Chapter-1

Introduction

1.1 Introduction

Species of *Enterobacter cloacae* complex (ECC) are Gram negative, rod-shaped and facultative anaerobes belonging to the *Enterobacteriaceae* family. These bacteria are commonly found in or on plants, insects, and many environmental sources such as water, sewage, or in soil. It is not only saprophytic in the environment but also a member of the human gut microbiome [1]. In recent decades, only *E. cloacae* in ECC have acquired clinical importance and emerged as a human pathogen [1]. They have become one of the leading causes of nosocomial infections (hospital-acquired infections) around the globe, reportedly accounting for 5% to 10 % of intensive care unit (ICU) infections [1]. Currently, ECC comprises six different species *Enterobacter aerogene, Enterobacter cloacae*, *Enterobacter hormaechei, Enterobacter kobei, Enterobacter ludwigii*, and *Enterobacter nimipressuralis.* Species of ECC are clubbed together due to the high percentage of genetic relatedness ranging from 61 to 67 % [1]. The taxonomy of the *E. cloacae* complex is mainly based on whole-genome DNA-DNA hybridizations and phenotypic characteristics [2].

Lately, *E. cloacae* has emerged as a troublesome pathogen for the healthcare institution. This well-known nosocomial pathogen contributes to respiratory tract infections, lower-abdominal infections, bacteremia, endocarditis, osteomyelitis, septic arthritis, and skin/soft tissue infections [1]. Furthermore, its emerging clinical significance and ability to acquire carbapenem resistance adds it to the pathogen priority list by WHO [3]. The present chapter reviews various aspects, including prevalence, pathogenicity, and pathogenic arsenals essential for pathogenicity and competition as well as antibiotic resistance of *E. cloacae.*

1.2 Clinical infestations and outbreaks

Over recent decades, *E. cloacae* have received clinical significance and emerged as nosocomial pathogens in ICU and primarily affect patients hospitalized for a prolonged period during their vulnerable age groups. It is best known as a human opportunistic pathogen frequently associated with hospital-acquired infections such as urinary tract infections, respiratory tract infections, meningitis, which primarily afflict immunocompromised patients [4]. As shown in Fig.1.1, *E. cloacae* is also responsible for infections at other systemic sites. Some of the genotypes of *E. cloacae* have previously been detected in clinical specimens, specifically in throat swab, sputum, rectal swab, and urine [5, 6]. Some of the clinical isolates of *E. cloacae* has been frequently isolated from the blood [7, 8] and are known to undergo systemic dissemination, i.e., from the blood it disseminates to the intestine and kidney, and cause bacteremia [9].

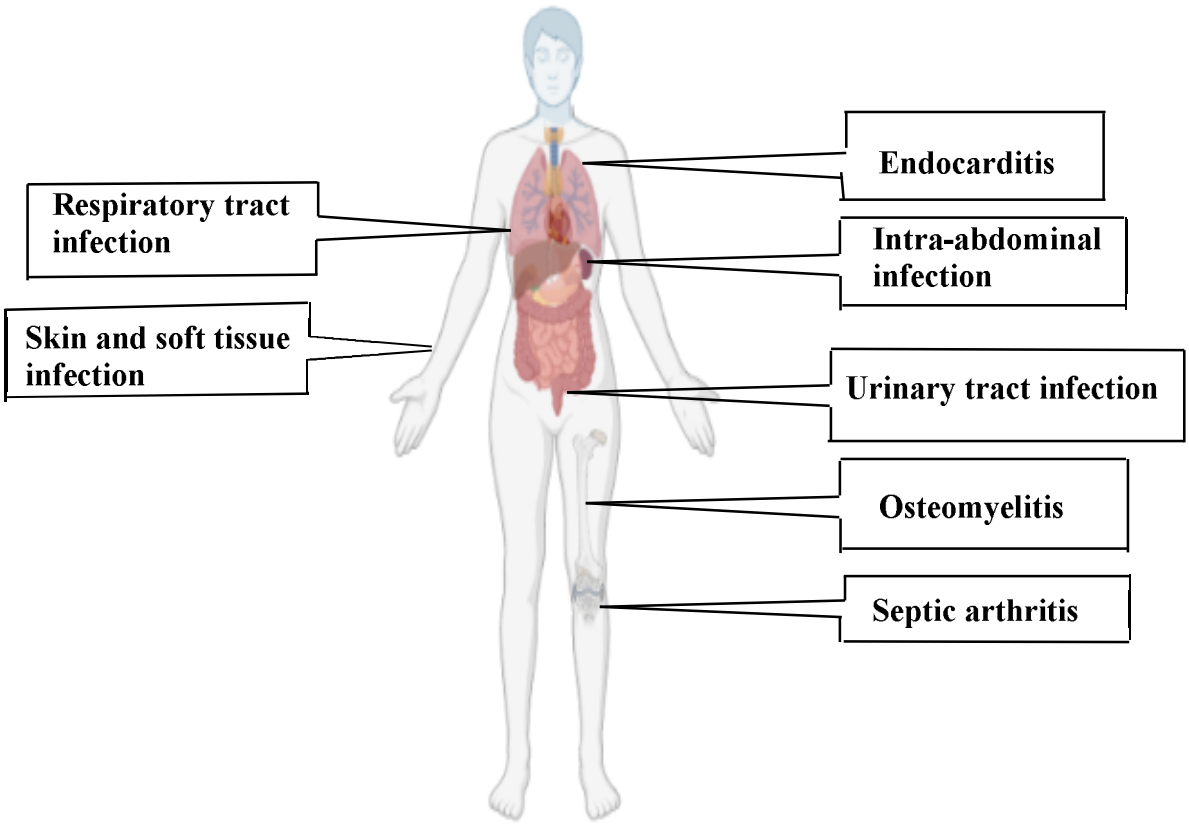

Fig.1.1. Schematic depiction of infections caused by *E. cloacae* in humans. *E. cloacae* is known to typically infect the respiratory and urogenital tract, but complications can lead to serious health issues, even leading to death. In addition, it has been found to cause endocarditis, intra-abdominal infection, osteomyelitis, septic arthritis and skin infections.

The outbreaks of *E. cloacae* infections is usually associated with its contamination in various medical, intravenous and, other surgical devices [10]. The nosocomial complications caused by *E. cloacae* include 5% sepsis, 4% urinary tract infections, 5% pneumonia, and 10% postsurgical peritonitis cases [1]. Over a decade, several outbreaks of *E. cloacae* have been reported in neonatal ICU [11,12]. In 1998, van Nierop *et al.* reported an outbreak of *E. cloacae* resulting in nine deaths in a neonatal ICU of a provincial hospital in South Africa [13]. In 2003, Kuboyama *et al.* reported three outbreaks in neonatal ICU of women hospital, Brazil with a mortality rate of 34% in 42 systemic infections of *E. cloacae*. [14]. The pathogenicity of *E. cloacae* is not restricted to humans, it is also known to adversely affect plants and cause various diseases in crops. It is reported as an onion pathogen and causes soft rot disease on dragon fruit [15]. It was reported as a causative agent for mung bean sprout diseases, coconut root wilt diseases and grey kernel diseases in macadamia [16-20]. It causes two additional plant diseases that occur in Hawaii, internal yellowing of papaya fruit [21] and rhizome rot of ginger [22].

1.3 Diagnosis and treatment of *E. cloacae* infections

The routine identification of *Enterobacter* spp. has been performed using commercial systems like API® 20E or Vitek®2, by evaluating their phenotypic characteristic. *E. cloacae* can unambiguously be identified by molecular techniques such as PCR amplification of *16S rRNA* gene, the *oriC* locus, and *gyrB* [23]. Furthermore, Genotyping of *hsp60*, *rpoB*, and *ERIC* appeared to be a promising novel method to differentiate *E. cloacae* from other species of *E. cloacae* complex [1]. In 2012, mass spectrometry was used to identify the *E. cloacae* complex, but it failed to differentiate the species within the group. Later on, a German team implemented a combination of MALDI-TOF MS and *E. cloacae* specific duplex real-time PCR to differentiate all the six species of the *E. cloacae* complex [24].

E. cloacae infections are usually treated using broad-spectrum antibiotics such as carbapenems, beta-lactams, along with beta-lactamase inhibitors, fluoroquinolones, aminoglycosides, and sulfamethoxazole/trimethoprim [24]. However, with the emergence of antibiotic resistance in various isolates, treating *E. cloacae* infections has become challenging. The majority of the *E. cloacae* strains show resistance to the first and second generation of cephalosporins. However, treatment with the third generation of cephalosporins can be effective. It can lead to multi-resistant infections due to reduced drug permeation through the outer cell membrane [24]. Therefore, fourth-generation cephalosporins (Fourth generation) are considered an acceptable treatment option. It is relatively stable among Amp-C beta- lactamase and have extra ammonium group, which allows them to rapidly penetrate through the outer

membrane [24]. However, all classes of cephalosporins are ineffective against ESBL (Extended-spectrum beta-lactamase). Earlier, carbapenems were reported as the most potent treatment for *Enterobacter* infection. Imipenem and meropenem were shown effective against *E. cloacae* [24]. Nevertheless, resistance is increasing in recent years due to horizontal gene transfer of the carbapenem resistance gene. Antibiotics such as polymyxins, fosfomycin, and tigecycline are considered as a possible treatment for carbapenem-resistant *Enterobacter* (CRE). Instead of monotherapy, a combination of antibiotics shows more effectiveness against *E. cloacae* infections.

1.4 Emergence of antibiotic resistance in *E. cloacae*

Antibiotic resistance is one of the major threats to public health. New resistance mechanism is being constantly evolving in microorganisms, hampering an effective treatment of many infections. It also increases the health care cost compared to treating non-resistant infections [25]. The prevalence of *E. cloacae* has increased dramatically due to the acquired resistance to various antibiotics and its extended-spectrum. It has become the third broad spectrum *Enterobacteriaceae* species involved in nosocomial infections after *Escherichia coli* and *Klebsiella pneumoniae* owing to the dissemination of β-lactamase, especially ESBL and carbapenemases [26, 27]. In general, most of the isolates of *E. cloacae* are susceptible to sulfamethoxazole/trimethoprim, chloramphenicol, tetracyclines, fluoroquinolones, aminoglycosides, and carbapenems but are intrinsically resistant to penicillin, ampicillin, amoxicillin, amoxicillin-clavulanate, and first-second generation cephalosporins [1]. However, many clinical and environmental isolates have developed resistance to the majority of the antibiotic classes. The production of β-lactamase is a major mechanism responsible for the resistance against β-lactam class of antibiotics. Till now, four molecular amber classes of β-lactamase were identified, and all four are found in most of the *E. cloacae* isolates: class A (penicillinases), class B (metalloenzymes), class C (cephalosporinases) and class D (oxacillinases).

E. cloacae is naturally resistant to various antibiotics due to the low permeability of its outer membrane [24]. Porins (water-filled channels) present in the outer membrane allow molecules, including antibiotics, to pass through. Porins such as OmpC and OmpF in *E. coli* allow entry of various non-specific large and charged molecules like bile salts and antibiotics. [28]. Similar to OmpC and OmpF of *E. coli, E. cloacae* has Omp35 and Omp36 porins that may perform similar functions. Indeed, mutations or loss of Omp35 and Omp36 inactivating these porins led to carbapenem resistance by blocking their uptake [29]. Furthermore, *Enterobacter* spp. Possess efflux pumps that export antibiotics such as tetracycline,

chloramphenicol and fluoroquinolones thereby enhancing antibiotic resistance [30]. Around 40 % of all multi-drug resistant clinical strains have an active efflux pump [31]. AcrAB-Tol efflux pump found in *Enterobacter* spp., majorly in *E. cloacae,* can flux out about 80-90% of norfloxacin in initial 10-15 min [31]. Several *E. cloacae* isolates has been reported for the presence of RND (Resistance-nodulation-cell division) and MATE (multi antimicrobial extrusion protein) families of efflux pumps [32]. The combination of porin-mediated slow diffusion of antibiotics across the membrane and its active export outside the cell *via* efflux pumps results in the high level of intrinsic resistance of *E. cloacae* to antibiotics.

The development of narrow as well as multi-drug resistance in *E. cloacae* can result due to horizontal gene transfer, long-term antibiotic treatment, inappropriate use of antibiotics, and other reasons. Fig.1.2 shows the antibiotic emergence of *E. cloacae* outbreak in France. The development of antibiotic resistance can be a major challenge in treating *E. cloacae* infections. Therefore, it is high time to investigate mechanisms by which *E. cloacae* exert its pathogenicity using an appropriate animal model organism so that the specific therapeutic targets can be identified to eliminate infections caused by *E. cloacae*.

1.5 Virulence determinants of *E. cloacae*

Despite the relevance of *E. cloacae* as an opportunistic pathogen, very little is known about its pathogenicity mechanism and the factors influencing its virulence. The mechanism of *E. cloacae* pathogenicity appears to be complex and multifactorial with the presence of different putative virulence factors whose role is still not clear in the development of disease. The genome analysis and molecular studies conducted by various research groups have implicated the role of siderophore, biofilm, toxins, hemolysins, resistance to antimicrobials, and secretion system [Fig1.3] in the pathogenesis of a given pathogen. [5, 34-].

Siderophores:The ability of the microorganism to assimilate iron through chelators is important for the bacterial pathogenesis and establishment of infection. Many microbes have evolved complex strategies for harvesting and stealing available iron. Among these, siderophores production and excretion is the most common. Till date, many siderophores have been identified that differ in their structure and are classified as catecholate, carboxylate, hydroxamate, phenolate, and mixed-ligand siderophore [37]. Generally, siderophore encoding genes were found in bacteria like *Pseudomonas, Shigella, Salmonella,* and *Yersinia* spp. [38].

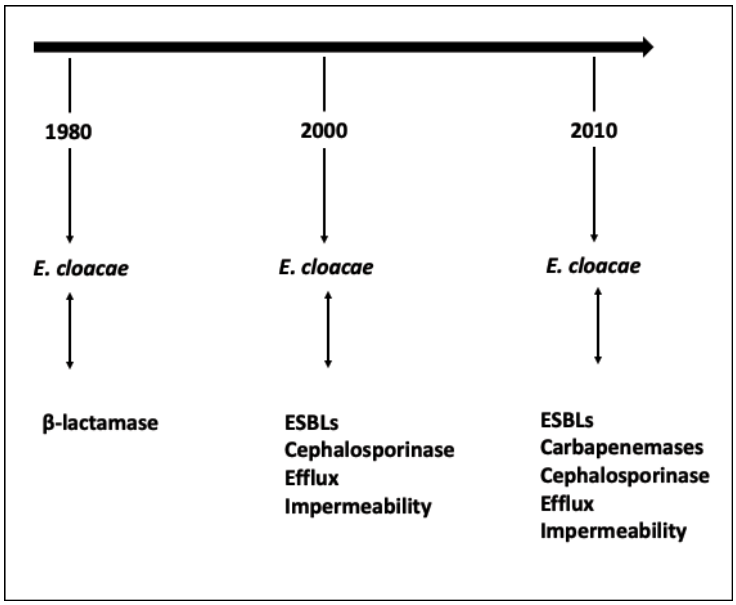

Fig.1.2 Schematic illustration of antibiotic emergence of *E. cloacae* outbreak in France [33]. In the last few decades, inappropriate use of antibiotics such as β-lactam, ESBLs, cephalosporin, and carbapenems against *E. cloacae* lead to the development of resistance mechanisms like the production of antibiotic degrading enzymes, efflux pumps, and reduced permeability to gain resistance against the above antibiotics.

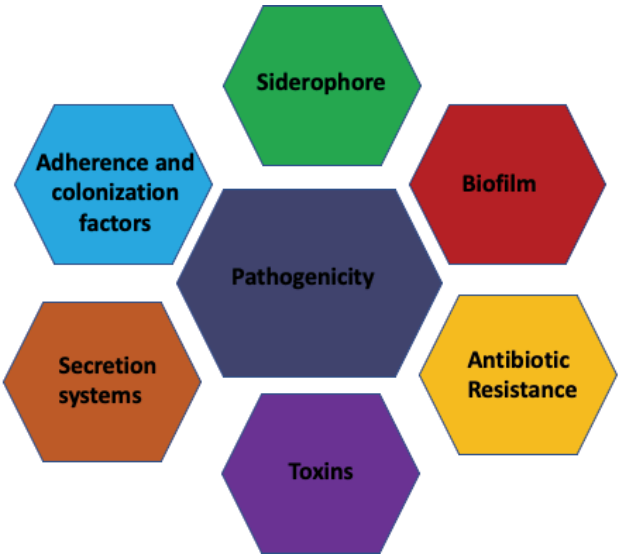

Fig.1.3 Diagrammatic representation of possible virulence factors involved in the pathogenicity of *E. cloacae*. This bacterium has both cell-associated (like the flagellum, LPS involved in adherence and colonization) and secreted virulence factors (secreted toxins, degrading enzymes, and siderophores). Its ability to form biofilm, transfer effector molecules to the target cells *via* secretion system, and antibiotic resistance has crucial role in pathogenicity.

In many cases, the genes encoding for siderophore biosynthesis are located on pathogenicity islands like the genes for aerobactin production are found in SH-1 pathogenic islands of *Shigella flexneri*[38], genes for yersiniabactin-mediated iron uptake on HPI (high-pathogenicity island) of *Yersinia* spp. and other iron transport proteins on SPI1 of *Salmonella enterica* subspecies *enterica* serovar Typhimurium [39]. The presence of these genes at pathogenic islands establishes a correlation of siderophore with bacterial virulence. Siderophore-like enterobactin and aerobactin synthesis were also reported in *E. cloacae* [40]. Both enterobactin and aerobactin have been proven as an important virulence factors in the clinical isolates of *E. coli* and other bacteria [41]. However, their role in the pathogenicity of *E. cloacae* is yet to be addressed. The presence of these siderophore-producing pathways in *E. cloacae*, may have a crucial role in its pathogenicity.

Biofilm: Bacteria form biofilm as a part of their survival mechanisms allowing them to survive in a hostile environment. Bacteria within the biofilm can withstand host immune response and are less susceptible to antibiotics. Bacterial biofilm is recognized as an important cause of various human infections, such as pneumonia in cystic fibrosis, endocarditis, and prosthetic devices [36]. One of the important component of biofilm is curli-fimbriae protein. Curli is reported to play a crucial role in host colonization, innate response activation, and cell invasion [36, 42-43]. The expression of *csgA* and *csgD* encoding the main subunit of curli and the operator of an operon showed a significant correlation with biofilm formation [162]. Recently, it has been shown that 78% of clinical isolates of *E. cloacae* (n=11) has on operon named *csgBA,* coding for curli [36].

Toxins: One of the important virulence factors required for pathogenesis is the production of toxins. *E. cloacae* secrete a variety of toxins, including enterotoxins and α-haemolysin. Enterotoxins assemble at the cell membrane forming a pore leading to cytotoxicity. While α-haemolysin bind to the outer membrane thereby oligomerizing to form water-filled channels [44]. Barnes *et al.* isolated thiol-activated pore-forming cytotoxins from *E. cloacae* that exerted a harmful effect on erythrocytes and leukocytes [36]. Later on, Paraje *et al* discovered that a high concentration of this toxin caused lysis of leukocytes before triggering apoptosis [164]. It was also demonstrated that thiol-activated cytotoxins of *E. cloacae* were required for the invasion, colonization, and destruction of host immune cells [5, 36]. Clinical strains of *E. cloacae* were reported to have *Stx-1*, encoding a shiga toxin 1 that plays an important role in the invasion of *Shigella dysenteriae* during infection [45]. Paraje *et al.* suggested that cytotoxic activity of *E. cloacae* was associated with a toxin of 13.3Kda [46]. Aerobactin and mannose-

sensitive hemagglutinin produced by *E. cloacae* are implicated to induce apoptosis in Hep-2 cells [5, 34, 47]. Thus, the bacterial ability to produce toxins is a general indicator of its virulence.

Efflux pump: Perez *et al* revealed that the deletion of *acrA* and *tolC,* which encode for AcrAB-TolC efflux pump, significantly affects its antimicrobial resistance [48]. In addition to its role in antimicrobial drug resistance, the efflux pumps like AcrAB-TolC system is also essential for systemic colonization in mice [48].

Secretion system: Several Gram positive and negative pathogenic bacteria employ complex machinery for colonization and delivery of virulence factors that thereby modulating host response. These machineries, called secretion systems, are a combination of different proteins with varied functions. The secretion systems are also known to enable pathogenic bacteria to destroy phagocytes and epithelial cells, facilitating their spread within the host [49, 50]. It has been reported that many pathogenic bacteria, such as *Francisella tularensisi* [51], *Burkholderia mallei* [52], *Campylobacter jejuni* [53], and *Yersinia enterocolitica* [54] utilize secretion systems to target eukaryotic cells and establish infection. The comparative genomic analysis of clinical *E. cloacae* conducted by Krzyminska *et al.* demonstrated 27% of these strains have T3SS [55]. Hence, the presence of these secretion systems is indicative of pathogenic potential and can be considered as a potential therapeutic target [56]. The following section highlights various secretion systems and explains type six secretion system (T6SS) in detail.

1.6 Bacterial Secretion systems

The function of the secretion system is to secrete proteins that may help establishment in a given niche and promote microbe-host interaction during commensal, mutualistic, or parasitic interactions [57]. The role of the secretion systems in pathogenesis ranges from intoxicating target cells to enhancing invasion on eukaryotic cells and/or scavenging resources in an environmental niche. Till date, nine types of bacterial secretion systems (T1SS to T9SS) have been identified. [58, 59]. All secretion systems: T1SS to T9SS are found in Gram negative bacteria except T7SS, which is specifically present in Gram positive bacteria like *Mycobacteria* and *Corynebacteria.* These secretions systems are an assembly of 1 to more than 20 different proteins depending upon the type and functionality of the secretion system.

Many pathogenic bacteria transfer effector molecules across the cell envelope employing a secretion system in one or two steps. T1SS, T3SS, T4SS, or type 6 (T6SS) are used to translocate effector molecules across an inner and outer membrane in a single step. On

the other hand, T2SS and T5SS export effector molecules first to periplasmic space and then across the outer membrane. T7SS of Gram-positive bacteria translocate proteins across both the membranes and the cell wall, but it is still unclear whether this translocation is a single-step or a multistep process. Recent studies support the fact that the T1SS plays an important role in plant-associated microbe-host interaction. T1SS contains three important components, i.e., ATP binding cassette (ABC) transporters, membrane fusion protein (MFP) and, outer membrane factors (OMFs) [60]. Some additional structural protein components are also required for the complete assembly of machinery across both membranes. T2SS operates in a two-step process and is only found in Gram negative proteobacteria phylum [61]. It is identified that T2SS is required for the virulence of human pathogens like *Vibrio cholerae*, enterotoxigenic *E. coli* and, some of the plant pathogens like *Ralstonia solanacearum* and *Xanthomonas campestris* pv. Campestris (Xcc) [61]. T3SS is also found in Gram negative bacteria that show interaction with both plant and animal cells as a mutualist or as a pathogen [62]. The apparatus of T3SS is termed as injectosome, which functions to deliver the effector molecules into the host cells and other bacteria. It is mainly involved in pathogenicity and colonization in the host. T3SS is also important for the beneficial association with the host, like the rhizobial nitrogen-fixing mutualists [62]. T4SS is more complex and unique in comparison to other secretion systems due to its ability to transfer nucleic acids in addition to proteinaceous molecules into both bacterial and plant cells [63]. T4SS is predominantly found in pathogenic bacteria [64] indicating presence of T4SS as a hallmark of pathogenicity. Among all the secretion systems found in Gram negative bacteria, T5SS is most widely distributed and generally associated with virulence [65]. T5SS mainly secrete adhesion molecules required for host cell adhesion, the initial step of bacterial infection. Bacteria utilize T7SS to transport protein across their inner and mycomembrane; a lipid-rich cell envelop similar to outer membrane. T7SS was reported to play a variety of roles in bacterial physiology and pathology [66]. T8SS of Gram-negative bacteria, also referred as curli biogenesis pathway, is responsible for the controlled formation and secretion of the curli protein, the functional amyloid [36]. The most recently identified type IX secretion system is exclusively present in species of phylum *Bacteroidetes*. It is responsible for providing gliding motility and is also involved in bacterial virulence [67]. T6SS is also one of the recently identified secretion systems. Its role in colonization and pathogenicity has been described in *V. cholerae* [59], *P. aeruginosa* [68], *B. pseudomallei* [69]. As the role of T6SS has attracted attention concerning its involvement in the pathogenicity of bacteria and complexity of structure, the present work also aims to investigate the role of T6SS in the pathogenicity and interbacterial competition of *E. cloacae*.

1.7 Type VI secretion system (T6SS)

Pukatzi *et al.* in 2006 reported an interaction mechanism between *V. chlorae* and its host cells through a contact-dependent transport of proteins *via* a proteinaceous apparatus known as Type VI secretion system (T6SS) [59]. This nano-machinery delivers effectors molecules directly into the opponent cells in a one-step process [70]. *In-silico* analysis of around 500 bacterial genomes revealed the presence of T6SS in 92 bacterial genomes [71]. It has been predicted that around 25% of Gram-negative sequenced bacteria have this nano-machine, making it the most widespread secretion system [72]. The role of T6SS in pathogenicity was initially reported in proteobacteria such as *V. cholerae* [73] and *P. aeruginosa* [74]. These bacteria target eukaryotic cells by delivering effectors and toxins such as DNase, phospholipase, and NAD^+ hydrolase, directly into the target cell *via* a syringe-like structure [75, 76]. Then, in 2010 "anti-bacterial" function of T6SS was also reported initially in *P. aeruginosa* and later in other bacteria like *V. cholerae* and *Serratia marcescens* [77]. Bacteria like *P. aeruginosa* [78] and *V. cholerae* [79] display both anti-bacterial and anti-eukaryotic activity through T6SS. Bacterial killing mediated by T6SS and its toxins can also result in acquired antibiotic resistance in predatory (killer) bacteria by gaining exogenous DNA of prey (killed) bacteria [80]. The T6SS containing bacteria (predator) also encodes cognate immunity proteins, which provide immunity against its own toxins (effector molecules) while such proteins are absent in the opponent cell [78]. Bioinformatics and structural analysis have provided strong evidence that the structural components of T6SS show homology with T4 bacteriophage tail [73, 81]. T6SS plays a significant role in reshaping bacterial communities and pathogenesis directly or indirectly.

1.8 Genetic organization of T6SS

Typically, this system is encoded by compact clusters of 13 core conserved genes required for the basic and functional structure of T6SS (TssA-M). The genetic organization of genes comprising T6SS of some clinical isolates is depicted in Fig.1.4. The T6SS genes are usually located in compact clusters within pathogenicity islands of the bacteria. For example, genes of T6SS of *S. enterica* subsp. *enterica* serovar Typhimurium, *P. aeruginosa,* EaEc (*Enteroaggregative E. coli*), and *F. tularensis* are found in respective pathogenic islands, *Salmonella* genomic island (SGI) [82], HSI (Hcp-secretion island) [78], *pheU* [83], and FPI (*Francisella* pathogenicity island) [51]. Genetic composition, including the G + C content of T6SS genes, is similar to the rest of chromosome, indicating these systems are not recently acquired by horizontal gene transfer [84].

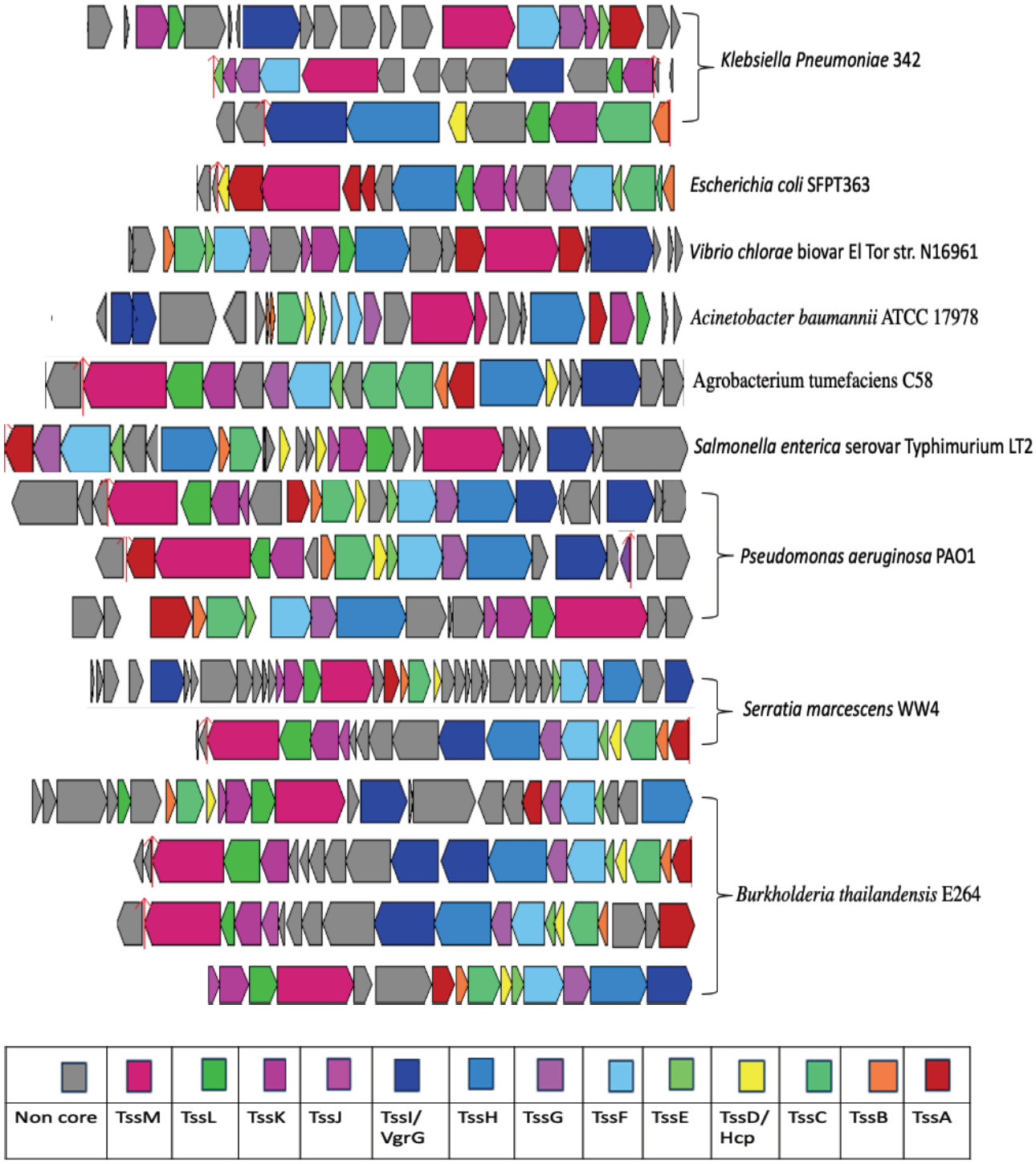

Fig.1.4. Genetic organization and T6SS-encoding gene clusters in different pathogenic bacteria. Genes are plotted as an arrow. The relative length of the arrow represents gene size. The forward arrow indicates genes are present on the positive strand, and the reverse arrow indicates genes on the negative strand**.** Components conserved across many T6SSs are represented in the same color. Color code for each gene is as provided as a key at the bottom. The 13 highly conserved core components are indicated by letter according to the Tss nomenclature.

Generally, most of the bacteria have a single cluster of T6SS like *V. chlorae* [84], whereas multiple copies of cluster are reported in several species. Interestingly, the functionality of these additional clusters doesn't seem to be redundant. For instance, *P. aeruginosa* have three T6SSs clusters (H1, H2, and H3 – T6SS) [84] of which H1 cluster is involved only in the bacterial killing while H2 and H3 T6SS target both prokaryotic and eukaryotic cells [68]. Similarly, *B. thailandensis* have five T6SSs, T6SS1-5. T6SS-1 is required for bacterial antagonism, while T6SS-5 is required for intramacrophage proliferation., Other clusters don't have any major role in bacterial competition or in virulence against eukaryotic cells [85].

1.9 Structural framework of T6SS

Apart from the 13 conserved genes encoding 'core' components of T6SS, accessory/additional components are also believed to be essential for the full-fledged activation of functional T6SS [74]. The core components form the basic functional unit of T6SS and are categorized as membrane complex, baseplate, and sheath/tube components [Fig.1.5] [72]. In this section, a detailed mechanism of T6SS functionality has been given.

1.9.1 The Membrane Complex

In most T6SSs, the trans-periplasmic membrane complex is a stable structure composed of three membrane-associated subunits, namely TssJ, TssL, and TssM (VasDFK in *V. cholerae*) and one additional subunit, i.e., TagL [70]. This complex provides the base for the recruitment of phage tail-like structure to the cell envelope and is considered to channelize the substrate (Hcp/VgrG) secretion upon sheath contraction [70, 72]. TssJ, an outer membrane lipoprotein [70], anchors to the outer membrane (OM) through acylation of the N-terminal cysteine. Certain TssL proteins have additional C-terminal periplasmic domains having similarities with the OmpA family peptidoglycan-binding motif. However, when this domain of TssL is missing, T6SS cluster encodes an additional protein, usually TagL, that substitutes TssL for peptidoglycan binding [87]. New progress has been made in understanding the role of TagL (formerly known as SciN) in *Enteroaggregative E. coli* (EAEC) [88]. TagL is an inner membrane protein with three transmembrane segments: a central cytoplasmic loop, C-terminal peptidoglycan binding motif domain, whereas the role of N-terminus part of TagL has not been addressed yet. It was demonstrated that TagL directly contacts TssL [87]. Aschtgen *et al.* precipitated a protein complex comprising of TagL, TssL, TssM, and TssJ by pull-down experiments, which shows that all four proteins are connected with each other and formed the membrane complex [87].

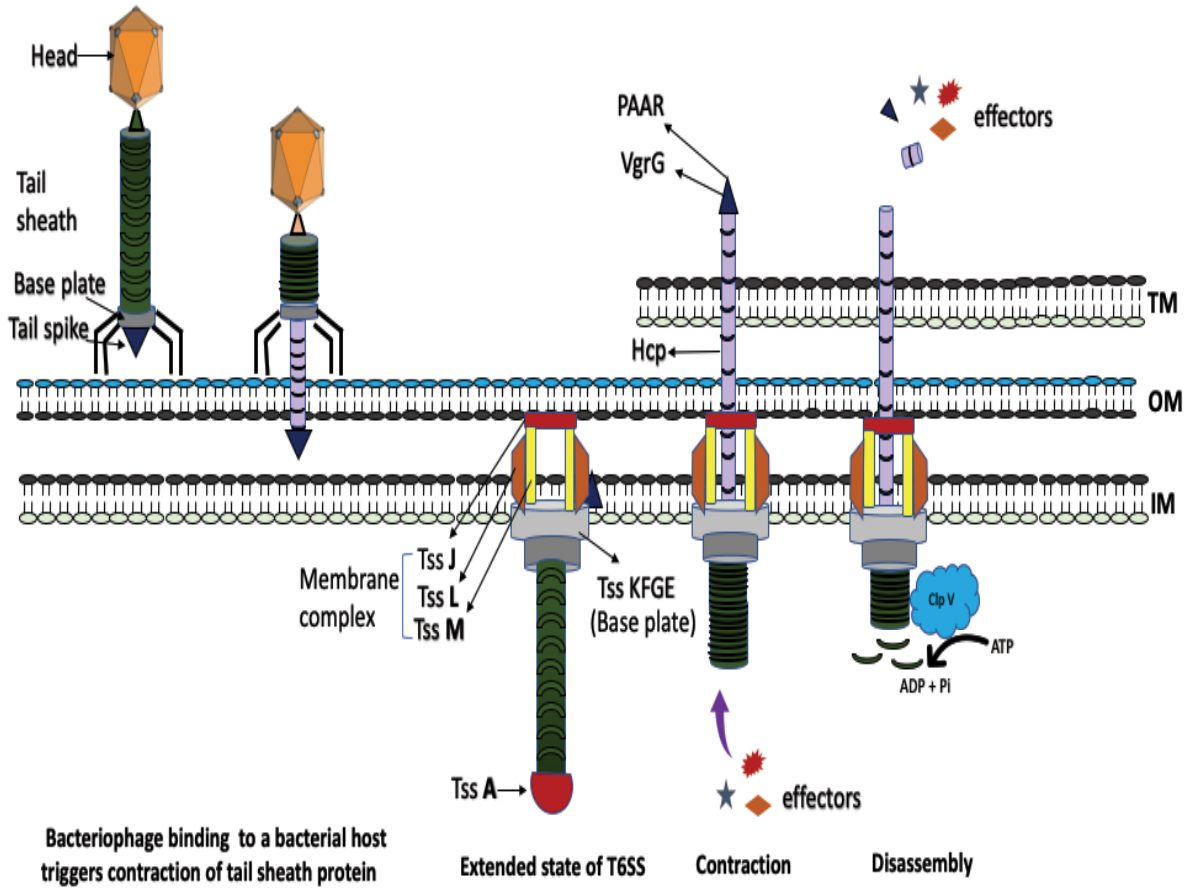

Fig.1.5. Schematic model of the overall structure of T6SS according to current information and its comparison with bacteriophage T4. The two images in the left panel show attachment in tubular proteins in the bacterial membrane. The images in the right panel indicate the assembly and sequential functioning of T6SS. The Core component of T6SS shares homology with bacteriophage. Homologous components are represented in common color. Bacteriophage attaches to the outer membrane of host cells by tail spikes connected to virion base plate. T6SS appears to be an inverted bacteriophage. T6SS attaches to the inner membrane, goes into the periplasm, and then to the outer membrane to penetrate target cells. Contraction of bacteriophage delivers tail spike into the bacterial cells. Contraction in T6SS delivers effector molecules across the target membrane. T6SS is usually made up of membrane complex, base plate and, tail sheath. TssJLM formed membrane complex that is connected to TssB-TssC of the tail sheath and Hcp by base plate component TssK, TssE and TssG. Effectors are recruited through VgrG and PAAR repeat proteins. Different environmental factors trigger contraction in T6SS, result in the ejection of spike tubes outside the cells into the target membrane to deliver effector proteins in the cells. Disassembly of the contracted sheath is regulated by an ATPase ClpV, which enables the T6SS for a new round of fire.

The role of TssM was shown to be the NTPase activity of TssM to fuel the secretion of hemolysin-coregulated protein (Hcp) in the plant pathogen *Agrobacterium tumefaciens* [89]. of TssM contacts the TssJ lipoprotein close to the OM [91]. In EAEC, the cytoplasmic domain of TssL dimerizes and interacts with TssM as well as TssK, and TssE proteins of baseplate components (described in 1.6.2). Studies based on negative-stain electron microscopy suggest that TssJLM complex arranges into double concentric rings of pillars and arches spanning the periplasm with ten copies of TssJ binding to ten copies of TssM. The N-terminus of TssMand ten TssL units are linked by the arches forming a structure with rotational five-fold symmetry [90]. However, recently, two independent groups have reported novel features to the existing model of membrane complex using high-resolution cryo-electron tomography [92]. An additional five copies of TssJ interact with two TssJ-TssM (cytoplasmic domain) sub-complexes making the stoichiometry 2:2 to 3:2 [90]. These groups also re-examined the mechanism of channel opening and identified an 11- amino acid loop in TssM, extending into the periplasmic space exhibiting two flexible hinges for MC tip opening [93]. Protein-protein interaction studies have highlighted systematic network formation during the assembly of membrane complex. It starts with the addition of the outer membrane lipoprotein TssJ, followed by TssM and TssL [90]. Another important aspect of membrane complex assembly is the point of contact in member proteins. The protruding loop of TssJ interacts with TssM in the inner membrane (IM), whereas the C-terminal periplasmic domain

1.9.2 Baseplate components

In T6SS, baseplate structure is like the final subcomplex part of the bacteriophage-like structure of T6SS. It is a cytoplasmic complex but anchored to the inner membrane and provides a The construction of the baseplate subcomplex has been elucidated based on the images obtained from cryo-electron microscopy at a resolution of 3.7Å. The peripheral wedge of the baseplates includes TssK, TssE, TssF, and TssG with a probable stoichiometry of $(TssK)_6$-TssE–$(TssF)_2$–TssG . Notably, the overall baseplate assembly is a six-fold symmetry which is unique from other complexes of T6SS [95]. In 2015, bioinformatics sequence analysis showed that the baseplate components TssG, TssF, and TssE have homologies with phage-like proteins. TssG corresponds to gp7, P2gpI or Mu Mup48; TssF to gp6,P2 gpJ or Mu Mup47; The T6SS assembly comprises six peripheral wedges with a central focal point.

TssF is a polypeptide with three predominant domains, an N-terminal α helix, central β barrels, and a mixed four-stranded β-sheet folded against hydrophobic α-helices [96].

Although TssF and TssG do not share noticeable sequence similarity, they are structurally similar. In addition, a central domain was found in TssF, which was absent in TssG. Though TssF and TssG do not have significant sequence similarity, they have perfectly aligned C-terminal domains. Based on this observation, Park *et al.* hypothesized that TssF and TssG might have emerged from the same evolutionary ancestor [96]. Pull-down assays demonstrated that the two domains of TssG interact with distinct modules, one with EPR motif (Glu-Arg-Pro motif) of TssE and the other with TssK and TssF.

TssK is a trimeric cytoplasmic connector protein with an external diameter of 155 Å (similar to sheath diameter). It interacts with two inner membrane proteins, namely TssM and the cytoplasmic domains of TssL, two phage-like tail components Hcp and TssC, and TssA, a key component [97]. N-terminal domains of TssK show homology to phage receptor-binding protein (RBP) of bacteriophage [95]. Hence, it expands the number of homologous proteins between bacteriophage and T6SS. TssK can adopt higher-ordered states and localizes at the outer edge of the cell in the vicinity of the counter-attacker [98]. The crystal structure data of TssK correspond to shoulder, neck, and head domains. Based on the bacterial two-hybrid and co-immunoprecipitation analysis, the shoulder-neck domains form higher states. The C-terminal head domain of TssK is connected with the cytoplasmic domain of TssL by the L3-L4 loop of TssK, while N-terminal domain of TssM connected with the preassembled TssFG complex. [95]. TssK foci formation is independent on TssE and TssA but greatly depends on TssF-G and VgrG, depicting TssKFG bound to central spike [99]. A new study showed that TssK foci stability depends on the essential membrane core protein TssM, but not directly for its assembly [100]

Unlike understanding the role of other baseplate units, not much is known about cytoplasmic protein TssE in biogenesis. Interestingly, microscopic fluorescence analysis of sheath assembly dynamics shows that deletion of TssA allowed sheath assembly at reduced levels whereas the absence of TssE and TssBC doesn't form functional T6SS assembly in *P. aeruginosa* and *V. cholerae* [101]. TssE functions to prevent detachment of TssB-sheath and its premature disassembly. TssE is recruited TssKFG complex is established networks and initiating the sheath polymerization [99].

1.9.3 Tail, Sheath /tube components and TssA-like proteins

In bacteriophage, the inner tube (T4 tail) is encased in a contractile sheath made up of gp18 subunit. Similar to the bacteriophage tail, two proteins, namely TssB and, TssC assemble and form tail sheath in T6SS, as shown in Fig.1.5. Both the proteins are encoded by *tssB* and *tssC*

genes respectively and co-occur in all T6SS clusters. 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 enclosed by a contractile sheath assembled from VipA/VipB (TssB/TssC) heterodimers. Researchers demonstrated that the TssBC proteins interact with the component of the tube, Hcp. Hcp, a secretory protein, gave the first clue of a regulatory cascade in *V. cholerae* [70]. An inner polymeric Hcp (hemolysin coregulated protein), a structural homologue of gpV, organizes into a tube of diameter 35- to 40-Å assembled inside the tail sheath in vitro [81]. Six Hcp molecules construct a hexameric ring of width 80–90 Å stabilized by an extended inter-subunit array [74].

The sheath forms an erect and tubular structure existing in an elongated 'extended' and shorter 'contracted' conformations oriented approximately perpendicular to the cell envelope [101]. Both extended and contracted conformation of the sheath were deciphered by cryoEM [102]. The core domain arises from three monomers, $(TssC)_2$-TssB built by four β-strands. The intermolecular interactions of N-terminal TssB and C-terminal TssC linkers contribute majorly to the sheath stability [102]. The sheath domains 1 and 2 share similarities with phage, but domain 3 is specific to T6SS with a ClpV binding pocket. Interactions between β-strands in domain 1 seemingly trigger the sheath polymerization, extension, and potentially also for sheath contraction [102]. VipA/B tubules form dodecameric gear wheel-like shapes which spontaneously disassemble by a process dependent on ClpV-mediated ATP hydrolysis [103]. Unlike one-time contraction of the sheath in phages, T6SS sheath is recycled *in vivo* by ClpV [101]. ClpV represents the hallmark of T6SS, as in TssM variants of *E. tarda* deficient in ATP binding and/or hydrolysis still support Hcp and VgrG export [104]. Interestingly, *Francisella novicida* lacks ClpV with an unusual five times longer sheath than *V. cholerae* [105]. In some of the bacterial species, the opposite end of the sheath is loaded with a capped-surface complex composed of 12 copies of TssA or its homologs. , plays an important role in coordinates tail tube and sheath biogenesis [106].

Importantly, it is noteworthy to understand the energy dynamics of the sheath contraction and disassembly. Sheath undergoes a transition from high energy to low energy states when contracted and disassembled. During contraction, the rotation increases, leading to alteration in overall structures. This results in the movement of the inner tube during contraction. About 420 nm push of 1 μm long inner tube releases ≳15000 kcal/mol of energy with ~10 rotations in less than 5 ms towards the prey [101]. As mentioned previously, the

disintegration of sheath components occurs in a few seconds (~10) once the AAA+ protein ClpV recognizes the exposed N-terminus of TssC [103].

It is in recent years when the functions of TssA were defined and categorized into three distinct protein classes. These proteins have a conserved N-terminal domain and variable C-terminal domains [107]. TssA proteins of Class A (proposed name TsaC) present in *E.coli*, *V. cholerae,* and act as chaperones facilitating sheath-tube copolymerization. In *E. coli*, the N-terminal part of TssA has ImpA domain which interacts with the base plate component, whereas the C terminal interacts with Hcp protein [106]. The second class, Class ImpA, a_N-terminal domain, forms hexameric foci in the distal end of the assembled sheath. It prevents excessive sheath formation and stabilizes the sheath during sheath extension [107]. In *V. cholerae*, TagA interacts with TssA, TssK, ClpV, VipA, and VipB. Surprisingly upon sheath extension, the TssA foci disappeared. Finally, the third class, Class C (proposed name TsaB) proteins, lack genes encoding TagA-like proteins. Its functional role is yet to be established. The assembly, contraction and, disassembly of this T6SS system was shown to be very rapid in *P. aeruginosa* forming short sheaths capable of effector release [108]. They interact with base plate components and initiate sheath assembly, which is seen in the H1-T6SS cluster of *P. aeruginosa* [109].

1.10 Regulatory pathways and environmental signals of T6SS

Assembly of T6SS is an energy-consuming process for the bacterial cell, thus is tightly regulated at the transcriptional or translational level in response to certain environmental signals and a variety of factors. For instance, in *P. aeruginosa* the T6SS regulation is strictly controlled post-transcriptionally by the Gac/Rsm pathway and post-translationally by threonine phosphorylation [74]. In some bacteria, transcriptional regulation of T6SS was observed *via* quorum sensing [110]. Other factors regulating the expression of T6SS include biofilm formation [111], variations in temperatures [112], availability of iron, [113], and stresses [114]. Here, we provide examples of regulatory pathways and signals that modulate T6SS expression.

1.11 Different regulators of T6SS across bacterial species

1.11.1 Fur and its regulatory networks

Many pathogens live in an iron-deficient host environment, where many gene transcripts are regulated by iron through the iron uptake regulatory protein (Fur). Fur protein acts as a salient transcriptional modulator for iron-regulated promoters [115]. It has been reported that this Fur

protein fine-tunes the expression of T6SS genes, as shown in Fig.1.6. In *P. aeruginosa,* two fur binding regions (fur boxes) were identified in the promoter of H2-T6SS. The expression of T6SS is repressed by iron at the transcriptional level [116]. Fur boxes are found to be overlapped with -10 box of the promoter, suggesting that binding of fur at this position in the presence of iron restricts RNA polymerase binding to -10 box and thus represses the transcription of T6SS genes. Similar fur-dependent regulation of the T6SS genes were discovered in other bacteria as described below.

In EAEC, T6SS is encoded by *sci1* gene cluster, which is important for biofilm formation, and its expression is regulated by iron availability through Dam (DNA adenine methyl transferase and fur repression. Two Fur boxes are present in the *sci-1*Type VI gene cluster in the promoter region, including one overlapping with the putative -10 box. Also, there is a target of the Dam methylase, a GATC site, GATC-32, which overlaps with the Fur-binding box. The methylation of GATC by Dam methylase is required for the expression of *sci-1.* Recent studies suggested a controlled switch regulatory mechanism with iron as a regulatory factor [117]. Recently, the presence of an internal promoter P_{4532} within the *sci1* T6SS gene cluster was encountered by the same group. The Fur protein represses the internal promoter on a Fur box overlapping with the -10 transcriptional element. Fur acts as a switch to on and off the expression of genes, and methylation stabilizes the ON phase [117]. In brief, under iron replete-conditions Fur will block the binding of RNA polymerase to the -10 box and prevents Dam methylase to methylate the GATC-I site. Thus, the expression of *sci1* genes is maintained in a repressed state or OFF phase. Phase reversal occurs when the Fur protein dislocates from the fur1 box (promoter region) under iron-deficient conditions. Now, the GATC-1 site is exposed for Dam-dependent methylation resulting in the expression of *sci1* genes or ON phase [113].

In *E. tarda,* Fur-mediated gene regulation of T3SS and T6SS overlaps with PhoB-PhoR two-component system. PhoB -PhoR sense fluctuations in Phosphate concentration, whereas Fur sense alteration in iron concentration. Cross-talk between these signaling pathways is implemented by EsrC (*E. tarda* secretion regulator) for regulating the expression of T3SS and T6SS [118]. There are two other response regulators, namely EsrA and EsrB, which directly activate *esrC*. The *esrC* encodes EsrC regulator, a homologue of AraC family of transcriptional regulators, regulates the expression of the T6SS gene cluster and certain T3SS genes of *E. tarda*. Regulation of this secretion system is controlled by transcription regulators like PhoPQ and EsrAB [118]. In EsrC-mediated gene regulation, PhoB interacts with PhoU to activate *esrC*

and supress Fur through unidentified regulators. On the other hand, high iron concentration in the environment makes Fur bind directly to the Fur box in the promoter region of *evpP* gene (T6SS gene cluster of *E. tarda*) and inhibit binding of EsrC to the same region, which eventually represses the transcription of T6SS genes. In addition, Fur also suppresses the transcription of PhoB and *esrC*, suggesting the negative cross-talk with the Pho regulon [118]. The expression of T6SS in *K. pneumoniae* is regulated via transcriptional control by various transcriptional regulators. The PhoPQ two-component system governs the regulation of T6SS in bacterial competitions. Many environmental factors such as temperature, oxygen tension, pH, iron levels, and Nacl regulates the expression of T6SS encoded by a hypervirulent *K. pneumoniae* strain. However, the detailed mechanism is not investigated yet [119].

1.11.2 Threonine-phosphorylation (TPP) dependent and independent regulatory networks

In *P. aeruginosa*, T6SS assembly is spatially controlled in response to the attack by neighbouring. Some accessory protein complex mediates this type of regulation through post translation regulation system known as the TPP pathway. Although TPP-dependent pathways are predominant in many bacterial species, they vary slightly from each other to address the needs of each species. The key molecules remain the same but possess distinct molecular interactions. The TPP-dependent regulation of T6SS has been characterized in a couple of bacteria, including *P. aeruginosa, S. marsecesns,* and *A. tumefaciens.*

In *P. aeruginosa*, TPP- dependent pathway regulates T6SS by post-translational modification via threonine phosphorylation of two key proteins PppA and PpkA, which exhibit phosphatase and kinase, respectively, which in turn regulates the activity of a regulator protein Fha 1. Briefly, p-Fha1 (phosphorylated Fha1) plays an instrumental role in the activation of H1-T6SS(T6SS cluster of *P. aeruginosa*), as shown in Fig.1.6. Recently, the crystal structure of PppA from *P. aeruginosa* was elucidated at 2.1Å, which revealed a catalytic pocket containing two manganese ions and a flexible flap subdomain [120]. Initially, in the resting phase, Fha1 protein is maintained in an unphosphorylated state due to a higher level of PppA expression against PpkA basal activity. Under appropriate environmental signal, PpkA with von Willebrand A (VWA) domain in its C- sensing protein complex comprising TagQRST [121]. On activation, PpkA gets autophosphorylated, interacts with, and phosphorylates Fha1 converting it to pFha1 [122]. This phosphorylates-Fha1 regulates the secretion of Hcp and triggers the T6SS. Upon removal of environmental signals, PppA dephosphorylates p-Fha 1, turning off the T6SS function [123].

Similar to *P. aeruginosa*, *S. marcescens* also exhibits TPP-dependent regulation via PpkA-mediated phosphorylation of Fha at Thr_{438}. The deletion of Fha homologue in *S. marsences* restricted Hcp secretion, demonstrating the involvement of Fha in the functionality of T6SS. TPP dependent regulation in *S. marcescens* differs from the *P. aeruginosa* in its signal activation and mechanism wherein cell-contact interaction is not required [124]. Thus, it is considered as an 'offensive' system, whereas *P. aeruginosa* exhibits "defensive behavior or T6SS dueling". *P. aeruginosa* fires its T6SS apparatus to target its neighboring cells. The attacked neighboring cell then builds its T6SS apparatus and fires a retaliatory T6SS counterattack. This initial attacker then senses this T6SS based counterattack and retaliates in turn; such a phenomenon is referred as T6SS dueling [121]. Recently, another protein RtkS, was identified in *S. marcescens* to form such a system. A novel protein was discovered named RtkS situates in the periplasm of *S. marcescens*. Later, it was shown that it is responsible for Fha phosphorylation, causing multiple Fha proteins to associate with the T6SS, which promotes the basal complex formation and allowing the entire T6SS structure to get assemble.[125].

Interestingly, in *A. tumefaciens* PpkA phosphorylates, a highly conserved N-terminal cytoplasmic domain site, Thr 14 of TssL, unlike Fha phosphorylation. A similar mechanism is observed in the marine pathogen *V. alginolyticus* where phosphorylation cascade by protein kinase PpkA2 phosphorylates DotU2 leading to the Fha2 interaction and thus, T6SS-2 firing [126].

1.11.3 TagF-mediated regulation

Research group that identified TPP pathway in *P. aeruginosa*, discovered a post-translational regulation protein, TagF, in upstream of the T6SS cluster of *P. aeruginosa*. TagF plays a role in repressing the activation of H1-T6SS. TagF regulates T6SS by interacting with Fha1. However, unlike TPP-dependent activation of Fha1, the interaction of TagF with Fha causes repression of T6SS in a TPP -independent manner [123, 124] *A. tumefaciens* and *R. leguminosarum* contain tagF and pppA fusion protein (dual expression in a single ORF). TagF binds to Fha when expressed at high levels and represses the latter from initiating T6SS assembly [123, 124, 126].

1.11.4 Quorum sensing in T6SS regulatory networks

In many Gram negative bacteria, quorum-sensing, a density-dependent process, is known to play an important role in various functions, including pathogenesis [127]. In recent years, the regulation of T6SS by QS at the community level has been demonstrated in various bacteria

including *V. cholerae, V. alginolyticus, Acinetobacter baumannii, B. thailandensis* [128,129]. Diagrammatic representation of QS regulation is shown in Fig. 1.6.

In *V. cholerae*, two distinct quorum sensing systems were reported, one activated by auto inducer-1(AI-2) and another by autoinducer-1 (CAI-1). It has two sensory kinases: LuxQ and CsqS, to detect signal molecules involved in quorum sensing. Another phosphotransfer protein LuxU, acts on LuxO (DNA- binding response protein) and phosphorylates it. Phosphorylated-LuxO activates the expression of *qrr1-4* sRNA molecules that repress the production of HapR. HapR seemed to regulate the Hcp expression level [128]. QS system regulates the expression of *hcp* gene and HapR transcriptional activator, linking QS and Hcp regulation. It has been noted that quorum sensing regulation of T6SS of *V. cholerae* O1 strain is stringently controlled by quorum sensing metabolic network involving cAMP-CRP, and the RpoN alternate sigma factor. In the endemic and pandemic *V. cholerae,* master regulators TsrA and VasH are involved in the interplay of T6SS and HapR QS pathways of *V. cholerae* [110]. In a recent work using ChIP-seq analysis and electrophoretic mobility shifts assays, RpoN, a major transcription factor for many biological pathways, was shown to directly influence upregulation of QS system *las.* Also, RpoN directly binds to the promoter regions of *hcpA* and *hcpB* of *P. aeruginosa* PAO and upregulates the expression of T6SS genes [130].

There are many other pathways unique to different bacterial species. However, a lot is uncertain in the regulatory mechanisms as we can see various pathways entwine to bring about the firing of T6SS. The regulatory mechanisms provide the depth of information regarding the infection and potential therapeutic targets, an ever-challenging work in progress.

1.12 Function of T6SS

The T6SS mediates the export of numerous effector proteins responsible for carrying out a variety of biological functions [75]. T6SS has been reported to be involved in the acquisition of micronutrients such as iron, manganese, and zinc. H3-T6SS of *P. aeruginosa* secretes TseF effectors, which facilitate iron acquisition. Similarly, T6SS of *B. thailandensis* secrete zinc and manganese scavenging protein to fulfill its demand [131]. Thus, T6SS confers a survival advantage to bacteria by delivering toxins to the neighboring cells and killing competing cells by acquiring micronutrients from a niche of multiple bacterial species. It also plays a crucial role in bacterial stress response and contributes to cell survival in adverse conditions in the host

132].

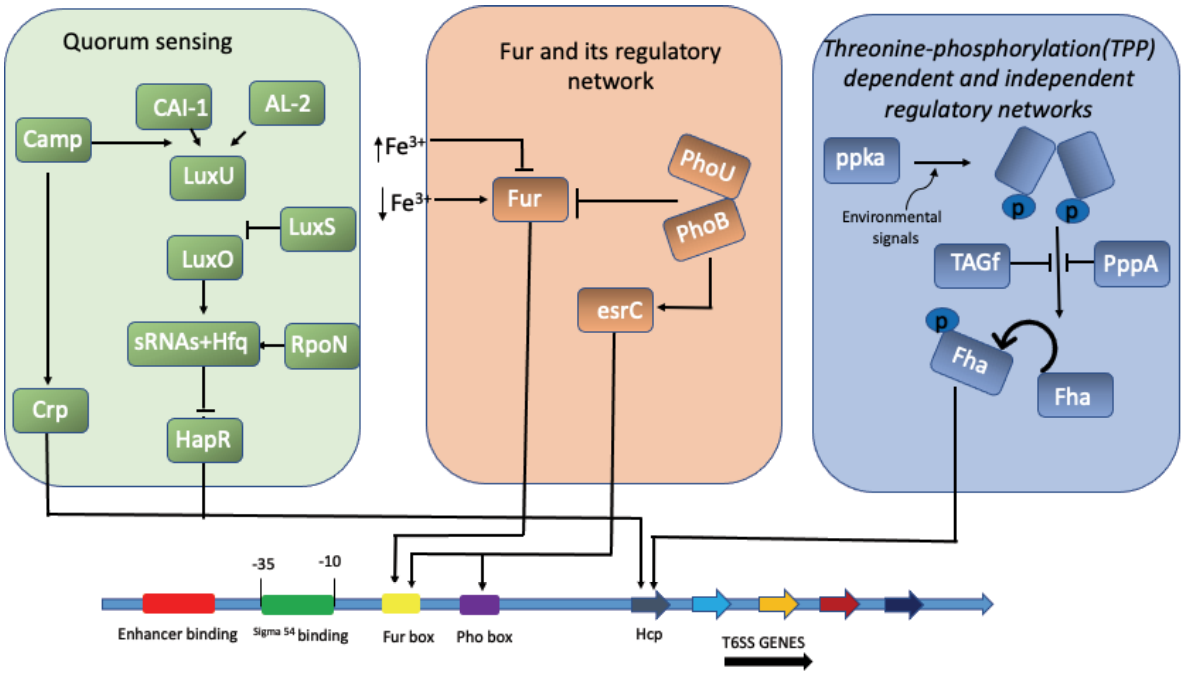

Fig.1.6. Schematic representation of different pathways involved in the regulation of T6SS. (A) shows crosstalk communication of quorum sensing and T6SS in *V. chlorae*. QS is regulated by two autoinducers, CAL-1 and AL-2. Sensory kinase CqsS and LuxQ phosphorylate LuxU. LuxU passes the phosphate to LuxO that activates sRNAs. This sRNA are assisted by sRNA chaperone Hfq. This complex destabilizes the HapR, which is the master regulator of QS. HapR activates T6SS and other virulence factors. (B) shows crosstalk communication between fur regulatory network and T6SS in *P. aeruginosa*. Low level of iron in the environment allows Fur to bind to the -10 region of Fur box. Thus binding of fur at this position restricts RNA polymerase binding to -10 box and thus represses the transcription of T6SS genes. Similarly, in *E. tarda*, T6SS PhoB-PhoR two-component system, sense fluctuations in Phosphate concentration PhoB interacts with PhoU to activate another regulator in this pathway and suppress Fur, lead to transcription of T6SS genes. (C) shows crosstalk communication of TPP regulatory network and T6SS in *P. aeruginosa*. TPP mediated T6SS regulation governed by two key proteins PppA and PpkA, which exhibit phosphatase and kinase, which in turn regulates the activity of a regulator protein Fha by its phosphorylation and de-phosphorylation. p-Fha1 plays an instrumental role in the expression of H1-T6SS of *P. aeruginosa*.

Initially, T6SS was associated with bacterial pathogenesis; later on, it was reported that , mainly in epithelial and macrophage cells. For instance, *P. aeruginosa* invades epithelial cells and interferes with microtubule nucleation by transferring VgrG effector protein *via* T6SS [73]. *V. cholerae* also delivers T6SS-based toxins that hamper the host cytoskeleton [49], *icmGCDJBF* a T6SS cluster of *Legionella pneumophila* is required for the killing human macrophages [50], and SGI (*Salmonella* genomic island) of *Salmonella* related to *icmF* gene cluster is also involved in intervention of eukaryotic cell invasion [82]. The following section provides a detailed description of T6SS secreted effectors molecules having a role in the pathogenesis.

1.13 T6SS effectors targeted into eukaryotic cells

T6SS plays an important role in the virulence of many bacteria by introducing effector molecules in a contact-dependent manner thereby intervening host cells' functions. Initial studies of T6SS in the context of pathogenesis were conducted in *P. aeruginosa* and *V. cholerae*. The most characterized effector molecule involved in T6SS-mediated pathogenesis is VgrG protein. One or more copies of VgrG encoding genes are found in the majority of T6SS. Studies in *P. aeruginosa* and *V. cholerae,* suggest VgrG exists as a trimeric protein. *V. cholerae* genome, possess three copies of *vgrG*, one of these codes for aVgrG with C-terminal extension. The function of this VgrG is similar to the RtxA toxin of cholera that has a actin cross-linking domain (ACD). Pukatzki *et al.* showed that this C terminal of VgrG protein is capable of cross-linking actin in the eukaryotic cell [73]. Deletion of C terminus of *vgrG1* abolished T6SS-mediated toxicity. VgrG1 ACD was responsible for inducing inflammation and associated pathology in the mice thus, confirming the role of *V. cholerae* T6SS in pathogenesis [133]. Similarly, *Aeromonas hydrophila* T6SS secrete VgrG effector proteins having vegetative insecticidal protein (VIP-1) domain at its C-terminal. VgrG of *A. hydrophila* has a cytotoxic effect on HeLa cells in which it disrupts actin cytoskeleton of the host cells [134]. However, VgrG of *B. pseudomallei* mediates membrane fusion and cell-cell spread. Though the VgrG is required for the majority of functional T6SS, non-VgrG effectors toxins like VasX, a novel virulence factor secreted by T6SS of *V. cholerae*, has role in virulence. A *vasX* mutant exhibits attenuated virulence toward *Dictyostelium discoideum* [59]. Similarly, *P. aeruginosa* used its T6SS to inject phospholipase, a non *vgrG* toxins, that targets membrane complex and are also crucial for host cell toxicity [68].

Apart from being part of T6SS assembly, Hcp is secreted out as an effector protein having role in virulence. Recently, a study on *Burkholderia mallei*, the causative agent of

glanders disease in donkey, mules, horse, and sometimes humans, also utilize Hcp effector molecules to infect its host. Consistent with this, Hcp knock out in *B. mallei* led to loss of its virulence in the hamster model of infection [135]. Similarly, role of *hcp* has also been found to be critical for *P. aeruginosa* pathogenesis. *P. aeruginosa,* is known to be associated with respiratory tract infections in cystic fibrosis patients. Patients suffering from chronic fibrosis show presence of antibodies to Hcp1.T6SS cluster of *P. aeruginosa* is located on Hcp secretion *island* (HSI) consists of three (HSI) loci HSI1, HSI2, and HSI3. Most of the studies have focused on the HSI1 and its role in virulence. Interestingly, Mougous *et al.* demonstrated that Hcp-1, encoded by HIS1, is a hexameric protein with 40 Å of diameter whose secretion is facilitated by ClpV1[108]. In addition to its association with cystic fibrosis in humans, it has been experimentally demonstrated that *P. aeruginosa* T6SS is also involved in inducing chronic lung infection in rats [74]. This suggests that T6SS is required for the virulence of *P. aeruginosa.* In another study conducted on *B. cenocepacia,* AtsR, a global virulence regulator, was reported to control T6SS. AtsR is a sensor kinase present in the inner bacterial membrane. AtsR mutant *B. cenocepacia* showed increased biofilm and altered T6SS function [136].

The T6SS may also modulate bacterial interaction with plants. A plant symbiotic bacteria *Rhizobium leguminosarum* that forms nitrogen-fixing nodule in the leguminous plant also encodes T6SS. Bladergroen *et al.* reported that *tssK* mutant of *R. leguminosarum* was unable to form functional nodules on the plant in comparison to the wildtype strain [137]. This mutant was unable to secrete a protein of about 27 kDa, later identified as Hcp. Later on, the genes of this T6SS were named as *impA* to *impN* (impaired in nodulation). Other bacteria like *C. jejuni*, *B. pseudomallei* also need T6SS for survival or toxicity towards host cells, however, the molecular basis for this requirement has not been completely characterized [53, 69].

1.14 Bacterial targeted T6SS toxins

A fundamental shift in the understanding of T6SS function happened when it was discovered that the T6SS of *P. aeruginosa* also exhibit anti-bacterial properties [78]. With the help of T6SS *P. aeruginosa* transport effectors or toxins like Tse1-3 in opponent bacteria in a contact-dependent manner [78]. These effector molecules are encoded by genes located on H1-T6SS. Out of these three effectors, Tse1 and Tse3 have amidase and muramidase activity, respectively, each of which targets different bonds within the peptidoglycan [75]. Tse2 is a bacteriostatic toxin targeted to bacterial cytoplasm, but the exact mechanism of toxicity is not known. [78]. Nevertheless, Tse2 of *P. aeruginosa* showed toxicity to yeast and mammalian cells as well [78]. The downstream region of Tse2 encodes a antitoxin protein, named Tsi2,

that prevents self-toxicity. *P. aeruginosa* lacking Tse2/Tsi2 were outcompeted and disrupted in co-culture with wild-type cells (78). The phylogenetic analysis showed that the Tse proteins of different bacteria belong to the phylogenetically diverse superfamily of T6SS-associated peptidoglycan-degrading effectors [138]. Similarly, *B. thailandensis* secrete an effector named $Tae^{BT,}$ which has peptidoglycan amidase activity and cause intoxication in neighbouring bacteria [138]. Like *P. aeruginosa*, *B. thailandensis* also encodes an immunity protein whose corresponding gene is located downstream to that of $Tae^{BT.}$). T6SS-1 also helps to outcompete *Pseudomonas putida* in competition experiments. It was evident from the observation that *B. thailandensis* strain carrying a deletion in T6SS-1 allows the *P. putida* strain to outcompete and dominate over *B. thailandensis* [85]). In addition, deletion of T6SS-1 also impaired biofilm formation by *B. thailandensis*. Similar anti-bacterial function of T6SS has subsequently been reported in the number of bacterial species [139-141].

VgrG, also exhibits anti-bacterial activity. For instance, VgrG3 of *V. cholerae* has peptidoglycan-degrading activity at its C-terminal extension [140]. Apart from VgrG3, other T6SS-dependent secreted peptidoglycan degrading effectors such as Tae (an amidase) and Tge (a glycoside hydrolase) have been identified, which shows the toxic effect to *E. coli* [77]. Another class of anti-bacterial effectors functioning as lipase and targeting cell membrane has also been identified [68]. To prevent the action of these effector molecules to host bacteria, their immunity proteins contain signal peptides, indicating these T6SS secreted lipases primarily act in the periplasm [142]. These lipid-targeting T6SS effectors, also called Tle effectors, have been classified into five subgroups- Tle1-5. Tle1 to Tle4 families exhibit GXSXG motifs (X refers to any amino acid) of alpha and beta hydrolases family commonly found in lipase and esterases. However, Tle5 has HxKxxxxD motif similar to the phospholipase D enzyme [76]. Till now, eight Tle proteins have been characterized so far, including Tle1- (*P. aeruginosa* TlePA, *B. thailandensis* TleBT, and *Enteroaggregative E. coli* TleEAEC), Tle2 (*V. cholerae* Tle2VC), Tle 4 (*P. aeruginosa* Tle4), or Tle5 (*P. aeruginosa* Tle5aPA and Tle 5b PA], *K. pneumoniae* Tle5bKP [76].

Overall T6SS plays a significant role in reshaping bacterial community and their pathogenesis, directly or indirectly. Thus, understanding the 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, 79], *S. enterica* subspecies *enterica* serovar Typhimurium [82], and *C. jejuni* [143]. It has been predicted that around 25% of the sequenced Gram-negative bacteria have this nanomachine,

making it the most widespread secretion system [144]. Though the T6SS have been characterized in a few species, including *V. cholerae* [59], *P. fluorescens* [145], and *P. aeruginosa* [78], the role of T6SS in pathogenesis and bacterial competition is poorly characterized in many opportunistic pathogens like *E. cloacae*. Considering the role of T6SS in pathogenesis, it could be a potential target to treat nosocomial infections.

Although there have been studies on the pathogenicity of a couple of clinical isolates understanding of *E. cloacae* pathogenicity is not investigated in detail. Moreover, studying the pathogenic potential of exogenous (non-clinical) isolates remains ignored. Research in our laboratory identified *E. cloacae* SBP-8 (Accession No. NAIMCC-B-02025), a rhizosphere bacterial isolate, obtained from *Sorghum bicolor* plant grown at sambhar-lake of Rajasthan. This strain ameliorated salt stress and promoted plant growth under abiotic stress (salt stress) conditions [146]. The genome analysis of *E. cloacae* SBP-8 (NCBI Accession no: NZ CP016906) showed the presence of set of genes for 1-aminocyclopropane-1-carboxylate deaminase (AcdS), siderophore, phosphate solubilization and IAA (indole acetic acid) production [147]. All these genes confer the ability to promote plant growth under abiotic stress. The strain also possesses genes responsible for colonization, including adherence, chemotaxis, and motility, all required for successful colonization into the host organisms. Apart from the genes mentioned above, the genome also harbors genes that have a role in the bacterial pathogenicity like antibiotic resistance genes (like *MarA, MarB MarC,* and *MarD*), genes encoding curli proteins, *pgaABCD* operon involved in the quorum-sensing mechanism, and other genes required for biofilm formation. In addition, *E. cloacae* SBP-8 have genes code for toxins like aerobactin and enterotoxins [147]. Detailed mining of the genome sequence of *E. cloacae* SBP-8 indicated the presence of four secretion systems, namely I, II, IV, and VI [147]. It has all genes required to form a complete and functional assembly of T6SS, a recently identified secretion system. T6SS is recognized as a feature of pathogenic or clinical isolates of Gram-negative bacteria. However, it has not been studied and reported in opportunistic pathogens like *E. cloacae* of exogenous origin.

The cases of nosocomial infections caused by opportunistic pathogens like *E. cloacae* are still prevalent. Most of the studies of *E. cloacae* infections have been conducted with clinical isolates of endogenous origin. However, environmental isolates having exogenous origin can also have pathogenic potential under amenable conditions. Therefore, understanding the pathogenic potential and mechanisms of pathogenesis of such isolates is essential to containing the fatalities resulting from *E. cloacae* infections. Thus, we aimed to characterize

the pathogenic potential of *E. cloacae* SBP-8 along with the role of T6SS in pathogenesis and bacterial competition.

1.15 Model organism for studying pathogenesis

Studying bacterial pathogenesis requires a suitable model host organism that can help gain mechanistic insights into the infection process. Previously, researchers have used Drosophila [148] and *Galleria mellonella* [149] to study the pathogenicity of *E. cloacae*. Few reports are also available that explore the systemic dissemination of *E. cloacae* in mice, where it is showed that *E. cloacae* colonize the intestine and reticuloendothelial organ like the kidney [9]. However, the model mentioned above did provide insight into the pathogenicity of *E. cloacae,* but host-*E. cloacae* interaction has not yet been characterized. Moreover, a suitable model for screening and characterizing the pathogenic potential of *E. cloacae* is yet to be characterized. The current study has attempted to optimize the following two animal models (as describe in chapter 2 and 3) for testing the pathogenic potential of *E. cloacae* SBP-8.

1.15.1 *Caenorhabditis elegans*

Caenorhabditis elegans (*C. elegans)* has been used as a favorable model organism to study the innate immunity of humans due to the conserved host defense mechanisms during bacterial infection. Its small size, short generation time, ease of handling, and transparent body provides an advantage over other model organisms for studying host-pathogen interaction. The life cycle of this nematode consists of embryonic development and 4 larval stages, completing in about 3 days [Fig.1.7]. In its natural habitat (soil), it frequently encounters a variety of pathogens, which activates host antimicrobial mechanisms to eliminate the infection. To counter the bacteria, nematodes have conserved signal transduction pathways such as the DAF-2/DAF-16 pathway and p38 MAPK pathway to protect worms from different pathogens. DAF-2/DAF-16 mediates the *C. elegans* host immune response by regulating the defence effectors required for the host survival during pathogen attack. While p38 MAPK signaling pathway activates the production of antimicrobial peptides. A remarkably large number of both Gram negative and Gram-positive human pathogens have been shown to infect *C. elegans,* such as *S. enterica* [150], *Y. pestis* [151], *P. aeruginosa* [152], *S. marcescens* [153], and *S. aureus* [154]. Thus, *C. elegans* can be used as a model host for studying the pathogenesis of *E. cloacae*.

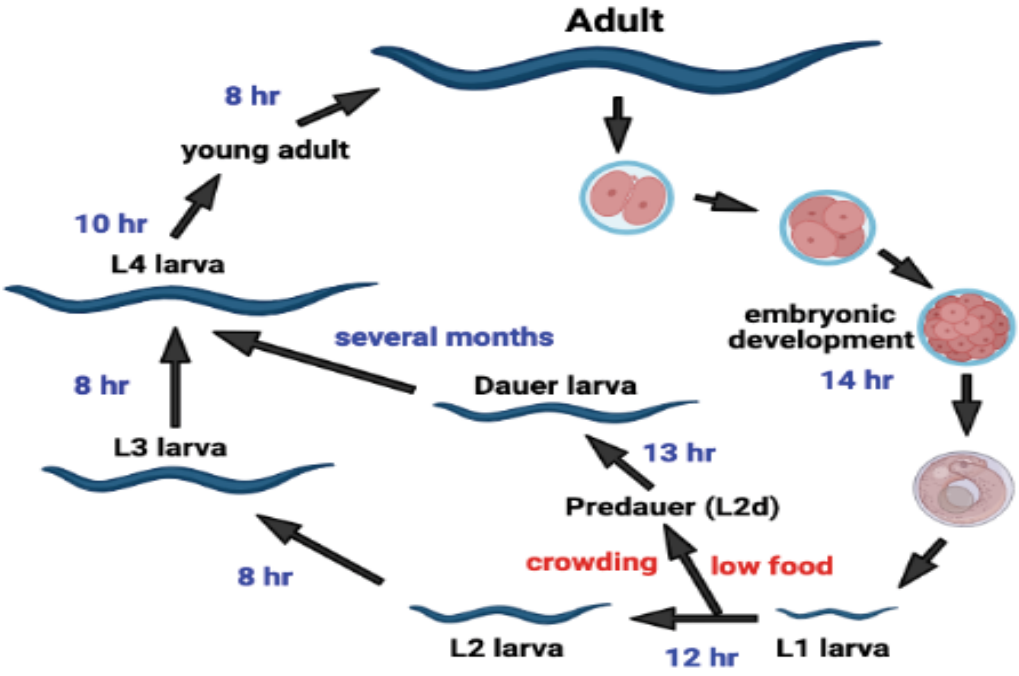

Fig.1.7. Life cycle of *C. elegans* at 22°C. The diagram shows that after hatching from the embryo, worms transit through 4 larval stages (L1-L4) before maturing to an adult. Considering 0 min is fertilization, the first cleavage occurs at 40-50 min while the embryo containing multiple cells (~30 cells) are laid out after 150 min. This embryo then progresses through the four larval (L1-L4) stages before becoming an adult. Hours in blue color indicate the length of time a worm spends at a particular stage of development, and the length of worm at a particular stage of development is marked in micrometer, next to the stage name.

1.15.2 Murine model

The mouse shows good similarity to humans in terms of anatomy, physiology, and genetics; thus, are extensively used to study pathogenies and immunity operational mechanisms during systemic infection. Generally, the immune system is well developed by six to eight weeks of age, and mice in this age group are used for infection studies [155]. Murine model allows the use of different inoculation routes while permitting the examination of different stages of the disease. They are advantageously used over other mammalian model organisms as they are relatively inexpensive, easy to maintain, easy to handle, and amenable for genetic manipulations [155]. Even the knockout mouse for key genes of different infectious and genetic diseases is available, providing an invaluable tool for understanding the host-pathogen interaction.

1.16 Gaps in Existing research

Nosocomial infections caused by various bacteria, including *E. cloacae* and other members of *E. cloacae* complex, has been a great concern. Its occurrence has further worsened due to the rise of resistance to antibiotic drugs in these bacteria, which makes the treatment difficult. In addition to its presence in a hospital environment and clinical samples, *E. cloacae* is also present in soil and other habitats. These bacterial isolates can exhibit some beneficial 146], biodegradation of organic contaminants [156], and bioremediation of toxic metals which attracts our attention to exploit them at the field level [157]. However, considering the pathogenic potential of these beneficial isolates, we must ensure biosafety concerns before their use for field application. In general, the pathogenic potential of environmental or exogenous isolates (other than clinical one) of such opportunistic pathogen are not tested, which can have adverse consequences. Unfortunately, despite the fact that nosocomial infections by this opportunistic bacterium still affects a number of immunologically compromised patients, and exogenous isolates of *E. cloacae* could have the pathogenic potential [158], few studies have been conducted to understand the detailed mechanisms and virulence factors for infestation of *E. cloacae* pathogenesis.

One of the reasons for the lack of such studies is the absence of a suitable animal model for this pathogen. Therefore, the present work aims to investigate the pathogenic potential of an exogenous isolate, *E. cloacae* SBP-8, originally isolated from *Sorghum bicolor*. The study aims to characterize *C. elegans* and mice as a model host for the pathogenesis of *E. cloacae* SBP-8 while comparing it with that of *E. cloacae* ATCC13047, a clinical isolate. Although

there are few reports, which showed the systemic infection of *E. cloacae* in mice, the host immune response is still uninvestigated. To the best of our knowledge, we are first to use *C. elegans* as a model host for studying *E. cloacae* pathogenicity and characterize host immune response during infection.

Based on the previous studies, it appears that T6SS plays an important role in the colonization and pathogenesis of many pathogenic bacteria, including *V. cholerae* [59] and *P. aeruginosa* [73]. However, the role of T6SS in pathogenesis and bacterial competition is poorly characterized in many hospitals acquired opportunistic pathogens, including *E. cloacae*. In 2020, Bustos *et al.,* characterized the T6SS of *E. cloacae* 13047, a clinical isolate, and demonstrated its role in virulence and pathogenesis [35]. However, exogenous or environmental strains of *E. cloacae* like *E. cloacae* SBP-8 may have the potential to cause pathogenesis owing to the presence of pathogenic genes like the genes for T6SS assembly and functionality. In the present study, we aimed to analyze the secretion system genes employing bioinformatics tools and check the functionality of T6SS of the given isolate. We also attempted to characterize the role of the T6SS of this exogenous isolate in pathogenesis and/or bacterial competition.

With this background, the following objectives were proposed to fulfil the aim of the research.

1.17 Objectives

Keeping the above-mentioned facts in mind, the present thesis aims to accomplish the following objective:

1. Elucidating the pathogenic potential of *E. cloacae* SBP-8 using *Caenorhabditis elegans* as a model host.
2. Studying the systemic dissemination of *E. cloacae* SBP-8 during infection in mammalian (murine) model host.
3. Characterizing the T6SS of *E. cloacae* SBP-8 and its role in pathogenesis and inter-bacterial competition.