Review

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Burkholderia cepacia complex: epithelial cell–pathogen confrontations and potential for therapeutic intervention

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Burkholderia cepacia complex (Bcc) is an important and virulent pathogen in cystic fibrosis patients. The interactions between this pathogen and the host lung epithelium are being widely investigated but remain to be elucidated. The complex is very versatile and its interactions with the lung epithelial cells are many and varied. The first steps in the interaction are penetration of the mucosal blanket and subsequent adherence to the epithelial cell surface. A range of epithelial receptors have been reported to bind to Bcc. The next step in pathogenesis is the invasion of the lung epithelial cell and also translocation across the epithelium to the serosal side. Furthermore, pathogenesis is mediated by a range of virulence factors that elicit their effects on the epithelial cells. This review outlines these interactions and examines the therapeutic implications of understanding the mechanisms of pathogenesis of this difficult, antibiotic-resistant, opportunistic pathogen.

Introduction

Burkholderia cepacia complex (Bcc) is a group of closely related Gram-negative bacteria that emerged as opportunistic pathogens in the 1980s in patients with cystic fibrosis (CF) and chronic granulomatous disease (CGD) (Isles *et al.*, 1984). *B. cepacia* was identified over 50 years ago as a plant pathogen (Burkholder, 1950), and is widely isolated from the biosphere. The *Burkholderia* genus now comprises at least 43 species, which are extremely diverse and versatile (Vial *et al.*, 2007). While Bcc has many potential uses in the environment, particularly in bioremediation, the pathogenesis of Bcc in susceptible populations is a major cause for concern (Parke & Gurian-Sherman, 2001). There are at least 15 species within the complex (Coenye *et al.*, 2001, 2003; Vanlaere *et al.*, 2008). Furthermore, isolates from two possible additional novel species are being examined and are currently categorized together as Group K (Mahenthiralingam *et al.*, 2006). Geographical differences in the prevalence of Bcc species exist: in North America, the most predominant strain is *Burkholderia cenocepacia*; however, in Europe, *Burkholderia multivorans* predominates (Govan *et al.*, 2007).

Although Bcc is reported to cause infections in only 3.5% of CF patients in the world, this still represents a significant problem, as CF patients colonized with Bcc experience a more rapid decline than those colonized with the more commonly acquired pathogen *Pseudomonas aeruginosa* (Courtney *et al.*, 2004). The first nine species identified have been extensively studied and differ greatly in terms of their virulence and transmissibility. All Bcc species and

Group K isolates have been isolated from CF patients; however, of these, *B. multivorans* and *B. cenocepacia* are particularly virulent (Mahenthiralingam *et al.*, 2005). Once a patient is colonized with a Bcc strain, the organism is rarely eradicated. Bcc infection is usually acquired late in the course of the disease and the outcome of infection can vary, ranging from maintenance of a stable respiratory function to a rapid, and ultimately fatal, clinical decline. Bcc has emerged as a serious pathogen for CF patients for the following reasons. (1) Infection can result in ‘cepacia syndrome’, a condition occurring in approximately 20% of CF patients infected with Bcc, characterized by fever, pneumonia and bacteraemia (Isles *et al.*, 1984). (2) Bcc is inherently resistant to antimicrobial treatment and increased resistance is observed on formation of Bcc biofilms *in vitro*. It is resistant to aminoglycosides, quinolones and β -lactams (Chernish & Aaron, 2003), can use penicillin G as a carbon source (Beckman & Lessie, 1979), and is also inherently resistant to antimicrobial peptides (Taylor *et al.*, 2007). Furthermore, formation of Bcc biofilms has been shown to further increase resistance to antibiotics *in vitro* (Caraher *et al.*, 2007b). (3) Finally, Bcc strains can be transmitted from patient-to-patient. There are strains of *B. multivorans* and *B. cenocepacia* which are highly transmissible, though other species, e.g. *Burkholderia dolosa*, have also been shown to be transmissible (LiPuma *et al.*, 1990). In particular, one strain of *B. cenocepacia*, referred to as the ET12 strain, has been isolated from patients in different countries on both sides of the Atlantic (Mahenthiralingam *et al.*, 2005). Transmission is considered to be due to direct contact,

but exactly how Bcc spreads from one CF patient to another has yet to be determined. McDowell *et al.* (2004) showed that while the greatest risk of transmission was associated with *B. cenocepacia*, other species have also been found to be involved in cross-infection. The UK CF Trust guidelines (http://www.cftrust.org.uk/aboutcf/publications/consensusdoc/C_Burkholderia_cepacia_Sep_2004.pdf) recommended that segregation measures should be put in place for all patients with Bcc infection, regardless of species or strain. Patient-to-patient spread of other Bcc species was subsequently confirmed (Agodi *et al.*, 2001; Biddick *et al.*, 2003). Indeed, a recent systematic review of isolation procedures would support the use of isolation measures to reduce the transmission of Bcc (Festini *et al.*, 2006).

While *B. cenocepacia* and *B. multivorans* predominate, there are unusual prevalences of certain species due to isolated incidences. A Portuguese CF centre in Lisbon reported that *B. cepacia* (formerly genomovar I) was isolated from 85% of Bcc-colonized CF patients, most likely due to contamination of a non-sterile saline preparation for nasal application (Cunha *et al.*, 2007). In addition, an outbreak of *B. dolosa* (genomovar VI) in a children's hospital in Boston was associated with an accelerated decline in lung function and a reduction in survival (Kalish *et al.*, 2006). Outbreaks also arise in non-CF populations; for example, *B. cenocepacia* emerged as a nosocomial pathogen in patients without CF and has resulted in substantial mortality due to bacteraemia (Woods *et al.*, 2004). Furthermore, *B. cepacia* was evident in blood cultures of children without CF following administration of a contaminated salbutamol nasal spray, and concomitant use of budesonide dramatically enhanced the risk of infection (Ghazal *et al.*, 2006).

In a recent study, it was shown using multilocus sequence typing that over 20% of clinical isolates were indistinguishable from environmental strains (Baldwin *et al.*,

2007), which raises a number of questions. What makes Bcc so successful? Furthermore, how has it managed to survive and flourish in both the environment and the CF lung? It is now apparent that human pathogens share many mechanisms of pathogenesis and that virulence factor genes tend to be clustered on distinct islands in bacterial chromosomes and on plasmids. These common mechanisms at the epithelium include host cell attachment, invasion, intracellular survival, acquisition of iron and virulence regulation (Finlay & Falkow, 1997). Indeed, a pathogenicity island (cci) has been identified on the *B. cenocepacia* genome which includes genes for persistence, virulence and metabolic mechanisms (Baldwin *et al.*, 2004).

The interactions between Bcc and lung epithelial cells share many common features with other mucosal pathogens (Fig. 1). In this review, the interactions that Bcc has with these cells are identified with a view to exploring its mechanisms of pathogenesis and its capacity to survive in the CF lung. The implications of these investigations to the development of better treatments against Bcc are also highlighted. Prior to this, a description of the host cell environment particular to Bcc infection will help put this in context.

Airway epithelial cell barrier

The function of the respiratory epithelium relies on the close association between neighbouring epithelial cells, forming a tight barrier (Godfrey, 1997). The trachea and bronchi are lined with ciliated columnar epithelial cells and lead into the bronchioles, which have a simpler structure with both ciliated columnar epithelial cells and non-ciliated epithelial cells. These cells are polarized, with distinct apical and basolateral protein and lipid expression. Above the epithelial cells lies a superficial liquid layer containing mucous-gland and goblet cell secretions and

Fig. 1. Schematic of lung epithelial cells and their interactions with Bcc. The bacteria attach to receptors on the lung epithelial cells and either invade via intracellular vacuoles or translocate through the epithelium. Bcc also forms biofilms, which have a different interaction with lung epithelial cells.

antibodies, which contribute to the protection against infection by inhaled bacteria. Mucus clearance is a major innate defence mechanism mediating the removal of virtually all inhaled substances from airways, and defects in this mechanism are associated with a range of chronic airway diseases in humans (Boucher, 2007). The integrity of the epithelial barrier is maintained by tight junctions, which are located in the apicolateral borders of epithelial cells of the lungs and are responsible for the selective regulation of ions, water, other small neutral molecules and immune cells via the paracellular route (Denker & Nigam, 1998). The tight junction is formed by numerous proteins including ZO-1, claudins and occludins that form contiguous rings around epithelial cells and attach to the actinomyosin rings of other cells. These proteins have different roles in the formation and function of the tight junction (Maher *et al.*, 2008). In particular, ZO-1 expression is inversely correlated with tight junction integrity in lung epithelial cells (Winton *et al.*, 1998). Further down the respiratory tract, the alveoli are lined with a single thin layer of cells: type I epithelial cells, which make up 93 % of the alveolar surface and are responsible for gaseous exchange; and surfactant-producing type II cells, which are involved in apical to basolateral sodium transport (Adamson & Bowden, 1974; Evans *et al.*, 1975).

Airway epithelia of the CF lung

The primary defect in CF is linked to mutations in the cystic fibrosis transmembrane regulator (CFTR) (Riordan *et al.*, 1989). Pathogenesis in CF is directly linked to CF airway pathology (Boucher, 2004). Increased mucus due to defective mucus clearance, abnormal submucosal gland function, in addition to an enhanced inflammatory response and reduced antimicrobial peptide efficacy, all contribute to the persistent infections that are the hallmark of CF lung disease. This build-up of mucus provides an ideal environment for the colonization of pathogens and is directly linked to the CFTR defect (Boucher, 2007). Indeed, five species of Bcc have been shown to bind to airway mucus, and treatment with dextran resulted in a reduction in thickness of this mucus and a decrease of Bcc adhesion (Sajjan *et al.*, 2004b).

The CFTR mutation leads to defective regulation of the sodium ion channel (ENaC) and the combination of defective CFTR with increased ENaC activity results in a depletion of airway surface liquid. This, in turn, results in a reduction in the height of the liquid layer above airway epithelial cells leading to flattened cilia, and consequently reduced transport of the already dehydrated mucus. This appears to be a primary symptom as early post-mortem studies showed that newborn infants had airway plugging of terminal bronchioles, prior to any indication of either infection or inflammation (Zuelzer & Newton, 1949). Another direct role of the CFTR mutation in CF pathogenesis has been attributed to CFTR acting as a pattern recognition molecule involved in clearance of *P.*

aeruginosa. The associated cellular activation and stimulation of transcription factor NF- κ B was absent in cells and transgenic mice with the Δ F508 mutation (Schroeder *et al.*, 2001).

Alterations in CF airway epithelial cells have also been described which further compound the issue of susceptibility to infection, by providing receptors for pathogens to adhere to. CF airways show increased sulfation of the high molecular mass glycoproteins, and in particular increased 6-sulfated sugars in CF mucins (Xia *et al.*, 2005). While this was originally thought to be due to an acquired abnormality, due to extensive inflammation, it has also been shown that it persists in primary culture and was a genetically determined characteristic of CF airway epithelial cells (Noah *et al.*, 1997). Increased fucosylation and reduced sialylation are other characteristics of CF and are directly linked to the CFTR defect (Rhim *et al.*, 2000). In particular, compared to non-CF cells CF cells have a higher ratio of asialylated GM1 (aGM1) to sialylated GM1, and more CF cells than non-CF cells express surface aGM1 (Saiman & Prince, 1993). This is significant as *P. aeruginosa* and Bcc have both been reported to bind to this asialylated glycolipid (Krivan *et al.*, 1988; Saiman & Prince, 1993). Moreover, increased fucosylation and alterations in specific fucosyl residues of membrane glycopeptides (Glick *et al.*, 2001) also have relevance to the microbiology of CF; for example, *P. aeruginosa* expresses a lectin which specifically recognizes α 1,3/4 fucose containing motifs of CF epithelial cells, which may explain, at least in part, the high incidence of that pathogen in the CF lung (Mitchell *et al.*, 2002). Finally, it was recently shown that CFTR deficiency led to a reduction in acid sphingomyelinase activity, allowing ceramide accumulation in lung tissue (Teichgraber *et al.*, 2008). This resulted in both enhanced inflammation and enhanced susceptibility to *P. aeruginosa* infection and appeared to be reversed by treatment of mice with amitriptyline.

Interactions between Bcc and protein receptors of lung epithelia

Following entrapment of Bcc in the mucous layer, the adhesion of the organism to the epithelial surface is the first step in the host cell interaction. Bcc adheres to epithelial cells via both protein and glycolipid receptors, in addition to secretory mucins. In an early study, 19 out of 22 Bcc isolates were shown to bind to purified mucins (Sajjan *et al.*, 1992); however, the exact speciation of these isolates was not identified. Isolates that were capable of binding to secretory mucins expressed the cable pilus phenotype. The epithelial cell receptor for the cable pili-associated adhesin (a 22 kDa protein) of certain *B. cenocepacia* strains was identified as cytokeratin 13, a 55 kDa protein which is enriched in CF epithelial cells (Sajjan *et al.*, 2000). Binding of piliated *B. cenocepacia* to lung explants from CF patients was higher than that for other *B. cenocepacia* isolates while other species within the

complex did not show any binding to CF lung explants. However, the cytokeratin 13/22 kDa adhesin interaction was not as clear-cut as it originally seemed. Later studies showed that *B. cenocepacia* strains which did not show Cbl pili on their surface also bound to cytokeratin 13 and also that expression of Cbl pili in *Escherichia coli* was not sufficient to mediate binding to this epithelial receptor (Sajjan *et al.*, 2001). In spite of this, the 22 kDa adhesin implicated in the attachment may still have a role in pathogenesis, as blocking *B. cenocepacia* strain BC7 with antibodies prior to infection of epithelial cells reduced both cytotoxicity and IL-8 secretion in primary cultures of bronchial epithelial cells (Sajjan *et al.*, 2002). The combined expression of the 22 kDa adhesin and the Cbl pili was required for optimal binding to cytokeratin 13 (Urban *et al.*, 2005). Interestingly, this mechanism of attachment is limited to certain *B. cenocepacia* strains and is not prevalent among all ET12 strains. More recently, it has emerged that *B. cenocepacia* strain BC7 also binds to a second receptor of 55 kDa, namely, the TNF receptor 1 (TNFR1). This interaction does not utilize the 22 kDa protein as an adhesin, but is considered partly responsible for the potent pro-inflammatory response elicited by this virulent Bcc strain (Sajjan *et al.*, 2008). It remains to be seen whether TNFR1 is also a receptor for other members of *B. cenocepacia*, or whether it is a more general receptor for other species within the complex, as a whole.

Adhesion of Bcc to glycolipid receptors

The second class of host cell receptors for binding are glycolipid receptors, though the evidence on which glycolipids are involved is conflicting. Krivan *et al.* (1988) demonstrated that members of the Bcc bound to glycosphingolipids containing GalNAc β 1-4Gal sequences either terminally or internally, including aGM1 and aGM2 (Table 1). The same strains did not bind to globosides, which contain a terminal GalNAc β 1-3Gal sequence, or to GM1 and GM2. In contrast, a later report identified that a non-piliated *B. cenocepacia* strain bound optimally to globotriosylceramides (Gb3, trihexosylceramides), which contain an unsubstituted galactose at the terminus, and showed relatively weaker binding to aGM1 and aGM2 (Sylvester *et al.*, 1996). While the earlier studies (Krivan *et al.*, 1988) had indicated that there was no binding to Gb3, the exact species of the strain used in this study was not identified, which allows for the possibility that glycolipid binding may be species-specific. Certainly, the absence or presence of Cbl pili

affected the affinity for glycolipid receptors (Sylvester *et al.*, 1996); however, the ultimate role of these receptors in pathogenesis was not explored. The glycolipid aGM1, which is a receptor for flagella of *P. aeruginosa* strain PAO1, is located at the apical surface of polarized 16HBE14o-epithelial lung cells and is therefore available for bacterial binding (Adamo *et al.*, 2004). While both *P. aeruginosa* and Bcc have shown binding to aGM1, no competition for binding was observed between these two pathogens on bovine tracheal cells; rather, the attachment of Bcc strains was enhanced in the presence of *P. aeruginosa* supernatants and whole cells (Saiman *et al.*, 1990). There has been much controversy regarding the attachment of *P. aeruginosa* to glycolipids. *P. aeruginosa* type IV pili were shown to mediate the interaction with aGM1 of polarized kidney epithelial cells, resulting subsequently in cytotoxicity and internalization of the bacteria (Comolli *et al.*, 1999). However, subsequent reports showed that clinical *P. aeruginosa* isolates did not bind aGM1 or aGM2 either on airway and kidney epithelial cells or in purified form (Emam *et al.*, 2006). In addition, use of anti-aGM1 antibodies to block binding sites has been suggested to be inappropriate, due to non-specific binding of these antibodies to LPS and other *P. aeruginosa* antigens (Schroeder *et al.*, 2001).

In polarized epithelia, aGM1 is generally present at a low level on the apical surface of healthy undamaged cells; however, this receptor becomes available following injury or in CF epithelia (de Bentzmann *et al.*, 1996; Saiman & Prince, 1993), providing enhanced receptors to CF pathogens. Other pathogens also exploit glycolipid receptors; for example, Shiga-like toxin binds to several Gal α 1-4Gal-containing glycolipid receptors, including Gb3 (Samuel *et al.*, 1990), while uropathogenic *E. coli* utilizes at least four globosides as receptors, including Gb3 and Gb4 (Stapleton *et al.*, 1998).

Furthermore, these glycolipids may elicit intracellular signalling, thereby prompting inflammatory responses. Lipid rafts are small membrane domains composed of glycosphingolipids and cholesterol, which play a role in host-pathogen interactions among many bacteria, including *P. aeruginosa* and *E. coli*, which includes both internalization and cytokine induction as recently reviewed by Riethmuller *et al.* (2006). Polarized lung epithelial cells that were stimulated with *P. aeruginosa* strain PAO1 mobilized TLR2 to the apical surface together with aGM1 (Soong *et al.*, 2004). Interaction of pathogens with this

Table 1. Examples of glycolipids which have been identified in epithelial binding of Bcc

Glycolipid	Other name	Terminal sugar	Reference
Asialo ganglioside (aGM1)	Gangliotetrosylceramide (Gg4)	Gal β 1-3GalNAc β 1-4Gal β 1-4Glc β 1-1Cer	Krivan <i>et al.</i> (1988)
aGM2	Gangliotriosylceramide Gg3	GalNAc β 1-4Gal β 1-4Glc β 1-1Cer	Krivan <i>et al.</i> (1988)
Gb2	Digalactosylceramide	Gal α 1-4Gal β 1-1Cer	Sylvester <i>et al.</i> (1996)
Gb3	Trihexosylceramide (THC)	Gal α 1-4Gal β 1-4Glc β 1-1Cer	Sylvester <i>et al.</i> (1996)

glycolipid could be central to the intense pro-inflammatory responses that they trigger.

Therapeutic implications of adhesion

Elucidating the binding of Bcc to host epithelial cells will allow development of anti-adherence strategies and should also allow better vaccine development. Three studies have examined the potential for inhibiting adherence of Bcc strains with carbohydrates. Dextran inhibited the binding of a range of Bcc species to A549 cells, but had a relatively lower effect on Cbl⁺ *B. cenocepacia* strains (Chiu *et al.*, 2001). Use of high-molecular-mass dextrans and a sugar alcohol, xylitol, was also suggested as a means of inhibiting adherence of Bcc before or after lung transplantation (Sajjan *et al.*, 2004a). In both cases, the inhibition was stated to be non-specific; however, in the latter case it was suggested that the inhibition in adherence was indirectly associated with removal or disruption of the surface mucus. Thomas & Brooks (2004) demonstrated that a number of di- and trisaccharides (including GalNAc β 1-3Gal, GalNAc β 1-4Gal, NeuAc α 2-3Gal β 1-4Glc) inhibited binding of *B. cenocepacia* strain 9091 to A549 epithelial cells by up to 80 %. In addition, polysaccharides such as dextran, dextran sulfate and heparin also reduced attachment to the same extent. The broad range of saccharide structures that were found to inhibit the attachment was suggested to be due to the requirement of Bcc to attach to many environmental surfaces. The therapeutic potential of inhibition of binding of Bcc to lung cells has yet to be established; however, aerosolized dextrans have reduced pneumonia in mice when challenged with *P. aeruginosa* (Bryan *et al.*, 1999). In addition, pneumococcal load was

reduced and protection from bacteraemia was conferred in a rabbit model following administration of oligosaccharides and sialylated derivatives (Idanpaan-Heikkilä *et al.*, 1997). While there is a long way to go, particularly in avoiding complications such as oedema, saccharide-based adhesion inhibitors might be exploited as therapies to prevent infection with a highly antibiotic resistant pathogen such as Bcc.

Intracellular invasion of epithelial cells

Many studies have shown that Bcc can invade and survive within epithelial cells *in vitro* (Cieri *et al.*, 2002; Duff *et al.*, 2006; Martin & Mohr, 2000). Using a panel of 29 strains, a clear correlation between invasion of A549 cells and infection in an *in vivo* mouse model has been shown (Cieri *et al.*, 2002). Intracellular invasion is mediated via a membrane-bound vacuole after initial alignment of bacteria along the epithelial membrane (Fig. 2). However, different routes of invasion of primary lung epithelial cultures have been observed for various species of Bcc, including invasion as a biofilm, rearrangement of the cytoskeleton and subsequent destruction of the cell and penetration through the epithelium by paracytosis (Schwab *et al.*, 2002). Our studies have also shown different mechanisms of invasion of Bcc species mediated by receptors at distinct locations within the polarized epithelial cell (Duff *et al.*, 2006). The process of actin rearrangement by *B. cenocepacia* was confirmed in separate studies, but was common to both viable and non-viable bacteria (Sajjan *et al.*, 2006). *B. multivorans* strains also promoted disruption of the actin filament network, and single cell entry and translocation of primary cultures of

Fig. 2. Interaction between Bcc bacteria and lung epithelial cells *in vitro*. Bacteria were examined by transmission electron microscopy and were observed aligning with the plasma membrane of the epithelial cell prior to invasion via a cellular vesicle. Bar, 1 μ m. Reproduced from Caraher *et al.* (2007a) with the kind permission of Elsevier.

lung epithelial cells by *B. multivorans* was facilitated by actin disruption (Schwab *et al.*, 2003). In contrast, intact actin filaments were required only for biofilm entry of *B. multivorans*, but not for single cell entry or paracytosis. Functional flagella are required for invasion, although they do not appear to be essential for adherence. Non-motile mutants of *B. cenocepacia* strain J2315 with a disruption in either the *fliG* or *fliI* genes both showed a three- to fourfold reduction in invasion of A549 cells, which was not due to an alteration in adherence (Tomich *et al.*, 2002). Furthermore, lipase appears to play a role in invasion of Bcc (Mullen *et al.*, 2007).

The intracellular fate of *B. cenocepacia* strain K-562 after invasion has been examined in the CF-derived airway epithelial cell line IB3 (Sajjan *et al.*, 2006). Live bacteria avoid lysosomal digestion by residing inside endoplasmic reticulum-derived autophagosomes and replicating within the endoplasmic reticulum. In contrast, non-viable K-562 cells were digested within 6 h of uptake into epithelial cells. The bacterial effectors of this process in other lung pathogens have been linked to type IV secretion systems, for example in *Legionella pneumophila* (Molofsky & Swanson, 2004).

Translocation across the epithelium

A major contributor to mortality in CF patients colonized with Bcc is the development of bacteraemia in a subgroup of patients, and their subsequent rapid decline (Isles *et al.*, 1984). In order to achieve this, Bcc has to penetrate through the lung epithelium in order to gain access to the blood system. Bcc disrupts the intercellular tight junctions and translocates from the apical side to the basolateral side of intact epithelial monolayers *in vitro* (Duff *et al.*, 2006; Kim *et al.*, 2005). Isolates from four different Bcc species showed comparable levels of translocation, demonstrating that the potential to cause septicaemia is not just limited to the two most virulent species (Duff *et al.*, 2006). In both studies, tight junction proteins were shown to be disrupted. Expression of ZO-1 was reduced in Calu-3 cells following Bcc application, while in 16HBE14o- cells, translocation was accompanied by dephosphorylation of occludin and its removal from the tight junction (Duff *et al.*, 2006; Kim *et al.*, 2005). Cell-free supernatants only contributed partially to this effect, and lipase, which is produced in large quantities by members of the complex, did not disrupt tight junction integrity of Calu-3 cells (Mullen *et al.*, 2007). Tight junction disruption is also a mechanism of pathogenesis and translocation of other bacteria, including *Salmonella enterica* and enteropathogenic *E. coli* (Jepson *et al.*, 2000; Simonovic *et al.*, 2001). *P. aeruginosa* also disrupts tight junction integrity and translocates across airway epithelia by a mechanism associated with rhamnolipid secretion and incorporation within the host cell membrane (Zulianello *et al.*, 2006). In contrast, translocation of *Mycobacterium tuberculosis* across bilayers of epithelial and endothelial cells is mediated by prior invasion of epithelial cells (Bermudez *et al.*, 2002).

Therapeutic implications of invasion and epithelial translocation of Bcc

The ability of Bcc to invade, overcome cellular degradation and translocate across the epithelium has implications for the development of future antibiotic therapies. It will be important to consider intracellular organisms and those that have penetrated beyond the epithelium in the fight against persistent, chronic Bcc infection.

Virulence factors

In addition to the processes of attachment and invasion, Bcc produces virulence factors which enhance its pathogenicity in epithelial cells (Table 2). Those that are not directly related to epithelial cell interactions are well-described elsewhere (Mahenthalingam *et al.*, 2005) and are therefore not included below.

Lipase

The *Burkholderia* genus is one of the most important lipase-producing bacterial genera in terms of biotechnology applications, and although lipases were identified in Bcc isolates as early as 1984 (McKevitt & Woods, 1984), little is known about their role in pathogenesis. A type II secretion pathway has been identified for lipase secretion, which like many type II secretion pathways is under quorum sensing control (Weingart & Hooke, 1999), although its regulation in Bcc is independent of the cepIR quorum sensing system (Gotschlich *et al.*, 2001). Lipase expression among Bcc isolates is highest among *B. multivorans* strains followed by *B. cenocepacia* (Mullen *et al.*, 2007). In addition, treatment of strains with a specific lipase inhibitor reduced the potential for invasion, suggesting that lipase played a role in invasion of lung epithelial cells. Other pathogens also utilize lipase as a virulence factor. The lipase of *Entamoeba histolytica* also enhances its invasion and intestinal colonization (Gilchrist *et al.*, 2006). Lipase also plays a role in the pathogenesis of other diverse pathogens including *Helicobacter pylori* and *Propionibacterium acnes* (Miskin *et al.*, 1997; Piotrowski *et al.*, 1994).

Metalloproteases

Bacterial metalloproteases can play a role in the pathogenesis of lung pathogens such as Bcc. Bcc strains secrete extracellular zinc metalloproteases that may play a role in virulence (Corbett *et al.*, 2003). One of these, ZmpA, has been identified in five species of the Bcc, including *B. cenocepacia*, but not in other species, including *B. multivorans* or *B. dolosa* (Gingues *et al.*, 2005). Another, ZmpB, is proteolytically active against many proteins found in the extracellular matrix, including type IV collagen and fibronectin, and against key members of the immune system, including neutrophil α -1 proteinase inhibitor and interferon- γ , illustrating its potential for damage to lung tissue and host response (Kooi *et al.*, 2006). Many other pathogens, for

Table 2. Bcc virulence factors that may act at the epithelial interface and their known effects *in vitro*

Virulence factor	Protein/gene	Species (strain)	Function	Reference
Proteases	<i>zmpA</i>	Many, not <i>B. multivorans</i> or <i>B. dolosa</i>	Proteolysis of extracellular matrix	Gingues <i>et al.</i> (2005)
	<i>zmpB</i>	Many, not <i>B. multivorans</i> or <i>B. dolosa</i>	Proteolysis of extracellular matrix	Kooi <i>et al.</i> (2006)
	<i>mgtC</i>	<i>B. cenocepacia</i> (K-562)	Survival in macrophages; virulence rat lung infection model	Maloney & Valvano (2006)
	HtrA protease	<i>B. cenocepacia</i> (K-562)	Growth under osmotic or thermal stress, survival rat infection model	Flannagan <i>et al.</i> (2007)
	Serine protease (homologous to subtilase family)	<i>B. cenocepacia</i> (J2315)	Digestion of ferritin to release iron	Whitby <i>et al.</i> (2006)
Lipases	Lipase	Multiple species	Role in invasion	Mullen <i>et al.</i> (2007)
	Phospholipase C	<i>B. cenocepacia</i> , <i>B. multivorans</i> , <i>B. vietnamiensis</i> , <i>B. ambifaria</i>	Digestion of phosphatidylcholine of lung surfactant – no correlation with virulence yet	Carvalho <i>et al.</i> (2007)
Exotoxin	LPS	Many species	Cytokine secretion, inflammatory response	Reddi <i>et al.</i> (2003)

example, *Vibrio cholerae*, secrete metalloproteinases which cause a loss of transepithelial resistance in epithelial monolayers (Fullner *et al.*, 2001). *P. aeruginosa* virulence factors, most likely elastase, have been shown to overactivate host metalloprotease and prevent the formation of TJ during repair (de Bentzmann *et al.*, 2000). Metalloproteases are also upregulated in the CF lung (Lopez-Boado *et al.*, 2001), which may further exacerbate the issue.

Serine proteases

Another protease, HtrA, a periplasmic serine protease, has recently been identified as a virulence factor in *B. cenocepacia* strain K-562 (Flannagan *et al.*, 2007). It has previously been demonstrated to be important for intracellular invasion and virulence in *Legionella pneumophila* and *Listeria monocytogenes* (Pedersen *et al.*, 2001; Wilson *et al.*, 2006). In general, *htrA*-negative K-562 mutants were unable to survive in a rat lung infection model. A serine protease has also been shown to be responsible for the ability of *B. cenocepacia* to utilize ferritin as an iron source. Ferritin, not previously determined among other pathogenic bacteria as an iron source, has been identified as a source for Bcc (Whitby *et al.*, 2006). The ability to utilize iron from ferritin, which can sequester over 4000 iron atoms, represents a significant advantage for Bcc, as the CF lung has significantly higher ferritin levels than the non-CF lung (Whitby *et al.*, 2006).

LPS

The LPS of Bcc is distinct from that of other Gram-negative bacteria due to differences in both core oligosaccharide and

the lipid A moiety (Silipo *et al.*, 2007). It plays a dual role in pathogenesis: contribution to antimicrobial peptide resistance and promotion of a potent pro-inflammatory response (Bamford *et al.*, 2007). Its interactions are not limited to host immune cells; *B. cenocepacia* LPS induced IL-8 secretion in airway epithelial cells and was shown to be more potent than *P. aeruginosa* LPS (Reddi *et al.*, 2003).

Other potential virulence factors need to be studied further to investigate the exact role that they play in pathogenesis and whether they interact with the lung epithelium. For example, two-dimensional electrophoresis studies have identified the loss of a hydroperoxide reductase subunit, a stress-related protein, in a *B. cenocepacia* strain variant which was more persistent in a mouse model (Chung & Speert, 2007). This variant also showed amplified motility. The link between stress-regulated proteins and Bcc persistence *in vivo* has yet to be fully explored.

Implications of virulence factors for potential therapy against Bcc

Virulence factors such as metalloproteases are being considered as targets for vaccine development. Immunization of rats with a conserved zinc metalloprotease peptide decreases the severity of Bcc infection (Corbett *et al.*, 2003). It was also shown that peptide epitopes of *P. aeruginosa* elastase decreased lung damage by 70 % (Sokol *et al.*, 2000). In addition, lung damage was reduced by 50 % on challenge with a Bcc strain after immunization with these peptides. Recently, a potential vaccine has been described which is a trisaccharide based on the repeating unit of a polysaccharide in the LPS of a clinical isolate of *B. cepacia* (Faure *et al.*, 2007).

Models to study epithelial cell interactions

Epithelial cell models

Intracellular invasion of lung epithelial cells is a simple model that has been widely used to examine the virulence and epithelial interactions of Bcc strains (Caraher *et al.*, 2007a; Martin & Mohr, 2000). It relies on the killing of extracellular bacteria and the protection of intracellular bacteria from antibiotics. As mentioned previously, a correlation was found between invasion of epithelial cells and infection in an *in vivo* mouse model (Cieri *et al.*, 2002). One limitation which is occasionally cited for this type of assay is the inherent antibiotic resistance of Bcc strains; however, this can be readily overcome by prior validation of the assay to ensure that all strains being examined are killed following the antibiotic treatment being used. Lung epithelial cells also represent a good model for studying the interactions with host cells, including disruption of epithelial tight junction integrity and translocation (Duff *et al.*, 2006; Kim *et al.*, 2005). Calu-3 cells and 16HBE14o-cells are well-characterized and form differentiated epithelial monolayers when grown on semipermeable supports under well-defined conditions. The degree of cell differentiation of airway epithelial cells is important in interpreting these types of studies. While A549 cells represent a useful model to compare the invasive potential of a range of strains, they do not readily polarize or form tight epithelial monolayers and are therefore not ideal for mechanistic studies of invasion or subsequent bacterial fate (Duff *et al.*, 2006). Polarization and tight junction formation has had significant effects on studies carried out on *P. aeruginosa*. Cells which were not polarized and did not have intact tight junctions readily internalized *P. aeruginosa*, while no bacteria were observed inside cells which had intact tight junctions (Plotkowski *et al.*, 1999). Polarized epithelial cells can also be used to examine the intracellular fate of internalized Bcc strains (Sajjan *et al.*, 2006).

Primary lung epithelial cells

Primary lung epithelial cells are the most powerful model for examining host-epithelial interactions at the molecular and cellular level. In particular, comparative experiments between lung cells from CF patients and lung cells from individuals without CF are useful in discerning the key interactions involved in microbial pathogenesis. A limitation of this type of work is access to good-quality lung tissue, usually isolated at the time of transplantation. In addition, obtaining sterile CF tissue is particularly difficult. When isolated and cultured on filters, the primary epithelial cells retain their differentiated ciliated mucus-producing phenotype for up to 2–4 weeks (Schwab *et al.*, 2002).

Other models used to examine host interactions of Bcc

The *Caenorhabditis elegans* model of cell killing has been used to identify the role of a quorum sensing system and to

examine the role of type III secretion systems of Bcc (Huber *et al.*, 2004; Markey *et al.*, 2006). More recently, *Galleria mellonella* larvae have been used to determine the virulence of Bcc strains *in vivo* (Seed & Dennis, 2008). Both systems have been well characterized and are particularly suitable for the examination of innate immune responses to pathogens or virulence factors but are unlikely to be suitable for studying interactions at the epithelial interface. Rat agar bead models and murine agar bead models have been utilized to evaluate the virulence of Bcc isolates and species. The rat agar bead model has been used, for example, to examine the role of HtrA as a virulence factor (Flannagan *et al.*, 2007). Both rat and murine models have an advantage over insect larvae and nematodes in that the interactions with lung epithelia and other host cells can be examined at the microscopic level. In recent Bcc infection studies, the CGD (gp91phox^{-/-}) mice also represented a good model for assessment of Bcc virulence (Sousa *et al.*, 2007). In particular, comparison of virulent strains with mutants for virulence factors such as EPS and quorum sensing showed significant differences in terms of survival time, c.f.u. isolated in the lung and mortality rate between virulent and avirulent strains. Although limitations in the model were identified as a lack of compartmentalization of infection and functioning mucociliary clearance, it appears to be suitable for the comparison of virulence factors.

Concluding remarks

The choice of isolates and strains used in these studies is the key to the successful elucidation of the mechanisms of pathogenesis of Bcc in CF patients. Once a particular mechanism has been observed and examined in detail using one isolate, the studies must be expanded to include clinical isolates from an individual species and also to include other species within the complex. Notwithstanding that, the clinical outcome of Bcc infection is difficult to predict in groups of CF patients infected with the same epidemic strain (Govan & Nelson, 1993) due to the role of multiple host factors, emphasizing the need for therapies which prevent colonization. Overall, these types of investigations will allow the development of alternative non-antibiotic treatments against multiple Bcc species and will be beneficial in enhancing the quality of life of people with CF.

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