Chapter-4

Characterizing Type Six Secretion System (T6SS) of E. cloacae SBP-8 and its role in pathogenesis and inter-bacterial competition.

4.1 Introduction

In a natural environment, bacteria interact positively or negatively with other organisms at intra-as well as inter-kingdom level for survival and proliferation. They are equipped with various arsenals to outcompete the microbial competitors or infect higher organisms. During infection and competition many bacteria efficiently translocate small molecules, DNA, and proteins from its cytosol into the target cell through dedicated macromolecular complexes [58]. These multi-protein complexes are termed secretion systems. Till date, nine different types of secretion systems (T1SS to T9SS) have been identified [58], requiring one to over twenty different proteins for their functionality. One of the recently identified systems, the type VI secretion system (T6SS) was first discovered in *V. cholerae*, exhibiting a contact-dependent interaction with the host cells [70]. This proteinaceous nano-machinery delivers effector molecules directly into the opponent cell in a one-step process [66]. Initially, T6SS of a few proteobacteria such as *V. cholerae* [217], *Pseudomonas aeruginosa* [74], and *Burkholderia spp.* [85] were reported to target eukaryotic cells injecting into them the effector proteins and toxins such as DNase, phospholipase, and NAD^+ hydrolase [75]. These effector molecules interfere with the cytoskeleton playing a role in pathogenesis [218]. Later in 2010, a study in *P. aeruginosa* identified T6SS as an "anti-bacterial" against other species, the concept being echoed in other bacteria like *V. cholerae* [140] and *Serratia marcescens* [141].

Typically, T6SS is comprised of at least 13 core conserved proteins (TssA-M) that are required for forming the basic and functional structure [70]. Hcp (TssD) is the most notable of these proteins, due to its abundance in bacterial culture supernatant [219]. *In-silico* and structural analysis provide strong evidence about significant homology between the structural components of T6SS and the T4 bacteriophage tail [70]. T6SS is a complex made up of large multiple proteins including cytoplasmic proteins (like ATPase ClpV), inner membrane proteins (like IcmF) and effectors molecules like Hcp/VgrG. Apart from above-mentioned proteins, genes like *tssJ, tssK, tssL,* and *tssM* are also involved in assembling the membrane-embedded complex [70]. This complex provides the base for the recruitment of phage tail-like structure to the cell envelope. It is considered to channelize the substrate (Hcp/VgrG) secretion upon sheath contraction while Tssk, Tsse, Tssf, and TssG forms a cytoplasmic complex that is anchored to the inner membrane providing a platform for sheath and tube assembly [70]. Two proteins, namely TssB and TssC assemble forming a tail sheath in T6SS. Electron microscopy of tail sheath reveals that TssB and TssC form tubular structures that share remarkable

similarities with bacteriophage sheath. The phage tail-like component constitutes a baseplate complex and a tube of Hcp hexamers is enclosed by a contractile sheath assembled from TssB/TssC heterodimers. Structural data of these two proteins indicate that they may be involved in forming cell-puncture device [70]. Two homologous proteins Hcp and VgrG are characteristically secreted to the extracellular medium. Since these two proteins are also necessary for the system to work properly, it is suggested that once secreted they form extracellular part of the secretion system. Based on structural similarities between T6SS and bacteriophage injectosome, current model for the mechanism of action of T6SS could be divided into three main steps, as shown in Fig.1.5 of chapter 1: (i) assembly of complete machinery: a phage tail like structure, including the base plate, the tube and the sheath which assemble on membrane complex (ii) translocation of effector molecule: contraction in basal plate induce the inner tube which is composed of Hcp tripped by VgrG protein towards the opponent cell to deliver effectors molecules (iii) disassembly of complete apparatus: ClpV is recruited to base plate having ATPase activity which leads to disassembly of T6SS apparatus. ClpV is also required for T6SS assembly facilitating TssB-TssC polymerization and it provides energy to contract the tail sheath. Detailed structure and function of T6SS is discussed in chapter 1.

T6SS-dependent effects operate *via* a syringe-like structure allowing the attacking bacteria to inject an array of effectors targeting various cellular processes of the target cell. Along with effector molecules, attacking species possess cognate immunity proteins providing immunity against their own toxins [78]. Overall, T6SS plays a significant role in reshaping bacterial community and their pathogenesis, directly or indirectly. Thus, understanding T6SS of various pathogens could be instrumental in targeted control of the pathogens. Previous studies have demonstrated that knocking out the genes of T6SS attenuated virulence and colonization of *V. chlorae* [59], *S. enterica* subspecies *enterica* serovar Typhimurium [82] and *Camphylobacter jejuni* [220]. It has been predicted that around 25% of the sequenced Gram-negative bacteria have this nanomachine, making it the most widespread secretion system [144]. However, T6SS have been characterized in a few species including *V. cholerae* [59], *P. fluorescens* [145], *S. marcescens* [141] and *P. aeruginosa* [78]. Yet the role of T6SS in pathogenesis and competition is poorly characterized in many opportunistic pathogens like *E. cloacae* which is responsible for many hospital-acquired infections [1].

E. cloacae is a Gram-negative opportunistic pathogen that has emerged as an important cause of severe nosocomial infections. Reportedly, *E. cloacae* infections are of endogenous origin and difficult to treat due to antibiotic resistance among several strains [221]. Its ability to form biofilm, and secrete siderophore as well as toxins like enterotoxins, pore-forming toxins and hemolysins, contribute to its invasion, colonization, and destruction of host immune cells [34]. Liu *et al.*, identified two T6SS loci, namely T6SS-1 and 2 along with genes encoding toxin production and cell-wall degrading enzymes in the genome of *E. cloacae* ATCC 13047, a clinical isolate from human cerebrospinal fluid [21]. This suggests they could compete with other bacteria either in the environment or host (facilitating host colonization). In 2020, Jorge *et al.* characterized T6SS clusters of *E. cloacae* ATCC 13047 and demonstrated their role in intestinal colonization, host cell adhesion, biofilm formation, and anti-bacterial activity [222]. In previous chapters, we demonstrated the pathogenic potential of a rhizospheric soil isolate *E. cloacae* SBP-8 using *C. elegans* [197] and a murine model. Whole-genome analysis of *E. cloacae* SBP-8 indicated presence of T6SS [147]. Therefore, we attempted to test the role of T6SS in the pathogenesis and inter-bacterial competition of an exogenous strain having a different genetic make-up. In this chapter, we describe and compare T6SS of *E. cloacae* SBP-8 while characterizing its role in pathogenesis as well as anti-bacterial inhibitions.

4.2 Materials and Methods

4.2.1 Bacterial strains and culture conditions

All bacterial strains and plasmids used in the study are detailed in table 1.

Table 4.1. List of bacterial strains and plasmids used in this study

Bacterial strains	*E. cloacae* SBP-8
	E. cloacae MTCC 509
	E. coli DH5α
	E. coli OP50
Plasmids	pACBSR-Hyg
	pKD-3
	pSW002-Pc-DsRed-Express2

All bacterial strains were routinely cultured in Luria broth (LB), Himedia, India at 37°C except *E. cloacae-pACBSR-Hyg*, which was grown at 30°C. *E. cloacae-pACBSR-Hyg* was obtained by electroporation of temperature-sensitive plasmid *pACBSR* having λ-Red recombination

system (a kind gift by Dr. Tzu-Wen Huang, Taipei Medical University, Taiwan) in *E. cloacae* SBP-8. *E. coli* OP50 was used as a food source for maintaining wildtype N2 strain of *C. elegans* (a kind gift from Vidya Devi Negi, National Institute of Technology, Rourkela) on nematode growth medium (NGM) at 25°C. For growth curve analysis, the medium was supplemented with ampicillin (100 µg/ml), chloramphenicol (25 µg/ml) or kanamycin (30 µg/ml, tetracycline (15 µg/ml) as per the resistance of the strains.

4.2.2 *In-silico* characterization of T6SS of *E. cloacae* SBP-8 genome

The complete genome sequence of *E. cloacae* SBP-8 [Accession No. CP016906] was screened for genes showing sequence similarity to genes associated with T6SS. T6SS-encoding gene clusters were identified by T6SS-Hammer of Secret6 online tool [223] with E- value <0.0001. T6SS genes were identified as a locus containing at least 9 T6SS component genes in a contiguous set with no more than 8 unrelated genes between each known T6SS gene and a maximum of 15000 bp between two T6SS gene cluster.

4.2.3 Extraction and analysis of secretome for functional characterization of T6SS

E. cloacae SBP-8 was grown to stationary phase (16 h) in LB at 30°C to identify the Hcp and other secretion system proteins in secretome as describe by Murdoch *et al.* [141]. Culture supernatant was collected at log and stationary phase and filtered with 0.22 µm syringe-driven filter unit. To precipitate secreted proteins, equal volume of chloroform-methanol mix (1:1) was added to the supernatant and allowed to incubate for 1 h at 4°C. The supernatant was then centrifuged to collect the protein pellet which was further washed with 1 ml of methanol and air-dried. Finally, the protein pellet was resuspended in 100 µl of sample lysis buffer (Tris-HCl, SDS, EDTA, glycerol, bromophenol blue, and 2.5 % ß-mercaptoethanol) and boiled for 10 min at 95 °C. The proteins were separated on SDS-PAGE (15%) and stained with Coomassie brilliant blue G250. The proteins were quantified by the Bradford method using Thermo scientific Multiscan reader (India).

4.2.4 Mass Spectrometric analysis of the secretome

The pellet of secreted proteins was reduced with 5mM TCEP (Tris (2-carboxyethyl) phosphine), alkylated with 50 mM of iodoacetamide, and digested with trypsin (Trypsin/lysate ratio,1:50) for 16 h at 37°C. A C18 silica cartriage was used to remove the salt and protein purification. Further purified protein pellet was dried using speed vac [224]. The dried pellet was resuspended in buffer (55% acetonitrile, 0.1% formic acid) and were subjected

to MALDI--TOF- MS/MS for analysis and identification of proteins at V-proteomics, New Delhi. The mass spectrometric analysis of peptide mixture was performed using EASY-nLC 1000 system (Thermo Fisher Scientific, India) coupled to Q Exactive (Thermo Fisher Scientific, India) equipped with a nano-electrospray ion source. 1.0 µg of the peptide mixture was resolved using 25 cm PicoFrit column (360 µm outer diameter, 75 µm inner diameter, 10 µm tip) filled with 1.8 µm of C18-resin (Dr. Maisch, Germany). Samples were processed, and generated RAW files were analyzed with Proteome Discoverer (v2.2) against the uniprot *E. cloacae* reference proteome database.

4.2.5 Construction of *clpV* knockout in *E. cloacae* SBP-8

We generated an in-frame deletion of *clpV*, encoding an ATPase of T6SS machinery in *E. cloacae* SBP-8 genome, as described by Datsenko and Warnner [225] with minor modifications. This method involves replacing the desired gene with an antibiotic (chloramphenicol) resistance cassette using the bacteriophage lambda red recombinase system. *E. cloacae* SBP-8 carrying the recombinase system in *pACBSR* plasmid was grown in LB containing 10 mM arabinose at 30°C till the OD_{600nm} of 0.3-0.4. Electrocompetent cells were then prepared. Chloramphenicol cassette was amplified from pkD3 plasmid with the primer set mention in table 2. The amplicon contained chloramphenicol cassette flanked with sequences upstream (38 bp) and downstream (21 bp) of *clpV*. Purified CmR cassette (200 ng) was electro transformed into *E. cloacae* SBP-8, having pACBSR, using a 2mm diameter bio rad cuvette using gene pulsar II (Bio-Rad) at 2.5 kV, 25 W for 5 ms. Transformants of *E. cloacae* SBP-8 with *clpV* deletion were screened using PCR from the LB plate supplemented with chloramphenicol and ampicillin at 37°C. The confirmatory primers, binding to a region flanking yet upstream of the knockout primers and within the chloramphenicol cassette, used for confirming the *E. cloacae* SBP-8 *clpV* knockout are mentioned in the table 2.

Table 4.2. List of primer used in this study

S.No	Gene name	Sequence
1.	cmR (chloramphenicol cassette)	FP5′GATACGCTGTTATTTACCTCTCTGGAAAGCGC GTGTAGGCTGGAGCTGCTTCG RP5'CAGTTCTCTTGATGATTATTACGCGGCCATAT GAATATCCTCCTTA 3'
2.	KO Confirmatory 1	FP5′GATACGCTGTTATTTACCTCTCTGGAAAGCGC GTGTAGGCTGGAGCTGCTTCG 3' RP5'CCTCCTTAGTTCCTATTCCG 3'
3.	KO Confirmatory 2	FP5′CGTGGCGATCTGGTGTATCG 3' RP5'CCTCCTTAGTTCCTATTCCG 3'
4.	*clp V*	FP5′AGCTTTCGCATCGCTACATT3' FP5′CGCTCCGCTATCTCAATTTC3'

4.2.6 *ERIC*-PCR fingerprinting

Enterobacterial Repetitive Intergenic Consensus (*ERIC*) PCR-based DNA fingerprinting was performed as described in previous chapters. *ERIC*-PCR was performed using bacterial DNA obtained by quick boiling lysis of the colonies in nuclease-free water, the universal primers - ERIC1R (5′ -ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC2 (5′ - AAGTAAGTGACTGGGGTGAGCG3′), and PCR Mix (Himedia, India). The banding pattern of the PCR products was captured with gel documentation system (Bio-Rad, USA).

4.2.7 Interbacterial competition assay

Bacterial cultures (*E. cloacae* SBP-8, *E. coli* DH5α, and *E. cloacae* Δ*clpV*) were each grown overnight and sub-cultured (1:100 dilution) in the LB media. The *E. cloacae* SBP-8 and *E. coli* DH5α cultures were grown up to 0.3 OD_{600nm} and then mixed in a 10:1 ratio. A 5 µl of above culture mix was spotted on 0.22 µm nitrocellulose membrane laid on LB agar supplemented with tetracycline as a selective marker for *E. coli* DH5α and incubated at 37°C for 5 h. The filter membrane on the agar surface was subsequently removed, vigorously vortexed in sterile PBS to resuspend the bacteria in PBS. Colony-forming unit (CFU) per ml of both the bacterial species/strains was estimated by plating 10-fold dilution on the LB-agar plate with appropriate antibiotics. *E. coli* DH5α having pSW002-Pc-DsRed-Express2 plasmid was selected on LB plate with tetracycline (15 µg/ml), *E. cloacae* SBP-8 having *bla* gene in its genome were selected on ampicillin (100 µg/ml) while *clpV* knockout *E. cloacae* SBP-8 having chloramphenicol cassette selected on LB plate with chloramphenicol (30 µg/ml). In order to verify the contact-dependent killing by *E. cloacae* SBP-8, a similar assay was performed by

interposing 0.22 μm filter between *E. cloacae* SBP-8 (or *E. cloacae* SBP-8 Δ*clpV*) and *E. coli* DH5α. After incubation, the membrane or membrane with media cut by cork borer was resuspended in sterile PBS and the above protocol was repeated for CFU count.

4.2.8 Bacterial internalization and proliferation assay

In order to determine bacterial internalization and proliferation by RAW 264.7 and HT-29, a gentamicin protection assay was performed as described previously [199]. Macrophage cell line derived from BALB/c (colorectal adenocarcinoma cell line with epithelial morphology (HT-29) was a generous gift from Prof. Rajdeep Chowdhury, Dept. of Biological Sciences, BITS Pilani, India. Both the cell lines were grown in DMEM (Dulbecco's Modified Eagle's medium) media supplemented with 10% FBS (Fetal Bovine serum). Around 1.5 X 10^5 cells were seeded in a 24-well plate and incubated at 37°C in 5% CO_2 for 24 h in DMEM. Cells were infected with wildtype and *clpV* knockout *E. cloacae* SBP-8 at a multiplicity of infection (MOI) of 50 at 37°C for 1 h and then washed three times with PBS and treated with 100 μg/ml gentamicin for 1 h to kill extracellular bacteria. The infected cells were washed three times with PBS and then incubated with 25 μg/ml of gentamicin. After 2 and 16 h post-infection (pi), the cells were washed and lysed with 0.5 ml of 0.1% Triton-X in PBS, and then serial dilutions of lysates were plated on LB-agar with required antibiotics and incubated overnight at 37°C to determine the number of viable bacteria. Subsequently, the internalization (at 2h) and fold proliferation (2 to 16h) was calculated.

4.2.9 *C. elegans* solid killing and bacterial colonization assay

The *C. elegans* solid killing assay and intestinal colonization assay were performed as described in chapter 2. Synchronized L4 worms were allowed to be infected with both *E. cloacae* SBP-8 wildtype and *clpV* knockout strain for 18 h, and a total of 20 worms from these plates were transferred to a fresh NGM plate having *E. coli* OP 50. Live and dead worms were scored every day followed by survival analysis of the infected worms.

To evaluate the intestinal colonization of *E. cloacae* SBP-8 wildtype and *clpV* knockout strain in *C. elegans*, worms were allowed to feed on wildtype and *clpV* knockout strains of *E. cloacae* SBP-8 for 6, 12, 24, and 48 h. After each time point, worms were transferred to NGM plate having *E. coli* OP50 and maintained for 24 h. The worms were then incubated with 100 μg/ml of gentamicin for 1 hr and washed 3 times with M9 buffer, followed by treatment with 25 μg/ml of gentamicin for 30 mins. Nematodes were further washed with M9 to remove

residual gentamicin and finally lysed with 0.1 % Triton X-100 using a Qiagen bead tissue lyzer LT (Qiagen, India) and plated on LB-agar plate with the required antibiotic.

4.2.10. Expression analysis of antimicrobial genes of *C. elegans*

Nematodes were treated as described for the intestinal colonization assay. A total of 50 worms were exposed to wildtype and *clpV* knockout *E. cloacae* SBP-8 strains for different periods (6, 12, 24, and 48 h). After infection, worms were picked and washed multiple times with M9 buffer to exclude the bacteria attached to the worm. TRIzol (Sigma, USA) was used for total RNA extraction, which was further quantified by using µCuvette G1.0 spectrophotometer

(Eppendorf, Germany). Around 200 ng of RNA was used for cDNA synthesis using Thermo scientific verso cDNA synthesis kit. Details of primers used in quantitative real-time PCR (qPCR) are listed in table 1 of chapter 2. A $2^{-\Delta\Delta Ct}$ method was used to calculate relative change

β-actin

4.2.11 Statistical analysis

Bacterial competition and bacterial colonization assay were performed at least three times with three biological replicates, while intracellular assay in cell lines was performed twice with three biological replicates. Data were expressed as mean ± standard deviation and analyzed using Graph Pad Prism 8. The Kaplan-Mayer log test was used to compare survival curves. Significant difference between means of the parameter was calculated using a nonparametric t-test.

4.3 Results

4.3.1 Identification of T6SS in *E. cloacae* SBP-8 and testing its functionality

Whole-genome sequence mining of *E. cloacae* SBP-8 revealed the presence of a single 32 kb long type VI secretion cluster. As shown in Fig.4.1 , the cluster contains 13 core, essential and conserved T6SS genes namely, *tssA* to *tssM*. Some of the T6SS structural genes are present as multiple copies including 4 copies of *tssH* and 2 of *tssD* but, these are located elsewhere in the genome. Details of T6SS structural genes of *E. cloacae* SBP-8 extracted from uniprot are listed in table 4.3.

The presence of TssD (Hcp) in bacterial culture supernatant is a hallmark of functional and active T6SS [141, 139]. To test if T6SS was functional in *E. cloacae* SBP-8, we assessed the secreted proteins (secretome) in the supernatants recovered from the bacterial cultures grown up to log and stationary phase for the presence of putative Hcp protein band. A protein band at

~17 kDa, probably corresponding to Hcp, was detected only in the secretome of stationary phase culture. This indicates the secretion of Hcp under nutrient-deprived conditions but not in nutrient rich. Mass spectrometry of the whole secretome of a stationary phase *E. cloacae* SBP8 showed the presence of TssD (Hcp) and TssI (VgrG) proteins of size 16.9 kDa and 71 kDa, respectively. Thus, the SDS-PAGE analysis followed by mass spectrometry result confirmed the presence of Hcp, which suggests that *E. cloacae* SBP-8 have functional and active T6SS.

To further confirm the functionality of T6SS in *E. cloacae* SBP-8, an in-frame null mutant of *clpV* (*clpV* knockout strain), encoding an ATPase, was constructed by replacing it with

chloramphenicol cassette using a λ-Red recombination system [Fig.4.2 A]. The production of knockout of *clpV* was confirmed by PCR based approach using three primer sets. Two sets amplifying the regions spanning sequence internal to the chloramphenicol cassette and just upstream the gene (set1)/homologous region used for knockout (set 2).

Fig 4.1. *In-silico* identification of T6SS in *E. cloacae* SBP-8. Genetic organization of T6SS gene cluster in *E. cloacae* SBP-8 genome. Genes in the various loci are color-coded and represented as blocked arrows showing the direction of transcription. Genes other than the components of T6SS are shown in grey

Table 4.3. List of T6SS orthologs of *E. cloacae* SBP-8 and their proposed function.

S. no	Unified T6SS nomenclature.	Position of T6SS genes in the genome, [strand], and gene size(bp)	Uniport protein ID	% Identity	Function
1.	TSSA	2826185…2827201/ [- strand]/ 1017 bp	Q9I750	48.06	Forms a base plate complex
2.	TSSB	2825585…2826109/ [-strand]/ 525 bp	Q9I749	73.17	Forms a tubular structure, homologous to T4 phage contractile tail sheath proteins
3.	TSSC	2824082…2825581/ [-strand]/ 1500 bp	Q9174 8	74.37	Forms a tubular structure, homologous to T4 phage contractile tail sheath proteins
4.	TSSD	282326…2823746/ [-strand]/ 483bp	Q9I747	46.30	Effector/structure; Homologous to T4 phage tube
5.	TSS E	2817332…2817895 / [-strand]/ 564bp	Q9I745	44.81	Base plate protein
6.	TSSF	2815458…2817335/ [-strand]/ 1878bp	Q9I744	44.27	Forms a base plate complex
7.	TSSG	2814418…2815461/ [-strand]/ 1044 bp	Q9I743	43.13	Forms a base plate complex
8.	TSS H	2811764…2814378/ [-strand]/ 2616bp	Q9I742	60.91	ATPase/effector chaperon
9.	TSSI	2802288…2808449 / [-strand]/ 6162 bp	Q9I741	40.65	Membrane penetrating effector protein
10.	TSSJ	2834194…2834697/ [-strand]/ 504bp	Q9I752	34.78	Outer membrane lipoprotein involved in stabilizing T6SS
11.	TSSK	2832825…2834168/ [- strand]/ 1334 bp	Q9I753	43.85	Forms a base plate complex
12.	TSSL	2831569…2833807 /[- strand]/ 1239 bp	Q9I757	42.79	Inner membrane lipoprotein involved in stabilizing T6SS
13.	TSSM	2827946…2831566/ [- strand]/ 3621bp	Q9I755	44.12	Essential base plate protein.

The size of the corresponding bands are 900 bp and 1 kb respectively [Fig.4.2 A]. *E. cloacae* SBP-8 and *E. cloacae clpV* knockout show similar growth kinetics suggesting *clpV* is dispensable for the growth [Fig.4.2 C]. The absence of band corresponding to Hcp in the secreted protein profile of *E. cloacae* SBP-*8 ΔclpV* [Fig.4.2 B] indicate T6SS is functionally inactive. Hence, the *E. cloacae* SBP-8 T6SS appears to be functional and active under laboratory conditions while losing its functionality on knocking out *clpV*.

4.3.2 T6SS does not have any role in the pathogenesis of *E. cloacae* SBP-8

To determine the role of T6SS in the pathogenicity of *E. cloacae* SBP-8, we compared the virulence of the *E. cloacae* SBP-8 *ΔclpV* with that of the wildtype *E. cloacae* SBP-8 using *C. elegans* as a model host. Worms infected with *the E. cloacae* SBP-8 *ΔclpV* showed around more 5% reduction in survival rate as compared to those infected with *the E. cloacae* SBP-8, though the result was not statistically significant [Fig.4.3 A, 50% and 45% mortality on infection with *E. cloacae* SBP-8 and *E. cloacae* SBP-8 *ΔclpV*, respectively). Similarly, the deletion of *clpV* did affect, though statistically insignificant, the intestinal colonization of *C. elegans* as well [Fig.4.3 B]. *ERIC*-PCR confirmed that the bacteria recovered from the infected nematodes are *E. cloacae* SBP-8/*E. cloacae* SBP-8 *ΔclpV* thereby proving Koch's postulates regarding infection with microorganisms [Fig.4.3 C].

To assess the induction of innate immune response in *C. elegans* infected with wildtype and *clpV* knockout *E. cloacae* SBP-8, temporal expression profile of some antimicrobial genes such as *clec-60*, *clec-85*, *clec-87*, *lys-1*, and *tol-1* was examined. Both, the wildtype and *clpV* knockout *E. cloacae* SBP-8 strain infected worms showed a similar expression pattern for the above genes. Temporal expression profile of *clec-60*, *clec-85* and *lys-1* showed an increasing trend up to 12 h followed by a decrease at 24 and 48 h in both *clpV* knockout and wildtype *E. cloacae* infected worms [Fig.4.4 A, B, C]. At 12 h, these genes displayed a lower expression in *E. cloacae* SBP-8 *ΔclpV* than the wildtype, but again it was not statistically significant.

In the infected worms, the expression of *clec-87* increased by 6 h while gradually decreasing up to 48 h [Fig.4.4 C]. Worms infected with *E. cloacae* SBP-8 *ΔclpV* showed a significant decrease in *clec-87* expression at 48 h as compared to the worms infected with *E. cloacae* SBP-8 [Fig.4.4 C]. Upregulation of the *tol-1* gene was observed as early as 6 h of infection followed by further upregulation at later timepoints implying continuous activation of immune response by the bacteria [Fig.4.4 D]. Though the *tol-1* expression was low for

worms infected with *E. cloacae* SBP-8 *ΔclpV* at 12 and 24 h post infection but it was not statistically significant.

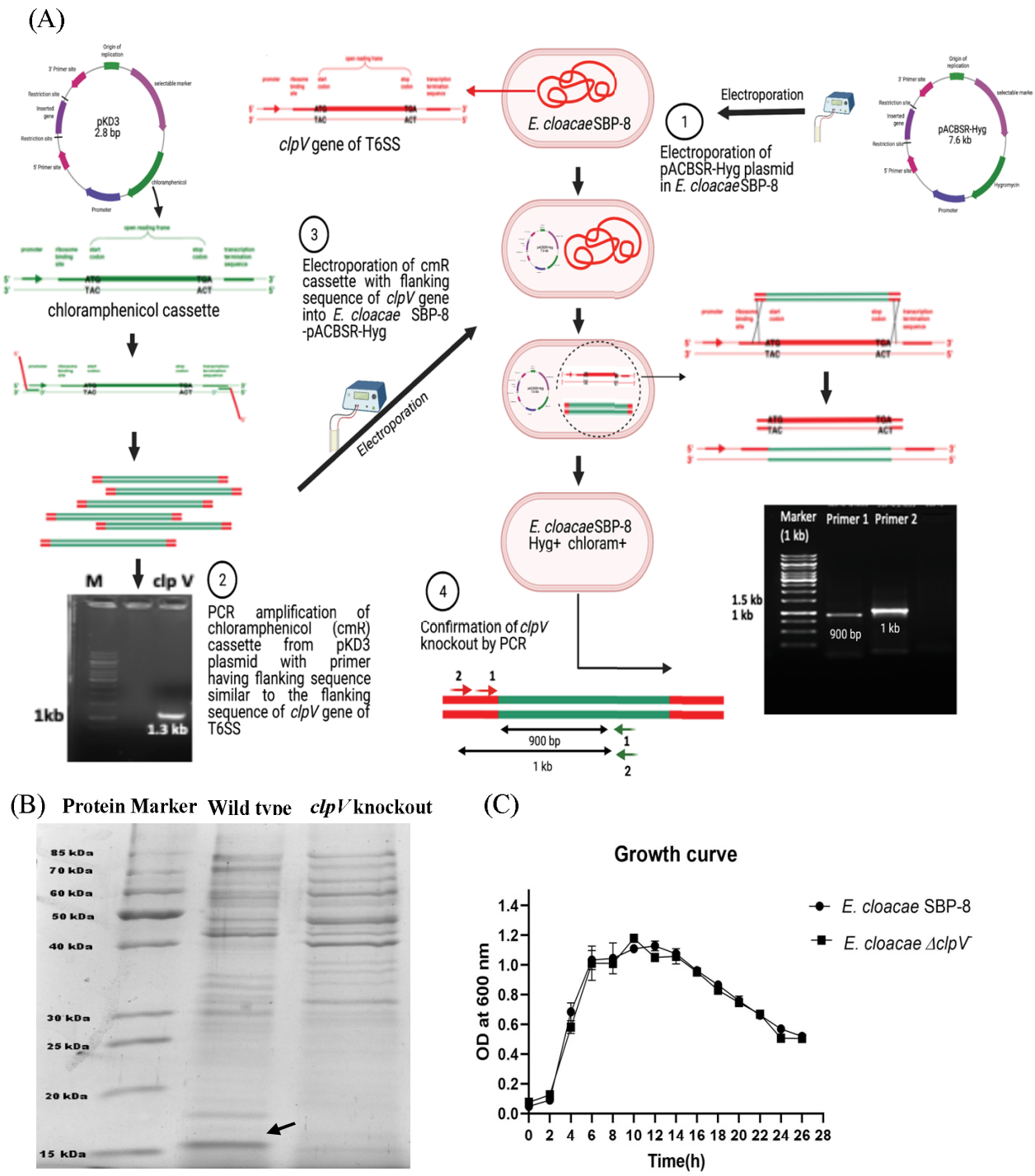

Fig 4.2. Identification of functional and active T6SS in *E. cloacae* SBP-8. (A) Genetic organization of T6SS gene cluster in *E. cloacae* SBP-8 genome. Genes in the various loci are color-coded and represented as blocked arrows showing the direction of transcription. Genes other than the components of T6SS are shown in grey (B) Diagram depicting the rationale and procedure for preparation *clpV* knockout by homologous recombination and its confirmation by PCR. (C) Total secreted proteins from wildtype and *clpV* knockout of *E. cloacae* SBP-8 were isolated from cultures grown to stationary phase in LB and separated by 15 % SDS-PAGE. (D) Growth curves kinetics of *E. cloacae* SBP-8 and *E. cloacae* SBP-8 *ΔclpV*. The growth curve was performed in two biological replicates, and error bars denote the standard error of the mean of the replicates

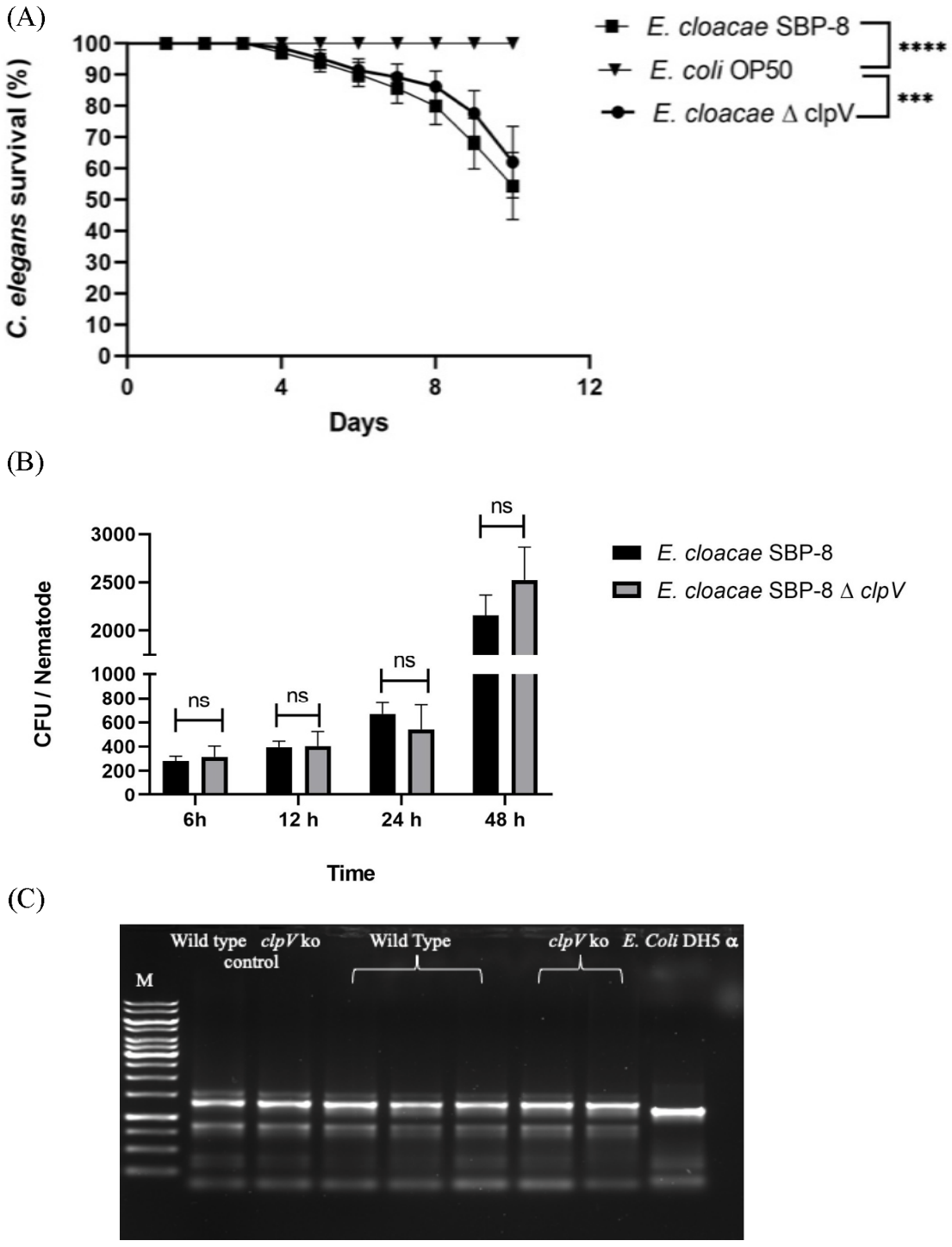

Fig.4.3 Role of the *E. cloacae* SBP-8 T6SS in virulence against *C. elegans*. Synchronized L4 worms (n=20) were fed on either *E. cloacae* SBP-8, *E. cloacae ΔclpV*, *E. coli* OP50, and the worms were analyzed for survival (A) and intestinal colonization of *E. cloacae* SBP-8 and *E. cloacae ΔclpV* in the worm's intestine was assessed using CFU analysis at a different time interval. (B). *ERIC* PCR of bacterial colonies obtained in CFU analysis of infected worms (C). Lane 1 to 7 shows a similar pattern of bands, confirm the *E. cloacae* SBP-8 colonization. Lane 1 and 2 have wildtype and knockout *E. cloacae* as a positive control. *E. coli* OP50 was used as a negative control in lane 8, negative.

Studies suggest that T6SS governs pathogenesis of many pathogenic bacteria like *E. coli* K1 and *Salmonella* by influencing host colonization, survival in the hostile host environment, and promoting invasion/intracellular proliferation in epithelial cells and phagocytic cells [82, 226-227]. To confirm the role of *E. cloacae* SBP-8 T6SS in these processes, we used HT-29 a colon carcinoma intestinal epithelial cell line, to evaluate the invasion and proliferation of the *E. cloacae* SBP-8 using gentamicin protection assay. Both the strains show similar invasion percent, and 2.5-fold proliferation was observed from 2 h to 16 h of infection. [Fig.4.5 A]. We also check its ability to survive in major phagocytosis cell-like macrophages using RAW264.7 cell lines. Likewise, the number of both the bacterial population within RAW 264.7 macrophages decreased by 4-fold from 2 to 16 h post-infection [Fig.4.5 B]. The results indicate that T6SS of *E. cloacae* doesn't have a role in the invasion and survival within the host cells.

4.3.3 *E. cloacae* SBP-8 utilizes T6SS to eliminate bacteria (*E. coli*) in a contact dependent manner.

To elucidate the role of *E. cloacae* SBP-8 T6SS in bacterial competition, we mixed *E. cloacae* SBP-8 (wildtype and *clpV* knockout *E. cloacae* SBP-8 separately) with *E. coli* DH5α and monitored for survival of each strain as described in the methodology. As shown in Fig.4.6 A, comparatively more *E. coli* DH5α cells were outcompeted/killed by wildtype *E. cloacae* SBP-8 than by *E. cloacae* SBP-8 *ΔclpV*. Quantitively, there was a 99% reduction in the number of viable *E. coli* 5 h post-incubation with wildtype *E. cloacae* SBP-8, while only 35% reduction when incubated with *clpV* knockout *E. cloacae* SBP-8 [Fig.4.6 B]. In both cases, the final population size of wildtype and *clpV* knockout *E. cloacae* SBP-8 was similar. The above results suggest that the *E. cloacae* T6SS apparatus is directly involved in inter-bacterial competition/bacterial killing.

To verify whether the T6SS-mediated inhibition of *E. coli* DH5α by *E. cloacae* SBP-8 is contact-dependent, the assay was repeated where the *E. coli* DH5α and *E. cloacae* SBP-8 were separated by 0.22 μm nylon membrane. As opposed to the 99% reduction in the number of viable *E. coli* DH5α when in direct contact with *E. cloacae* SBP-8 no reduction in the viable *E. coli* DH5α was observed when separated by a nylon membrane [Fig.4.6 C]. This was irrespective of whether the *E. coli* DH5α was above or below the membrane on the LB-agar plate. All these results indicate that *E. cloacae* SBP-8 utilizes T6SS for bacterial competition and kills the competitor in a contact dependent manner, probably injecting the effectors/toxins into the competitor.

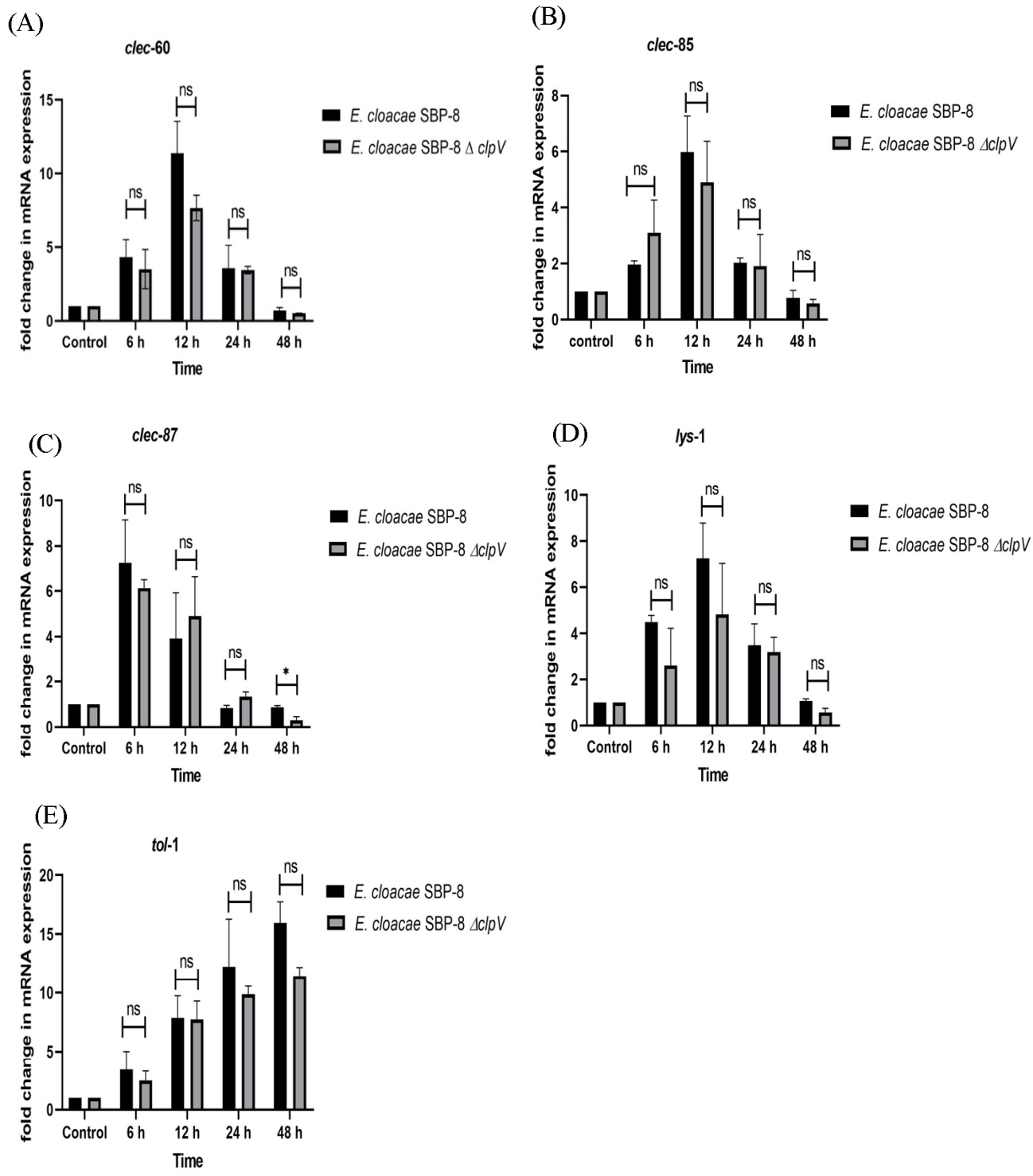

Fig.4.4 Relative expression of the antimicrobial and *tol-1* gene in *E. cloacae* SBP-8 wild type and *clpV* knockout infected worms. Quantitative expression profiling was performed for the antimicrobial peptide genes *celc60*, *clec85*, *clec87*, *lys-1* (D-G), and\ immune response gene *tol-1* (H) of *C. elegans*. *β-actin,* a housekeeping gene was used to normalized the relative expression of the above genes. The level of significance was analyzed by Paired t-test (*p<0.05).

(A) (B)

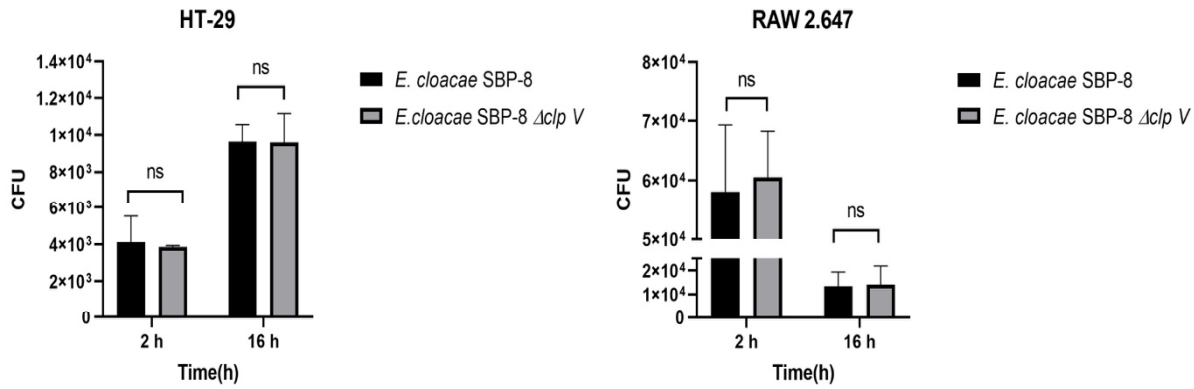

Fig.4.5. Role of the *E. cloacae* SBP-8 T6SS in intracellular proliferation. Images (A and B) represent multiplication of *E. cloacae* SBP-8 and *clpV* knockout *E. cloacae* in epithelial cells (HT-29) and macrophages (RAW 264.7).

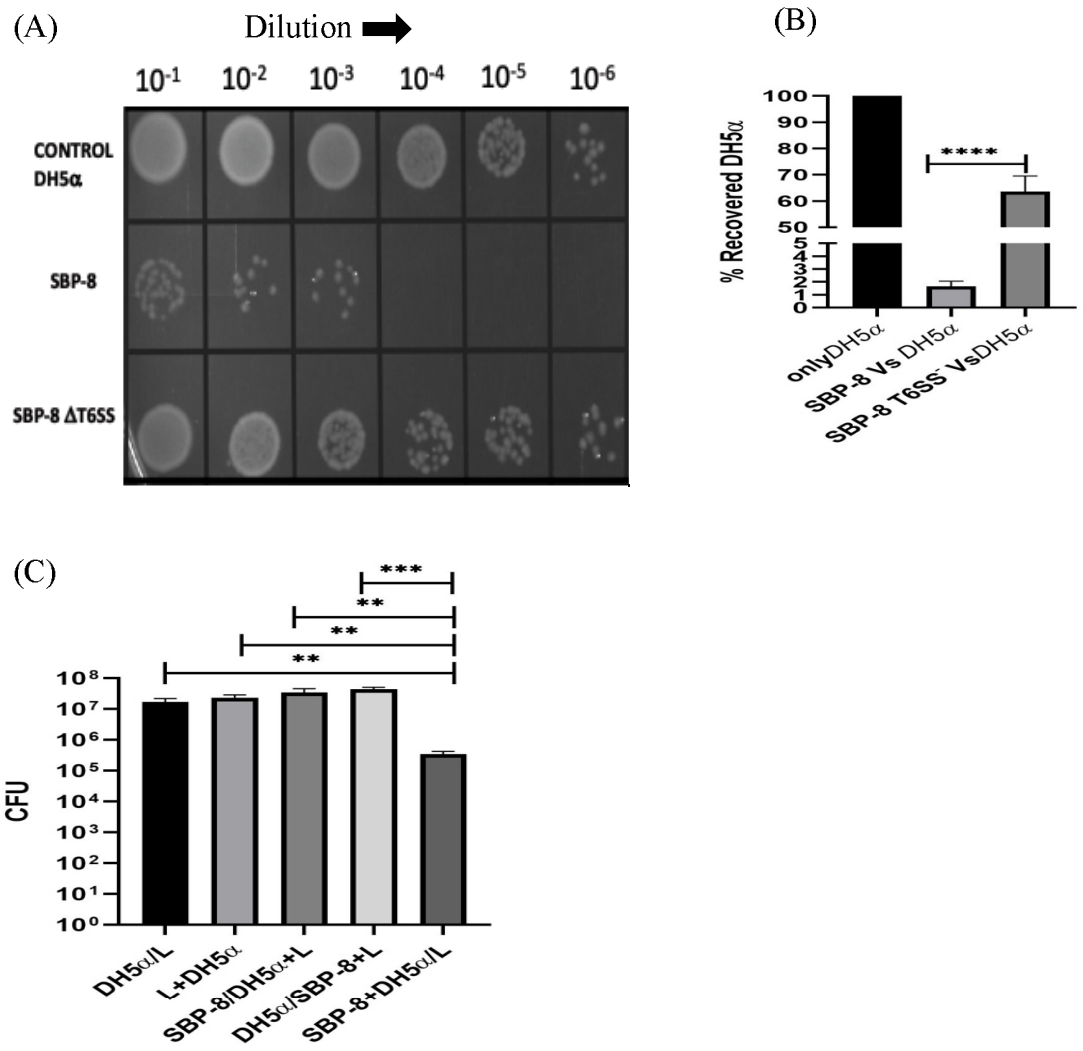

Fig.4.6. *E. cloacae* SBP-8 T6SS plays role in bacterial competition. (A) Semi-quantitative assessment of surviving *E. coli* DH5α after 5 h of competition with *E. cloacae* SBP-8, *E. cloacae* SBP-8 *ΔclpV*. (B). Quantitative assessment of *E. coli* DH5α using CFU analysis. The cultures from competition experiment were serially diluted to evaluate CFU of *E. coli* DH5α. Only DH5α is a control and refers to growth of *E. coli* DH5α in media alone (C). Quantitative assessment of *E. coli* DH5α populations when grown in either direct contact with or separated by nylon membrane from *E. cloacae* SBP-8. The slash between strains (DH5α. = *E. coli* DH5α, SBP-8 = *E. cloacae* SBP-8) or L (L-broth) indicate the presence of a 0.22 μm nylon filter membrane. Data represents mean of 3 biological replicates performed in triplicates. Error bars denote the standard error of the mean of the replicates.

4.4 Discussion

It has been speculated that many pathogenic bacteria have evolved molecular mechanisms to kill the host cells and bacterial competitors to establish infection. T6SS is a highly regulated system of Gram-negative bacteria contributing to a variety of functions, including inter-bacterial competition and virulence of pathogenic bacteria [85, 145, 220]. The role of T6SS in pathogenicity and competition has been demonstrated in clinical isolates of various bacteria, including that of *E. cloacae*. However, less is known about the T6SS in environmental or exogenous bacterial isolates, which may have pathogenic potential. In the previous chapter, we demonstrated the pathogenic potential of an exogenous isolate of *E. cloacae* SBP-8 using *C. elegans* [197] and mice as model hosts. In the present study, we attempted to characterize T6SS and its role in the virulence of *E. cloacae* SBP-8.

Examination of the genetic organization of *E. cloacae* SBP-8 revealed that it has one T6SS cluster, which appears to be complete having all the required conserved core genes for active structural and functional T6SS. However, its clinical counterpart, *E. cloacae* ATCC 13047, has two clusters of T6SS [35]. Nonetheless, similar to our observation, the environmental isolates of *E. cloacae,* namely *E. cloacae* SDM and EcWSU1 recovered from soil and onion, respectively, encode only one cluster of T6SS [21]. The T6SS cluster of *E. cloacae* SBP-8 and other environmental isolates resembles with T6SS-1 cluster of *E. cloacae* ATCC 13047, encoding the T6SS apparatus involved in bacterial competition [222]. Different pathogenic bacteria like *Burkholderia thailandensis* [85] and *Pseudomonas aeruginosa* [228] have more than one T6SS cluster. This could imply that *E. cloacae* strains having more than one distinct T6SS cluster are likely to have a fitness advantage in terms of pathogenesis and survival in different environments.

The presence of Hcp in the culture supernatant of several bacterial pathogens, including *Burkholderia mallei* [135], *Serretia marcescens* [141], *Edwardsiella tarda* [229], and *Enteroaggregative Escherichia coli* (EAEC) [83] has been considered as a trademark of T6SS functionality. Coomassie-stained SDS-PAGE analysis of culture supernatants of *E. cloacae* SBP-8 showed a Hcp band at expected size of 16.9 kDa. However, this was only at stationary phase, where the nutrients in the media are exhausted. Further, mass spectrometric analysis of this secretome showed the presence of key T6SS effectors molecules including Hcp and VgrG indicating a functionally active T6SS. Hcp functions as an effector molecule and also assembles into hexameric units wrapped into a sheath forming a filamentous tube-like structure

similar to T4 bacteriophage tail facilitating the translocation of effector proteins [70]. VgrG shows structural homology with the bacteriophage tail proteins gp27-gp5 forming a pointed needle/spike at the end of the tube-like structure. When the tail sheath contracts, effectors molecules loaded in nanotube are injected into the target cells [230]. After translocating the effector molecules, T6SS assemblies are recycled by ClpV ATPase to initiate a new round of effector injection into the target cell.

In-frame deletion *clpV,* led to the loss of T6SS functionality in *E. cloacae* SBP-8 as confirmed by the absence of 16.9 kDa Hcp band in SDS-PAGE in the knockout strain. The *clpV* deletion did not affect the growth kinetics suggesting T6SS is despensable for growth, atleast in nutrient rich media. Nonetheless, the secretion system is essential for bacterial competition/killing. The *clpV* knockout *E. cloacae* SBP-8 showed decreased contact-dependent killing or reduced competitive ability against *E. coli* DH5α (35% reduction verses the 99% reduction exhibited by wildtype strain). This killing was abrogated on placing a nylon filter between the competing bacteria. Our results are similar to the previous reports for the T6SS dependent competition of *Acetobacter baumannii* [139], *S. marcescens* [141], *V. cholerae* [140] and clinical isolate of *E. cloacae* [35].

Considering the pathogenic potential of *E. cloacae* SBP-8, we used *C. elegans* as a eukaryotic host to investigate the involvement of *E. cloacae* T6SS in its pathogenicity. During infection *in vivo* survivability of enteric pathogen depends upon its ability to colonize and interact with the target host [231]. Functional inactivation of T6SS (*clpV* knockout strain of *E. cloacae* SBP-8) did not have any significant impact on colonization efficiency in worm gut nor did it led to any remarkable change in the mortality rate of the infected worms. In *C. elegans,* the p38 MAPK pathway is crucial in mediating immune response against most of the pathogen through the production of antimicrobial peptides. We explored if T6SS could possibly regulate these antimicrobial genes either directly or indirectly through the p38 MAPK pathway. The expression of genes, *clec-60*, *clec-85*, *clec-87*, *lys-1*, and *tol-1*, which are considered as indicators of host innate immune response against various pathogens like *V. cholerae* [186], *Shigella flexneri* [185], and *Klebsiella pneumoniae* [178] was monitored in the worms infected with wildtype and *clpV* knockout strain of *E. cloacae*. In both the cases similar temporal pattern of expression was observed suggesting that *E. cloacae* T6SS trigger the inname immune resposne in *C. elegans*. We further investigated if any other step of pathogenesis is mediated by T6SS. T6SS is known to influence cell adhesion, and invasion in many enteric pathogens

such as *Camphylobacter jejuni* [220], *Burkholderia pseudomallei* [69], *Aeromonas hydrophila* [134], *V. chlorae* [59] *S.* typhimurium [226] and *E. cloacae* ATCC13047 [35]. However, in contrast to the above reports, the wildtype and *clpV* knockout strain of *E. cloacae* SBP-8 displayed comparable invasion and proliferation in HT-29, intestinal epithelial cells, and RAW 264.7 macrophages. These results indicate that the *E. cloacae* SBP-8 T6SS neither contribute to the invasion nor to the survival in the host intestinal-epithelial cells and macrophages. However, *E. cloacae* SBP-8 *ΔclpV* exhibited reduced potency in outcompeting other bacteria like *E. coli* DH5α. This could mean that T6SS provide an advantage to the wildtype in outcompeting the gut bacteria and aid in invasion and colonization in mammalian host. But this needs to be explored using an appropriate model organisms like mice. Similarly, T6SS-1, which is similar to T6SS of *E. cloacae* ATCC 13047 was not involved in adherence to epithelial (HeLa) cells but in the bacterial competition and colonization of mice gut [35]. However, *E. cloacae* ATCC 13047 T6SS-2, absent from *E. cloacae* SBP-8, was involved in adherence to HeLa, mice-gut colonization and bacterial competition [35]. Thus, it could be said that T6SS-2 could contribute to the adherence and maybe invasion into the host cells. Murdoch *et al.*, also reported that T6SS of *Serretia marcescens* is not required for virulence in eukaryotic hosts like *C. elegans* and *Galleria mellonellas* but have direct involvement in its anti-bacterial ability [141]. It has been reported earlier that out of three clusters, the H2 T6SS cluster of *P. aeruginosa* has a direct role in invasion in HeLa cells and also in virulence against *C. elegans,* whereas cluster H1 is responsible for inhibition of bacterial competitors [116]. From above observations, it appears that T6SS of *E. cloacae* SBP-8 is mainly deployed for combating its competitors in the natural environment, and this isolate may employ alternative mechanisms for exerting virulence in the eukaryotic host.

In conclusion, *E. cloacae* SBP-8, an environmental (non-clinical) isolate acting as an opportunistic pathogen possess a functionally active T6SS with no detected role of this T6SS in the virulence in *C. elegans.* However, *via* its T6SS it exhibits inhibitory action against its bacterial competitors which may prove beneficial in colonizing the gut of higher eukaryotes like mice. *E. cloacae* SBP-8 kills competing bacteria like *E. coli* in contact-dependent manner. However, deletion of *clpV* does not affect the adhesion and proliferation of *E. cloacae* SBP-8 in eukaryotic cell lines.