity of pUCP19/*HindIII*1500*PstI* (□), pCon2(3) (▒), pCon2(3)-Kan (■), pCon2(3)-Zeo (▤), pCon4 (▨) and pCon5 (▩) to that of pUCP19 (fold = 1.0) in their respective hosts and conditions as stated in Table 2. Elastinolytic activities were not detected in *P. aeruginosa* S5/pCon4, S5/pCon5, PAO1/pCon4 and PAO1/pCon5 as the constructs were unable to replicate in the hosts.

mL^{-1} and 92.33 (Fig. 2), respectively, compared to the control. Similar overexpression profile was obtained for KRX/pCon2(3)-Kan and KRX/pCon2(3)-Zeo with their respective activities of 2.68 and $2.76 A_{495} \cdot \text{h}^{-1} \cdot \text{mL}^{-1}$. The former and latter constructs had yielded respective fold of 89.33 and 92.0 in respect to their controls (Fig. 2). With the presence of extra *lac* repressor gene, KRX/pCon5 had recorded a lower activity of $0.14 A_{495} \cdot \text{h}^{-1} \cdot \text{mL}^{-1}$ compared to KRX/pCon4 ($0.26 A_{495} \cdot \text{h}^{-1} \cdot \text{mL}^{-1}$), which possessed only low amounts of repressor from bacterial genome. Elastinolytic activity in KRX/pUCP19/*HindIII*1500*PstI*, in contrast, had recorded approximately 50 % of its counterparts with double expression cassettes under the same host.

Meanwhile, pUCP19/*HindIII*1500*PstI* in *E. coli* TunerTM (DE3) pLacI displayed the greatest expression of elastase activity of $4.33 A_{495} \cdot \text{h}^{-1} \cdot \text{mL}^{-1}$ compared to other constructs transformed under the same host with 11.70 increase in fold. Increment of activities was also viewed on pCon2(3), pCon2(3)-Kan and pCon2(3)-Zeo by 8.59-, 8.76- and 2.86-fold, respectively, compared to their controls (Fig. 2). From Table 2, a similar observation was detected for construct pCon4 and pCon5 with elastinolytic activity of 0.43 and $0.39 A_{495} \cdot \text{h}^{-1} \cdot \text{mL}^{-1}$, respectively. The expression of these two constructs were lower than that of constructs with two expression cassettes. This might be due to the presence of two *lac* operator sites for binding of Lac repressor that conferred to tight repression of the promoter in addition to the presence of *lac* repressor gene carried by plasmid TunerTM (DE3) pLacI and pCon5. However,

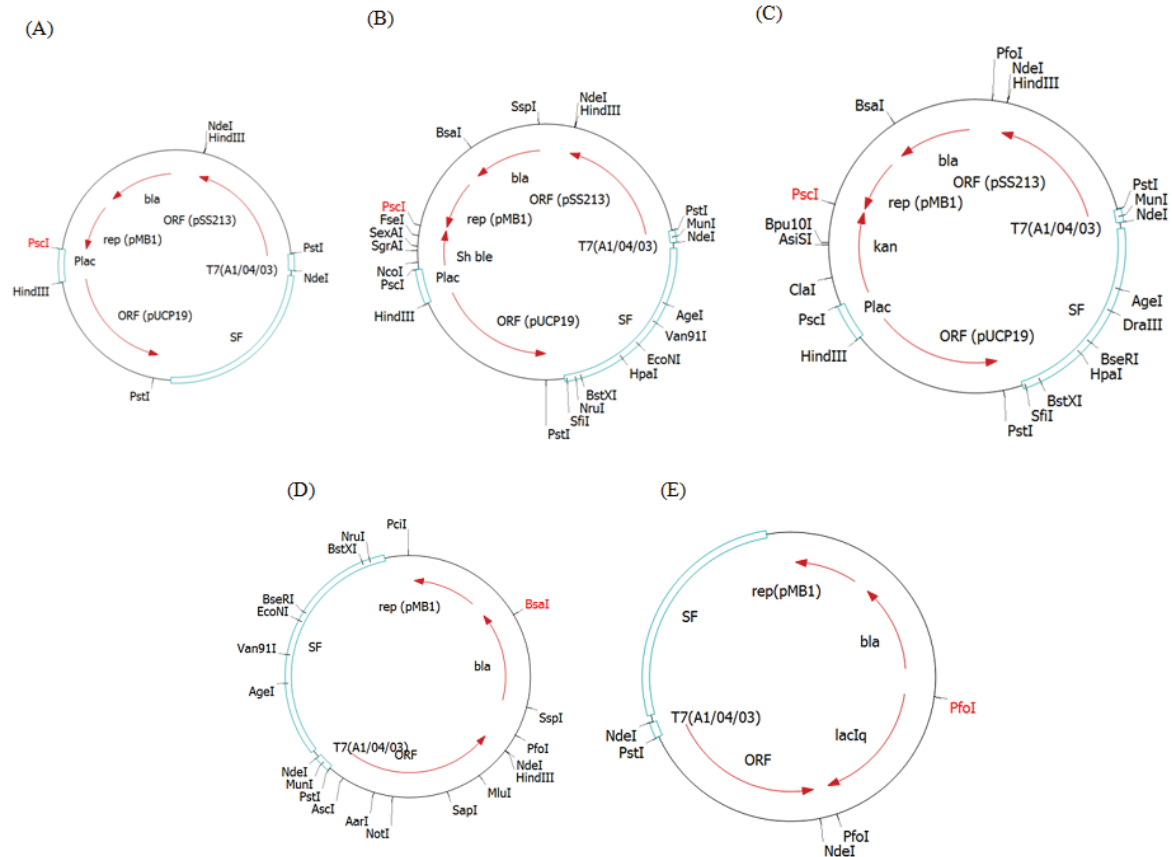


Figure 3. Physical maps of constructed shuttle vectors carrying P_{lac} and $P_{T7(A1/04/03)}$ expression cassettes. (A) pCon2(3), (B) pCon2(3)-Zeo, (C) pCon2(3)-Kan, (D) pCon4 and (E) pCon5. The maps were generated by Clone Manager 9 Basic Edition (Scientific and Educational Software, USA) and not drawn according to scale.

higher expression levels were noticed, when the *lac* and $T7_{(A1/04/03)}$ promoters appeared simultaneously in *E. coli*. Unlike *E. coli*, only several constructs namely pUCP19/*HindIII*1500*PstI*, pCon2(3), pCon2(3)-Kan and pCon2(3)-Zeo were able to replicate in *P. aeruginosa* S5 and PAO1. Similar to the expression of recombinant elastase strain K in *P. aeruginosa* as reported earlier, elastinolytic activities exhibited by these constructs were also detected at the extracellular culture media of S5 and PAO1. With the exception of pUCP19/*HindIII*1500*PstI* and pCon2(3)-Zeo of S5, which showed significantly higher expression levels of 5.31 and 4.85 $A_{495} \cdot h^{-1} \cdot mL^{-1}$, respectively, the elastinolytic activities exhibited by S5/pCon2(3) and S5/pCon2(3)-Kan remained indifferent to their controls (Table 2). As a comparison, *P. aeruginosa* PAO1/pCon2(3) had overexpressed and recorded an elastinolytic activity of 0.35 $A_{495} \cdot h^{-1} \cdot mL^{-1}$ (Table 2 and Fig. 2; 17.50 \times greater than its control). Additionally, other PAO1 constructs such as pUCP19/*HindIII*1500*PstI*, pCon2(3)-Kan and pCon2(3)-Zeo had also documented respective

substantial expression level of 0.22, 0.31 and 0.11 $A_{495} \cdot h^{-1} \cdot mL^{-1}$.

5. Discussion

Escherichia-Pseudomonas shuttle vectors, pCon2(3) [Fig. 3(A)], pCon2(3)-Zeo [Fig. 3(B)] and pCon2(3)-Kan [Fig. 3(C)] were generated from several sources, namely pUCP19/*HindIII*1500*PstI*, *PstI*1500*HindIII*/ $T7_{(A1/04/03)}$, *EM7/Sh ble* and *kan/pro*. In which all of the DNA fragments were originated from pUCP19, pSS213, pSTBlue-1 and pPICZ α A, respectively. Both of the pUCP19 and pSS213 carried the organic solvent tolerant elastase gene from *P. aeruginosa* strain K. pSS213 is a versatile vector that carries gentamicin resistant gene, a p15A replicon, MCS of pUC18, modified phage T7 early gene promoter $T7_{(A1/04/03)}$ and *lacI^q* gene. The plasmid can be used for screening, plasmid maintenance, gene insertion, gene expression and repression of the $T7_{(A1/04/03)}$ promoter, respectively. Suh, et al. (8) had tested the efficiency of $T7_{(A1/04/03)}$ by placing a promoterless and 3'-truncated autoinducer for elastase and pyocynin,

rhlR', into pSS213 for intergration into *P. aeruginosa* PAO1 genome by homologous recombination of *rhlR* sequences in addition to *moriT* to allow mobilization into *P. aeruginosa* via conjugation. This resulted in an elastase activity of 102.5 A₄₉₅.min⁻¹.g⁻¹ from 86.7 A₄₉₅.min⁻¹.g⁻¹ (control), an increment of 18% following the induction by 1 mM of IPTG. In PAO1, the construct pCon2(3) was able to overexpress greater elastinolytic fold with an increment of 1464.4%. in respect to the one reported by Suh and colleagues (8).

Expression vectors are designed to achieve regulated expression of cloned genes from inducible promoters. The corresponding regulatory genes are either contained on the same plasmid or are provided *in trans*, either encoded by a compatible plasmid or a chromosomally integrated element (5). The *lac* operon-based promoters including *P_{lac}* and the T7 promoter (*P_{T7}*) are the prominent inducible promoter/regulatory elements. This is due to the fact that these two are most easily adapt with the host condition especially in Pseudomonads and *E. coli*. The constructed vectors are made up of double expression cassettes from pUCP19/*HindIII*1500*PstI* and pSS213/*PstI*1500*HindIII*, each controlled by *P_{lac}* and *P_{T7(A1/O4/O3)}*, respectively. Thus, the transcription and translation processes of the desired gene will be aimed to be twice as effective as the native vectors. Thereby, it can be expected to have an expression fold increase of at least two times more than the unmodified plasmid with single cassette.

Two genetic tools, pCon4 [Fig. 3(D)] and pCon5 [Fig. 3(E)], were developed in order to facilitate the study of protein expression in different promoter/repressor system by opportunistic bacterial pathogen *P. aeruginosa* and *E. coli*. The absence of MCS of pUCP19 and *lac* promoter in pLac⁻ will greatly assist in promoter/repressor substitution by modified T7 promoter, *T7_(A1/O4/O3)*, MCS of pUC18 and *lac* repressor, *lacI^q*, in pCon4 and pCon5. In our study, pCon4 was aimed to visualize the functionality of tightly regulated T7 promoter system in controlling the expression level due to the presence of two *lac* operator sites, *O3* and *O4*. This may result in higher rate of promoter clearance that lowers the occupancy of the promoter by RNA polymerase, thereby increases the repression factor (13). While the construction of pCon5 will display the repression level of *lacI^q* on *T7_(A1/O4/O3)* promoter at the *O3* and *O4* operator sites, but addition of IPTG relieves repression and the gene of interest can be expressed. This construct, in fact, will reduce the level of basal transcription or leakiness that typically occurred in *lac*-mediated promoter (absence of induction) in *Pseudomonas* sp. and *E. coli* (1).

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