ABSTRACT

Over recent decades, *Enterobacter cloacae* have received clinical significance and emerged as nosocomial pathogen. *E. cloacae* reported to possess different virulence factors that could potentially influence its pathogenesis. Generally, the *E. cloacae* infections are of endogenous origin occurring in immunocompromised patients. However, the pathogenic potential of exogenous isolates are not explored. Moreover, the mechanisms of its pathogenicity also remain elusive, possibly due to the absence of established model hosts. Thus, we explored the utility of *Caenorhabditis elegans* as a model host to test the pathogenicity of *E. cloacae* SBP-8, a soil isolate. *E. cloacae* SBP-8 progressively colonized the intestine of *C. elegans*. It induced cell death (as assessed through DNA damage), reproductive defect and caused reduction of lifespan, comparable to a clinical isolate, *E. cloacae* (MTCC 509). Observation with Nomarski microscope revealed significant anterior pharyngeal distention and altered egg arrangement with internal egg hatching in 70% infected worms. The internal egg hatching was observed as early as 48 h post infection. *E. cloacae* SBP-8 infection reduced the brood size by 16%. A 2′,7′-dichlorodihydrofluorescein diacetate staining confirmed the 10-fold induction of reactive oxygen species implicating either mitochondrial damage or septic shock in infected worms. Expression analysis through RT-PCR indicated stimulation of immune response by *E. cloacae* SBP-8 in worms by upregulating *tol-1*, a Toll-like receptor, within 6 h of exposure. During the initial phase of infection (up to 24 h) the nematodes exhibited protective immune response by upregulating antimicrobial peptide genes, *lys-1*, *clec-60*, *clec-85,* and *clec-87*. However, these genes were downregulated at later hours (48 h), indicating the nematodes surrendered to the infection. A similar trend was observed for reproductive genes (*lin-29* and *let-23*), suggesting a struggle to maintain functional reproduction by the nematodes. These results clearly demonstrated that *E. cloacae* SBP-8 colonization in *C. elegans* affecting its physiology, reproduction, and survival.

In order to investigate the systemic dissemination of *E. cloacae* SBP-8, which is not possible with *C. elegans*,we used mouse as the model host. Our study showed that *E. cloacae* SBP-8 progressively colonized the reticuloendothelial organs when infected with intra-peritoneal and intra-gastric route. Bacterial numbers increased from 6 to 24 hour post-infection (hpi) in organs like liver, lungs, spleen, and kidney when infected intra-peritoneally. Colonization of *E. cloacae* SBP-8 in these organs was confirmed by *ERIC*-PCR DNA fingerprinting Enterobacterial Repetitive Intergenic Consensus) . The infection induced the recruitement of neutrophils and other innate immune cells in kidneys and lungs. However,

congestion, interalveolar septa thickening with mild fibrosis, and infiltration of immune cells were observed only in the lungs. Biochemical serum analysis revealed increased levels of bile, AST/SGOT, AST/SGP in infected mice indicating liver damage. The bacterial infection induced immune response by systemic production of proinflammatory cytokines such as IL-1α, IL-12, IFN-γ, TNF-α, GM-CSF and IL-17A as well as anti-inflammatory cytokines such as IL-4 and IL-10. The given results of pro-inflammatory and anti-inflammatory cytokines suggested the activation of cell mediated immunity and host response to control the infection-induced inflammation, respectively. All the above-mentioned parameters imply the severity of infection. Further, the intarcellular survival assay demonstrated that *E. cloacae* SBP-8 survived inside the macrophages (for upto at least 16 h), invaded and multiplied (2.5-fold) within the epithelial cells. Hence, *E. cloacae* SBP-8 appears to be an intracellular pathogen which probably disseminates by surviving within the phagocytic cells. The colocalization studies employing confocal microscopy showed around 40% reduction in lysosomal fusion of *E. cloacae* SBP-8 from early to late time point (2 to 16 h) of infection in RAW 264.7, macrophage cell line. On the contray, most of the *E. cloacae* SBP-8 inhibited lysosomal fusion in HT-29, intestinal epithelial cell line

After elucidating the pathogenic potential of a rhizospheric soil isolate *E. cloacae* SBP-8 using *C. elegans* and a murine model, we systematically characterized and investigated the role of type six secretion system (T6SS) of *E. cloacae* SBP-8 in eukaryotic and bacterial cell interaction. Analysis of genome sequence of *E. cloacae* SBP-8 (Accession No: NAIMCC-B-02025) revealed the presence of all essential genes required for a functional T6SS. It has one complete T6SS gene cluster which is similar to one of the clusters (T6SS-1) of *E. cloacae* ATCC 13047 (a clinical isolate). The functionality of T6SS was confirmed by secretome analysis employing SDS-PAGE followed by mass spectroscopic detection ofan Hcp effector protein.. As the secretion of Hcp depends on ClpV, an ATPase, we knocked out *clpV* and used the strain for further study. Loss of Hcp protein in the secretome of *E. cloacae* SBP-*8ΔclpV* inferred the presence of functional T6SS. Infection with *E. cloacae* SBP-*8ΔclpV* did not show any significant change in the life span and rate of colonization in infected *C. elegans* as compared to its wild type counterpart. Similarly, no major significant change was observed in expression profiling of antimicrobial genes (*clec-60*, *clec-85*, *clec-87* and *lys-1)* and toll like receptor *(toll-1)* gene, involved in stimulating immune response against pathogen. Further, the mutant did not exhibit significant difference in the ability to invade and proliferate in intestinal cells and phagocytosis by macrophages. The above results ruled out the role of T6SS in

pathogenicity and invasion in host organism. However, the competition assay showing decreased inhibition of *E. coli* growth by mutant (*E. cloacae* SBP-8*ΔclpV*)demonstrated that T6SS is required for contact-dependent inhibition of competing bacteria.

Overall, this study demonstrated that the exogenous isolate of of *E. cloacae* SBP-8 is pathogenic as tested using model hosts namely *C. elegans* and mice. The T6SS, though does not play significant role in pathogenesis, is required for interbacterial competition.