Supplementary Tables

Table S1: List of strains used in this study

Bacterial strains	Characterization	Source/ Reference
Acinetobacter baumannii	ATCC 17978	(Baumann *et al.*, 1968)
Escherichia coli strains:		
TOP10	Cloning host for maintaining recombinant plasmids	Department of Microbiology culture collection
NEB 5-alpha	Cloning host for maintaining recombinant plasmids	New England Biolab, US
XL-1 Blue	Cloning host for maintaining recombinant plasmids	Agilent, US
BL21(DE3)	Expression host	Novagen, USA

109 **Table S2: List of primers used in this study**

Name	Sequence (5′ -3′)	Function
RT_*cptA*-F	ATGCCGTTACTATGGTGGTTAG	Transcriptional analysis in *A. baumannii*
RT_*cptA*-R	CGAGAAATGGTGTTCTGTGAATG	Transcriptional analysis in *A. baumannii*
RT_*cptB*-F	GGTCTTCCTGTTCTACCAACTC	Transcriptional analysis in *A. baumannii*
RT_*cptB*-R	CGTCGTGGTTTAAAGGAAATTGA	Transcriptional analysis in *A. baumannii*
RT_*cptBA*-F	CTGAGTTGGTAGAACAGGAAGAC	Transcriptional analysis in *A. baumannii*
RT_*cptBA*-R	AGCTGATTACAACTAACCACCATA	Transcriptional analysis in *A. baumannii*
16S-rRNA-F	TGAGATGTTGGGTTAAGTCCCGCA	Housekeeping gene for transcriptional analysis in *A. baumannii*
16S-rRNA-R	TGTAGCAACCCTTTGTACCGACCA	
ihfB-F	GATAGAAAGACTTGCCACCCA	Integration host beta subunit used as housekeeping gene for transcriptional analysis in *E. coli*.
ihfB-R	CCAGTTCTACTTTATCGCCAG	
cptB-F	GATACCATGGTCATGTGATCAGGTAAACT (*Nco*I)	Amplification of *cptB* fragment together with 50bp at 5' end of *cptB* for cloning in pET28b (+).
cptB-R	TCAAGAATTCTTAACCATGAACATAGTGTT (*Eco*RI)	
RBS-F	GATAGAGCTCTCATGTGATCAGGTAAACT (*Sac*I)	For amplification of the 50bp (ribosome binding site) upstream to the *cptBA* system used in the overlap extension PCR with *cptA* ORF. The underlined segment represents the first 15 nucleotides of *cptA* ORF, while the shaded segment is the last 20 nucleotides in the ribosome binding site.
RBS-R	CAAATGGTTGATCAATTATTTCCCCATCTGCGTTG	
cptA-F	GCAGATGGGGAAATAATTGATCAACCATTTGAATT	For amplification of *cptA* used in the overlap extension PCR with the ribosome binding site. The shaded segment is the last 16 nucleotides in the ribosome binding site while the underlined segment represents the first 22 nucleotides of *cptA* ORF. RBS-F and *cptA*-R primers were used for overlap extension PCR of *cptA* with RBS region (50bp).
cptA-R	TGTCGAATTCTTAATAGAGTTTTTCTAAAG (*Eco*RI)	
T7-F	TAATACGACTCACTATAGGG	For checking cloning in pET28b (+).
T7-R	GCTAGTTATTGCTCAGCGG	
GTApBAD-F	TTCGAGCTCGGTACCCGGGGATCCTGGTACCTCATGTGATCAGGTAAACTAAGCG	For amplification of *cptA-cptB-*P_{T7} promoter and *lacI* gene, and cloning in pBAD18 ori using Gibson assembly cloning kit.
GTApBAD-R	AAGCTTGCATGCCTGCAGGTCGACTACTGCCCGCTTTCCAGTC	
pBAD-F	ATGCCATAGCATTTTTATCC	For checking cloning in pBAD18 ori.
pBAD-R	GATTTAATCTGTATCAGGF	
lacI-R	ACTGCCCGCTTTCCAGTC	Used with pBAD-F to confirm the orientation of *cptA-cptB-*P_{T7}*-lacI* template within the pBAD18 ori.

cptBA-F	GAACGAATTCATTGTTCTGGCATGGTGG (*Eco*RI)	For amplification of *A. baumannii cptBA* TA system together with its upstream promoter (401bp) for cloning in pBAD42.
cptBA-R	TGTCGAATTCTTAATAGAGTTTTTCTAAAG (*Eco*RI)	

110

Supplementary Figures

Figure S1:

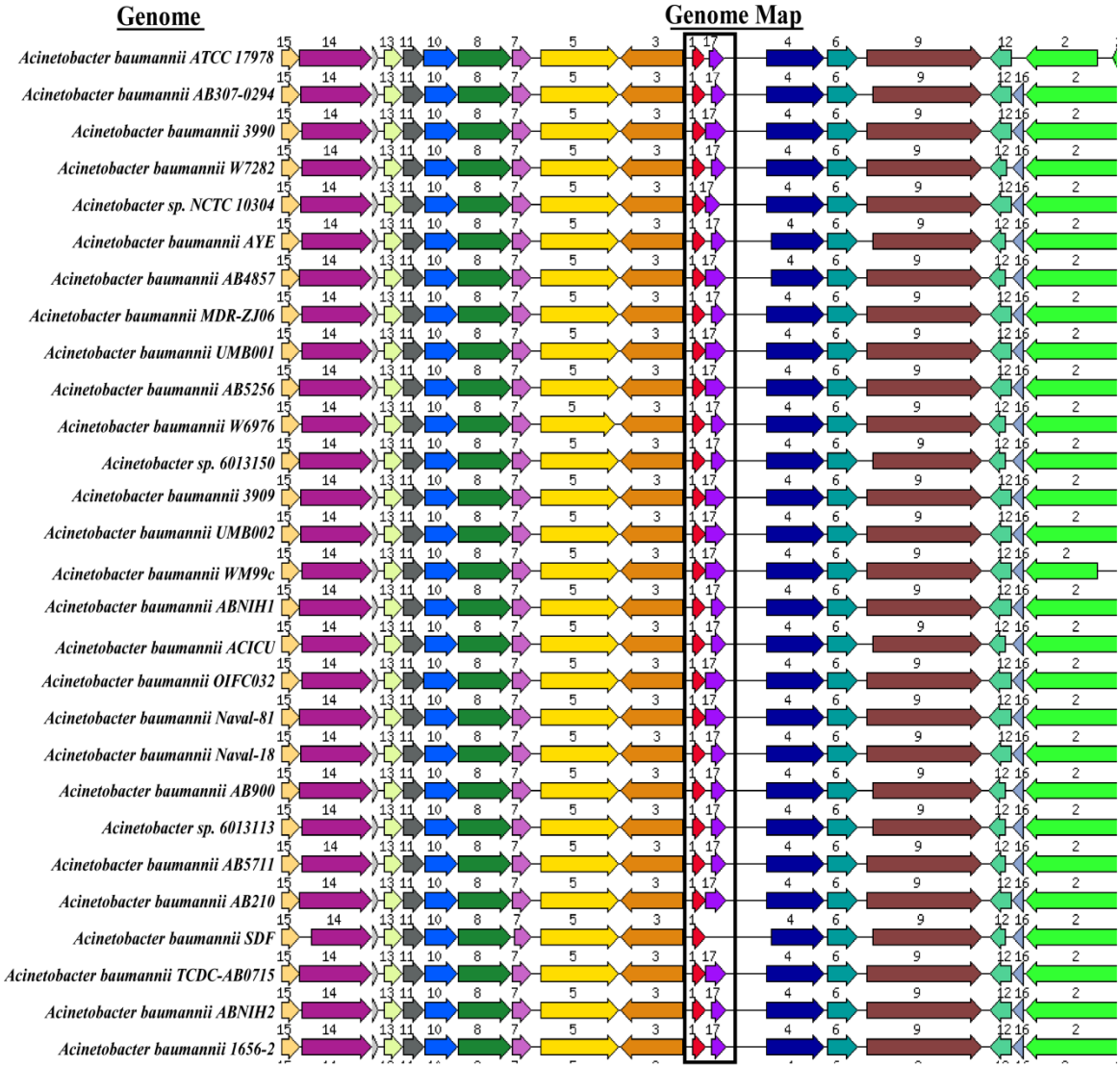


Figure S1: Abundance and gene orientation of *cptBA* alleles among different *Acinetobacter* spp.

Genetic maps of different *Acinetobacter* spp. showing the conservation of the corresponding *cptA* and *cptB* genes alleles in 195 strains of *A. baumannii* in the same synthetic order. The red arrow (labeled "1") and the purple arrow (labeled "17") represent the antitoxin and the toxin genes, respectively. The result was retrieved from the SEED Viewer Compare Region tool and NCBI database.

Figure S2:

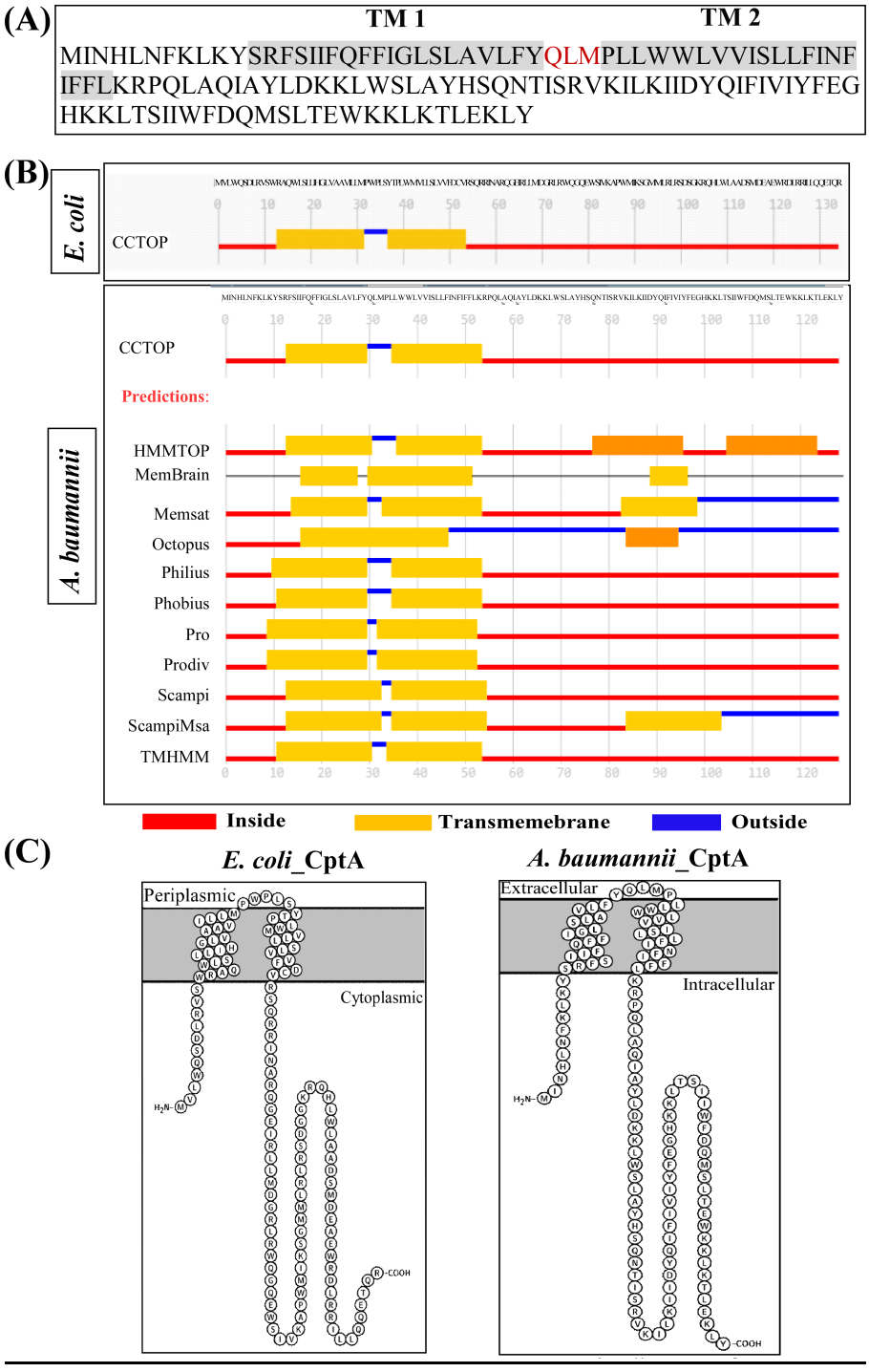


Figure S2: Predicted topology of the hypothetical *A. baumannii* toxin simulating the transmembrane nature of *E. coli* CptA.

(A) Amino acid sequence of *A. baumannii* CptA-like toxin showing two transmembrane domains (TM1 and TM2). (B) Output from the CCTOP server showing all possible topologies of the *A. baumannii* toxin candidates versus *E. coli* CptA, (C) A Protter-generated schematic diagram of

CptA toxin in both *E. coli* and *A. baumannii* showing similar distribution of amino acids through the bacterial membrane.

Figure S3:

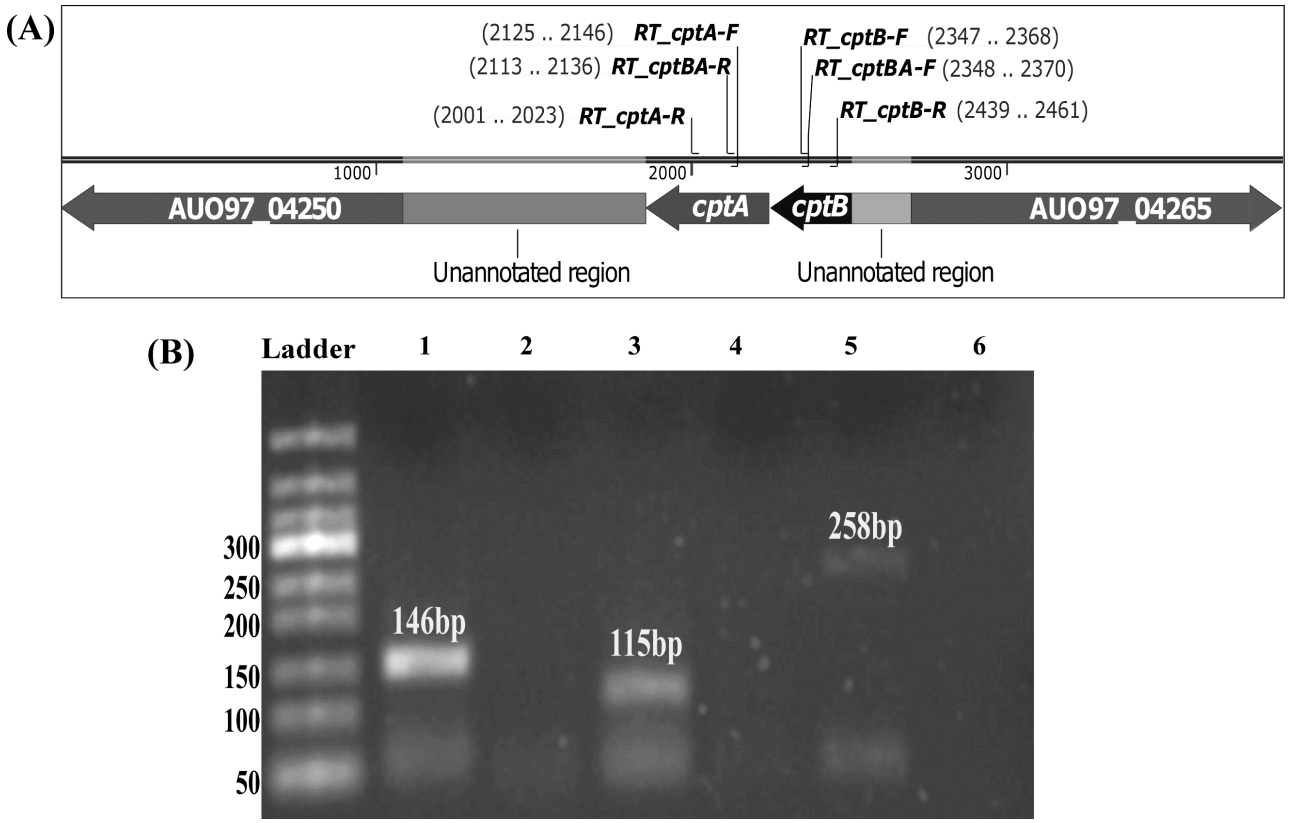


Figure S3: Transcriptional analysis of *cptBA* in *A. baumannii* ATCC 17978.

(A) Genetic map showing about 3.86 Kb of *A. baumannii* chromosomal DNA including *cptB* (the antitoxin) and *cptA* (the toxin) encoding genes together with primer- binding sites used in RT-PCR experiment. This map was generated by Snapgene® viewer software. (B) A photograph of a 2 % wt/vol agarose gel showing the positive RT-PCR products of the *cptB* antitoxin (115 bp), the *cptA* toxin (146 bp) and part of the polycistronic *cptBA* cDNA (258 bp) at lanes 1, 3, and 5, respectively. A non-RT RNA sample was used as a negative control to rule out any DNA traces (lanes 2, 4, and 6). The first lane contained 50 bp DNA ladder (Qiagen, Germany) for size comparison.

Figure S4:

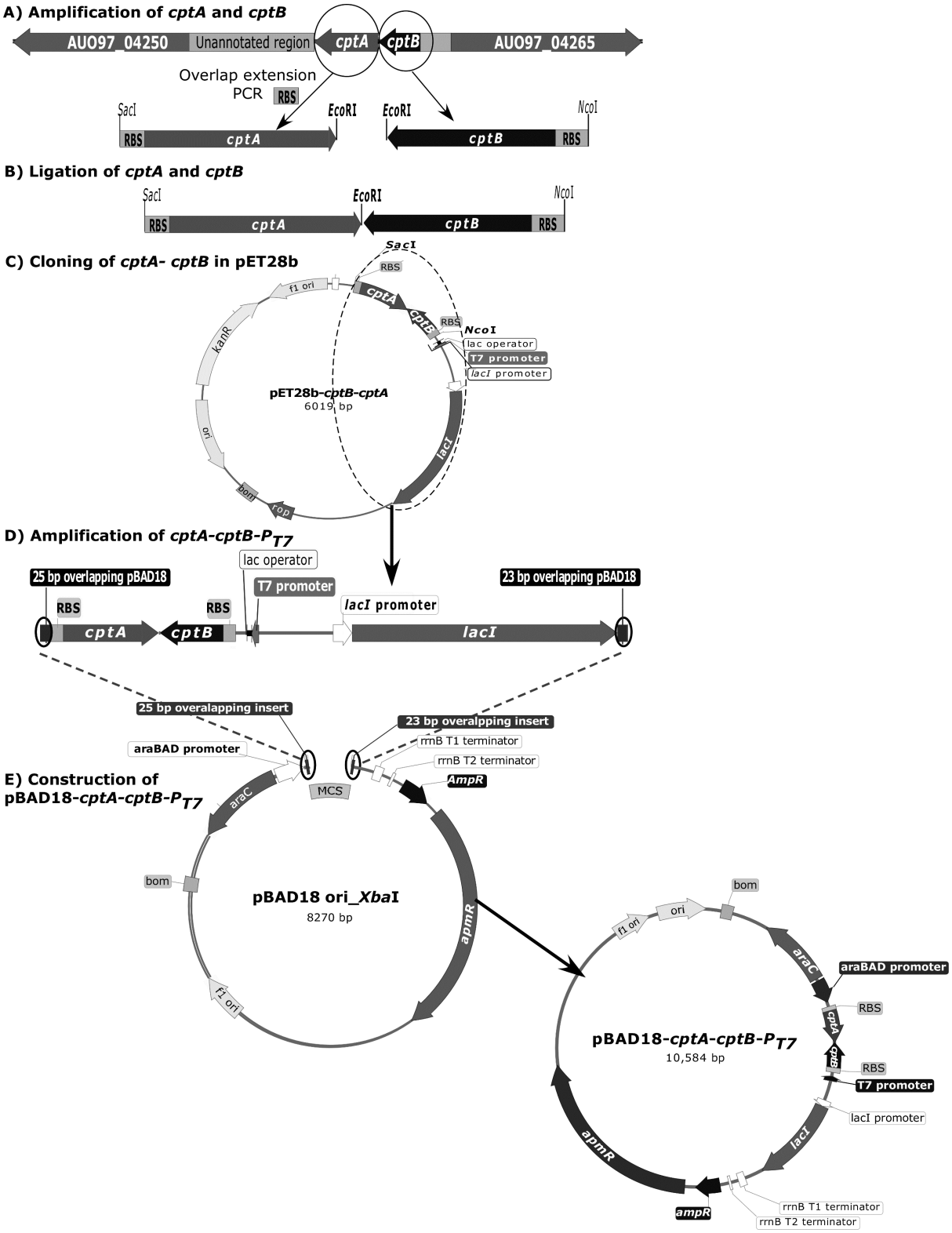
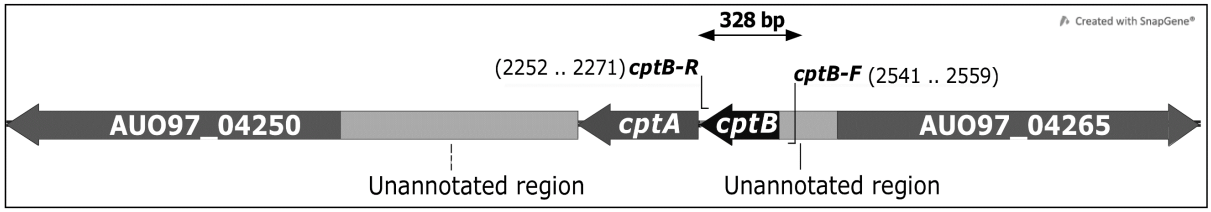


Figure S4: Schematic diagram for the construction of dual promoter pBAD18-*cptA-cptB-*P_{T7} vector.

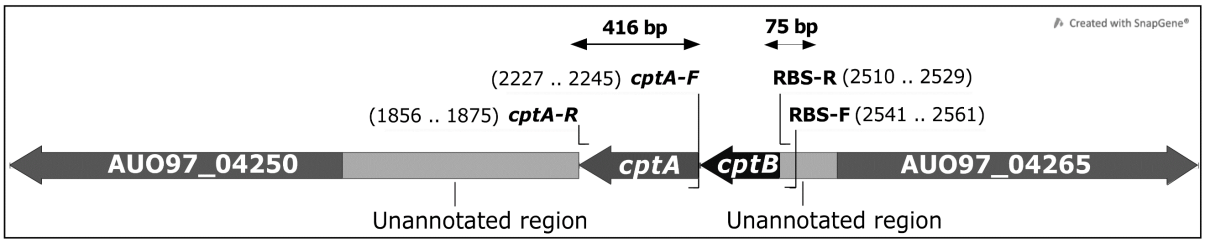
The diagram showed the steps carried out for the construction of dual promoter vector of pBAD18-*cptA-cptB-*P_{T7} in which the *cptB* antitoxin was under the control of P_{T7} promoter while the *cptA* toxin was controlled by araBAD P_{BAD} promoter.

Figure S5:

(A) Amplification of *cptB* (antitoxin, AUO97_04260) with upstream RBS



(B) Amplification of *cptA* (toxin, AUO97_04255) and RBS fragment



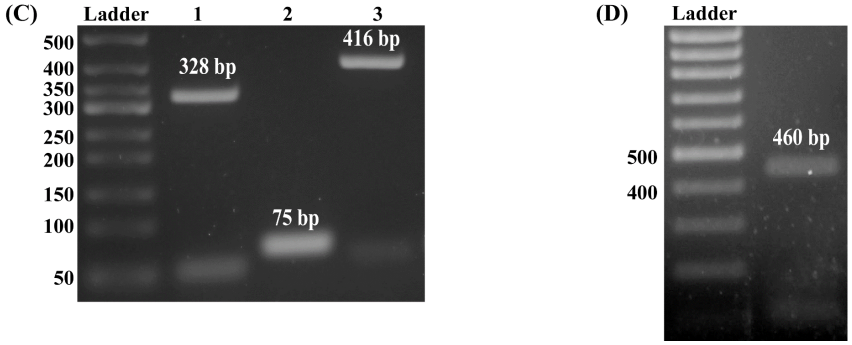


Figure S5: The *cptBA* cassette locus within *A. baumannii* ATCC 17978 genome.

(A) Genetic map of the antitoxin and the upstream region, containing the ribosome-binding site, showing the binding sites of *cptB*-F/R primers and the expected size of the amplicon obtained using them. (B) Genetic map of the toxin and the upstream region, containing the ribosome-binding site, showing the binding sites of *cptA*-F/R primers in addition to RBS-F/R and the expected sizes of the amplicons obtained using them. Maps were generated with SnapGene® viewer software. (C) A photograph of a 2 % wt/vol agarose gel showing the PCR products that were obtained using the following primers: *cptB*-F/R primer pair with overhanging *Nco*I and *Eco*RI restriction sites (lane 1, 328 bp), RBS-F/R primer pair (lane 2, 75 bp), and RBS-F/ *cptA*-R primer pair with overhanging *Sac*I and *Eco*RI restriction sites (lane 4, 416 bp). The first lane contained 50 bp DNA ladder (Qiagen, Germany). (D) A photograph of a 2 % wt/vol agarose gel showing the overlap extension PCR products (460 bp) of toxin and RBS obtained using the RBS-F/*cptA*-R primer pair. The first lane contained a 100-bp DNA ladder (Thermo Scientific™ GeneRuler 100 bp Plus) for size comparison.

Figure S6:

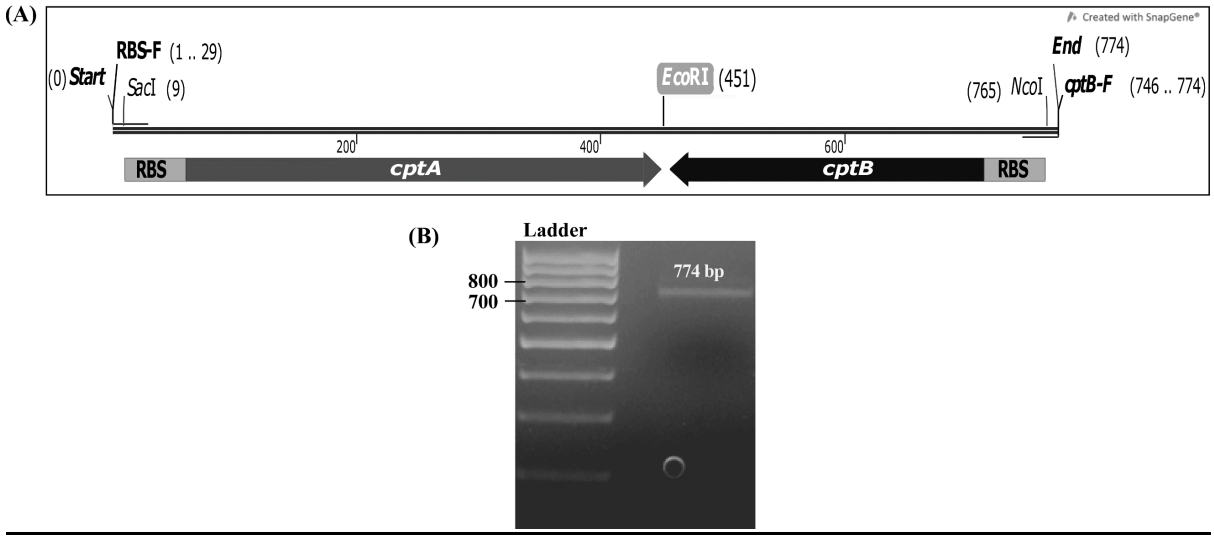


Figure S6: Ligation of the amplified RBS-Toxin and the RBS-Antitoxin products by their 3' ends.

(A) Genetic map of the ligated *A. baumannii cptA-cptB* TA system in reverse orientation by their 3'ends through the overhang *Eco*RI sites. The map was generated by SnapGene® viewer software. (B) A photograph of a 1% wt/vol agarose gel showing PCR product of the ligated *cptA-cptB* (774 bp) using *cptB*-F/RBS-F primer pair with overhanging *Nco*I and *Sac*I restriction sites. The first lane contained a DNA ladder (Thermo Scientific™ GeneRuler 100 bp Plus) for size comparison.

Figure S7:

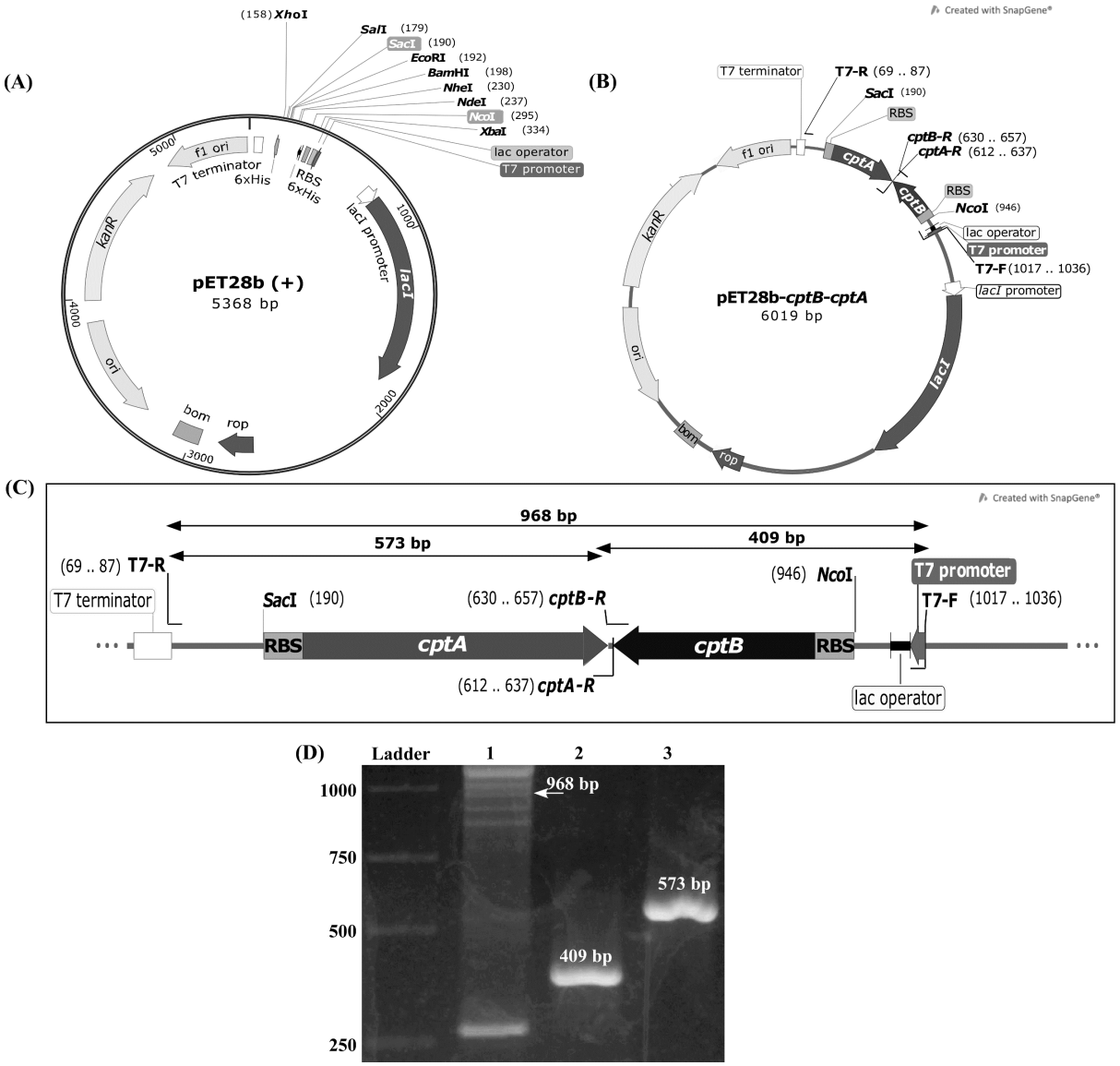
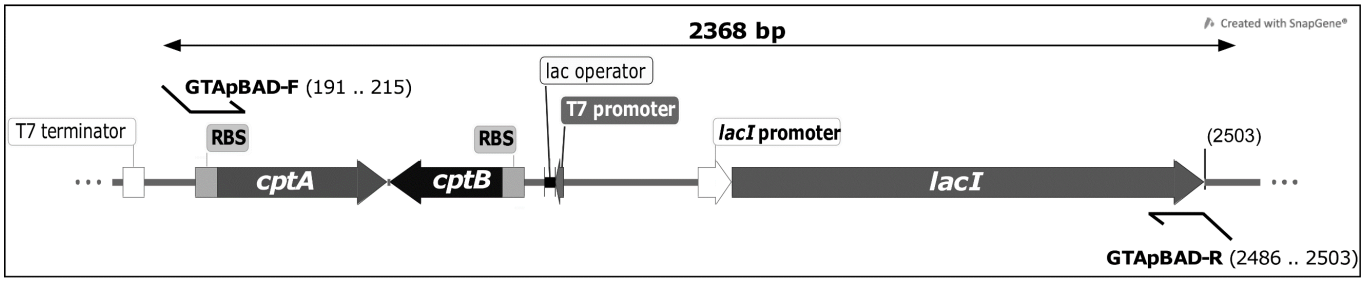


Figure S7: Cloning of *cptB-cptA* in pET28b (+).

(A) Genetic map of the pET28b (+) plasmid, showing the multiple cloning site (MCS). (B) Genetic map of the pET28b plasmid with *cptB-cptA*, inserted through *Sac*I and *Nco*I ends so that antitoxin will be under the control of T7 promoter (P_{T7}). (C) Genetic map of the MCS with *cptA-cptB* in the cloned pET28b. The map shows binding sites of primers used for detection of insertion and correct orientation—with the expected product sizes. This map was generated by SnapGene® viewer software. (D) A photograph of a 1 % wt/vol agarose gel showing the PCR products of 968 bp, 409 bp and 573 bp obtained using the T7-F/R primer pair (lane 2), T7-F/*cptB*-R primer pair (lane 3) and T7-R/ *cptA*-R primer pair (lane 4), respectively. The first lane contained 1 Kb DNA ladder (Solis Biodyne, Estonia) for size comparison.

Figure S8:

(A)



(B)

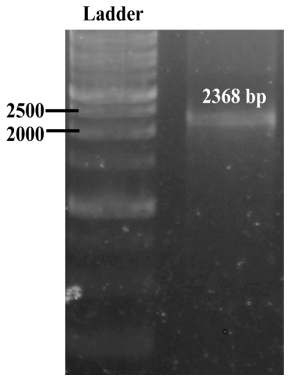
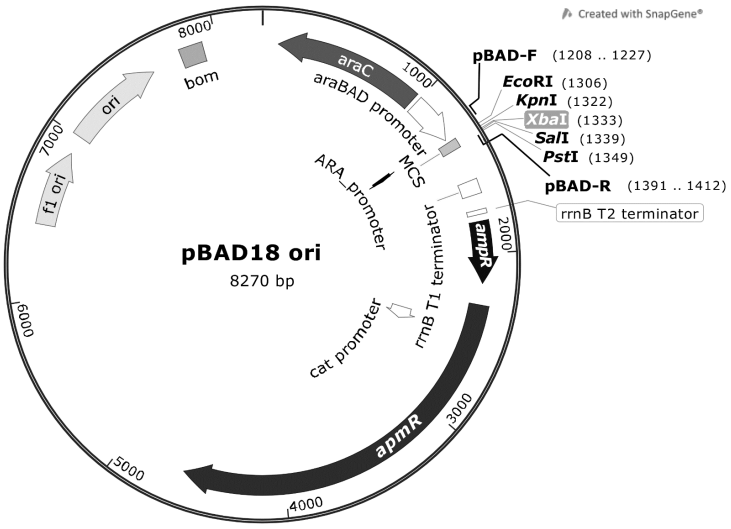


Figure S8: Amplification of *cptA-cptB-* P_{T7} promoter and *lacI* gene using the cloned pET28b-*cptA-cptB* as template.

(A) Genetic map of a part of the cloned pET28b-*cptB-cptA* showing the *cptA-cptB-*P_{T7} and *lacI* gene, and the binding sites for the GTApBAD-F/R primers, used in the amplification of the *cptA-cptB-*P_{T7} and *lacI* gene, for cloning of pBAD18 ori. This map was generated by SnapGene® viewer software. (B) A photograph of a 0.8 % wt/vol agarose gel showing the PCR product (2368 bp) of the *cptA-cptB-*P_{T7} promoter with the *lacI* gene obtained using the GTApBAD-F/R primer pair. The first lane contained 1 Kb DNA ladder (Solis Biodyne, Estonia) for size comparison.

Figure S9:

(A)



(B)

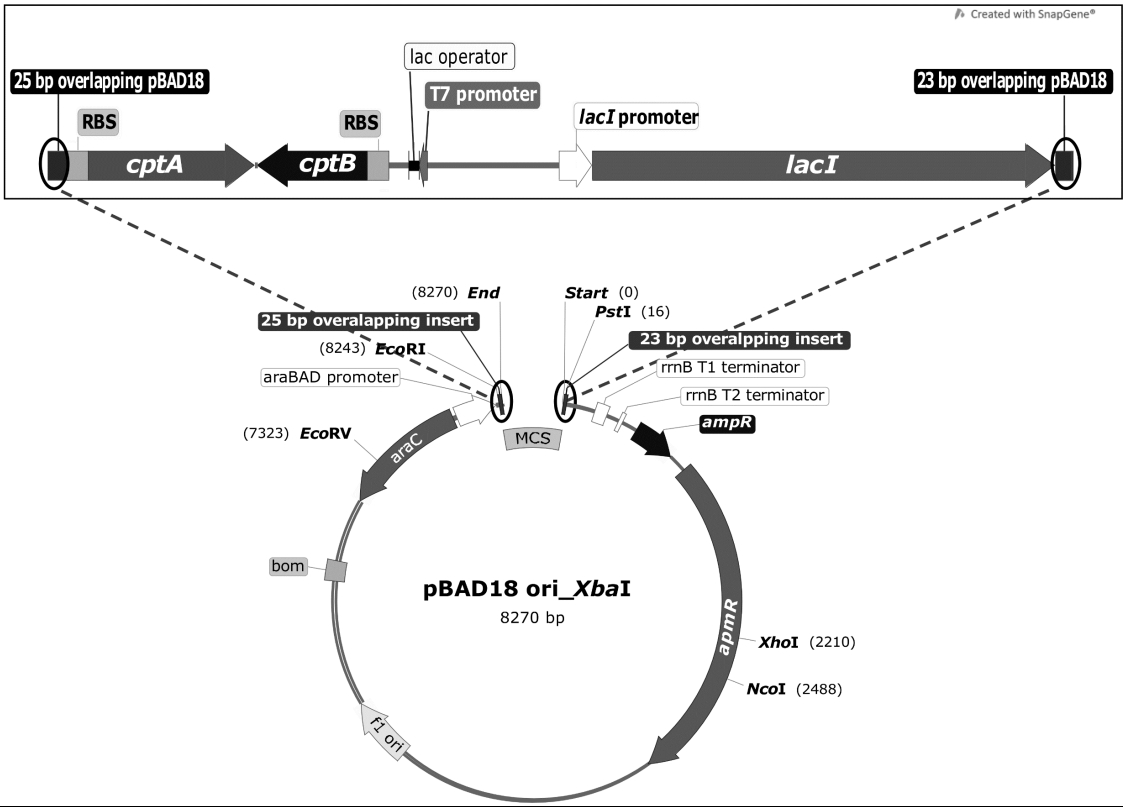


Figure S9: Diagrammatic illustration of insertion of *cptA-cptB-*P_{T7}*-lacI* fragment in pBAD18 ori using Gibson assembly cloning technique.

(A) A Genetic map for the pBAD18 ori showing the MCS. (B) A schematic map for the amplified *cptA-cptB-* P_{T7} promoter-*lacI* gene using GTApBAD-F/R primer pairs with 23-25 bp overlapping regions with the *Xba*I digested pBAD18 ori. The maps were generated by SnapGene® viewer software.

Figure S10:

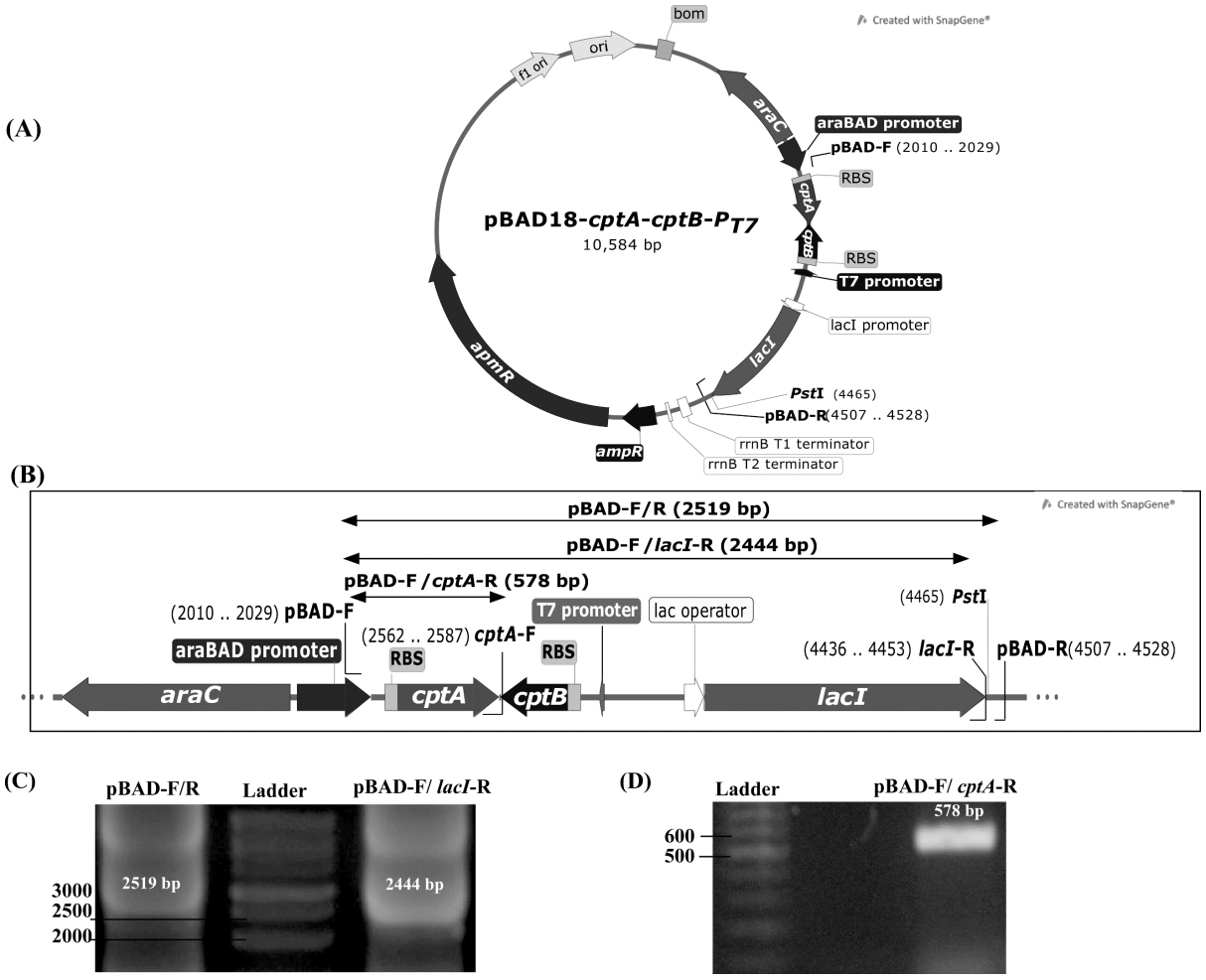


Figure S10: Construction and confirmation of the dual promoter vector, pBAD18-*cptA-cptB-P*$_{T7}$.

(A) Genetic map for dual promoter vector pBAD18-*cptA-cptB-P*$_{T7}$. (B) A detailed schematic map for *cptA-cptB-P*$_{T7}$ region within pBAD18 ori showing the presence of *cptA* under the control of araBAD promoter. The diagram shows the binding sites of primers used for detection of insertion and correct orientation together with the expected product sizes. These maps were generated by SnapGene® viewer software. (C) A photograph of a 0.8 % wt/vol agarose gel showing the PCR products of 2519 bp and 2444 bp obtained using the pBAD-F/R primer pair (lane 1) and pBAD-F/ *lacI*-R primer pair (lane 3), respectively. The first lane contained 1 Kb DNA ladder (Solis Biodyne, Estonia). (D) A photograph of a 1.5 % wt/vol agarose gel showing the PCR products of 578 bp obtained using the pBAD-F/*cptA*-R primer pair (lane 3). The first lane contained DNA ladder (Thermo Scientific™ GeneRuler 100 bp Plus).

Figure S11:

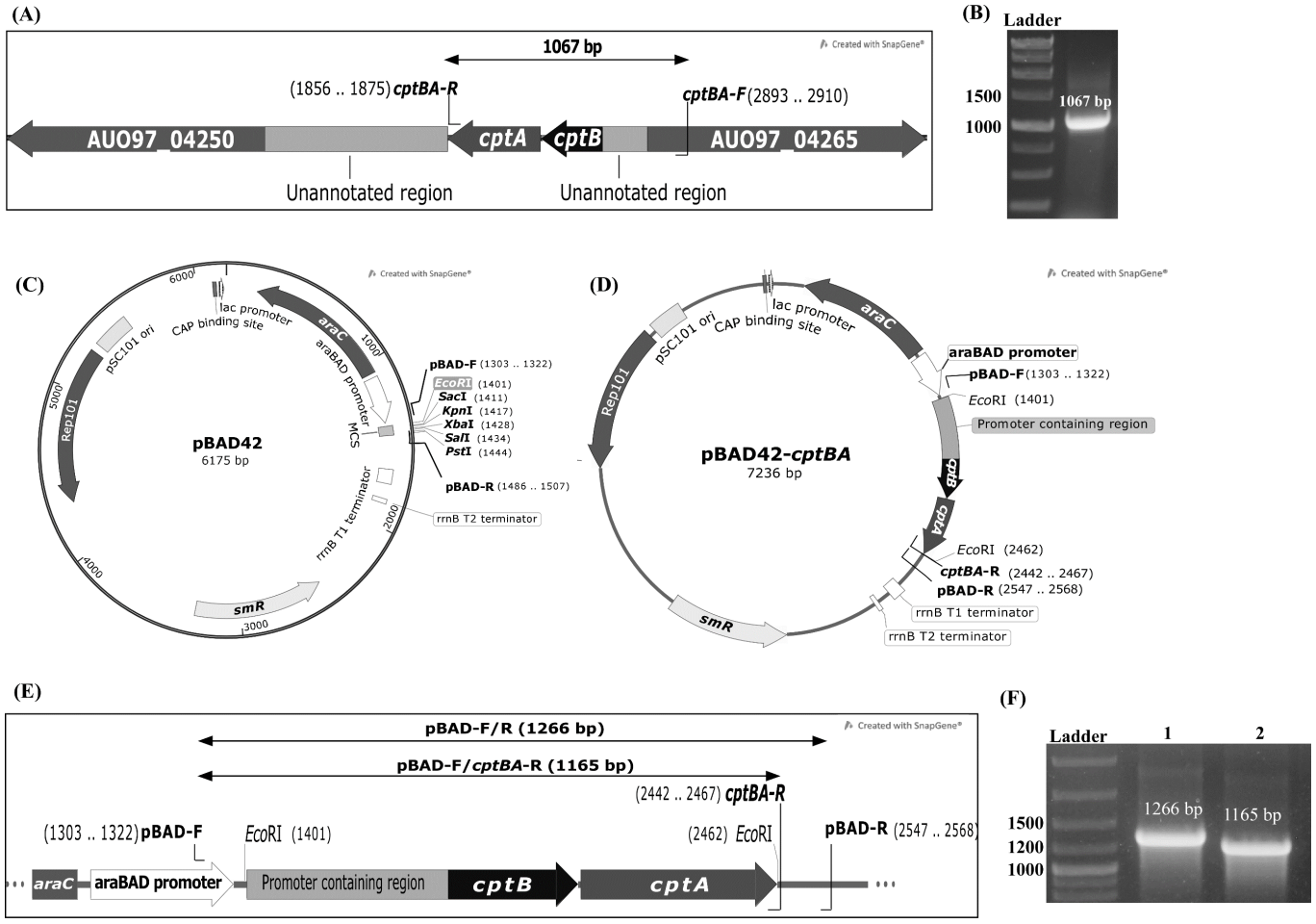


Figure S11:Construction and confirmation of pBAD42-*cptBA*.

(A) Genetic map showing about 3.86 Kb subunits of *A. baumannii* chromosomal DNA including *cptBA* locus together with binding sites of *cptBA*-F/R primers (with overhang *Eco*RI site) used in the amplification of *cptBA* with 401 bp upstream region, that is expected to contain the promoter. (B) A photograph of a 0.8 % wt/vol agarose gel showing the product of the *cptBA* locus with upstream promoter containing region (1067 bp). The first lane contained 1 Kb DNA ladder (Solis Biodyne, Estonia). (C) Genetic map of the pBAD42 showing the MCS. (D) Genetic map of the cloned pBAD42-*cptBA* so that the antitoxin precedes the toxin and the whole system is under the control of araBAD promoter. (E) Detailed genetic map for the MCS with *cptBA* cloned in pBAD42. It shows binding sites of primers used for detection of insertion and correct orientation with the expected product sizes. (F) A photograph of a 1 % wt/vol agarose gel showing the PCR products of 1266 bp and 1165 bp obtained using the pBAD-F/R primer pair (lane 1) and pBAD-F/*cptBA*-R primer pair (lane 2), respectively. The first lane contained DNA ladder (Thermo Scientific™ GeneRuler 100 bp Plus). All maps were generated by SnapGene® viewer software.

Figure S12:

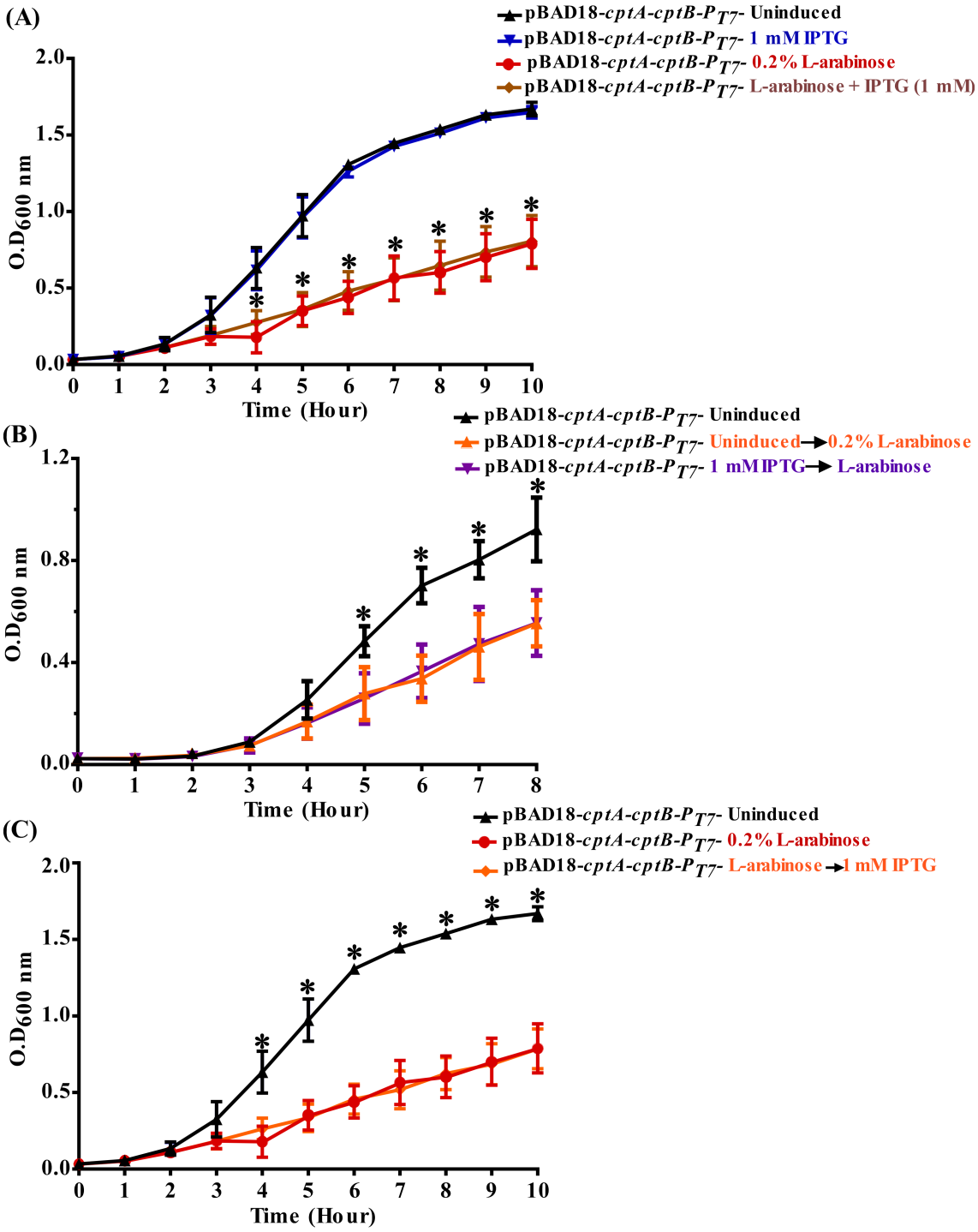


Figure S12: Overproduction of *A. baumannii* CptA toxin *vs.* the CptB antitoxin in *E. coli*.

Growth curves of *E. coli* Bl21(DE3) cells harboring the dual promoter plasmid pBAD18-*cptA-cptB-P*$_{T7}$ *vs.* uninduced cells (control) at the following conditions: (A) induction by either 0.2% L-arabinose (CptA inducer), 1 mM IPTG (CptB inducer), or both; (B) induction of antitoxin expression prior to toxin expression; (C) induction of toxin expression prior to that of the antitoxin. OD_{600nm} was measured every hour up to 10 hours. The data represent the means of three independent experiments. Error bars represent the standard error of the means. Asterisks (*) indicate statistically significant differences

(p<0.05) between induced and uninduced cells. All comparisons were evaluated by ANOVA followed by *post hoc t*-tests with Holm-Sidek correction for multiple testing.

Figure S13:

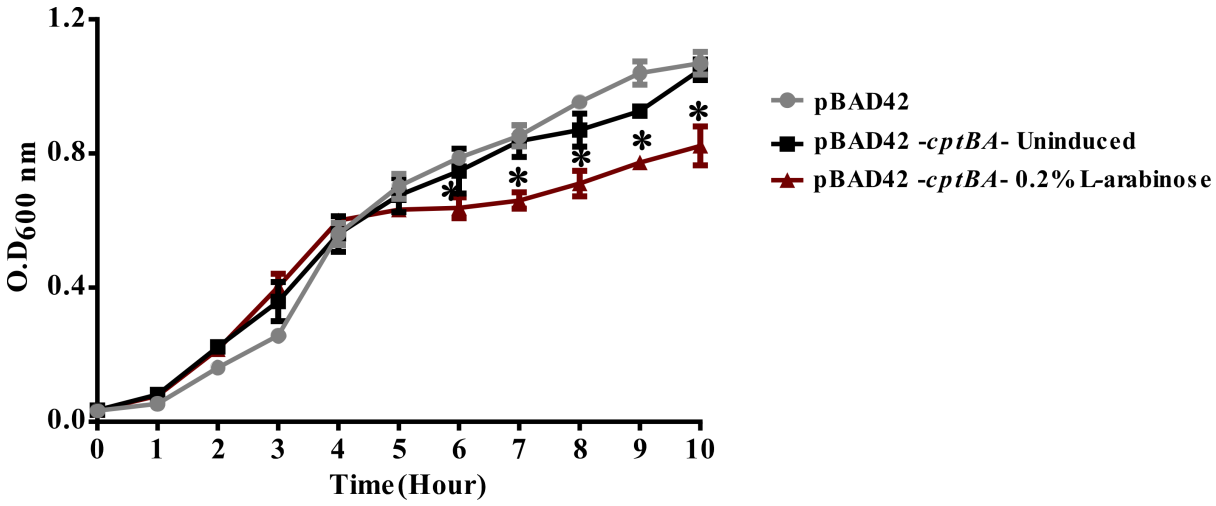


Figure S13: Effect of overproduction of *A. baumannii* CptBA TA pair on the growth of *E. coli*.

Growth curve of *E. coli* Bl21(DE3) cells harboring the single promoter plasmid pBAD42-*cptBA* induced by 0.2% L-arabinose (inducing *cptBA*) *vs.* uninduced cells, and those carrying pBAD42 (control vector). Samples were drawn every hour for 10 hours. The data represent the means of three independent experiments. The error bars represent the standard error of the means. Asterisks (*) indicate statistically significant differences against uninduced cells (at $p<0.05$), as determined by ANOVA followed by *post hoc t* test.

Figure S14:

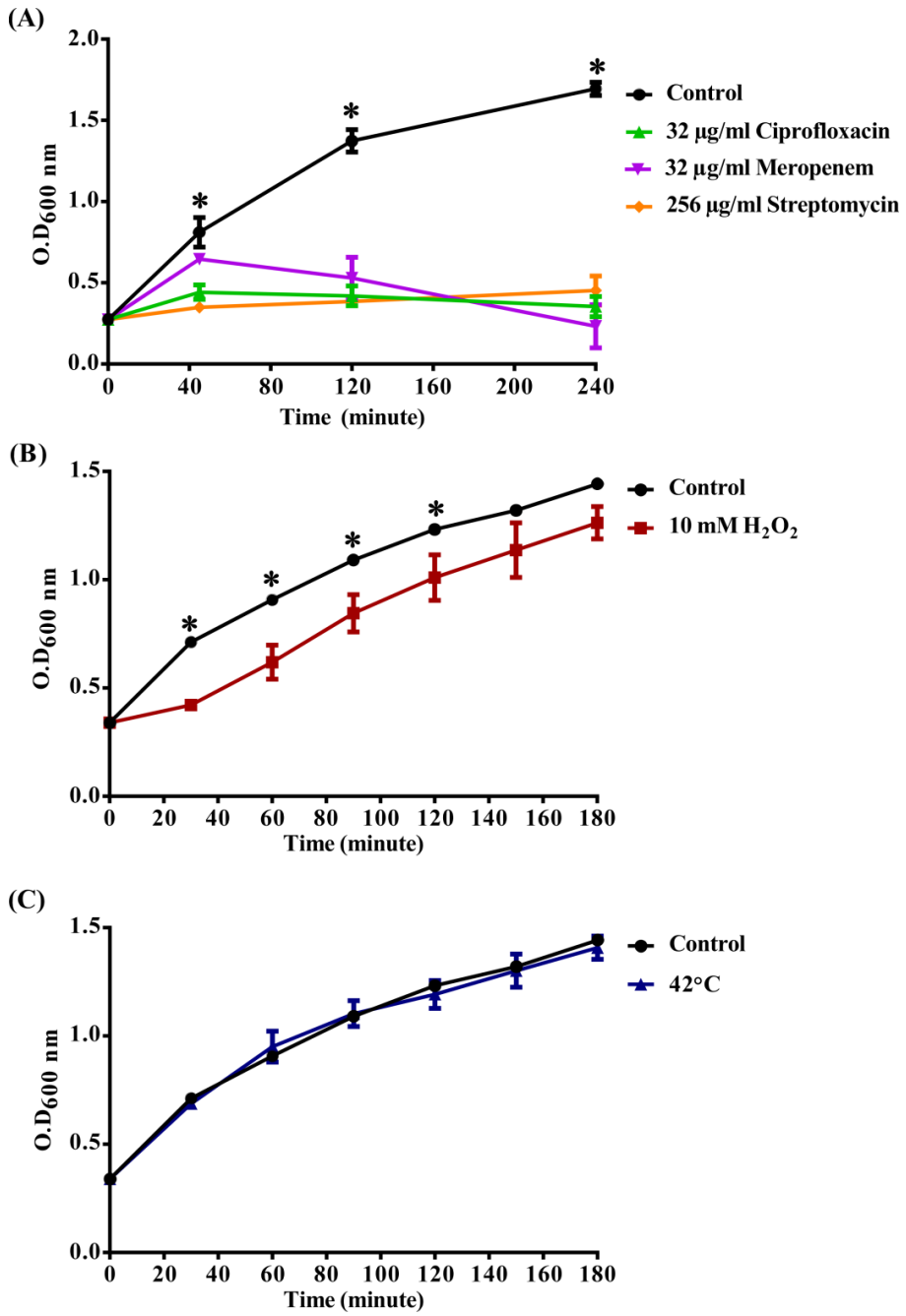


Figure S14: Effect of exposing *A. baumannii* to different stress factors.

Growth curves of *A. baumannii* ATCC 17879 upon exposure to (A) different antibiotics, (B) oxidative stress and (C) high temperature (42 ºC *vs.* 37 ºC). Samples were withdrawn at different time intervals. (A) Significant decrease in the growth rate was observed in the presence of all studied antibiotics that cannot be reversed for 4 hours with except to meropenem that affects the growth significantly after 2 hours. (B) While the growth rate was significantly decreased under the effect of 10 mM H_2O_2 *vs.* unexposed cells (control) that was specifically clear until 30 min of exposure to H_2O_2, then the growth restarts to increase slowly but remained lower than the unexposed cells. The data represent the means of three independent experiments. The error bars represent the standard error of the means. Asterisks (*) indicate statistically significant differences (at $p<0.05$), as determined by ANOVA followed by *post hoc* multiple *t* test.

Figure S15:

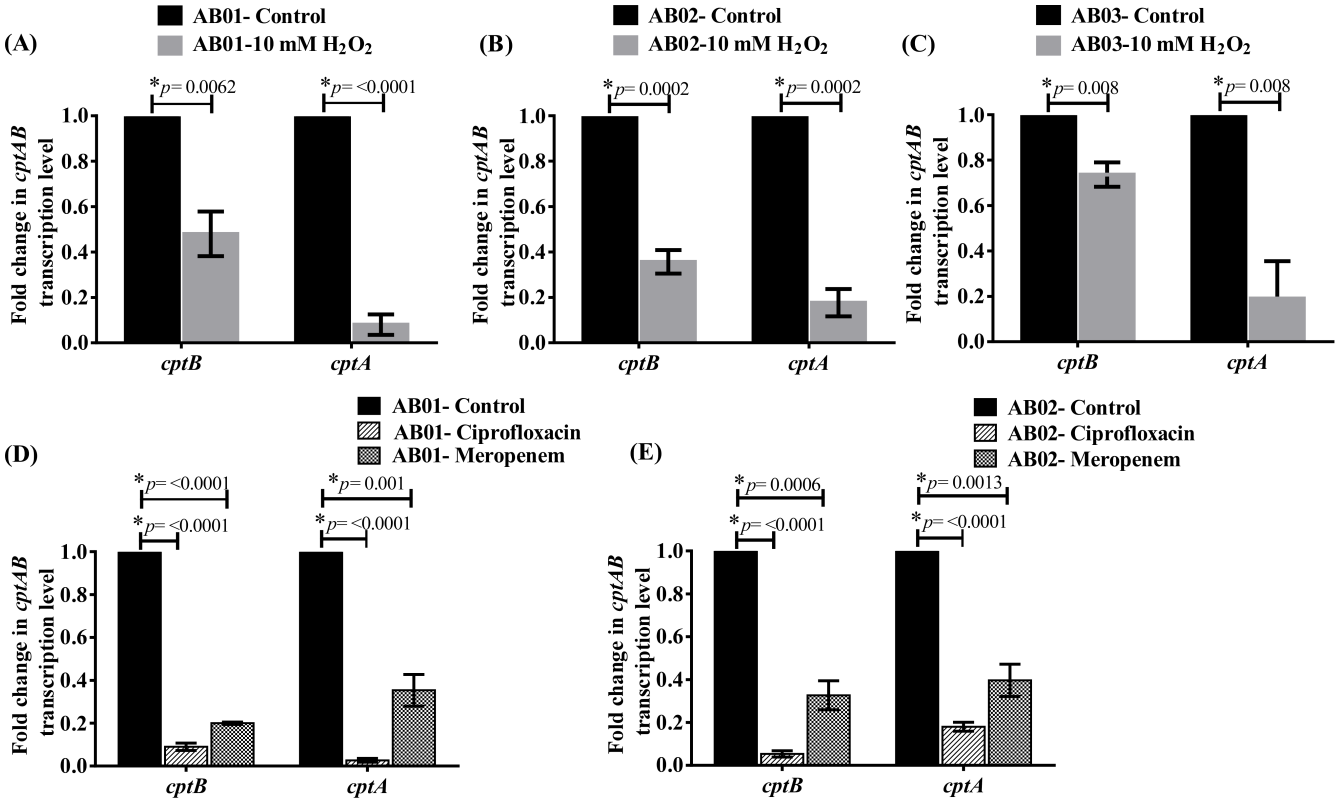


Figure S15: Expression of CptBA system under different stressful conditions in *A. baumannii* clinical isolates AB01, AB02 and AB03.

Fold change in the transcription levels of *cptA* and *cptB* genes upon exposure of three isolates to (A-C) oxidative stress in addition to exposure of AB01 and AB02 to (D and E) different antibiotics. Significant downregulation was observed in *cptBA* under the effect of 10 mM H_2O_2 in the three isolates, and in the presence of ciprofloxacin (32 µg/ml) and meropenem (32 µg/ml) for AB01 and AB02. Fold change was calculated using the ΔΔCt method. The data represent the means of three independent experiments. The error bars represent the standard error of the means. Asterisks (*) indicate statistically significant differences (at $p<0.05$), as determined by the unpaired student *t* test.

294

295

296 *The graphical abstract was created with BioRender.com and SnapGene® viewer software.