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***Burkholderia cepacia* Complex Infections Among Cystic Fibrosis Patients: Perspectives and Challenges**

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Abstract

The *Burkholderia cepacia* complex (Bcc) is a group of closely related bacterial species that emerged in the 1980s as the etiological agents of severe and often lethal respiratory infections among cystic fibrosis (CF) patients. After several outbreaks in CF centers in Europe and North America, segregation measures were introduced to avoid patient-to-patient transmission. Presently, the prevalence of Bcc infections among CF patients worldwide is below 5% in the majority of CF centers, although exceptions are registered in some European countries. Infections by these pathogens remain problematic due to the high resistance to antimicrobials, the easy patient-to-patient transmission, and the unpredictable outcome of infections that range from asymptomatic carriage to the cepacia syndrome, a fulminating pneumonia often associated with septicemia that can lead to the decease of patients within a period of time as short as 1 week. In this chapter, we review the evolving epidemiology of Bcc infections in CF patients, the virulence traits and mechanisms used by these bacteria, and the recent developments in vaccine and vaccine components research to prevent Bcc infections.

Keywords: *Burkholderia cepacia* complex, emerging species, evolving epidemiology, virulence determinants, immunoreactive proteins, vaccine development

1. Introduction

The *Burkholderia cepacia* complex (hereafter referred to as Bcc) is a group of closely related bacteria that emerged in the 1980s as problematic pathogens to cystic fibrosis (CF) patients [1]. Infections by Bcc are particularly feared due to (1) the easy patient-to-patient transmission of

specific strains; (2) the ability to resist to multiple antibiotics; and (3) the unpredictable outcome of infections, which ranges from asymptomatic carriage to the so-called cepacia syndrome, an often lethal necrotizing pneumonia accompanied with septicemia [1, 2]. Initially described in the 1950s by Burkholder [3] as the cause of soft rot in onions, the species then named *Pseudomonas cepacia* was moved into the new genus *Burkholderia* after the work of Yabuuchi and colleagues in 1992 [4]. However, the most impressive developments on the taxonomy of this group of bacteria have been achieved after the seminal work of Vandamme and colleagues who proposed the division of the species into distinct genomovars [5]. Presently, the Bcc comprises 20 species (Table 1), and the genome sequence of several strains is publicly available in databases such as the Burkholderia Genome DB and the Integrated Microbial Genomes & Microbiomes [6, 7].

Bcc species	Genome sequence availability	Reference
<i>B. ambifaria</i>	4 complete genomes (strains AMMD, MC40-6, MEX-5, IOP-120)	[8]
<i>B. anthina</i>	In progress	[9]
<i>B. arboris</i>	In progress	[10]
<i>B. cenocepacia</i>	18 complete genomes (strains J2315, H111, AU1054, B1, MCO-3, PC184, HI2424, DDS 22E-1, DWS 37E-2, ST32, 842, 895, MSMB384 WGS, 6, 7, CEIB, 869T2, TAtl-371)	[11]
<i>B. cepacia</i>	8 complete genomes (strains 383, AMMD, ATCC 25416; Bu72, DDS 7H-2, GG4, JBK9, LO6)	[4]
<i>B. contaminans</i>	1 complete genome (strain MS14)	[12]
<i>B. diffusa</i>	In progress	[10]
<i>B. dolosa</i>	1 complete genome (strain AU0158)	[13]
<i>B. lata</i>	1 complete genome (strain 383)	[12]
<i>B. latens</i>	In progress	[10]
<i>B. metallica</i>	No information	[10]
<i>B. multivorans</i>	3 complete genomes (ATCC17616, ATCC BAA-247, DDS 15A-1)	[5]
<i>B. pseudomultivorans</i>	In progress	[14]
<i>B. pyrrocinia</i>	1 complete genome (strain DSM 10685)	[9]
<i>B. seminalis</i>	In progress	[10]
<i>B. stabilis</i>	No information	[15]
<i>B. stagnalis</i>	In progress	[16]
<i>B. territorii</i>	In progress	[16]
<i>B. ubonensis</i>	1 complete genome (strain MSMB22)	[17]
<i>B. vietnamiensis</i>	3 complete genomes (strains G4, LMG10929, WPB)	[18]

Databases were assessed by the end of July 2016.

Table 1. *Burkholderia cepacia* complex species names and genome sequence availability in the databases Burkholderia Genome DB and Integrated Microbial Genomes & Microbiomes [6, 7].

2. Evolving epidemiology of Bcc infections

All Bcc species are virtually potential pathogens to CF patients. However, epidemiology studies have shown an uneven geographical and regional distribution of clinical isolates among the Bcc species, with the predominance of *Burkholderia cenocepacia*, followed by *Burkholderia multivorans*. Early studies performed during the 1980s and 1990s have shown that in addition to cases of chronic infection due to specific strains, many outbreaks reported in Europe and North America were due to the spread of particularly virulent strains that easily disseminated within a given CF center [1]. Although the environment is thought to be the natural reservoir of these strains, a definitive proof is still lacking.

A few particularly epidemic strains became notorious for the worst reasons. Perhaps, the best-known strain is the Edinburgh-Toronto lineage also known as the ET12 clone, an intercontinental clone responsible for several infections and fatalities in CF centers in the UK and Canada [19]. The best-known representative strain of this highly transmissible clone is the *B. cenocepacia* J2315 strain, the first Bcc strain with its genome sequence publicly available (**Table 1**) and one of the best studied Bcc strains [20]. Another example of a strain that disseminated within centers and even among centers is the PHDC strain. The strain, responsible for almost 20% prevalence in one CF center in the USA, was later found in another CF center, where an increase in Bcc prevalence was experienced. The dissemination of the strain was associated with the transfer of an infected patient from the initial center to the second one [21]. A later study by Coenye et al. [22] showed that the PHDC strain was also present in European patients (namely in France, Italy, and the UK), concluding that the PHDC strain was the second-identified Bcc transatlantic clone. Interestingly, both intercontinental clones belong to the *B. cenocepacia* species, although the ET12 belongs to subgroup IIIA and the PHDC belongs to subgroup IIIB. The *B. cenocepacia* species includes other clones that spread among CF centers, namely the Midwest American clone and the CZI Czech epidemic clone [23, 24]. Evidence of transmission of particularly epidemic strains of *B. cenocepacia* led to the introduction of segregation measures in CF centers in Europe and America, with a significant reduction of prevalence of infections [1, 25–27]. However, these segregation policies had a devastating impact on patients infected with Bcc due to social isolation and stigma and negative psychological impacts [28]. Although effective in interrupting strain transmission, segregation measures do not prevent new acquisitions. Nevertheless, these measures led to a reduction of prevalence of Bcc infections from more than 20% in several centers to less than 5% both in the USA and the majority of European countries [29, 30]. However, prevalence of chronic Bcc infections is still ranging 5–10% in Denmark, Portugal, Slovak Republic, Russian Federation, and Latvia, reaching values of 15 and 23% in Serbia and Lithuania, respectively [30].

Although the Bcc strains responsible for the vast majority of infections both in Europe and North America belong to the *B. cenocepacia* species, recent evidence indicates a changing epidemiology. *B. multivorans* emerged as the dominant species in France by 2004 and as the second most important species in the USA [31, 32]. Recent reports also indicate *Burkholderia contaminans* as an emerging Bcc species associated with CF infections. Early reports of a high incidence of the species among CF patients came from Portugal and Argentina [33–35].

Interestingly, in the case of the Portuguese CF population, two *B. contaminans* clones infecting CF patients were found as indistinguishable from two *B. contaminans* strains isolated from nonsterile nasal saline solutions of commercial origin during routine surveillance by the Portuguese Medicines and Health Products Authority [36]. A recent work by Medina-Pascual and colleagues on the surveillance of Bcc infections in Spanish CF patients also reported a *B. contaminans* overall incidence of 36.5% in the period 2008–2012, surpassing the previously dominant species *B. cenocepacia* and *B. multivorans* [37]. The emergence of *B. contaminans* among Spanish CF patients was hypothesized to be due to unspecified ecological advantages that enable the species to increase its presence in hospitals or in the environment [37]. In the case of Swiss CF-patients, *B. cenocepacia* was the most frequently isolated species in the period 1998–2013, but *B. multivorans* and *B. contaminans* emerged during the last years of the study period [38]. A 30-year study of Bcc infections among CF patients from British Columbia (Canada) evidenced a major impact of segregation measures in Bcc epidemiology; while *B. cenocepacia* was dominant before the introduction of these measures, *B. multivorans* strains became dominant after implementation of novel infection control measures in 1995 [39]. This study and others highlight the impact of infection control measures on Bcc species recovered from CF patients. It is now apparent that while epidemic *B. cenocepacia* strains dominated in early years, nonclonal *B. multivorans* and *B. contaminans* strains are emerging.

3. Bcc virulence factors and traits

Over the last 20 years, substantial progress has been achieved on the knowledge of Bcc virulence factors and determinants, although the exact contribution of some of them to the success of infection remains to be fully understood. It is currently accepted that Bcc virulence does not rely on a single virulence factor, being multifactorial. Bacterial structures such as flagella, the cable pili, and the 22-kDa adhesin are considered virulence factors since they play important roles in the initial steps of interaction with the host cell, promoting the adherence to the lung surface and the invasion of lung epithelial cells [39–41]. In addition, the majority of *B. cenocepacia* strains are able to survive and replicate intracellularly in airway epithelial cells and macrophages, evading the primary cellular defense mechanisms of the lung and avoiding clearance. The factors involved in this ability, exopolysaccharide (EPS) biosynthesis, biofilm formation, resistance to antibiotics, and oxidative stress resistance, as well as the iron acquisition ability are also among virulence determinants described for Bcc [20, 42, 43]. Some of these virulence factors are further detailed below.

3.1. Alternative sigma factors

RpoE and RpoN are two alternative sigma factors involved in the regulation of the ability of intracellular *B. cenocepacia* to delay phagolysosomal fusion in murine macrophages [44, 45]. RpoE is the extra-cytoplasmic stress response regulator required by *B. cenocepacia* to grow under conditions of high osmolarity and high temperature [44]. RpoN, or sigma factor σ^{54} , is best known for its involvement in nitrogen-related gene regulation. In *B. cenocepacia*, σ^{54} is involved

in motility and biofilm formation [45]. Results from the mapping of σ^{54} regulon and the characterization of a *B. cenocepacia* H111-derived σ^{54} mutant suggest that this alternative sigma factor plays an important role in the control of nitrogen metabolism, in the metabolic adaptation of *B. cenocepacia* H111 to stressful and nutrient-limited environments and in virulence toward the nematode *Caenorhabditis elegans* [46]. In addition, it was also reported that RpoN regulates genes involved in exopolysaccharide production, biofilm formation, motility, and virulence [46]. A *B. cenocepacia* mutant defective in a gene encoding a putative σ^{54} -related transcription regulator (BCAL1536) was found as attenuated in the rat agar bead infection model [47].

3.2. Lipopolysaccharides and extracellular polysaccharides

One of the central components of the outer membrane in Gram-negative bacteria is the lipopolysaccharide (LPS), a complex molecule composed by the lipid A, the core oligosaccharide, and the O-antigen moieties (reviewed in Ref. [48]). The genes involved in LPS production by *B. cenocepacia* are located in chromosome I, organized in three main clusters, one for each LPS component (lipid A: BCAL1929 to BCAL1935; core: BCAL2402 to BCAL2408; O antigen: BCAL3110 to BCAL3125) together with additional genes encoding sugar modification enzymes [49, 50]. Bcc bacteria LPS differs from other Gram-negative bacteria LPS due to the complete lack of negatively charged residues and the presence of the heterodimeric disaccharide D-glycero-D-talo-oct-2-ulosonic acid-(2-4)-3-deoxy-D-manno-oct-2-ulosonic acid (Ko-(2-4)-Kdo) in the core region; the presence of a 4-amino-4-deoxyarabinose (Ara4N) residue, either in the core or in lipid A; and the structure of O-antigen [50, 51]. This particular composition changes the bacterial surface charge, inhibiting the binding and successful action of antibiotics, contributing to the persistence of bacterial infection [51]. Recently, it was demonstrated that although L-Ara4N modifications do not affect recognition, they are critical for the establishment of infection [52]. Several studies have demonstrated that when neutrophils interact with Bcc LPS, the expression of CD11b on their surface increases, stimulating neutrophil respiratory burst response [53]. In addition, macrophages and human blood cells are also stimulated by Bcc LPS, producing pro-inflammatory cytokines such as TNF- α , IL-6, and IL-8 [54, 55].

B. cenocepacia J2315 is unable to produce the O-antigen. In this particular strain, this is due to an interruption in the *wbcE* gene-encoding BCAL 3125 [56]. The expression of O-antigen by Bcc strains was demonstrated to reduce phagocytosis by macrophages without interfering with the intracellular survival of bacteria [56].

The production of exopolysaccharides (EPSs) was described for several *Burkholderia* species. EPS production by Bcc is regarded as playing an important role in the chronicity of Bcc infections [57–62]. Cepacian is the most common EPS produced by Bcc and non-Bcc species, both from clinical and environmental sources [59, 63]. Cepacian interferes with phagocytosis by human neutrophils, facilitating the bacterial persistence in a mouse model of infection [64, 65]. The EPS was shown to inhibit the production of ROS by neutrophils and to scavenge reactive oxygen species (ROS), playing a role in the survival of cepacian-producing strains in different environments [64–67]. As a result of a frameshift mutation in the *bceB* gene (BCAM0856) encoding a putative

glycosyltransferase, Cepacian is not produced by the *B. cenocepacia* ET12 representative strain J2315 [49, 62].

3.3. Biofilms

Bcc bacteria were found to persist in biofilms *in vitro*. Biofilm formation and maturation depend on many factors, including EPS production, motility, iron availability, and multiple gene regulatory systems, such as quorum sensing, alternative sigma factors, or global regulators such as the ShvR and AtsR [45, 58, 68–73]. In addition, Bcc can form small colony variants *in vitro*, a colony morphology that is associated with enhanced biofilm formation, antibiotic resistance, and persistence [74].

Several studies have been performed to understand the importance and relevance of biofilm formation in Bcc biology. Bcc bacteria growing in biofilms are usually more tolerant to multiple antibiotics, although similar susceptibilities were reported for planktonic and biofilm cells to the antibiotics kanamycin, amikacin, and ciprofloxacin [75, 76]. Recently, Bcc biofilms were shown to contain persister cells that are able to survive in the presence of high concentrations of antibiotics by avoiding production of reactive oxygen species [77]. In addition, using neutrophil-like dHL60 cells, it was shown that the presence of these immune system cells enhanced biofilm formation that protected Bcc bacteria against neutrophils by inducing their necrosis, acting as a barrier to the migration of neutrophils, and masking the bacteria from being recognized by neutrophils [78]. Although some evidence suggests that biofilm formation plays a role in bacterial persistence in the CF airways, this topic needs to be further studied.

3.4. Quorum sensing

Quorum sensing is a mode of regulation of gene expression that is dependent on the density of the bacterial population. Bcc bacteria have at least four quorum sensing systems. The CepIR quorum sensing system is homologous to the LuxIR system of *Vibrio fischeri* (reviewed in Ref. [79]). The CepIR system positively regulates the virulence of *B. cenocepacia* toward model organisms like *C. elegans*, *Galleria mellonella*, rodents, zebrafish, alfalfa, and onions [80–83]. In addition to the CepIR, *B. cenocepacia* encodes the CciIR, the CepR2, and the BDSF quorum sensing systems [84, 85]. While the CepIR and CciIR quorum sensing systems rely on acyl homoserine lactones as signaling molecules, the BDSF system uses cis-2-dodecenoic acid as the signaling molecule, and the CepR2 is an orphan quorum sensing system [85]. An arsenal of genes regulated by quorum sensing in Bcc bacteria was described, including the negatively regulated siderophore synthesis and the positively regulated expression of the genes encoding zinc metalloproteases (Zmps), swarming motility and biofilm formation, all thought to have an impact when the bacterium is infecting the CF patient [71, 80, 86, 87].

3.5. Protein secretion systems

Both Gram-negative and positive bacteria use protein secretion systems to secrete toxins or other proteins, either directly into the environment or into host cells. These systems are

particularly well studied in the CF pathogens Bcc and *Pseudomonas aeruginosa*. For instance, Bcc strains of the ET12 lineage and *Burkholderia vietnamiensis* harbor type I and II secretion systems (T1SS, T2SS) implicated, for instance, in the secretion of hemolytic proteins [88, 89]. The T2SS is also involved in *B. cenocepacia* secretion of two zinc metalloproteases, ZmpA and ZmpB, which play a role in virulence [80, 90]. Two T4SSs are encoded by *B. cenocepacia*; the T4SS-1 encoded in a plasmid, and the T4SS-2 encoded in chromosome 2 [91]. Until now, only the T4SS-1 was identified in *B. cenocepacia* strains as necessary for virulence in onions and intracellular survival in phagocytes [92].

In a mouse agar bead infection model, the T3SS has been shown to be important for bacterial pathogenesis [93]. Although the precise mechanism is still not clear, T3SS seems to play no role in intracellular survival of *B. cenocepacia* [94].

Four type V secretion systems are encoded within the genome of *B. cenocepacia* J2315 [49]. Proteins transported by this type of transporters contain pertactin and hemagglutinin domains and are thought to play a role in bacterial adhesion [49].

B. cenocepacia also encodes a T6SS, which was shown to affect the actin cytoskeleton of macrophages and the assembly of the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex in *B. cepacia*-containing vacuoles (BcCV's) by inactivation of Rac1 and Cdc42 [73, 95, 96]. *B. cenocepacia* was found to efficiently activate the inflammasome by a yet uncharacterized T6SS effector [97]. Consequently, monocytes and THP-1 cells release IL-1 β in a pyrin-, Asc-, and T6SS-dependent manner [97]. The T6SS also enhances caspase-1 activation, negatively regulated by the sensor kinase-response regulator AtsR [73]. In addition, a recent paper suggests that the T6SS might be important for the secretion of T2SS effectors into the host cytoplasm, such as ZmpA and ZmpB, revealing an unanticipated role for type II secretion systems in intracellular survival and replication of *B. cenocepacia* [96]. Although membrane vesicles cannot be considered a canonical secretion system, they can effectively allow the secretion of several hydrolytic enzymes and toxins [98]. **Table 2** summarizes and compares the most relevant information available about secretion systems of Bcc bacteria and their counterparts in the major CF pathogen *P. aeruginosa*.

3.6. Iron uptake

In order to carry out iron chelation and uptake, members of the Bcc can produce up to four distinct siderophores: ornibactin, pyochelin, cepabactin, and cepaciachelin [122]. Ornibactin appears to be the most important and abundant siderophore produced by *B. cenocepacia* strains [123, 124]. The pathways and regulatory mechanisms of ornibactin synthesis and uptake are relatively well known [87, 125–127]. The requirement of this siderophore for *B. cenocepacia* virulence was demonstrated in different infection models, including the rat agar bead, *G. mellonella*, and *C. elegans* [82, 125, 127].

The competition for available iron by Bcc bacteria and other CF lung colonizing organisms such as *P. aeruginosa* was reported to occur in the CF lung, although it is not completely clear how Bcc organisms acquire iron from host proteins [128, 129].

Secretion system	<i>Burkholderia cepacia</i> complex	<i>P. aeruginosa</i>
T1SS	Hemolytic proteins [88, 89]	HasAp (heme-binding) [99]; AprA and AprX (alkaline proteases) [100, 101]
T2SS	ZmpA and ZmpB [80, 90]	LasB (Major extracellular protease) [102], Staphylolysin LasA [102], Aminopeptidase PaAP [103], Protease IV [104], Lipases LipA, LipC, phospholipase C, PlcH, and PlcN [105, 106], CbpD Chitin-binding protein CbpD [107]; Exotoxin A [108]
T3SS	No effector described yet, plays a role in evasion of the host immune system [93, 94]	GTPase-activator ExoS and ADP-ribosyltransferase ExoT [109], adenylate cyclase ExoY [110], phospholipase A2 ExoU and ExoS [111]
T4SS	T4SS-1: Plant cytotoxic proteins, T4SS-2: Plasmid mobilization [91]	Integrative and conjugative elements (ICEs): ICE _{clc} [112], Pathogenicity islands: pKLC102 (includes the type IV sex pili-encoding pil cluster and the <i>chvB</i> gene encoding a virulence factor) [113], and PAP-I (includes several virulence factors, such as CupD type fimbriae, and the PvrSR/RcsCB regulatory system) [114]
T5SS	Four T5SS: two containing pertactin domains involved in adhesion, other two contain haemagglutinin repeats [49]	Autotransporter: EstA (esterase activity) [115]; Two-partner secretion systems LepA/LepB [116] and CupB [117], and the PdtA/PdtB system [118]
T6SS	Hcp and VgrGs [73, 95, 96]	Hcp and VgrGs [119, 120]
Membrane vesicles (MV)	MV-associated (metallo)proteases, (phospho)lipases, peptidoglycan-degrading enzymes [98]	Multiple virulence factors: Alkaline phosphatase, hemolytic phospholipase C; the Cif toxin that inhibits CFTR-mediated chloride secretion in the airways [121]

Table 2. Summary of secretion systems from Bcc and the respective counterparts from the CF major pathogen *P. aeruginosa*.

3.7. Resistance to antimicrobials

Difficulties in eradicating Bcc infections mainly result from their intrinsic resistance to multiple antibiotics, including polymyxins, aminoglycosides, and most β -lactams. In addition, these bacteria have the ability to develop *in vivo* resistance to virtually all classes of antibiotics [20, 130, 131]. Antibiotics administration to CF patients was also reported to affect resistance profiles of Bcc bacteria [132]. Various mechanisms involved in the resistance of Bcc to multiple antibiotics have been described and include enzymatic inactivation (β -lactamases, aminoglycoside-inactivating enzymes, dihydrofolate reductase), alteration of drug targets, integrons, cell wall impermeability, and active efflux pumps [88, 133–140]. However, major contributions to intrinsic and acquired multidrug resistance by Bcc seem to be due to efflux pumps of the resistance nodulation cell division (RND) family. In fact, the *B. cenocepacia* J2315 genome encodes at least 16 efflux systems of the RND family [141]. At least six of these RND efflux pumps were implicated in drug resistance—RND-1, RND-3, RND-4, RND-8, RND-9, and RND-10 [138–140, 142,

143]. RND-3 and RND-4 efflux pumps were described as being involved in the resistance to various antimicrobial drugs including tobramycin and ciprofloxacin; the RND-3, RND-8, and RND-9 efflux systems protect biofilm-grown cells against tobramycin; the RND-8 and RND-9 efflux pumps are not involved in ciprofloxacin resistance; and RND-10 efflux pump seems to confer resistance to chloramphenicol, fluoroquinolones, and trimethoprim [140, 143]. It was suggested that mutations in the RND-3 regulator-encoding gene may be responsible for the prevalent overexpression of this efflux pump in clinical Bcc isolates, contributing to their high levels of antibiotics resistance [144].

3.8. Motility

Genes involved in the synthesis and assembly of *B. cenocepacia* flagella are located in chromosome I, distributed within five clusters, with two additional genes found on chromosomes 2 and 3 [49]. These genes were found as being upregulated when the organism was incubated in CF sputum, contributing to its virulence in a murine agar bead infection model [145, 146]. More recently, flagellin expression and flagellar morphology of *B. cenocepacia* grown in a medium mimicking the CF sputum was analyzed [147]. Those nutritional conditions led to increased motility and flagellin expression, by inducing the synthesis of multiple flagella on the cell surface of *B. cenocepacia* K56-2 [147]. A link between the loss of bacterial motility and the development of the cepacia syndrome was recently established based on a transcriptomics analysis comparing the *B. cenocepacia* ST32 CF isolates recovered from bloodstream, at the time of cepacia syndrome, with their sputum counterparts, recovered prior to the development of this syndrome, revealing that flagellar genes were downregulated in isolates recovered from the bloodstream [148].

3.9. Intracellular survival

Infection assays using free-living amoeba demonstrated that *B. cenocepacia* can survive in an acidified intracellular compartment [94, 149]. These bacteria were also demonstrated to have the ability to delay the maturation of phagolysosomes in murine macrophages [94–96, 150]. Although the *B. cenocepacia* containing vacuoles (BcCVs) progress normally to the early phagosomal stage, the fusion of the BcCV's with late endosomes and subsequent maturation is significantly delayed comparing with vacuoles containing heat-killed bacteria [94]. In contrast to heat-killed bacteria that ended up in phagolysosomes with a pH of 4.5, BcCVs did not acidify normally maintaining a luminal pH around 6.4 [94]. This ability of *B. cenocepacia* to alter the acidification of the vacuole seems to be correlated with the delay in recruitment or assembly on the BcCV membrane of both the 16-kDa subunit of the phagosomal vacuolar ATPase (vATPase) and the NADPH phagocyte oxidase [96, 151]. In contrast, Al-Khodor and colleagues demonstrated that *B. cenocepacia* J2315 only transiently interacts with the endocytic pathway, event after which the bacterium is able to rapidly escape to the cytosol [152]. Escaped bacteria are afterward targeted by the host autophagy pathway, through the recruitment to the bacterial vicinity of the ubiquitin conjugation system, the autophagy adaptors p62 and NDP52, and the autophagosome membrane-associated protein LC3B. However, apparently, this host cell control through autophagy ultimately fails in a high proportion of infected cells,

being *B. cenocepacia* able to block the autophagosome completion and replicate in the cytosol of the host cell [152].

To better understand the intracellular behavior of *B. cenocepacia* in CF infected patients, studies have also been performed in Cystic fibrosis transmembrane conductance regulator (CFTR)-defective macrophages. Remarkably, the delayed maturation arresting of BcCV's is more exaggerated in CFTR-defective macrophages than in normal macrophages and is specific to live *B. cenocepacia* [153]. Although it is not clear how the CFTR defect enhances the *B. cenocepacia* intracellular survival, there is evidence of a link between the defective CFTR with autophagy deficiency and decreased clearance of protein aggregates and inflammation [154]. The elucidation of these survival details, especially the ability of *B. cenocepacia* to synergize with the CFTR defect and its consequences on the mechanism of autophagy will provide new avenues to explore novel therapeutic approaches for CF patients [155].

4. Toward a vaccine to prevent Bcc infections

No objective guidelines for eradication strategies are available for Bcc infections, as these pathogens are intrinsically resistant to the majority of the clinical available antimicrobials [156]. Currently, no immunotherapeutic strategy to protect CF patients from Bcc infections is available. Several studies on the immune response elicited by Bcc species in CF patients have been performed; however, they are challenging due to the ability of this bacteria to modulate and overcome the host immune responses and the ability to survive intracellularly in phagocytes and epithelial cells [157, 158].

An important aspect to consider during vaccine design is the optimal balance of Th1 and Th2 responses required for effective pathogen clearance. For example, a Th1 bias elicits a cell-mediated response, while Th2 induces a humoral immune response [159]. In the case of CF, their immune phenotype appears to be skewed toward Th2 responses [160]. In the case of Bcc, the type of host response necessary to clear the pathogen is still not fully understood, making it difficult to develop a protective vaccine (**Table 3**). Recently, BALB/c mice immunized intraperitoneally with the proteins Linocin and OmpW showed a significant reduction of *B. cenocepacia* and *B. multivorans* cells in the lung and lower dissemination of bacteria to the spleen [161]. While Linocin led to a robust Th1 response, the OmpW led to a mixed Th1/Th2 response [161]. The protection achieved with these proteins was greater against *B. cenocepacia* infection, and OmpW immunization was more efficient in reducing the lung bacterial load [161].

Nonpurified outer membrane proteins (OMP) from *B. multivorans*, supplemented with the mucosal adjuvant adamantylamide dipeptide (AdDP) that promotes a robust Th2 response, were tested for immunization of BALB/c mice [162]. A statistically significant increase in IgG and in mucosal IgA OMP-specific antibodies was observed, together with a reduction of *B. multivorans* burden and lung pathology, but only a moderate cross protection to *B. cenocepacia* was reported. The specificity of the immune response was found to be against

90, 72, 66, and 60 kDa proteins. Elicitation of specific IgA antibodies by mucosal immunization was also reported to be important to prevent the colonization of the respiratory tract by Bcc bacteria. In another study, the intranasal immunization of CD-1 mice with outer membrane proteins (OMP) from *B. cenocepacia* was described to originate a Th2-biased response with the maintenance of the bacterial burden, while mice immunized with OMP and the noninflammatory mucosal adjuvant nanoemulsion (NE) elicited a Th1/Th2-balanced response that led to a significant reduction of the *B. cenocepacia* cell burden [163]. The serum derived from mice vaccinated with OMP-NE could also inhibit *B. multivorans* growth by 80.1%, showing that induction of cross-reactive antibodies occurred after mice immunization. Additionally, a highly conserved 17-kDa OmpA-like protein was recently identified as a new immune-dominant epitope in mucosal immunization [163].

Metalloproteases are also considered as potential effective candidates for vaccine development [90]. It was demonstrated that immunizations of rats using a conserved zinc metalloprotease peptide 15 (PSCP) decreased the severity of *B. cenocepacia* infection and the lung damage was reduced by 50% upon challenge with a *B. cenocepacia* strain after immunization [90].

In 2012, it was shown that the bacterial surface polysaccharide poly- β -(1-6)-N-acetyl-glucosamine (PNAG) confers protective immunity against Bcc infection in a lethal peritonitis mice model [164]. In this study by Skurnik and colleagues using opsonophagocytic assays, it was observed that goat-raised antibodies against PNAG could kill Bcc strains (>80%) of the *B. ceno-*

Antigen	Immune response	Bcc animal model	In vitro models	References
OmpW	Mixed Th1/Th2	BALB/c mice immunosuppressed with cyclophosphamide	Spleen cells from mice	[161]
Linocin	Th1	BALB/c mice immunosuppressed with cyclophosphamide	Spleen cells from mice	[162]
OMP plus NE	Mixed Th1/Th2	CD-1 mice	Murine splenocytes	[163]
OMP plus AdDP	Higher IgG and IgA titers	BALB/c mice immunosuppressed with cyclophosphamide	ND	[162]
PNAG	ND	FVB/N mice	Opsonophagocytic assay	[164]
Zinc metalloprotease peptide 15 (PSCP)	Higher IgG and IgA titers	Sprague-Dawley rat agar bead model	ND	[90]
FliC	ND	ND	T cell hybridoma assays	[165]
BCAL2958	High IgG titers in human CF serum samples	ND	Human neutrophils	[166]

ND—Not determined.

Table 3. Summary of vaccine development against Bcc infections.

cepacia, *Burkholderia dolosa* and *B. multivorans* species. Furthermore, bacterial killing was found to depend of the presence of the complement [164].

Other proteins of putative immunogenic activity have been reported as potential vaccine candidates. However, studies in a Bcc infection animal model are still lacking (**Table 3**). One of these promising antigens is the OmpA-like BCAL2958 protein that was shown to be highly conserved in Bcc, to elicit IgG antibodies in CF patients and to elicit an increase of TNF α , elastase, NO, and MPO in neutrophils [166].

Musson and colleagues have shown that T-cell hybridomas against the *Burkholderia pseudomallei* flagellar protein FliC epitope cross-reacted with orthologous FliC sequences from *B. multivorans* and *B. cenocepacia* [165]. FliC epitopes were accessible for processing and presentation from live or heat-killed *B. cenocepacia* bacteria, demonstrating that flagellin enters the HLA class II Ag presentation pathway during infection of macrophages with *B. cenocepacia*.

Studies referred above revealed that subunit vaccines that only produce an antibody response cannot fully prevent an infection caused by Bcc bacteria [157, 161, 164]. Therefore, Bcc vaccines containing multiple antigens that elicit a balanced Th1 and Th2 response are expected to be effective in preventing Bcc infections. With this aim, immunoproteomics approaches have been performed. For instance, Mariappan and colleagues identified 18 immunogenic proteins from culture supernatants of *B. cepacia* that reacted with mice antibodies raised against inactivated *B. cepacia* whole cells [167]. More recently, the analysis of the immunoproteome of two clinical relevant strains of *B. cenocepacia* and *B. multivorans* revealed 15 common immunoreactive proteins that reacted with CF human serum samples [168].

5. Concluding remarks

An overview of Bcc infections in CF from early 1980s until the more recent available data was presented. The prevalence of Bcc species in CF patients worldwide is still evolving, most probably as a result of infection control measures and segregation policies. Many virulence factors have been identified, and the resulting wealth of information prompted the establishment of new research lines envisaging the development of novel protective strategies and products, namely vaccines and vaccine components.

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