Received: 20 September 2019 | Revised: 18 November 2019 | Accepted: 21 November 2019

DOI: 10.1111/1348-0421.12762

Microbiology and Immunology WILEY

REVIEW

Intracellular survival and innate immune evasion of *Burkholderia cepacia*: Improved understanding of quorum sensing-controlled virulence factors, biofilm, and inhibitors

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Abstract

Burkholderia cepacia complex (*Bcc*) are opportunistic pathogens implicated with nosocomial infections, and high rates of morbidity and mortality, especially in individuals with cystic fibrosis (CF). *B. cepacia* are naturally resistant to different classes of antibiotics, and can subvert the host innate immune responses by producing quorum sensing (QS) controlled virulence factors and biofilms. It still remains a conundrum as to how exactly the bacterium survives the intracellular environment within the host cells of CF patients and immunocompromised individuals although the bacterium can invade human lung epithelial cells, neutrophils, and murine macrophages. The mechanisms associated with intracellular survival in the airway epithelial cells and the role of QS and virulence factors in *B. cepacia* infections in cystic fibrosis remain largely unclear. The current review focuses on understanding the role of QS-controlled virulence factors and biofilms, and provides additional impetus to understanding the potentials of QS-inhibitory strategies against *B. cepacia*.

KEYWORDS

biofilms, *Burkholderia cepacia*, quorum sensing, virulence

1 | INTRODUCTION

Burkholderia cenocepacia is a common member of the *Burkholderia cepacia* complex (*Bcc*). Similar groups of ~20 gram-negative bacterial species (*B. cepacia, B. multivorans, B. cenocepacia, B. stabilis, B. vietnamiensis, B. dolosa, B. ambifaria, B. anthina, B. pyrrocinia, B. ubonensis, B. lateens, B. diffusa, B. arboris, B. seminalis, B. metallica*, and *B. lata*) are reported to be widely distributed in the natural environment.[1] Clinically, only three of the above have been under the spotlight of cystic fibrosis (CF), which includes *B. cenocepacia,*[2]

Abbreviations: AI, autoinducers; AHL, acyl homoserine lactones; Bcc, *Burkholderia cepacia complex*; BDSF, Burkholderia diffusible signal factor; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; C6-HSL, *N*-hexanoyl-homoserine lactone; C8-HSL, *N*-octanoyl-homoserine lactone; C10-HSL, *N*-decanoyl-homoserine lactone; DC, dendritic cell; c-di-GMP, cyclic dimeric guanosine monophosphate; EPS, exopolysaccharides; IL, interleukin; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MDR, multidrug resistance; NF-κB, nuclear factor-kappa B; PGE2, 3OC6-HSL, *N*-3-oxo-hexanoly-homoserine lactone; prostaglandin E2; QS, quorum sensing; REC, receiver domain; T3SS, type-3 secretion system; TLR, Toll-like receptor; TNF, tumor necrosis factor; Zmp, zinc-dependent metalloproteases.

B. multivorans,[3] and *B. dolosa*.[4] *B. cepacia* is normally found in soil, water, amoebae, and nematodes. *B. cepacia* complex (*Bcc*) occur as opportunistic pathogens in human and plants. *B. cepacia* is implicated with nosocomial infections, and in individuals admitted with chronic granulomatous disease, cystic fibrosis, in particular.[5] In clinical settings, *Bcc* is linked to high rates of human morbidity and mortality due to respiratory manifestations associated with cystic fibrosis.[6] A vast majority of cystic fibrosis patients develop chronic disease with *Pseudomonas aeruginosa* and *B. cepacia,* which are often associated with impaired respiratory functions.[7] Similarly, in tertiary care settings, *B. cepacia* causes severe infection among immunocompromised individuals and HIV-infected patients.[5,8] Recent lines of evidence indicate that *B. cepacia* is an emerging nosocomial pathogen.[5,8] The dissemination of *B. cepacia* in health care settings has been linked to cross-transmission, central venous access, and frequent pulmonary procedures.[9] In the hospital environment, *B. cepacia* can spread by contact with infected individuals as well as contaminated fomites.[10]

Upon entry, the bacterium proliferates in the local host tissue and initiates primary infection in the respiratory mucosa before disseminating into adjacent organs whilst manifesting the "cepacia syndrome."[11] In cepacia syndrome, >20% of cystic fibrosis patients infected with *B. cepacia* present with fever, acute pneumonia, and bacteremia.[12] It has also been reported that *Bcc* strains become converted to resistor cells due to the production of quorum sensing (QS)-controlled virulence factors and biofilm.[13] *Bcc* are often resistant to quinolones,[14] aminoglycosides,[15] β-lactams,[16] and several host antimicrobial peptides especially β-defensins.[17] *B. cepacia* uses a QS signaling molecule controlled by the QS system.[18] It is known that most gram-negative bacteria communicate with other bacterial cells in the closer vicinity via secretion of small signaling molecules called autoinducers (AIs), which are under the control of the QS system.[18,19] Once the threshold concentration is reached to a certain cell density, the bacteria acquire the ability to release virulence factors, and form biofilms.[18] *B. cepacia* employs two QS-systems, which play a paramount role in the synthesis of virulence factors and in the development of resistance to many conventional antibiotics to mislead the host immune system. *B. cepacia* are naturally resistant to different classes of antibiotics, and can subvert the host innate immune responses by producing QS-controlled virulence factors and biofilms,[20,21] which complicate disease severity, and therefore treatment for *B. cepacia* infection appears to be highly challenging.

2 | CYSTIC FIBROSIS

Cystic fibrosis is a genetic disorder arising from an inborn error of metabolism, which is mainly considered as a risk factor for acquisition of infection involving the lungs. A mutation occurring in the CFTR gene interrupts the activity of chloride channels, and blocks them from regulating the flow of chloride ions and water across the cell membrane.[22] A specific mutation within the CFTR gene called Delta F508 (ΔF508) produces a deletion of three nucleotides in the CFTR gene on chromosome 7 resulting in the loss of a single codon for phenylalanine. As a result of the mutation, an abnormal CFTR protein lacking phenylalanine residue is produced due to which the protein is mis-folded. The mis-folded protein becomes trapped in the endoplasmic reticulum and is not processed further resulting in the loss of function.[23] The hallmark of cystic fibrosis is the production of a thick and sticky mass of mucus, which can cause severe lung injury, also involving the pancreas, and other viscera.[24,25] Because of the loss of function, chloride ion conductance is affected, which is followed by reduced levels of chloride–potassium ion secretion and elevated sodium and water absorption in the apical membranes of lung epithelial cells. This results in the accumulation of thick mucus in the CF airways, creating a suitable niche for *B. cepacia* to thrive. CFTR abnormality leads to defective mucociliary clearance and impaired innate immune responses along the respiratory airway, resulting in patients becoming vulnerable to infection by chronic respiratory pathogens and acute exacerbations.[7] Hospital admitted cystic fibrosis patients who are immunocompromised are more often colonized by gram-negative pathogens, for instance *P. aeruginosa, Bcc*, and *Haemophilus influenzae*.[26] Notably, *B. cepacia* and *P. aeruginosa* are known to co-aggregate in the lungs, causing severe respiratory illness. The *Bcc* bacteria can be transmitted from human to human. In cystic fibrosis patients, the bacteria are reported to cause a fatal necrotizing pneumonia associated with septicemia, wherein which it can reside intracellularly within dust cells[27,28] and respiratory epithelial cells.[29] Formation of biofilm occurs in the CF lung initially mediated by motility and secretion of various virulence factors. Gradually the production of exopolysaccharides (EPS) occurs causing chronic inflammation. Prolonged inflammation generated by the organism leads to severe lung damage. The organism cannot be completely eliminated from the CF lung because of the natural antibiotic resistance it exerts.[30] *B. cepacia* is naturally resistant to β-lactams, quinolones, aminoglycosides,[31] and antimicrobial peptides.[17] Antibiotic resistance increases also because of the formation of a drug impermeable biofilm by the organism.[32]

3 | INVASION AND INTRACELLULAR SURVIVAL OF *B. CEPACIA*

3.1 | Epithelial cells

The human epithelium plays a significant role in maintaining the conduit of air to and from the lung alveoli. Columnar epithelial cells are major components of the respiratory epithelium, which play a crucial role in protecting the respiratory airway from pathogens by mucociliary clearance. There are three types of airway epithelial cells, namely, the ciliated cells, goblet cells, and the basal cells. Ciliated cells facilitate the mucociliary clearance, whereas the goblet cells produce mucins, a major component of mucus. The basal cells have the ability to differentiate into other epithelial cells and function during airway injuries.[33] The epithelial layer acts as a protective barrier against infections as these cells are closely adherent to each other by tight junctions and adherins.[34] It produces antimicrobial components such as defensins, lysozyme, and nitric oxide to eradicate airway pathogens.[35] It is also involved in the secretion of certain cytokines, which mediate the activation of other immune cells.[36] The respiratory epithelium is the primary layer of defense against airway pathogens[37] because of which it is more prone to infections relative to other respiratory cells.

Pathogenic bacteria enter the pulmonary compartment via inhalation and subsequently into the airway epithelial cells of the host. Following inhalation, the epithelial cells offers the first line of barrier defense, followed by innate immune mechanisms put forth by dust cells, neutrophils, and dendritic cells (DCs).[38] Nonetheless, *B. cepacia* has strong invasive and migratory potentials in the airway across the epithelial barriers, and therefore can invade the lumen as well as blood capillaries.[27] *B. cepacia* is an intracellular pathogen that can remain within the endosomes of alveolar phagocytes and respiratory epithelial cells.[37] However, it still remains a conundrum as to how exactly the bacterium survives the intracellular environment within the host cells of CF patients and immunocompromised individuals although *B. cepacia* can invade human lung epithelial cells, neutrophils, and murine macrophages.[13,39]

B. cepacia is highly invasive, and disseminates through the airway across the epithelial barriers causing septicemia.[40] The intracellular invasion of *B. cepacia* is mainly mediated via membrane-bound vacuoles after initial attachment of the bacterium to the epithelial cell membranes. After invading the lung epithelial cells, the bacterium forms a biofilm, rearranges the cell's cytoskeleton, causes cellular destruction, and releases a plethora of virulence factors into the host cells.[41] *B. cepacia* expresses cable pilin protein adhesion, which binds to the host mucins of the buccal epithelial cells.[42] The type-3 secretion (T3SS) system of *B. cepacia* is of paramount importance for its invasion into epithelial cells. It is also involved in the disruption of actin filaments of primary lung epithelial cells.[43] Certain virulence factors such as flagellin also assists bacterial invasion into epithelial cells. Experimental evidence suggests that mutations in the *fliC* gene of *B. cepacia* has led to reduced interactions with A549 epithelial cells.[44] Burns et al.[37] have reported that *B. cepacia* CEPO40 survives and replicates intracellularly in human respiratory epithelial cells. Environmental isolates of *B. cepacia* J2540, *B. cepacia* J2315 rapidly enter macrophages, and the frequency of such invasion was high, although less invasive than clinical strains in epithelial cells.[28] *B. cepacia* C1359 and NCTC10743 multiply and survive within A549 epithelial cells for >24 hr and *P. aeruginosa* PAO1 lacks the ability to multiply within the lung epithelial cells (A549), which significantly decreases after 24 hr.[45] *B. cepacia* C1359 multiplies intracellularly in type II pneumocytes as shown by a seven-fold increase in the number of bacteria (*B. cepacia* C1359) from 4 to 24 hr, whereas *B. cepacia* 10661 was unable to multiply for at least 24 hr.[46] A similar strategy has also been reported in *B. pseudomallei*.[47] Martin and Nohrr,[28] have reported that an environmental isolate of *B. cepacia* (J2540) has the ability to invade and survive intracellularly in a human cell line culture. A clinical strain and environmental isolate (J2540) of *B. cepacia* entered into macrophages with an invasion frequency similar to that of the clinical strain. But, when compared with clinical strains, the environmental isolate of J2540 was less invasive in epithelial cells. *B. cepacia* and its secreted proteins (virulence) are effectively invasive in A549 epithelial cells and during the infection *B. cepacia* induces a homeostatic response in the host cells for their prolonged survival within epithelial cells.[48]

3.2 | Macrophages

Macrophages are potent antigen-presenting cells that play a major role in the removal of pathogenic invaders. They are involved in phagocytosis, processing, and presentation of the pathogen to B and T lymphocytes. *B. cepacia* enters macrophages via macropinocytosis, where the organism moves freely in a macropinosome-like compartment. Macropinocytosis-mediated infection and the formation of a macropinosome-like compartment depends on the expression of T3SS.[49] *B. cepacia* has the ability to survive inside macrophages[50] due to their

resistance towards oxidative damage[51] and also because of the delay in phago-lysosomal fusion,[52] which acts as an important survival strategy for *B. cepacia* inside the lung. Saldias et al.[53] reported that the O-antigen moiety of *B. cepacia* lipopolysaccharide (LPS) reduced *Bcc* bacterial engulfment by phagocytosis. Bcc bacteria release several types of LecA-like surface lectins which may help in its entry into epithelial cells.[54] The oligosaccharide region of the lipid A core present in *B. cepacia* LPS plays a major role in the secretion of proinflammatory cytokines in addition to cell signalling. Interaction of the bacteria with macrophages leads to the production of interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α), which further recruits other phagocytic cells to the site leading to chronic infection.[55] IL-1β is a significant mediator of the acute-phase response and is involved in necessitating lung damage.[56] TNF-α produced by *B. cepacia* is more potent when compared with *P. aeruginosa*.[57] Persistence of these proinflammatory cytokines in cystic fibrosis could lead to prolonged inflammation, a rapid decline in lung function followed by early death.[58]

3.3 | Proinflammatory cytokines

B. cepacia is reported to cause cepacia syndrome in ~20% of cystic fibrosis patients. *B. cepacia* infections cause rapid inflammation in which infiltration of polymorphonuclear leukocytes followed by necrosis of respiratory epithelium could lead to high mortality rates.[59] Rapid inflammation is mediated by the production of proinflammatory cytokines by infected cells. Proinflammatory cytokines produced by the *B. cepacia*-affected cells recruit other inflammatory cells to the site of infection leading to disease exacerbation. Extracellular substances released by *B. cepacia* also trigger the release of IL-8 by respiratory epithelial cells.[60]

The cell-free supernatants of *B. cepacia* reportedly induce the production of IL-8, IL-6, and prostaglandin E2 (PGE2) in significant concentrations. Comparison between wild-type CFTR epithelial cells (A549) and CFTR mutant cells (IB3, S9, C38) has revealed that CFTR mutant cells produce increased concentrations of proinflammatory cytokines compared with the wild-type cells. Activation of nuclear factor-kappa B (NF-κB) is required for the cytokine production which appears to be stimulated by the cell-free supernatants of *B. cepacia*. The activity of NF-κB is also required for the production of cyclo-oxygenase-2, which is key to prostanoid synthesis.[61] Inhibition of *B. cepacia* induced NF-κB reporter by dexamethasone has revealed that a functional CFTR is not required for a NF-κB response to *B. cepacia*.[62] Though the exact composition of the cell-free supernatant of *B. cepacia* remains largely unknown, several virulence factors appear to trigger cytokine production. The LPS complex and flagella of *B. cepacia* are two known stimulators of the proinflammatory response leading to severe lung damage, and notably both components are QS-regulated structures of *B. cepacia*.[63]

Research suggests that highly purified LPS of *B. cepacia,* via TLR4 sensing, induces NF-κB activation and IL-1β production.[13,64] The lipid A component of LPS induces the IL-6 and TNF-α production by macrophages.[65] IL-1β is a key cytokine required for tissue destruction and pyroptosis[66] whose production during *B. cepacia* infection in macrophages requires TLR4 and caspase-1, mediated by the O antigen of the LPS complex.[67] Constant phagocytosis followed by macrophage activation can result in chronic inflammation. The flagella of bacteria contribute towards the host inflammatory responses together with motility, biofilm formation, adhesion, and invasion into epithelial cells.[68] *B. cepacia* flagellin can interact with TLR5, which can induce NF-κB and mitogen-activated protein kinase (MAPK) pathways that signal the production of IL-8, IL- 6, TNF-α, and IL- 1.[69] IL-8 plays a major role in tissue damage and recruitment of neutrophils. Infiltration of neutrophils causes excessive release of neutrophil oxidants and enzymes in the lung environment, stimulating mucus production, which worsens the condition in CF.[70] Dendritic cells infected with *B. cenocepacia* appears to have an over-expression of IL-8 messenger RNA (mRNA) levels. Prolonged infection leads to a significant increase in the levels of IL-6, IL-1β, IL-12, TNF-α, and IL-23 mRNA suggestive of inflammation.[71]

4 | OVERVIEW OF BACTERIAL QS

The production of virulence factors and biofilm in gram-negative organisms is controlled by the QS system.[72] QS refers to a phenomenon whereby bacteria are able to communicate with neighboring cells via autoinducers (AIs). Once AI molecules accumulate in the medium, the population density increases and the bacteria are able to sense the information to track changes in their numbers and also to communally modify gene expression and regulation of diverse physiological functions.[73] QS appears to control the production of various virulence factors, toxins, biofilm formation, symbiosis, antibiotic production, sporulation, competence, conjugation, bioluminescence, and is important for disease pathogenesis.[74] Several types of QS signals exist across many gram-negative bacteria, which utilize acyl homoserine lactones (AHLs) as signaling molecules.[75]

4.1 | Gram-negative bacterial QS

The QS system was first described in the 1960s and 1970s in *Vibrio fischeri,* a bioluminescent marine bacterium.[76] Once the bacterial threshold concentration is reached in the medium, the bacterium releases acyl homoserine lactones (AHL), which controls bioluminescence via QS proteins, LuxR (repressor), and LuxI (inducer). LuxI is an autoinducer synthase that produces *N*-3-oxo-hexanoly-homoserine lactone (3OC6-HSL), diffuses back into the cell, and specifically binds to LuxR. LuxR protein inducing the C-terminal domain activates the expression of the *luxCDABEG* operon, which contains the genes that facilitate bioluminescence in *V. fischeri*.[77] Besides, prototypes of the QS system have also been characterized in several other gram-negative pathogens such as *P. aeruginosa, Escherichia coli, B. cepacia, B. pseudomallei*, and *Yersinia pseudotuberculosis.* Of note, *P. aeruginosa* has two QS systems, namely *las* and *rhl,* which utilize AHLs as signaling molecules. *Bcc* are capable of utilizing two different types of signaling molecules, namely, the AHLs and *cis-2-* unsaturated fatty acids (Table 1).

5 | QUORUM SENSING IN *B. CEPACIA*

Many gram-negative bacteria release signaling molecules used in the secretion of virulence factors via a cell-density-dependent mechanism called the QS circuit.[82] *B. cenocepacia* consist of two set of QS genes (*cepIR* and *cciIR*) responsible for the synthesis and regulation of QS-controlled virulence factors and biofilm formation. The *CepI* and *CepR* are global regulatory cell-to-cell communication systems widely distributed across all Bcc species. The CepIR and CciIR QS systems normally interact with CepR, positively regulating the expression of the CciIR and CciR system and negatively regulating its own CepI expression. *CepI* is an AHL synthase, contributing to the synthesis of *N*-octanoyl-homoserine lactone (C8-HSL) and also synthesizes small amounts of *N*-hexanoyl-homoserine lactone (C6-HSL).[85] CepR is a transcriptional regulator that has both positive and negative regulatory properties. In *B. vietnamiensis* strains, CepR is essential for the control and expression of yet another QS system called BviIR, which utilizes *N*-decanoyl-homoserine lactone (C10-HSL).[20] These two QS systems (*CepI/CepR*) positively regulate, as well as are controlled by, the expression of extracellular proteases (zinc-dependent metalloproteases ZmpA and ZmpB),[86] swarming motility,[87] biofilm formation,[88] chitinases,[89] lipopeptide toxins,[90] and endo-polygalacturonase.[91] Similarly, the QS system negatively regulates, and synthesizes siderophores (pyochelin, salicylic acid, cepabactin, and ornibactins).[84,92] The *CepR2* QS system is present in *B. cenocepacia,* but not in other *Bcc* strains, and besides, this system is not involved in the regulation of any virulence factors (depicted in Figure 1).

5.1 | Fatty acid signal (diffusible signal factors)

The bacterium uses different types of QS signaling molecules for necessitating disease pathogenesis, and releases myriad virulence factors. Particularly, *Bcc* strains employ *cis-2-* unsaturated fatty acids referred to as *Burkholderia* diffusible signal factor (BDSF) signaling molecule, which is used to communicate with other bacteria in the vicinity.[93] The DSF molecule, *cis*-11-methyl-2-dodecenoic acid was first isolated from the supernatants of *Xanthomonas campestris,* and is also produced by *B. multivorans*.[94] The DSF signal has also been identified in *B. cenocepacia. Stenotrophomonas maltophilia* alters the antibiotic resistance, virulence factors, and persistence of *P. aeruginosa* in the airway

TABLE 1 Regulation of autoinducer molecules and QS system in gram-negative bacteria

S. No	Gram-negative bacteria	QS system/peptides	References
1	*V. fischeri*	LuxI/LuxR (Acyl homoserine lactones)	Dunlap[78]
2	*E. coli*	LsrABCD (Autoinducer 2)	Li et al.[79]
3	*S. typhimurium*	LuxS/AI-2 ((2R,4s)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran	Meijler et al.[80]
4	*P. aeruginosa*	Las and Rhl (*N*-(3-oxododecanoyl) homoserine lactone & *N*-butyryl homoserine lactone	Pesci and Iglewski,[81]
5	*B. cepacia* K56-12	cepIR (*N*-octanoylhomoserine lactone)	Lawenza et al.[82]
6	*A. baumannii*	LuxIR/AbaIR (*N*-3-hydroxy-dodecanoyl-homoserine lactone)	Niu et al.[83]
7	*A. hydrophila*	AhyRI/AsaRI (*N*-(butanoyl)-L-homoserine lactone & *N*-hexanoyl-L-homoserine lactone)	Swift et al.[84]

Abbreviation: QS, quorum sensing.

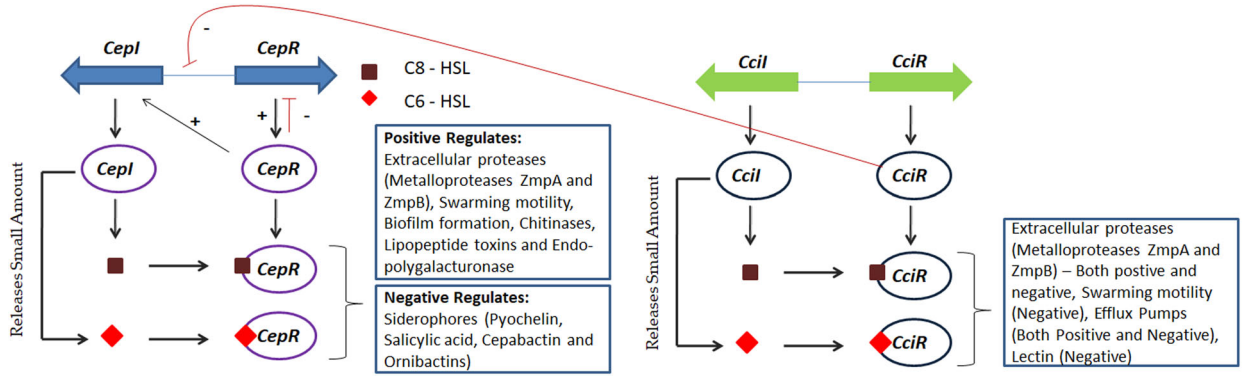

FIGURE 1 Quorum sensing systems in *Burkholderia cepacia* (C8-HSL and C6-HSL) [Color figure can be viewed at wileyonlinelibrary.com]

of patients with cystic fibrosis.[95] DSFs are involved in the regulation of bacterial motility, virulence factors, and biofilm formation in *Bcc*. BDSF is synthesized by the enoyl-CoA hydrates RpfFBc and is sensed by the receptor protein RpfR, which consists of Per/Arnt/Sim (PAS)-GGDEF-EAL domains. BDSF bind to the PAS domain and stimulates the cyclic dimeric guanosine monophosphate (c-di-GMP) phosphodiesterase activity of RpfR, which in turn lowers the intracellular c-di-GMP level. In *X. campestris,* the DSF receptor, RpfC, is a hybrid sensor kinase that phosphorylates the cognate response regulator RpfG.[96] Similarly, in addition to a receiver (REC) domain, this regulator consists of a HD-GYP domain, which is responsible for the c-di-GMP phosphodiesterase activity of the protein. RpfR regulates and controls the same phenotypes of BDSF that includes swarming motility, virulence, biofilm formation, and resistance to many antibiotics.[93,97]

6 | INTRINSIC RESISTANCE TO ANTIBIOTICS IN CLINICAL CYSTIC FIBROSIS

The emergence of multidrug resistance (MDR) in biofilm forming strains of *Bcc* poses a major threat and significant challenges in the therapeutic setting. *Bcc* strains are intrinsically resistant to β-lactams, chloramphenicol, quinolones, first and second generations of cephalosporins, trimethoprim, polymyxins and aminoglycosides, piperacillin, ticarcillin, and disinfectants.[98,99] In *Bcc* strains, multidrug resistance occurs due to the presence of various efflux pumps, which effectively splits antibiotics from the cells, prevents, and dispenses the entry of antibiotics into the bacterial cell surface and changes the cell envelope to lower the permeability to antibiotics. Similarly, multiple antibiotic resistance mechanisms have also been reported in the *Bcc* strains, including reduced outer membrane permeability, integrons, and alteration in drug targets. Tseng and others[100] have reported that ceftazidime- and chloramphenicol-resistance in clinical *Bcc* strains could be due to the increased expression of the RND-3 efflux pump and a specific mutation in the RND-3 efflux pump regulator gene. The exopolysaccharide, EPS, is one of the other key virulence factors involved in biofilm formation. An important characteristic of biofilm includes conferring resistance to antimicrobial agents and evasion from host immune responses, leading to an onset of recalcitrant infections.[101] The production of numerous virulence factors, extracellular degradative enzymes, and biofilm formation in bacteria are extremely difficult to negate and warrants effective alternate strategies.

7 | PRODUCTION OF VIRULENCE FACTORS AND FORMATION OF BIOFILM IN *B. CEPACIA*

Bcc plays a vital role in causing diseases in humans and animals. They possess a multitude of virulence factors, which enhances pathogenicity in different stages of infection in host cells, particularly in lung epithelial cells.[102] The production of extracellular virulence factors that include protease, haemolysin, lipase, and gelatinase, iron-chelating siderophores, T3SS and T4SS, LPS, pili and flagella and capsule, are identified by the use of different infection models.[103] Although *Bcc* is not known to produce toxin A and exoenzyme S, they are indeed capable of producing cytotoxic effects in vitro.

7.1 | Zinc metalloproteases

In *Bcc* strains, the *cepIR* genes and *cciIR* genes influence the control of extracellular proteases. The production of extracellular proteases may contribute to increased

potentials to cause disease pathogenesis. The *ccilR* genes found in *B. cenocepacia* contain the *cci* genomic island.[104] *Bcc* produces two different Zmps that include ZmpA and ZmpB, which are not genetically linked. A 36 kDa Zmp has been purified from *B. cepacia* genomovar III strain Pc715j.[105] *Bcc* also secrete ZmpA and ZmpB proteases, which are involved in disrupting tissue integrity and host defense mechanisms. ZmpA protease reportedly cleaves gamma interferon, IL-37, elafin, and secretory leukocyte inhibitor, and ZmpB cleaves human immunoglobulin, β-defensin-1, and lactoferrin. Most of the bacteria release proteases, which are inhibited by α_2-macroglobulin. ZmpA and ZmpB are able to digest α_2 macroglobulin, α-1 proteinase and inactivate antimicrobial peptide.[106]

B. cepacia complex produces two types of extracellular proteases that can inactivate mammalian protease inhibitors and can cleave gelatin, and human collagen types I, IV, and V. ZmpA and ZmpB are involved in synthesizing and releasing virulence factors, but are not required for bacterial survival in host models. Gingues and others[107] suggested that *zmpA* genes are present only in *B. cepacia, B. cenocepacia, B. subtilis, Bambifara*, and *B. pyrrocinia* but not in *B. multivorans, B. vietnamiensis, B. dolosa*, and *B. anthina*. McKevitt and others[105] reported that the intracheal instillation of purified proteinase injected into rat lungs leads to the onset of a form of bronchopneumonia characterized by polymorphonuclear cell infiltration and proteinaceous exudation into large airways.

7.2 | Lipases

Bcc are one of the most important lipase-producing bacteria. A type II secretion pathway is operational under the control of the *cepIR* QS system involved in the synthesis of a large number of lipases in *Bcc*.[108] The expression and production of extracellular lipase among the *Bcc* isolates is highest among *B. multivorans* and *B. cenocepacia*.[109] The production of lipase establishes the potential role of *Bcc* to invade the lung epithelial cells,[109] which has already been reported in *Helicobacter pylori* and *Propionibacterium acnes*.[110,111]

7.3 | Siderophores

Bcc bacteria possess an efficient mechanism for iron acquisition, and have four different types of siderophores that include ornibactin, pyochelin, cepabactin, and cepaciachelin that employ the full range of iron-binding groups present in naturally occurring siderophores. In cystic fibrosis, the *Bcc* bacteria have been reported to colonize the lungs, and require the expression of high-affinity iron uptake systems due to decreased iron availability.[112,113] The bacterium commonly secretes low molecular-weight Fe (III)-bound compounds known as siderophores, which are then transported into the bacterial cells through specific outer membrane receptors via the TonB-ExbB-ExbD protein complex.[114] Ornibactin is a modified tetrapeptide siderophore, consisting of a L-ornithine-D-hydroxyaspartate-L-serine-L-ornithine backbone that requires two types of nonribosomal peptide synthetases, Orbl and Orbj for its assembly. A linear hydroxamate/hydroxycarboxylate siderophore has been isolated and purified from *B. cepacia*.[115] The yellow-green fluorescent pyochelin phenolic siderophore promotes the removal of iron from host cell transferrin. The pyochelin siderophore is synthesized from salicylate by the successive addition of two molecules of cysteine, and the process requires NRPSs, PchE, and PchF.[116,117]

7.4 | Lipopolysaccharide

Lipopolysaccharide has biologically active outer membrane components that play a paramount role in host–pathogen interactions. LPS is a major virulence factor released by *Bcc,* and is mainly associated with an increase in the severity of disease pathogenesis in cystic fibrosis. LPS production in *Bcc* is controlled by the QS system. The O polysaccharide structure of *B. cepacia* serotype O4 consists of two polymers such as a major polymer, composed of Gal_2-$GalNAc_1$ and a minor polymer, composed of $GalNAc_2$–Rha.[118] It has an inner core saccharide region adjacent to the lipid A. The LPS is constructed by one to three molecules of D-glucose, L-glycero-D-manno-heptose, D-glycero-D-manno-heptose, 3-deoxy-D-manno-octulosonic acid, and D-glycero-D-talo-2-octulosonic acid, which are well conserved among the various bacterial species.[119] LPS mainly contributes to antimicrobial peptide resistance and the promotion of a potent proinflammatory cytokine response (by endogenous pyrogens TNF-α, IL-6, and IL-8) in humans. *Bcc* LPS elicits a strong immune response contributing to host cell damage and immunopathogenesis.[120] *Bcc* strains are intrinsically resistant to many antibiotics including aminoglycosides/sulphamethoxazole, polymyxin, and chloramphenicol resulting from decreased site-specific drug binding of these cationic drugs to LPS reduced outer membrane permeability and efflux pump.

8 | BIOFILM FORMATION BY *B. CEPACIA* AND BIOFILM INHIBITORS

Bcc bacteria form biofilms on abiotic surfaces such as glass and plastics. Similarly, they can form biofilms on

biotic surfaces such as human lung epithelial cells, leading to the notion that biofilmogenesis do play a paramount role in persistent lung infection in clinical cystic fibrosis.[1] *Bcc* bacteria form a biofilm matrix, which presumably supports their survival in the environment as well as in host cells, and confers increased resistance to synthetic drugs relative to planktonic cells. Biofilm production is controlled by the AHL-mediated QS system in several gram-negative pathogens, notably *Bcc* bacteria possess a CepIR QS system. The AHL-mediated biofilm synthesis was first reported in *B. cenocepacia* H111.[121] Biofilm development also involves the adhesion of cells to the surface via pili or flagella that often act as adhesins. *Bcc* bacteria are equipped with five types of pili, namely mesh (Msh), filamentous (Fil), spine (Spn), spike (Spk), and cable pili.[20] Of all the pili, the cable pilus (encoded by the cblA gene) plays a crucial role in adhesion to lung epithelial cells. Although it is not widely distributed in the *Bcc*, not all cblA-positive strains produce the pili. *B. cenocepacia* is controlled by the RpfFR QS system.[122] EPS is one of the major constituents of the biofilm matrix, which is involved in cell attachment and the mechanical stability of the biofilm.

There are limited numbers of biofilm inhibitors identified, which are active against biofilm formation by *B. cepacia*. Several plant derivatives, marine bioproducts, and synthetic biofilm inhibitors are reported to inhibit the QS-controlled virulence factor production and biofilm formation in drug resistant *B. cepacia*. Cellulase significantly inhibits QS and reduces biofilm growth against *B. cepacia*.[123] Narayanaswamy and colleagues[124] reported that the poly (acetyl, arginyl) glucosamine (glycopolymer) reduced the thickness of biofilm formation in *B. cepacia*. Diketopiperazine inhibitors have anti-virulence properties and these inhibitors interfere with protease, siderophore, and biofilm formation in *B. cenocepacia*.[125] At sub-inhibitory concentrations, carboxymethyl chitosan combined with metallic salts effectively inhibits ~80% biofilm formation and reduces the toxicity levels in *B. cepacia*.[126] Similarly, a combined therapy of baicalin hydrate and cinnamaldehyde with tobramycin significantly reduced the QS controlled virulence factors and biofilm formation in *Bcc* bacteria.[127]

9 | CONCLUSIONS

In healthcare settings, the treatment of CF-associated infections attributed to *Bcc* remains a major challenge as it produces a wide array of QS controlled virulence factors and biofilm, which impacts disease progression. Furthermore, due to the production of several virulence factors, the *Bcc* bacteria acquire the ability to invade the airway epithelial cells besides being resistant to many of the conventional antibiotics used in the treatment and management of cystic fibrosis. Despite the challenges prevailing in combating QS-controlled virulence factors and biofilm formation by *Bcc* bacteria, there are indeed anti-bacterial compounds and biofilm inhibitors to treat *Bcc* infection. The current concerns looming over QS-controlled virulence factors and biofilm forming drug resistant *Bcc* bacterial colonization in cystic fibrosis warrants the need to develop newer compounds, which can effectively inactivate both QS-controlled virulence factors, and biofilm formation to effectively prevent bacterial establishment in the respiratory compartment in clinical cystic fibrosis.

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How to cite this article: Ganesh PS, Vishnupriya S, Vadivelu J, Mariappan V, Vellasamy KM, Shankar EM. Intracellular survival and innate immune evasion of *Burkholderia cepacia*: Improved understanding of quorum sensing-controlled virulence factors, biofilm, and inhibitors. *Microbiology and Immunology*. 2020;64:87–98. https://doi.org/10.1111/1348-0421.12762