Chapter 2

Elucidating the pathogenic potential of E. cloacae SBP-8 using Caenorhabditis elegans as a model host.

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2.1 Introduction

E. cloacae, an opportunistic nosocomial pathogen, is one of the two clinically most important species within the *Enterobacter* genus [1,159]. *E. cloacae* infections are of endogenous origin occurring as a side effect of antibiotic therapy or compromised immune system [160]. It accounts for several nosocomial complications, including 5% sepsis, 4% urinary tract infections, 5% pneumonia, and 10% postsurgical peritonitis cases and neonatal outbreaks [1]. Despite this, very few reports exist describing its pathogenic potential and the ability to produce virulence factors. It produces toxins like hemolysins, leukotoxins, and thiol activated pore forming cytotoxins that destroy host immune cells [6,34]. *E. cloacae* adhere, invade, and induce apoptosis/necrosis in HEp-2 cells [161]. Type III secretion system (T3SS) is supposed to deliver toxins and virulence factors into the host cells. Around 27% of the clinical *E. cloacae* isolates are reported to possess T3SS genes [162]. *E. cloacae* also possess the AcrAB-TolC system that protects itself from antimicrobial agents and help systemic colonization in mice infected intraperitoneally [8]. The mechanism of *E. cloacae* pathogenesis is intricate, involving several factors with poorly defined roles. Many *E. cloacae* strains are antibiotic resistant posing difficulty in treating infections [1]. Therefore, to identify novel drug targets, it is essential to understand pathogenesis that, in turn, requires a suitable host to study the infection process.

Many researchers rely on mice and *Drosophila melanogaster* as common model organisms to study host-pathogen interactions [163, 164]. Off-late, *Caenorhabditis elegans* has emerged as an attractive model host due to its simple anatomy, shorter generation time, and similarity with the mammalian innate immune system [165, 166]. Similar to the infection studies with mice and the *Drosophila* model, the role of Tol, p38 MAPK, JNK pathways, *etc.* in regulating infections has been elucidated using *C. elegans* as a model host [165, 166]. Therefore, we utilized *C. elegans* as a model to study the pathogenicity of *E. cloacae*.

E. cloacae strain SBP-8 was used as a test pathogen as it harbors genes encoding virulence factors like toxins, siderophores, secretion systems (I, II, IV, and VI), adherence and colonization factors [146]. We investigated the pathogenic potential of *E. cloacae* SBP-8 by analyzing the host (*C. elegans*) response at the physiological and molecular levels. Through this study, we prove that against the established nosocomial origin of *E. cloacae* infections, the infections could also arise from environmental isolates.

2.2 Materials and methods

2.2.1 Bacterial strains and growth conditions

E. cloacae strain SBP-8, a soil isolate from our lab [147], was used as a test isolate for the infection studies. Known bacterial pathogen, *E. cloacae* (MTCC 509, clinical isolate from human cerebrospinal fluid, equivalent to NCTC 10005) and *Pseudomonas aeruginosa* PAO1 (MTCC 3541), a pathogenic bacteria, were obtained from the Institute of Microbial Technology, Chandigarh, India. Fluorescent derivatives, *E. cloacae* SBP-8-GFP and *Escherichia coli* OP50-GFP were obtained by electro-transformation of *pGB-5* plasmid (a kind gift from Dr. Shankar Manoharan lab, Indian Institute of Technology, Jodhpur, India). All the strains were grown in Luria Bertani (LB) broth (Himedia, India). Bacterial cultures were routinely maintained on LB-Agar media. Wild-type N2 strain of *C. elegans* (a kind gift from Vidya Devi Negi, National Institute of Technology, Rourkela) was routinely maintained at 25°C on nematode growth medium (NGM) agar plate with *E. coli* OP50 as a food source as per the standard method.

2.2.2 Electro transformation of *pGB-5* plasmid

Fluorescent derivatives of *E. cloacae* SBP-8-GFP and *Escherichia coli* OP50-GFP were obtained by electro-transformation of *pGB-5* plasmid (a kind gift from Dr. Shankar Manoharan lab, Indian Institute of Technology, Jodhpur, India). For electrocompetent cells preparation 10 ml of Prewarmed lb media was inoculated with 1 percent of overnight of *E. coli* OP50 and *E. cloacae* SBP-8. When OD_{600} reached 0.4, bacterial cells were harvested and resuspend in 10 ml of 10% ice-cold glycerol before washing with Ice cold Milli-Q. After two washes in 10 % ice-cold glycerol, cells were resuspended in 100 ul of 10 % ice-cold glycerol. 100 ng of *pGB-5* plasmid was used for Electro-transformation using Bio-Rad Electroporator.

2.2.3 *C. elegans* solid killing assays

The *C. elegans* solid killing assay was performed as previously described by Sim and Hibbert, 2016 with minor modifications [167]. Synchronized L4 worms (larval stage 4) were used for the infection studies. After 18 h of infection with the test strains (*E. cloacae* SBP-8 and *P. aeruginosa* PAO1), a total of 20 worms were transferred to fresh NGM plates supplied with *E. coli* OP50. Dead and live animals were scored every day. After every 48 h, live worms were transferred to fresh NGM plates seeded with *E. coli* OP50 to exclude the freshly hatched larvae from the analysis. The nematodes were scored as dead when they failed to respond to a gentle touch with a platinum wire. The nematodes that died by being stuck to the wall of the plate were excluded from the assay. *E. coli* DH5α and *E. coli* OP50 were used as negative control

while *E. cloacae* (MTCC 509) and *P. aeruginosa* PAO1 were used as a positive control. For statistical analyses, each experiment was carried out in triplicates.

2.2.4. Bacterial colonization assay

To measure the intestinal colonization of the test strains in *C. elegans,* the synchronized L4 larvae were allowed to feed on *E. cloacae* SBP-8 for 3, 6, 12, 24 and 48 h, and then transferred to the NGM plate containing *E. coli* OP50. After 24 h, the worms were washed thrice with M9 buffer (KH_2PO_4, Na_2HPO_4, 5 g NaCl and 1 M $MgSO_4$), and treated with 100 µg/ml of gentamicin for 1 hr followed by treatment with 25 µg/ml of gentamicin for 30 mins to remove surface colonized bacteria. Finally, the worms were washed with M9 buffer and lysed with 0.1 % triton X-100 in a Qiagen bead tissue lyzer LT (Qiagen, India). The lysates were serially diluted 10 times and plated on LB-agar containing 100 µg/ml ampicillin to estimate bacterial burden by counting CFU (colony forming units) analysis. For fluorescence microscopy, *C. elegans* were grown on NGM-agar plates having lawn of GFP-tagged *E. cloacae* SBP-8 for 6, 12, 24 and 48 h and then were placed in a 1 mM sodium azide solution on 2 % agarose for imaging under upright fluorescent microscope (Zeiss, Germany).

2.2.5. *ERIC*-PCR fingerprinting

nterobacterial Repetitive Intergenic Consensus (*ERIC*) PCR-based DNA fingerprinting was carried out as described by Shuhaimi *et al.* [168]. *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 (Biorad, USA).

2.2.6. Binary choice assays

The cultures (100 µl of 0.3 OD_{600}) of *E. cloacae* SBP-8, *E. coli* OP50 and *P. aeruginosa* PAO1 were spotted on NGM-agar plates, two strains on one plate [169]. The synchronized L4 worms were washed with M9 buffer and then 10 worms placed equidistant from the bacterial spots. The number of worms entering each bacterial spot were counted every 2 h and the percent migration of worms at each spot was calculated.

2.2.7. Reactive oxygen species (ROS) assay

The L4 worms were infected as described in solid killing assay. After 24 h of infection, worms were picked and washed several times with M9 buffer. Worms were subsequently incubated with 10 mM H_2DCFDA (2′,7′-Dichlorofluorescein diacetate) in M9 buffer for 30 min in dark

at 20°C [170]. Worms were washed with M9 buffer after staining with H_2DCFDA to remove excessive dye, mounted on agarose pad with 25 mM sodium azide and imaged with fluorescent microscope using GFP filter cube (Zeiss, Germany). The fluorescent intensity was quantified with ImageJ software.

2.2.8 DNA damaged assay To visualize the fragmented and condensed DNA which is an hallmark for programmed cell death, synchronized L4 worms were exposed to *E. cloacae* SBP-8, *E. cloacae* (MTCC 509) and *E. coli* OP50 for 48 h. After infection worms were washed with M9 buffer and fixed in 4% paraformaldehyde for 25 min at room temperature. Worms were washed with M9 buffer to remove excessive paraformaldehyde and incubated with 100 ng/ml of 4',6'-diamidino-2-phenylindole hydrochloride (DAPI) in M9 buffer for 1 h. After washing worms were mounted on agarose pad and imaged using UV filter in Apotome fluorescent microscope (Zeiss Germany).

2.2.9. Assay for analysing physiological and reproductive defect due to bacterial colonization

A batch of synchronized L4 worms were exposed to *E. cloacae* SBP-8 for 72 h and then mounted on 2% agarose pad with 25 mM sodium azide. The worms were imaged for physiological and reproductive defects under Nomarski microscope (Zeiss, Germany). Egg laying assay was performed as described by previously [171]. The nematodes were fed with *E. cloacae* SBP-8 and *P. aeruginosa* PAO1 for 24 h and transferred to *E. coli* OP50 plates. Total number of progenies produced by *E. cloacae* SBP-8 and *P. aeruginosa* PAO1 infected worms were counted for about 10 days until adult worms stopped laying eggs.

2.2.10. Expression analysis of antimicrobial and reproductive genes

Total RNA was extracted from 50 nematodes exposed to *E. cloacae* SBP-8 for different time periods (6, 12, 24, and 48 h) as previously described [171] with some minor modifications. Briefly, RNA was extracted using TRIzol (Sigma,USA) and quantified using μCuvette G1.0 spectrophotometer (Eppendorf, Germany). The cDNA was generated from 200 ng of RNA using Thermo scientific verso cDNA synthesis kit as per the instructions by the manufacturer. Quantitative real-time PCR (qPCR) was performed using Bio-Rad SYBR green dye in Bio-Rad thermocycler by using primers listed in table 1. Relative fold change in target gene expression was calculated using $2^{-\Delta\Delta}$Ct method by norma *β-actin.*

Table. 1.1 List of primers sequences used for RT-PCR experiments

Gene name	Sequence	Reference
β -*actin*	FP-5′ATCGTCCTCGACTCTGGAGATG3′ RP-5′TCACGTCCAGCCAAGTCAAG3′	[178]
let23	FP-5′GGAGACGAGGTTTTTCACGA3′ RP-5′CGGATCCCCAACATTTATCA3′	
lin29	FP-5′TCTCGATGCCATCAAACTGA3′ RP-5′GCTTTGTCATGTGCTTTTGC3′	
clec60	FP-5′TGT CTG CAT TCT TCC AGT CG3′ RP-5′CCC ATA CCC AGA CAC CTT TG3′	
clec85	FP-5′GAGCACGCTGAATGGAAAAT3′ RP-5′GCTCCAGAAGCTGGTGAGTC3′	
celc87	FP-5′GAGCACGCTGAATGGAAAAT3′ RP-5′GCTCCAGAAGCTGGTGAGTC3′	
tol-1	FP-5′CGTCAAATGAGCTTCCATCA3′ RP-5′ATTGGCTTCAGCATCGAGTT3′	
lys-1	FP-5′CTTTTTGCTTTGGCTTCTGC3′ RP-5′CTGCGTAGGCATATGAAGCA3′	

2.2.11 Statistical analysis

Most of the experiments were performed at least three times. Data were expressed as mean ± standard deviation and analyzed using Graph Pad Prism 8. The Kaplan-Mayer log test compared survival curves. One way ANOVA was used for statistical comparison between different groups and the significant difference between means of the parameter was calculated using a nonparametric t-test.

2.3 Results

2.3.1 *E. cloacae* SBP-8 infection decreases the lifespan of *C. elegans* through intestinal colonization, and induction of reactive oxygen species.

The pathogenic potential of *E. cloacae* SBP-8 was tested in comparison to two clinical isolates, *E. cloacae* (MTCC 509) and *P. aeruginosa* PAO1, in *C. elegans* using the solid killing assay. We observed a significant ($p < 0.001$) reduction in the survival of nematodes fed on *E. cloacae* SBP-8, *E. cloacae* (MTCC 509) and *P. aeruginosa* PAO1 [Fig.2.1 , 40% mortality with *E. cloacae* SBP-8, 55% with *E. cloacae* (MTCC 509) and 70% with *P. aeruginosa* PAO1, ten days post feeding].

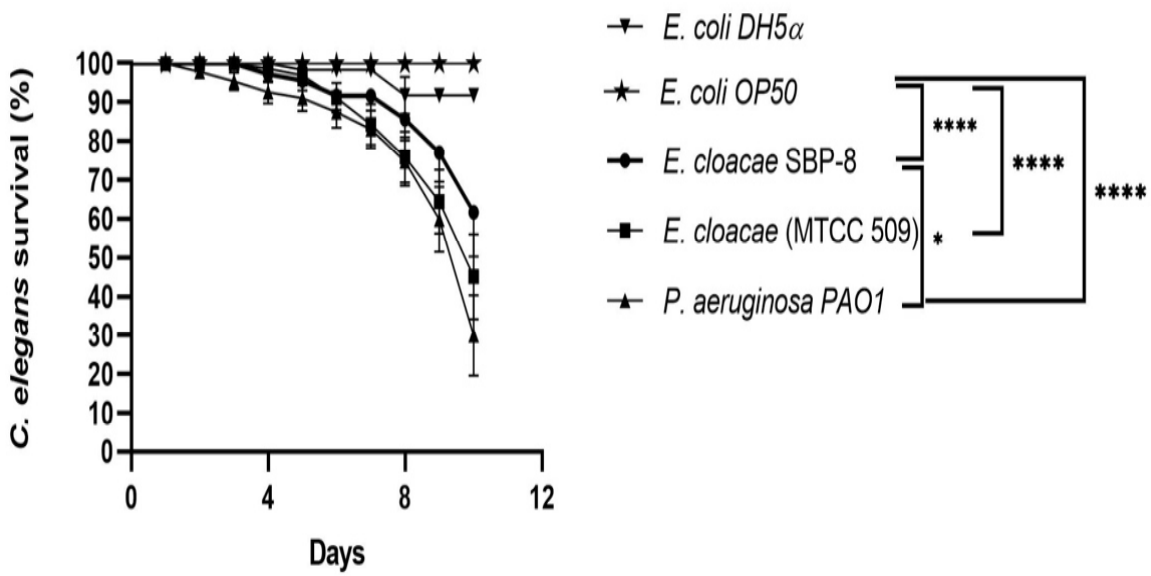


Fig.2.1. *E. cloacae* SBP-8 decreases the lifespan of *C. elegans.* Synchronized L4 worms (n=20) were fed on either *E. cloacae* SBP-8, *E. coli* DH5α, *E. coli* OP50, *E. cloacae* (MTCC 509) and *P. aeruginosa* PAO1 and the worms were analyzed for survival

The correlation between intestinal colonization and the reduction in lifespan of *E. cloacae* SBP-8 fed worms was evaluated by bacterial colonization assay followed by DNA fingerprinting of the recovered colonies. The *E. cloacae* SBP-8 and *E. cloacae* (MTCC 509) count within the nematodes increased with an increase in the feeding time [Fig.2.2 A], indicating the intestinal colonization by *E. cloacae* SBP-8 and *E. cloacae* (MTCC 509). Intestinal accumulation of *E. cloacae* SBP-8 increased significantly [from 6 h to 48 h, Fig.2.2 B]. It was less compared to that of *E. cloacae* (MTCC 509) and *P. aeruginosa* PAO1 [Fig.2.2 C], correlating well with the results of survival assay [Fig.2.1]. However, we did not observe significant difference in the survival of *E. cloacae* SBP-8 and *E. cloacae* (MTCC 509) infected worms [Fig 2.1]. ERIC-PCR based DNA fingerprinting analysis confirmed that the bacteria recovered from the infected nematodes are *E. cloacae* SBP-8 [Fig.2.3 A]. This experiment proves Koch's postulates regarding infection with microorganisms.

Further, we verified the intestinal colonization of *E. cloacae* SBP-8 using fluorescence microscopy [Fig.2.4 A-D]. We deduce that *E. cloacae* SBP-8 avoids pharyngeal grinding and gradually colonizes the entire gut. The bacteria colonized the distal pharyngeal region by 6 h, reached the lower gut by 12 h, colonized the intestine by 24 h, and then colonized the entire gut (pharynx to anus) by 48 h [Fig.2.4 A-D]. Since most animals, including *C. elegans* respond to pathogen invasion by producing ROS [170,172], we analyzed the extent of ROS production in the worms exposed to *E. cloacae* SBP-8 [Fig.2.5 A] or *P. aeruginosa* PAO1 [Fig.2.5 B]. As anticipated, the ROS production in *P. aeruginosa* PAO1-infected worms was higher than *E. cloacae* SBP-8 infected ones (fifteen fold versus ten fold) [Fig.2.5 C].

Oxidative stress could induce cell damage and cell death [173]. We next assessed the possibility of *E. cloacae*-induced cell damage in *C. elegans* through DAPI staining. Most of the *E. cloacae* SBP-8 and *E. cloacae* (MTCC 509) infected worms had damaged nuclei (Fig.2.6 A) as indicated by the intensive DAPI staining of different cells [Fig.2.6 B and C]. This signifies DNA damage indicating initiation of cell death. Some cells, especially intestinal, in *E. cloacae* (SBP-8 and MTCC 509) infected worms had fragmented nuclei suggesting apoptotic cell death. We also observed alterations in the germline with accumulated DNA breaks (intense DAPI staining) in *E. cloacae* SBP-8 and *E. cloacae* (MTCC 509) infected worms. The severity of DNA/cell damage was comparable between *E. cloacae* SBP-8 and *E. cloacae* (MTCC 509) infected worms. Even though some of the *E. coli* OP50 fed worms showed DNA damage [Fig.2.6 A] the severity of damage was less compared to *E. cloacae* SBP-8 and *E. cloacae* (MTCC 509) infected worms.

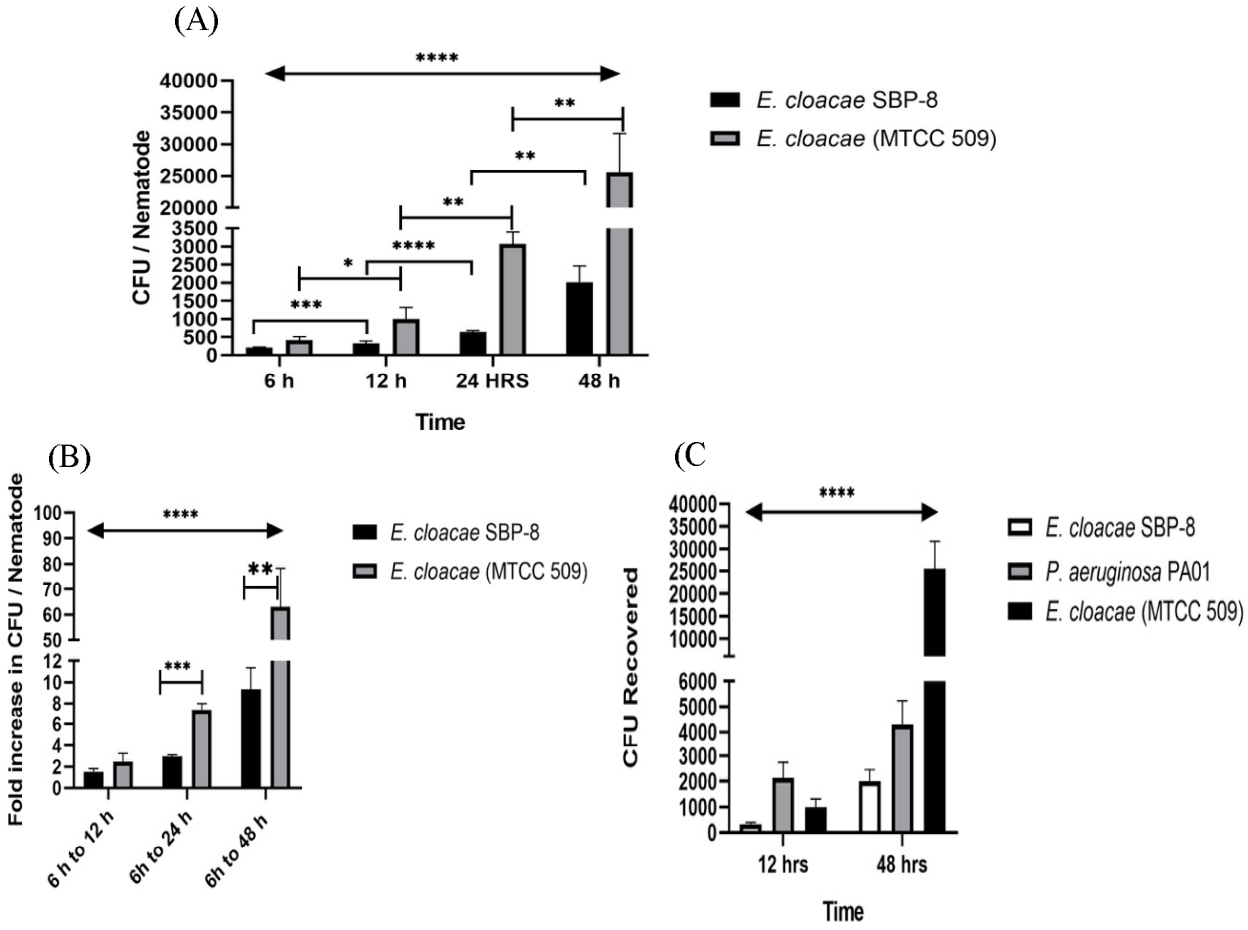


Fig.2.2. *E. cloacae* SBP-8 colonize in worm intestine. (A) Accumulation of *E. cloacae* SBP-8 and *E. cloacae* (MTCC 509) in the nematode intestine was assessed using CFU analysis by plating the lysates of the infected worms picked at different time intervals. (B) Fold change in *E. cloacae* SBP-8 and *E. cloacae* (MTCC 509) recovered per nematode at different time-intervals of exposure with respect to 6 h. (C) CFU recovered per nematode for *E. cloacae* SBP-8 and *P. aeruginosa* PAO1 and *E. cloacae* (MTCC 509) after 12 and 48 h of feeding

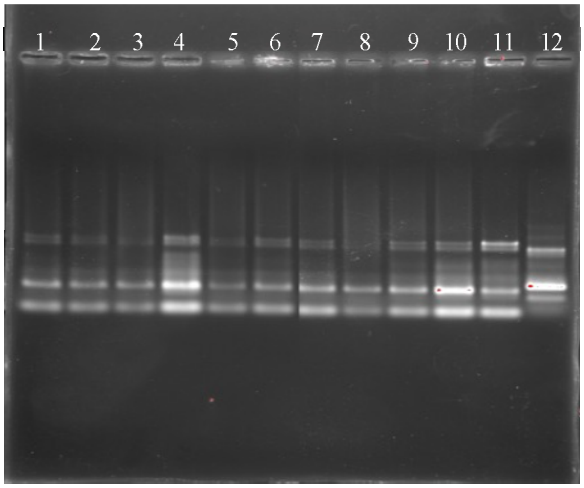


Fig. 2.3 Confirmation of Koch's Postulates. *ERIC* PCR of bacterial colonies obtained in CFU analysis of infected worms. Lane 1 -11 shows similar pattern of bands, confirm the *E. cloacae* SBP-8 colonization. *E. coli* OP50 was used as a negative control in lane 12 of bacterial colonies obtained in CFU analysis.

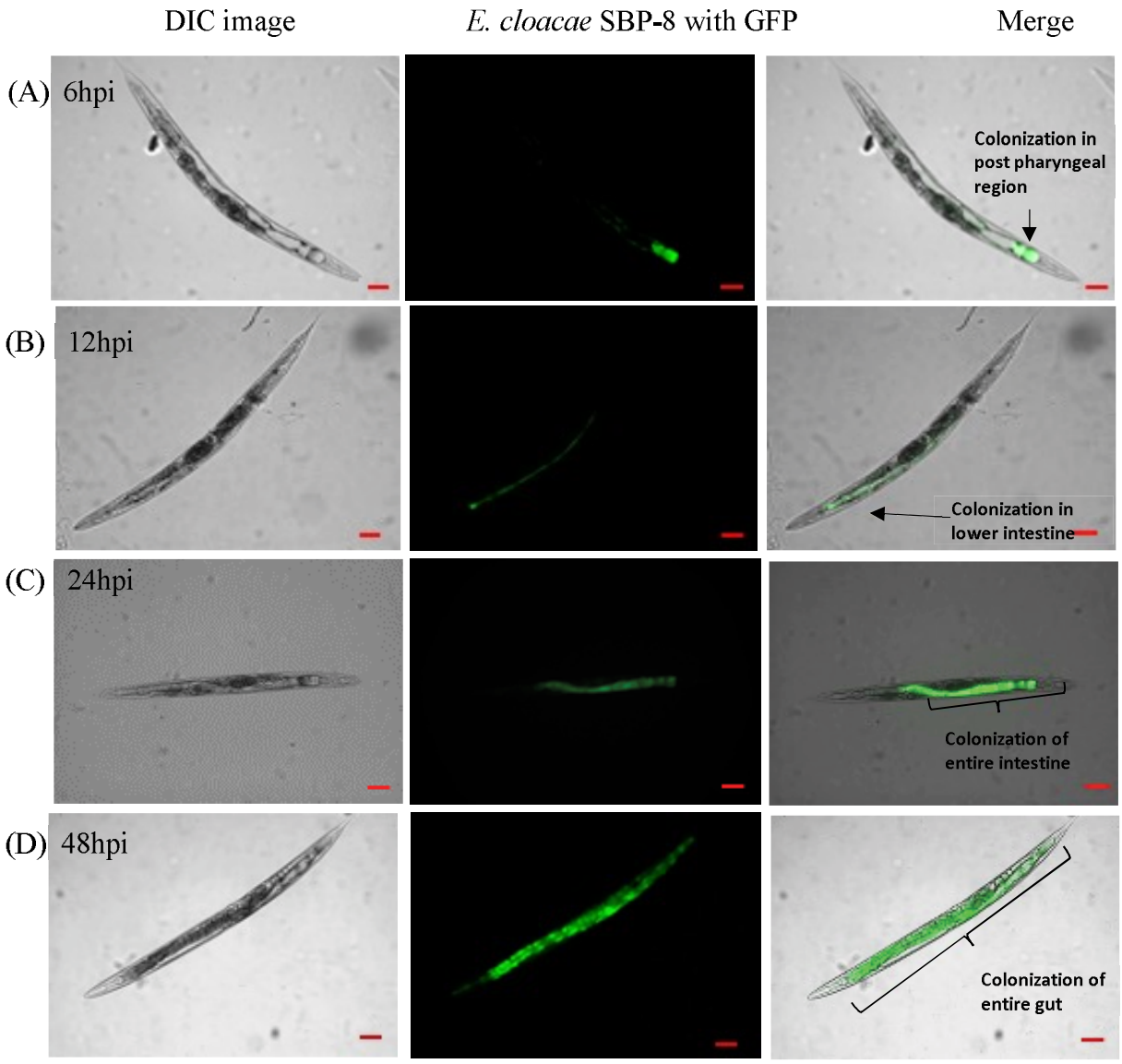


Fig.2.4. *E. cloacae* SBP-8-GFP colonization in intestine of *C. elegans*. (A to D) Representative fluorescence microscopic image showing *E. cloacae* SBP-8-GFP colonization in post pharyngeal region of *C. elegans* 6 hours post infection (hpi) (A), lower gut by 12 hpi (B), of the entire intestine of by 24 hpi (C) and entire gut from the pharynx to anus by 48 hpi (D). The scale bar represents 100 µm.

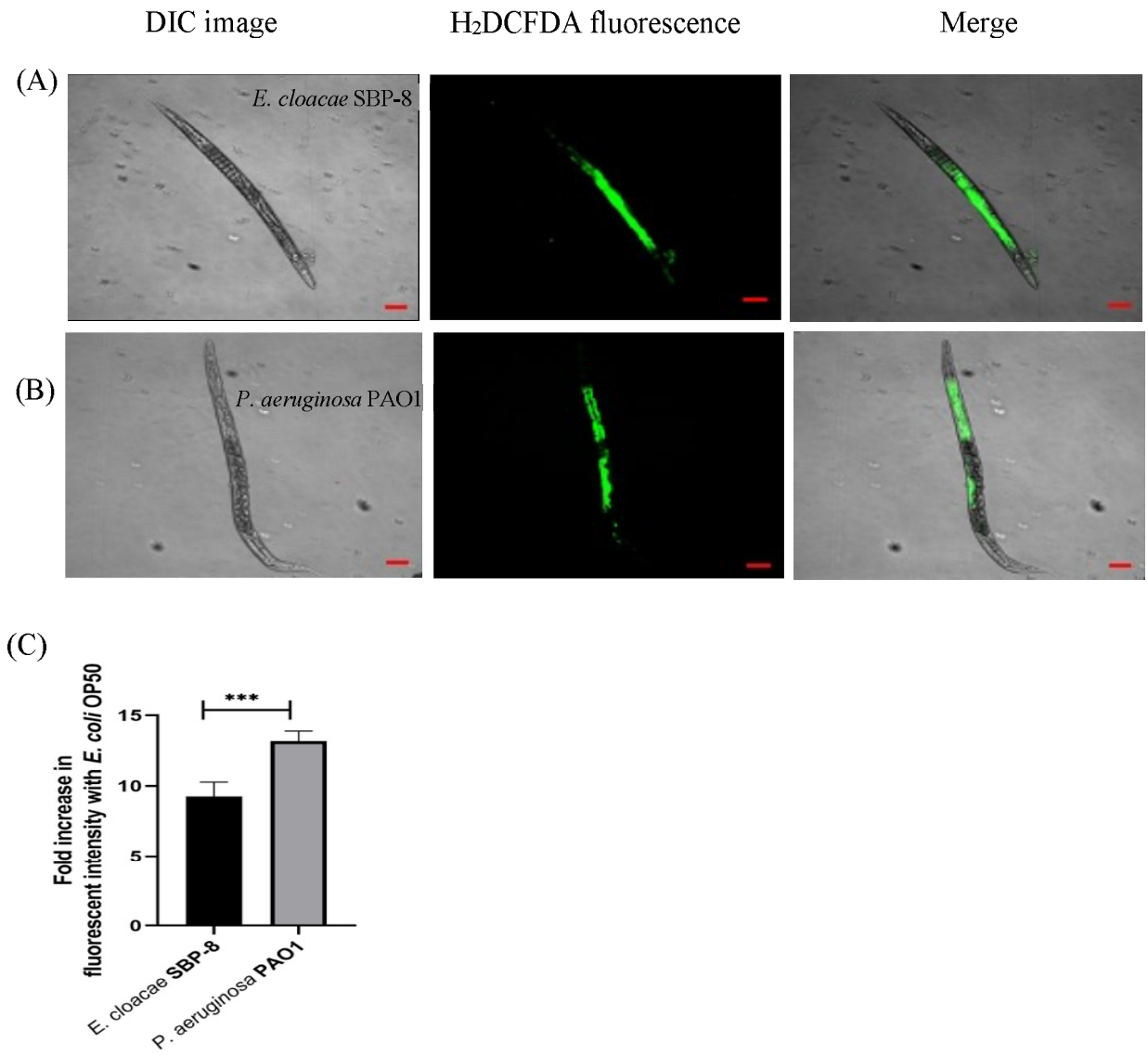
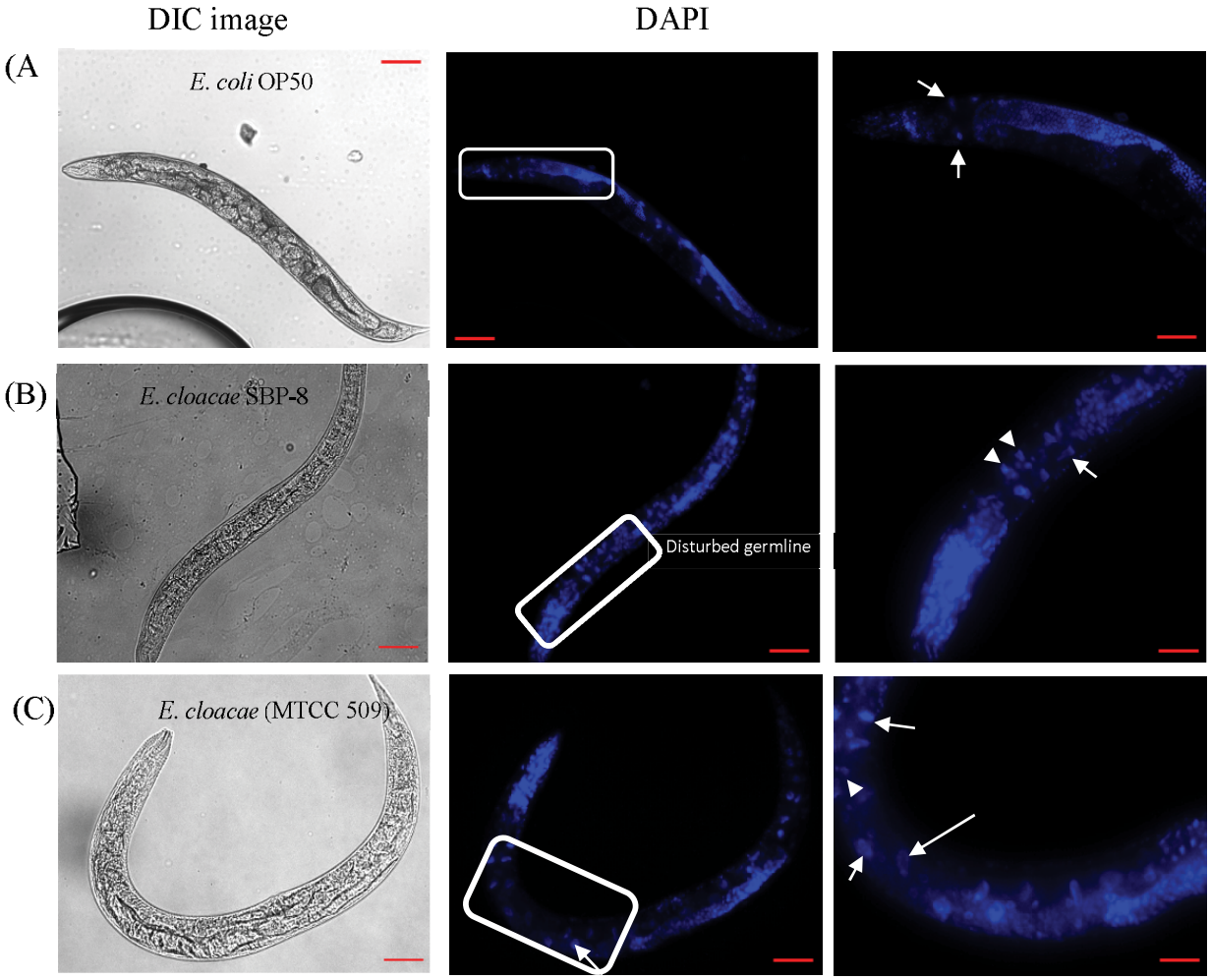


Fig.2.5. Exposure to *E. cloacae* SBP-8 induce ROS production. Representative fluorescent microscopic images of *C. elegans* displaying 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) fluorescence on infection with *E. cloacae* SBP-8 (A) and *P. aeruginosa* PAO1 (B). After 24 h of infection the nematodes were stained with H_2DCFDA to detect intracellular ROS. The scale bar represents 100 µm. (C) Fold increase in the fluorescence intensity of H_2DCFDA in *C .elegans* infected with *E. cloacae* SBP-8 and *P. aeruginosa* PAO1 for 24 h with reference worms fed on *E. coli* OP50. *** $p < 0.001$. The scale bar: 100 µm.



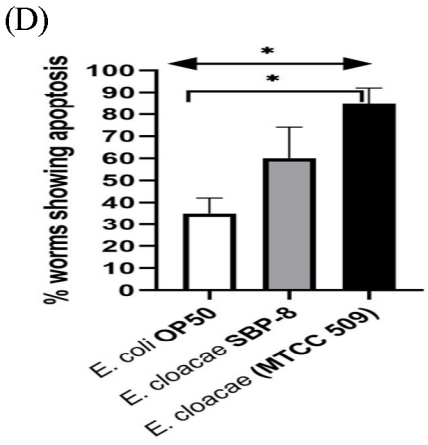


Fig.2.6. Exposure to *E. cloacae* SBP-8 induce DNA damage in the worms. (A - C) Representative fluorescent microscopic image of *C. elegans* 4′,6-diamidino-2-phenylindole) after 48 hpi with *E. coli* OP50 (A) *E. cloacae* SBP-8 (B) and *E. cloacae* (MTCC 509) (C). Arrows indicate the nuclei of intestinal cells and arrowheads point at fragmented nuclei. The scale bar: 50 µm (20 µm for magnified images). (G) Percentage of worms showing damaged intestinal DNA (intense DAPI staining signifying apoptosis). Percent apoptosis was calculated based on worms having DAPI stained nuclei.

2.3.2 *C. elegans* does not show any behavioural changes to minimize contact with *E. cloacae* SBP-8.

When exposed to some pathogenic bacteria, *C. elegans* alter their olfactory preferences to avoid the pathogen [174]. To test if *C. elegans* could avoid *E. cloacae* SBP-8 and prefer nonpathogenic *E. coli* OP50, we performed a binary choice assay. The worms migrated to lawns of both, *E. coli* OP50 and *E. cloacae* SBP-8 after 4 h of spotting though the number was higher in *E. cloacae* SBP-8. Six hours onwards till 12 h equal number of worms were found in both *E. coli* OP50 and *E. cloacae* SBP-8 zones [Fig.2.7 A]. When analysed against *P. aeruginosa* PAO1, almost all worms were seen in *E. cloacae* SBP-8 zone after 4 h of spotting. At 6 h, a few (10 to 15 %) worms also migrated to *P. aeruginosa* PAO1 spot and most (90%) to *E. cloacae* SBP-8 spot [Fig.2.7 B]. At the later time points (8 h and 12 h) all the worms were found in *E. cloacae* zone suggesting that worms at *P. aeruginosa* PAO1 spot (at 6 h) migrated out towards *E. cloacae* SBP-8. This result indicates that the nematodes do not avoid *E. cloacae* SBP-8 but *P. aeruginosa* PAO1.

2.3.3 *E. cloacae* SBP-8 induce physiological defects in infected worms.

To test whether *E. cloacae* SBP-8 induces physiological defects in *C. elegans*, we examined the infected worms under the Nomarski microscope. We observed damage to the pharyngeal bulb [Fig.2.8 B] and intestinal distension (a two fold increase compared to uninfected control) [Fig.2.8 A and B]. *E. cloacae* (MTCC 509) infected worms showed damaged pharyngeal bulb and distended intestine as early as 48 h [Fig.2.8 E]. Intestinal distension could compromise the diet of the worms, thus leading to starvation affecting its brood size [175]. Hence, we measured the brood size of *E. cloacae* SBP-8 and *P. aeruginosa* PAO1 infected worms observed that the infected worms had significantly ($p<0.001$) smaller brood size compared to that of *E. coli* OP50-fed worms [Fig.2.8 G]. Analysis of the egg arrangement in *E. cloacae* SBP-8 infected worms revealed disturbed egg arrangement pattern [Fig.2.8 D] with 70% worms showing internal hatching [Fig.2.8 D and H].

2.3.4 Differential regulation of immune regulatory and reproductive genes of *C. elegans* during *E. cloacae* SBP-8 infection.

To assess the induction of innate immunity, we examined the expression of *clec-60*, *clec-85*, *clec-87*, *lys-1*, and *tol-1* in *E. cloacae* SBP-8 infected worms at different time intervals. The expression of *clec-60*, *clec-85*, and *lys-1* increased at 12 h and then gradually decreased at 24 h and 48 h [Fig.2.9 A, 2.9 B, 2.9 D]. The *clec-87* gene had a similar expression pattern except that the expression peaked at 6 h and then gradually decreased [Fig.2.9 C]. The *tol-1* gene was

upregulated in infected *C. elegans* as early as 6 h, and its expression increased gradually with time up to 48 h [Fig.2.9 E], indicating that the worms sense *E. cloacae* SBP-8. The *E. cloacae* SBP-8-infected worms also displayed compromised reproductive behaviour. Hence, we monitored the expression of the reproductive genes like *let-23* and *lin-29* required for vulva development and egg laying, respectively. The expression of *let-23* and *lin-29* was significantly high at 12 h but decreased gradually, and at 48 h, it was less than or equal to that of the uninfected worms [Fig.2.9 F and 2.9 G].

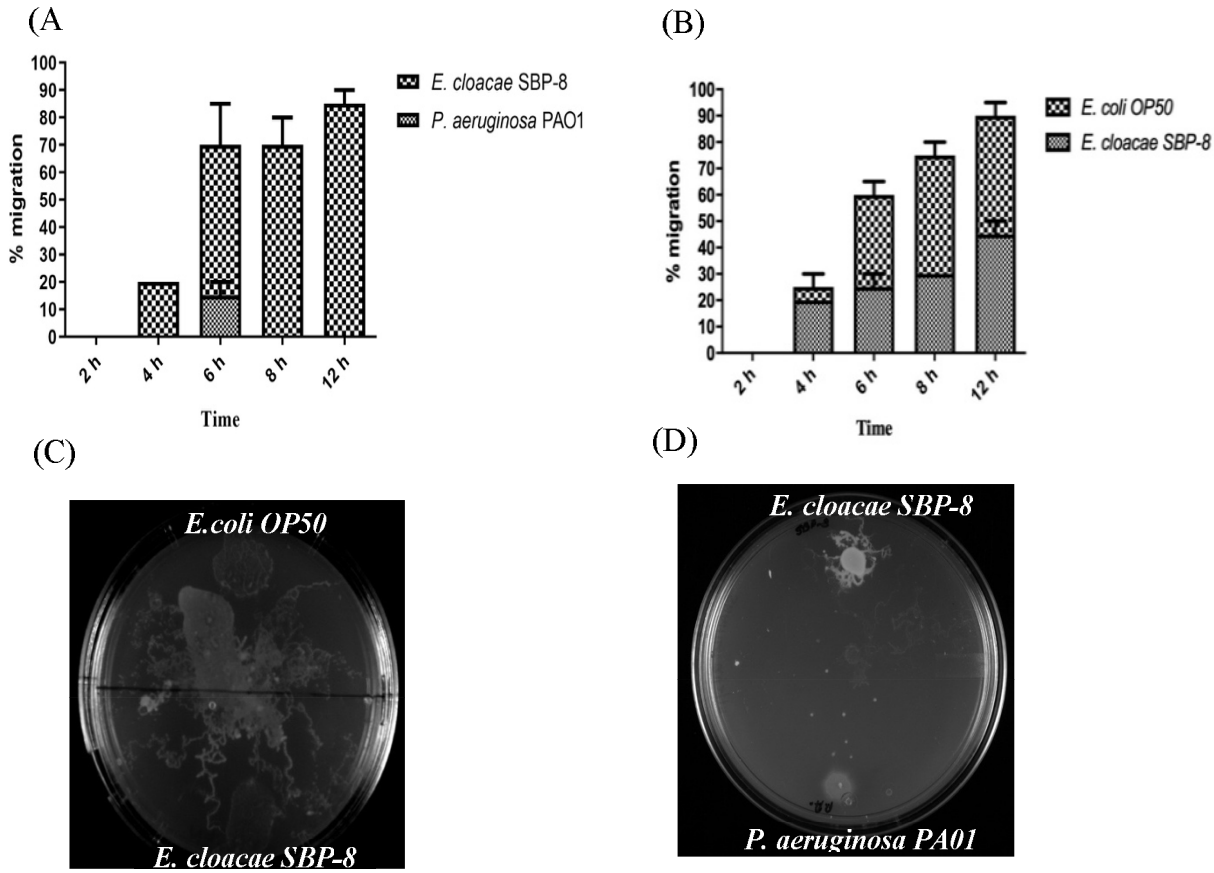


Fig. 2.7 *C. elegans* does not show any behavioural changes to minimize contact with *E. cloacae* SBP-8. Ten worms were placed at the center of a plate equidistant from each bacterial spot and the percentage of worms migrated to each spot was calculated every 2 h. Percentage migration of worms toward *E. cloacae* SBP-8 or *E. coli* OP50 (A and B), and *E. cloacae* SBP-8 or *P. aeruginosa* PAO1 (C and D) was calculated and plotted.

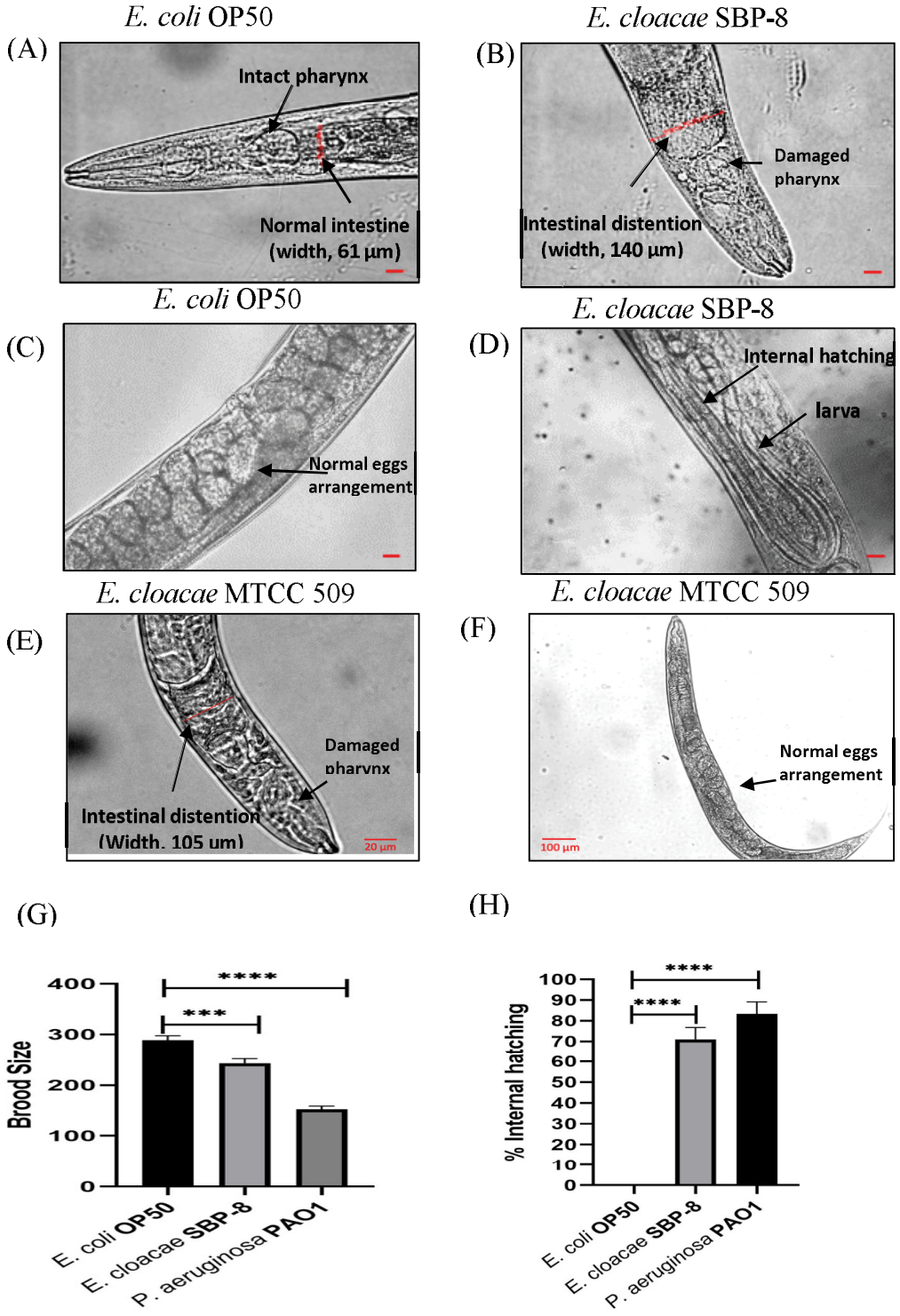


Fig.2.8. Physiological and reproductive defects in worms infected with *E. cloacae* SBP-8. *C. elegans* exposed to *E. cloacae* SBP-8 for 72 h were imaged under Nomarski microscope. *C. elegans* fed on *E. coli* OP50 showed intact pharynx with normal intestine (A) and normal egg development (C). *C. elegans* fed on *E. cloacae* SBP-8 had distended intestine (B) and internal hatching (D). *C. elegans* were exposed to *E. cloacae* (MTCC 509) for 48 h. The worms showed damaged pharynx with distention in intestine (E) while eggs arrangement was normal (F). Brood size of worms (n=10) fed with *E. coli* OP50 and *E. cloacae* SBP-8 and *P. aeruginosa* PAO1 (G). Percentage internal hatching in worms (n=10) fed with *E. coli* OP50 and *E. cloacae* SBP-8 and *P. aeruginosa* PAO1 72 hpi (H) was calculated and plotted. The scale bar: 20 µm.

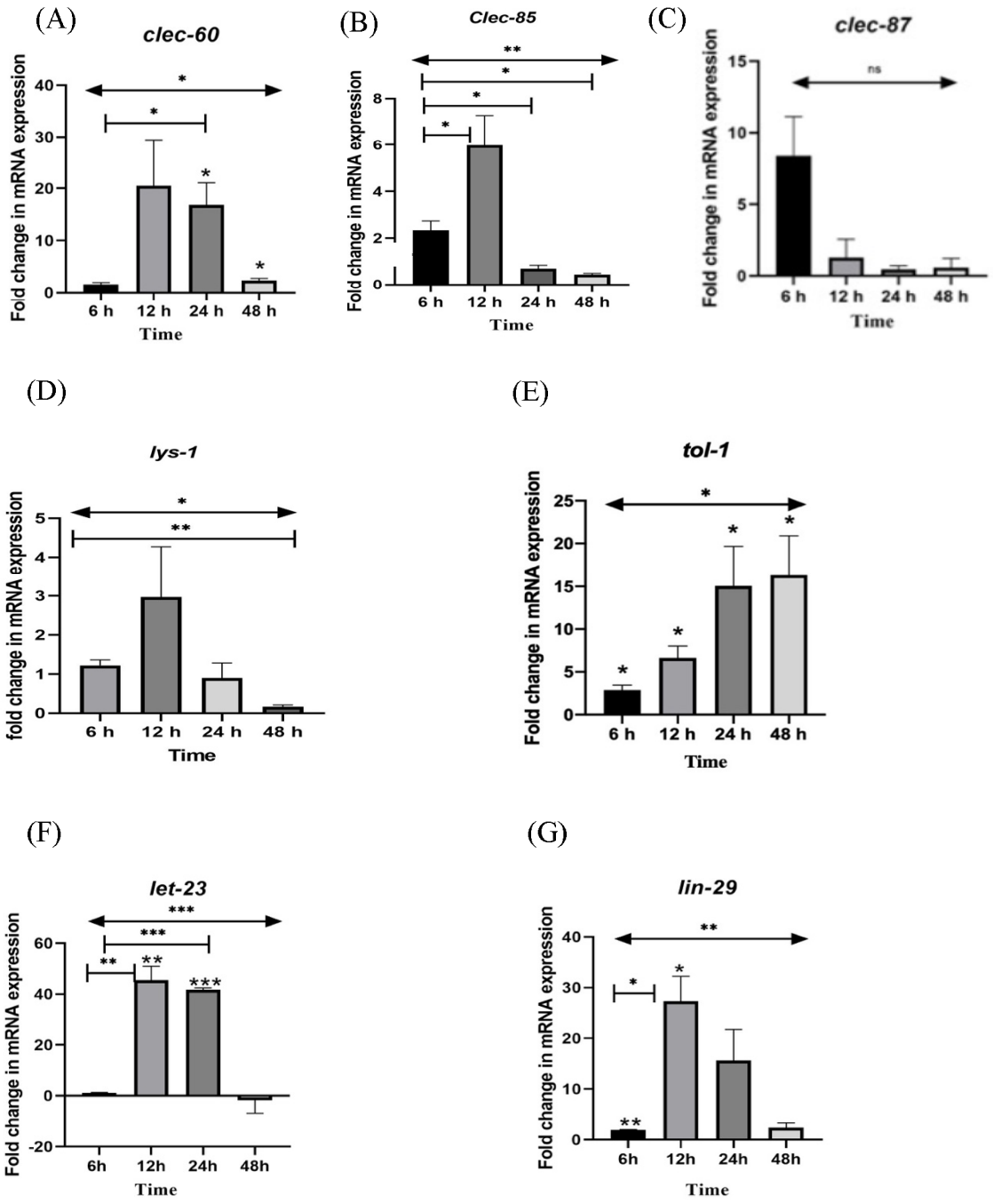


Fig.2.9. Relative expression of the antimicrobial and reproduction related genes in *E. cloacae* SBP-8 infected worms. Quantitative real time PCR was performed for the antimicrobial peptide genes *celc60*, *clec85*, *clec87*, *lys-1* (A-D), immune response gene *tol-1* (E), a zinc finger transcription factor involved in egg laying, *lin29* (F) and an EGF factor required for vulval development, *let23* (G). The relative expression of the genes was normalized over the housekeeping gene, *β-actin* was calculated and plotted. Level of significance was analyzed by one way ANOVA (↔) followed by Paired t-test (—).(*p<0.05)

2.4 Discussion

It is challenging to treat *E. cloacae* infections due to emerging antibiotic resistance and poorly studied virulence mechanisms [1]. Investigating its pathogenesis may unveil novel drug targets, former of which necessitates the development of a favourable model host. Detailed genome analysis of *E. cloacae* SBP-8 revealed potential pathogenic traits [147]. Therefore, we explored the pathogenic potential of *E. cloacae* SBP-8 using *C. elegans* as a model host. *E. cloacae* SBP-8 efficiently infected the nematode, ultimately killing it, however, to a lesser extent than *P. aeruginosa* PAO1 and *E. cloacae* (MTCC 509) (though not significantly different). Nevertheless, when such exogenous bacteria (from the soil) encounter immunocompromised patients, they may cause severe infection that are not of nosocomial origin. Though *E. cloacae* (MTCC 509) colonized the worms maximally, its effect on survival of worms was comparable to that of *E. cloacae* SBP-8.

E. cloacae SBP-8 gradually accumulated within the nematode gut, suggesting that the bacteria survived the pharyngeal grinding and host antimicrobials. By 72 h, it damaged the pharyngeal bulb and distended the intestine affecting nematode diet and nutrition, which further influences egg development and brood-size. *E. cloacae* (MTCC 509) infected worms showed damaged pharyngeal bulb and distended intestine as early as 48 h, but egg arrangement was normal at this time-point [Fig.2.8 E and F]. Similar to our observation, infections with pathogens like *Microbacterium nematophilum* [177], *K. pneumoniae* [178], *Xanthomonas oryzae* [175], *Vibrio alginolyticus* [171] and *Enterococcus faecalis* [179] or exposure to chemical stress and diet also caused similar reproductive defects [176, 180] including egg development and brood size, digestive tract alterations, metabolic profile, and life span of *C. elegans* [171,175]. Based on the study by Kamaldevi *et al.* where *K. pneumoniae* affected reproductive function by inhibiting *let-23* and *lin-29* [178], we hypothesize that *E. cloacae* SBP-8 infected worms manage the reproductive defects up to 24 h by increasing expression of *let-23,* a transmembrane tyrosine kinase that governs vulval development, and *lin-29,* a zinc finger transcriptional factor required for egg laying. Later (after 48 h), the worms cede to infection with a gradual decrease in gene expression resulting in compromised egg laying, internal hatching, and bagging of worms. These defects in reproduction could be linked to abnormal vulval development in *E. cloacae* SBP-8 infected worms, which is supported from the earlier reports where reproductive defects in worms caused vulva damage in *C. elegans* infected with *V. alginolyticus* [171] and *K. pneumoniae* [178].

No significant change was observed in the development and physiology of infected worms until 24 h suggesting that *E. cloacae* SBP-8 count at 24 h was insufficient to cause infection related defects. Nevertheless, the defense system was activated by this time point. The *tol-1* gene (a Toll homolog in *C. elegans*) was induced by 6 h and showed a progressive increase implying the detection of *E. cloacae* SBP-8 either by sensing the pathogen associated molecular patterns or virulence factors. This goes well according to the previous reported studies which inferred that a conserved Toll-like receptor is required for innate immunity in *C. elegans* against pathogens such as *P. aeruginosa* [181], *K. pneumoniae* [178] and *S. enterica* [182].

Tol-1 is Toll homolog in *C. elegans* governing its development and innate immunity, and also promoting pathogen avoidance behaviour [183, 184]. Thus, *tol-1* upregulation may be an effort by the nematodes to avoid the bacteria and mount a protective immune response against it. We propose that activation of *tol-1* could have induced *pmk*-1, a key regulator of p38 MAPK pathway, that regulates the expression of antimicrobial peptides like Lys-1 and C-type lectins. Some genes of the C-type lectin family, like *clec-85*, *clec-60,* etc. and the lysozyme genes like *lys-1* are reported as the key players of host innate immunity against different pathogens like *Shigella flexneri* [185], *V. cholerae* [186] and *Cronobacter sakazakii* [169] [165,166,187]. These genes were upregulated as early as 6 h post exposure to *E. cloacae* SBP-8 suggesting a counterattack by the nematodes. However, the expression of these genes gradually declined, indicating surrendering of the host to the pathogen attack as observed for pathogens like *Staphylococcus aureus* and *K. pneumoniae* in earlier studies [178,188]. Activation of *lys-1* during early hours followed by gradual repression indicates the role of p38 MAPK pathway against *E. cloacae* SBP-8 infection. Our result is corroborated with the findings which suggested that p38 MAPK and DAF pathways control the expression of antimicrobial peptides like *lys-1* under infection with pathogens like *K. pneumoniae* [178,189] and ETEC (Enterotoxigenic *E. coli*) [190].

The virulence factors of *E. cloacae* SBP-8 could be responsible for downregulating the antimicrobial genes either directly by inhibiting the p38 MAPK pathway or indirectly through some unknown pathway. Further studies are required to confirm the role of p38 MAPK pathway and the virulence factors, if any, in the gene regulation. Even though *tol-1*, required for pathogen avoidance behaviour, was upregulated, no avoidance behaviour was observed against *E. cloacae* SBP-8 for up-to 12 h. This suggests that the bacteria do not secrete any aversive compounds for the worms.

Accumulation of the *E. cloacae* SBP-8 in the gut of the worms triggered the production of ROS at 24 h. Such a pathogen-induced ROS production in the worms is regarded as a primitive form of septic shock, which is also indicative of the pathogenic potential of the bacteria [170]. The production of ROS is also linked to the killing of the worms by enteric pathogen, like *Salmonella* through primordial septic shock [170]. The production of ROS in infected worms could contribute to the reduction in lifespan of the worms. The induction of ROS may also be related to the production of thiol-activated toxins that induce ROS [34] or siderophores that damage the mitochondria [191,192]. The genome of *E. cloacae* SBP-8 does possess genes encoding a siderophore enterobactin which may be damaging to host mitochondria [147]. However, further experiments are needed to prove the siderophore production and its role under *in-vivo* conditions.

Induction of oxidative stress can cause nuclear damage leading to cell death primarily through programmed cell death (apoptosis) [20]. The ability of *E. cloacae* to induce apoptosis *via* DNA fragmentation was demonstrated against HEp-2 intestinal cells [173]. In our study, intensified nuclear staining of intestinal epithelial cells and germline cells in infected worms support induction of DNA damage by *E. cloacae* (both soil and clinical isolate). The damage to the germline may explain the observed reproductive defect in *E. cloacae* SBP-8 infected worms. Further, we also observed the fragmentation of nuclei especially in the intestinal cells indicating cell injury and apoptosis. Here, we highlight that the extent of cell-damage was similar in *E. cloacae* SBP-8 and *E. cloacae* (MTCC 509) infected worms, in fact a bit higher on *E. cloacae* SBP-8 infection. This could be one of the reasons why we did not observe significant difference in the survival of *E. cloacae* SBP-8 and *E. cloacae* (MTCC 509) infected worms though these bacteria differed in their colonization efficiency (a 10 fold difference).

To summarize, we prove the pathogenic potential of *E. cloacae* SBP-8 and demonstrated its colonization in *C. elegans* affecting its physiology, reproduction, and survival. It activated the innate immune system of the nematode through the Tol-1 pathway. We hypothesize the role of toll dependent MAP kinase pathway in *E. cloacae* infection. Finally, we conclude that *C. elegans* can be used as favourable a model host to study host pathogen interaction of *E. cloacae*.