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MINIREVIEW

A Decade of *Burkholderia cenocepacia* Virulence Determinant Research▽

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The *Burkholderia cepacia* complex (Bcc) is a group of genetically related environmental bacteria that can cause chronic opportunistic infections in patients with cystic fibrosis (CF) and other underlying diseases. These infections are difficult to treat due to the inherent resistance of the bacteria to antibiotics. Bacteria can spread between CF patients through social contact and sometimes cause cepacia syndrome, a fatal pneumonia accompanied by septicemia. *Burkholderia cenocepacia* has been the focus of attention because initially it was the most common Bcc species isolated from patients with CF in North America and Europe. Today, *B. cenocepacia*, along with *Burkholderia multivorans*, is the most prevalent Bcc species in patients with CF. Given the progress that has been made in our understanding of *B. cenocepacia* over the past decade, we thought that it was an appropriate time to review our knowledge of the pathogenesis of *B. cenocepacia*, paying particular attention to the characterization of virulence determinants and the new tools that have been developed to study them. A common theme emerging from these studies is that *B. cenocepacia* establishes chronic infections in immunocompromised patients, which depend more on determinants mediating host niche adaptation than those involved directly in host cells and tissue damage.

Burkholderia cenocepacia is a motile, rod-shaped, metabolically diverse Gram-negative betaproteobacterium (168, 169) that is widespread in the environment, particularly within the rhizosphere (8), and is also an opportunistic pathogen causing chronic lung infections in patients with cystic fibrosis (CF) as well as other immunocompromised patients (169). Using *recA* sequencing and multilocus sequence typing, *B. cenocepacia* isolates can be subdivided into four distinct lineages, IIIA, IIIB, IIIC, and IIID (168). To date the majority of clinical isolates belong to the IIIA, IIIB, and IIID lineages (4, 96, 110, 168). Isolates from the IIIB and IIIC lineages may be readily cultivated from the natural environment (8, 96, 127, 168). However, even in the absence of culturable IIIA and IIID lineage bacteria from soil, members of these lineages can be detected in soil using non-culture-based methods (127), suggesting that they may also be present in soil but in low abundance. *B. cenocepacia* is one of at least 17 phenotypically similar species known as the *Burkholderia cepacia* complex (Bcc) (168–171). Although almost all the Bcc species have been isolated from CF patients, *B. cenocepacia* was initially the species most commonly isolated from patients with CF (97, 153) and associated with epidemic spread between CF patients (96). For these reasons, *B. cenocepacia* was the main focus of research groups studying the molecular biology, pathogenesis, and antibiotic resistance of Bcc bacteria. However, in recent years, *Burkholderia multivorans* has overtaken *B. cenocepacia* as the most common Bcc isolate in American and United Kingdom CF patients (102, 133). Some *B. multivorans* strains are widely distributed and have been associated with outbreaks (9). This article reviews our current understanding of the virulence determinants of *B. cenocepacia* as well as the tools developed to study them. Since Bcc bacteria are resistant to many clinically useful antibiotics (1, 22, 57, 118, 163), the study of virulence determinants is important for identifying bacterial processes that could be targeted by novel antibiotics or alternative anti-infective therapies.

(A portion of this work appears in S.A.L.'s Ph.D. thesis.)

***Burkholderia* in the environment.** *Burkholderia* sp. live in diverse ecological niches, often in either beneficial or pathogenic relationships with other organisms (for a recent review, see the work of Compant et al. [36]). *Burkholderia* spp. have been described as plant pathogens (7, 21, 72), symbiotic rhizospheric or endophytic plant growth promoters (35, 130), endosymbionts of fungi (5, 56, 70) and insects (77, 144), and animal pathogens (31, 59). They can degrade pollutants (25, 30, 83, 84, 147), fix nitrogen and solubilize metals for use by their symbiotic partners (25, 73), produce compounds that protect their host-associated partners from pathogenic fungi, bacteria, protozoa, and nematodes (26, 111, 114), and even induce plant host defense mechanisms (37). *B. cenocepacia* can be associated with plants, including onions, sugarcane, maize, wheat, and legumes (8, 96, 112). Conceivably, such diverse biological interactions exert selective pressure, giving rise to highly adaptable bacteria. In turn, this ability to adapt to different conditions could contribute significantly to the antibiotic resistance and pathogenesis of *Burkholderia* spp., including *B. cenocepacia*. Indeed, many factors discussed below that are required for *B. cenocepacia* pathogenesis have more to do with adaptation for survival under changing conditions (e.g., metabolic path-

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ways, host antimicrobial molecule resistance mechanisms, and regulatory proteins required for the control of bacterial stress responses), which is likely necessary for establishing chronic infections, than with mounting a potent acute infection.

Genetics of *B. cenocepacia*. *Burkholderia* species have some of the largest, most complex bacterial genomes described to date (93, 105). They are high in percent G+C content, characterized by a multireplicon structure, and possess numerous gene duplications, insertion sequences, and mobile elements. These elements are thought to contribute to the plasticity of *Burkholderia* genomes and their ability to acquire a wide range of metabolic pathways (93). *Burkholderia* genomes can also mutate rapidly when the organisms are subject to *in vitro* stress conditions or during infections (46, 125, 128). The genome sequence of *B. cenocepacia* strain J2315 was published in 2009 (65), although the Wellcome Trust Sanger Institute made initial sequencing and annotation of the genome available to researchers since 2000. J2315 is a member of the electrophoretic type 12 lineage of strains that caused transmissible infections in CF patients in Canada, the United Kingdom, and Europe (58, 103, 168); strain J2315 is also a member of the IIIA phylogenetic lineage of *B. cenocepacia* (65, 168). The genome has over 8 Mbp and consists of three circular chromosomes and a plasmid (65). Most studies of *B. cenocepacia* conducted utilize strain J2315 or another CF clinical isolate, strain K56-2, which is clonally related to J2315 by pulsed-field gel electrophoresis (104) and is a double-locus variant by multilocus sequence typing (65). In this case, the available genome sequence of J2315 is applied to K56-2; however, there are some differences between these two strains, including differences in lipopolysaccharide (LPS) structure (120), pigment production (75), and interactions with eukaryotic cells (142). Strain K56-2 has often been used because it has lower resistance to antimicrobial agents than J2315, which facilitates genetic selection and makes it more amenable to genetic manipulation. The U.S. Department of Energy Joint Genome Institute has sequenced the genomes of three other *B. cenocepacia* strains from lineage IIIB. They include one isolate from the blood of a CF patient with cepacia syndrome and two rhizosphere isolates. These strains may be useful for genomic comparisons to IIIA lineage strains. The *Burkholderia* Genome Database (http://www.burkholderia.com) provides users with an excellent resource for annotations of genomes and comparative genome analysis and includes all of the sequenced *B. cenocepacia* genomes as well as genomes from other *Burkholderia* spp. (177). For the genes described below that contribute to virulence in *B. cenocepacia*, we have listed in Table 1 the systematic gene numbers from *B. cenocepacia* strain J2315.

TABLE 1. *B. cenocepacia* strain J2315 systematic gene names for virulence determinants described in this review

Gene or function	Systematic gene name(s) in J2315
rpoE	BCAL0998 and BCAL2872 (two copies)
rpoN	BCAL0813
htrA	BCAL2829
rpoN-EBP	BCAL1536
cepRI	BCAM1868/BCAM1870
aidA	BCAS0293
cciRI	BCAM0239a/BCAM0240
cepR2	BCAM0188
BDSF[a] synthase (*rpfF*)	BCAM0581
shvR	BCAS0225
atsR	BCAM0379
Type 3 secretion system	BCAM2045 to BCAM2057
Type 4 secretion systems	pBCA020 to pBCA059 (virulence related) BCAM0324 to BCAM0340 (plasmid mobilization)
Type 6 secretion system	BCAL0337 to BCAL0351
zmpA	BCAS0409
zmpB	BCAM2307
katA	BCAM2107
katB	BCAL3299
sodC	BCAL2643
hppD	BCAL0207
Ornibactin synthesis	BCAL1688 to BCAL1702
LPS core oligosaccharide[b]	
hldA	BCAL2945
waaC	BCAL3112
Ara4N biosynthesis	BCAL1929 to BCAL1935
Flagellum[b]	
fliC	BCAL0114
fliJ	BCAL0521
Cable pili	BCAM2756 to BCAM2762
acp	BCAL0995 and BCAL2875 (two copies)
mgtC	BCAM1867
Phenylacetic acid catabolism[b]	
paaE	BCAL0212
paaA	BCAL0216
amiI	BCAM0265
opcI	BCAM0267
pbr	Not present

[a] BDSF, *cis*-2-dodecenoic acid, diffusible, nonhomoserine lactone signal molecule.

[b] Numerous genes located in multiple clusters are required for these determinants; those listed have been mutated for characterization of virulence determinants.

Genetic tools for use with *B. cenocepacia*. Genetic manipulation of *Burkholderia* strains is challenging, and this initially hampered rapid progress in the field. However, a series of constantly improving mutagenesis systems specifically tailored for *B. cenocepacia* were developed over the last decade. Most of these systems employed a strategy in which nonreplicative plasmids are integrated into specific locations in the chromosome via homologous recombination, resulting in polar (51), nonpolar (53), or conditional (122) mutations. Recently, five systems for the creation of unmarked gene deletions have been published for *Burkholderia* spp., including *B. cenocepacia* (12, 33, 52, 61, 98). These systems allow the creation of multiple deletions within a strain and can also be used to integrate DNA into heterologous locations in the chromosome for *cis* complementation experiments and other applications. Other genetic tools for the study of *B. cenocepacia* include transposon mutagenesis (28, 68), microarray technology (47), subtractive hybridization (16), and high-throughput sequencing of RNA (RNA-seq) for quantification of transcriptional responses (179). Together, these tools facilitate much more rapid and refined manipulation of *B. cenocepacia* today than was possible a decade ago.

***In vivo* and *ex vivo* models of infection.** *In vivo* and *ex vivo* models have been used to study the ability of *B. cenocepacia* to cause disease. The most widely used animal model for studies

of members of the Bcc is the rat agar bead model of chronic lung infection that was originally developed for *Pseudomonas aeruginosa* infections (29). In this model, bacteria are embedded in agar beads, which are then inserted into rat lungs via the trachea, where they establish a chronic but nonlethal infection (29). Similar experiments using Bcc bacteria in agar beads have also been conducted in mice (159). A mouse model of chronic granulomatous disease, another condition in which patients are susceptible to opportunistic Bcc infections (176), has also been established for the study of Bcc virulence (148). Three simpler, less expensive animal models, the nematode *Caenorhabditis elegans* (82), *Galleria mellonella* moth larvae (146), and zebra fish (44) models, have also been used for the study of Bcc pathogenicity. As members of the Bcc are also plant pathogens, plant models developed for measuring the virulence of Bcc bacteria include the alfalfa seedling (15) and onion tissue (71) models.

The ability of *B. cenocepacia* to infect and survive in macrophages, both with and without a functional cystic fibrosis transmembrane conductance regulator (CFTR) protein (86, 88), epithelial cells (140, 145), dendritic cells (101), neutrophils (23, 24, 141), and amoeba (87), has been investigated; and these models have also been used for the study of putative virulence factors (reviewed in reference 143). A common theme that emerges from these studies is that after phagocytosis, the internalized *B. cenocepacia* cells delay the maturation of bacterium-containing vacuoles. In macrophages, the maturation delay results in reduced acidification of the lumen of bacterium-containing vacuoles and inhibition of the assembly of the NADPH oxidase complex on the vacuolar membrane, and these phenotypes are greatly exaggerated in the absence of a functional CFTR protein (74, 86, 88, 137). The *B. cenocepacia* organinisms in infected epithelial cells also interfere with the normal endocytic pathway, but intracellular bacteria eventually enter the endoplasmic reticulum, where they replicate. In dendritic cells, engulfed *B. cenocepacia* cells can alter cell function and induce necrosis (101). Studies of tissue culture systems have been hindered by the inability to complete traditional gentamicin protection assays due to bacterial resistance to gentamicin. However, new strains that are gentamicin sensitive but otherwise behave as the wild type during macrophage infections were recently constructed (60) and should facilitate these types of experiments.

VIRULENCE DETERMINANTS OF *B. CENOCEPACIA*

Several virulence determinants that may play a role in the ability of *B. cenocepacia* to cause disease have been proposed (Fig. 1). A signature tagged mutagenesis study of *B. cenocepacia* using the rat agar bead model of chronic lung infection identified over 100 genes that were required by *B. cenocepacia* for survival in this model (68). All three chromosomes and the plasmid possessed genes required for *B. cenocepacia* survival. These genes encoded proteins with diverse predicted functions, including regulatory, transport, and metabolism proteins, proteins involved in cell surface biogenesis, and conserved proteins of unknown function (68).

Putative virulence determinants were also identified through comparative transcriptomic studies using either microarray or high-throughput sequencing of RNA from closely related soil and clinical isolates of *B. cenocepacia* from the IIIB lineage, which were grown under conditions mimicking soil and CF sputum (179, 180). Similar microarray experiments were conducted with the IIIA lineage strain J2315 (47). These studies revealed hundreds of genes that were differentially regulated under conditions mimicking CF sputum. However, there was little convergence between the data obtained for CF isolates from the two different lineages, with only nine genes being differentially regulated in a similar fashion in the two studies (47, 179). This could be due to differences in experimental procedures or the result of important differences between strains of *B. cenocepacia*. The latter possibility is conceivable, since several virulence properties, described in more detail below, are strain specific. Therefore, we have listed in Table 2 virulence determinants of *B. cenocepacia* that have been characterized in multiple infection models and in Table 3 those that have been tested in only single infection models.

Alternative sigma factors and related proteins. Alternative sigma factors are regulatory proteins that activate transcription of particular gene subsets by binding at sites within promoter regions and interacting with the RNA polymerase complex, to allow the initiation of transcription (2). Two alternative sigma factors, RpoE and RpoN, are required for the ability of engulfed *B. cenocepacia* to delay phagolysosomal fusion in murine macrophages (53, 141).

RpoE is also required for the ability of *B. cenocepacia* to grow under conditions of high osmolarity and high temperature (53). In Gram-negative bacteria, RpoE mediates gene activation in response to extracytoplasmic stress (2), and individual components of the extracytoplasmic stress response are also likely to be important for the virulence of *B. cenocepacia*. These include HtrA-like periplasmic proteases, of which *B. cenocepacia* is predicted to encode four (51). Under conditions of extracytoplasmic stress, HtrA can act either as a chaperone to aid in the refolding of misfolded periplasmic proteins or as a protease to degrade misfolded proteins (154). Mutation of one of the HtrA proteases (BCAL2829) of *B. cenocepacia* resulted in a strain that is avirulent in the rat agar bead infection model and has increased sensitivity to osmotic and thermal stress (51).

RpoN is necessary for *B. cenocepacia* motility and biofilm formation (141). In other bacteria, RpoN activates the expression of genes necessary for a wide range of functions, and unlike the activation of other sigma factors, this activation also requires an enhancer binding protein (113). A mutant defective in 1 of the 20 predicted enhancer binding proteins (BCAL1536) of *B. cenocepacia* is attenuated in the rat agar bead infection model (68).

Quorum sensing. Quorum sensing allows regulation of gene expression on the basis of the density of the bacterial population. Bacteria secrete compounds that accumulate outside the cell, and once sufficient cell densities are reached, the concentration of the diffusible compound reaches a threshold and the bacteria begin to alter gene expression (54). These systems represent a way for bacterial cells to communicate with one another and coordinate multicellular behavior.

The *cepRI* quorum-sensing system, which mediates production of *N*-octanoylhomoserine lactone, regulates numerous functions in *B. cenocepacia*. Initial studies showed that disruption of the system leads to increased siderophore production

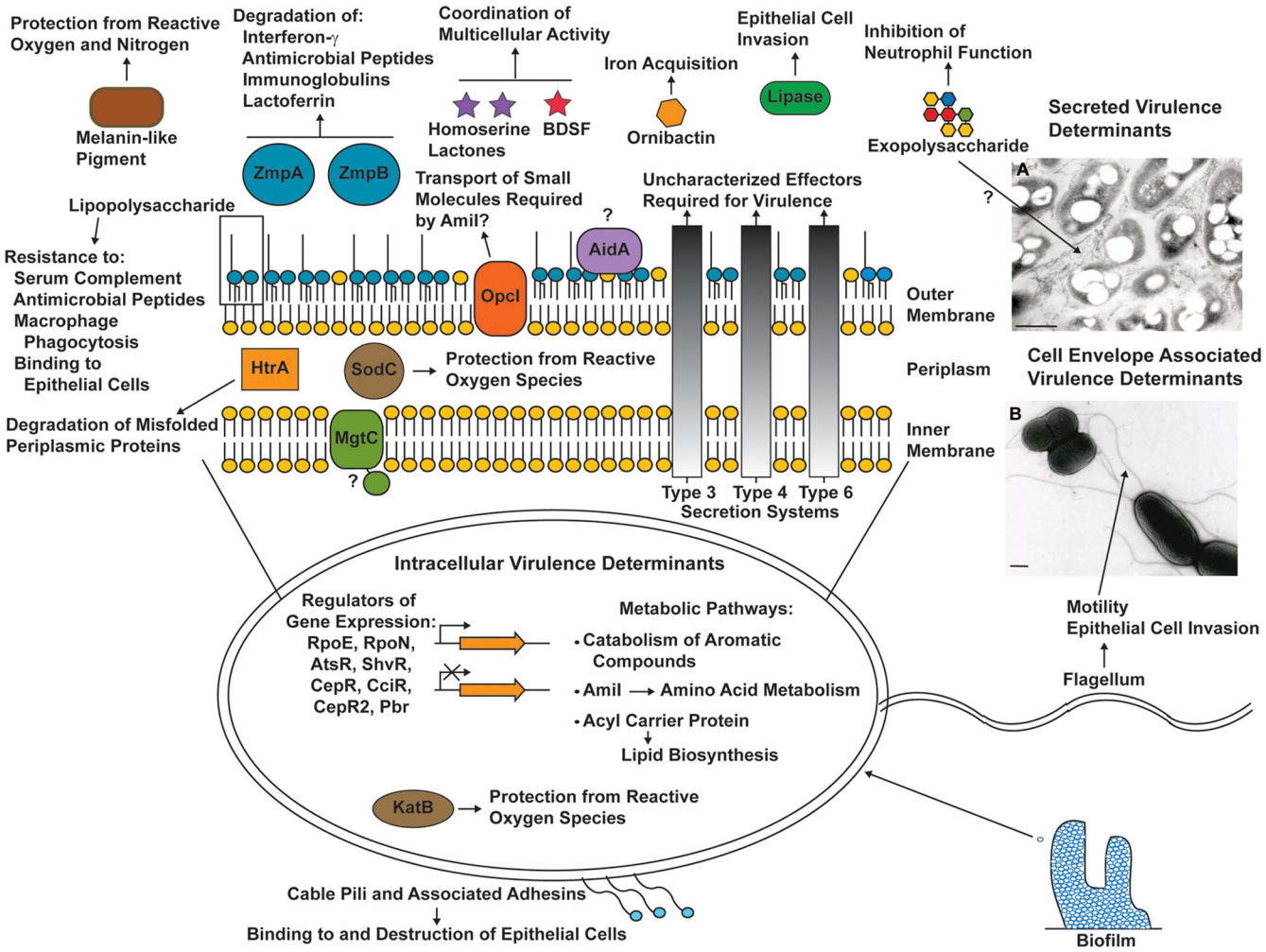

FIG. 1. Representation of the localization and known functions of *B. cenocepacia* virulence determinants. All of the established and proposed virulence determinants are discussed in the appropriate subsections of this review. BDSF denotes *cis*-2-dodecenoic acid, diffusible, nonhomoserine lactone signal molecule. Virulence determinants with proposed or unknown functions are denoted with question marks. (Inset A) Bacteria fixed in the presence of alcian blue and stained with uranyl acetate and lead citrate to visualize the extracellular matrix produced by bacterial cells from the virulent rough colony morphology phenotype described by Bernier et al. (14). The exact composition of this extracellular matrix is unknown but is proposed to consist of exopolysaccharide and possibly lipopolysaccharide and proteins (14). (Inset B) Bacterial cells negatively stained with uranyl acetate to visualize flagella. Bars, 0.5 μm. (Electron micrographs courtesy of Maria Soledad Saldías, reproduced with permission.)

and decreased extracellular protease and lipase activities (94). Further studies showed that this system was required for motility (95), biofilm stability (162), virulence in the rat agar bead model of chronic lung infection (151), and killing of *C. elegans* (82). A random promoter library screen identified almost 90 *B. cenocepacia* promoters regulated by CepR (157), and evidence also suggests that the *cepRI* system could potentially be involved in cross-species communication with *P. aeruginosa* (95). One molecule that is controlled by the *cepRI* system and that has been implicated in virulence is AidA, a surface protein required for the killing of *C. elegans* by an unknown mechanism (66).

B. cenocepacia also possesses genes for a second homoserine lactone-producing quorum-sensing system, designated *cciRI*, and an orphan regulator, designated *cepR2*, that is not encoded by a homoserine lactone synthase gene (108, 109). These systems also contribute to the regulation of many of the same functions as *cepRI*, though often in reverse to the regulation by *cepRI*, suggesting a complex network of gene regulation in response to bacterial cell density (108, 109, 119, 157).

Bacterial quorum-sensing systems can also utilize nonhomoserine lactone compounds, an example of which is the diffusible signal factor of *Xanthomonas campestris* (63). *B. cenocepacia* has one such system and produces *cis*-2-dodecenoic acid in a cell density-dependent manner (19). This diffusible signal molecule alters many of the same functions controlled by *cepRI*, *cciRI*, and *cepR2*; mutants unable to synthesize *cis*-2-dodecenoic acid have decreased motility, biofilm synthesis, and virulence in the *G. mellonella* moth larvae and zebra fish infection models (19, 44, 136). This molecule may also be involved in communication between *B. cenocepacia* and other

TABLE 2. Virulence determinants tested in multiple infection models

Virulence determinant	Mutant phenotype(s)				References
	Vertebrate models	Invertebrate models	Plant models	Eukaryotic cell culture models	
Ornibactin siderophore	3-log decrease in bacterial burden, rat agar bead model	Decreased virulence in *C. elegans* model, avirulent in *G. mellonella* model	Full virulence in alfalfa seedling model	No data	150, 164
LPS core oligosaccharide	Avirulent in rat agar bead model	Decreased virulence in *C. elegans* and *G. mellonella* models	Full virulence in alfalfa seedling model	Does not survive in murine macrophages	99, 121, 164
CepRI quorum-sensing system	Less lung tissue inflammation in rat agar bead model, decreased virulence after intranasal infection in *Cftr*$^{-/-}$ mice	Decreased virulence in *C. elegans* model (strain independent), decreased virulence in *G. mellonella* model (strain dependent)	Decreased virulence in alfalfa seedling model (strain dependent)	No data	82, 151, 164
CciRI quorum-sensing system	Less lung tissue inflammation, rat agar bead model	Full virulence in *C. elegans* and *G. mellonella* models	Full virulence in alfalfa seedling model	No data	10, 164
HtrA protease	Avirulent in rat agar bead model	Full virulence in *C. elegans* and *G. mellonella* models	Full virulence in alfalfa seedling model	No data	51, 164
AidA	Virulent in rat agar bead model	Decreased virulence in *C. elegans* model, full virulence in *G. mellonella* model	Virulent in alfalfa seedling model	No data	66, 164
BDSF[a]	Decreased virulence in zebra fish	Decreased virulence in *G. mellonella* model	No data	Does not inhibit *C. albicans* germ tube formation	19, 44, 136
Type 3 secretion system	3-log decrease in bacterial burden in rat agar bead model	Decreased virulence in *C. elegans* model, full virulence in *G. mellonella* model	Full virulence in alfalfa seedling model	Virulent in murine macrophages	86, 159, 164
Type 4 secretion system	No data	No data	Does not cause tissue destruction in onions	Decreased virulence in airway epithelial and macrophage cells	48, 138
Type 6 secretion system	Decreased virulence in rat agar bead model	No data	No data	Decreased survival in the presence of amoeba	6, 68
ZmpA protease	3- to 4-log decrease in bacterial burden, rat agar bead model (strain dependent)	Full virulence in *C. elegans* and *G. mellonella* models	Virulent in alfalfa seedling model with decreased bacterial burden	No data	15, 40, 81, 164
ZmpB protease	Less lung tissue inflammation in rat agar bead model	Full virulence in *C. elegans* and *G. mellonella* models	Full virulence in alfalfa seedling model	No data	81, 164
ShvR regulator	Less lung tissue inflammation in rat agar bead model	Full virulence in *C. elegans* and *G. mellonella* models	Avirulent in alfalfa seedling model	No data	14, 164
OpcI outer membrane porin	Less lung tissue inflammation in rat agar bead model	Full virulence in *C. elegans* and *G. mellonella* models	Full virulence in alfalfa seedling model	No data	10, 164
MgtC	Decreased virulence in rat agar bead model	No data	No data	Does not survive in murine macrophages	68, 107
Phenylacetic acid catabolic pathway	Decreased virulence in rat agar bead model	Decreased virulence in *C. elegans* model	No data	No data	68, 90
Cable pili	No data	Decreased virulence in *G. mellonella* model	No data	Does not kill airway epithelial cells	32, 136
Flagellum	Avirulent in mouse agar bead model	Decreased virulence in *G. mellonella* model	No data	Reduced invasion of epithelial cells	136, 160, 165

[a] BDSF, *cis*-2-dodecenoic acid, diffusible, nonhomoserine lactone signal molecule.

TABLE 3. Virulence determinants tested in only one infection model

Virulence determinant	Mutant phenotype in selected virulence model	Reference
RpoE alternative sigma factor	Does not survive in murine macrophages	53
RpoN alternative sigma factor	Does not survive in murine macrophages	141
RpoN enhancer binding protein	Decreased virulence in rat agar bead model	68
Secreted lipase	Decreased epithelial cell invasion	116
KatB catalase/peroxidase	Decreased survival in murine macrophages	—[a]
SodC superoxide dismutase	Does not survive in murine macrophages	76
Melanin-like pigment	Does not survive in murine macrophages	75
Pbr regulator	Decreased virulence in *C. elegans*	131
AmiI amidase	Decrease (1 log unit) in bacterial burden in rat agar bead infections	10
Acyl carrier protein	Decreased virulence in *C. elegans*	152

[a] —, K. E. Keith and M. A. Valvano, unpublished results.

bacteria as well as in interkingdom communication with *Candida albicans* (19).

Biofilms. Biofilms are complex, multicellular bacterial communities that can protect bacteria from antibiotics and the host immune system (50). Bcc bacteria are thought to live in CF lungs in biofilms, including mixed biofilms with *P. aeruginosa*, where they may even communicate with *P. aeruginosa* via quorum-sensing systems (134, 161). *B. cenocepacia* can also form biofilms *in vitro* (39), and the various genes required for the formation of these multicellular bacterial aggregates have been identified (67). Biofilm formation can be affected by multiple gene regulation systems, including quorum sensing (162), the alternative sigma factor RpoN (141), ShvR, a LysR-type regulator (14), and AtsR, a hybrid sensor kinase-response regulator that acts as a negative regulator of multiple virulence properties (6). Biofilm formation is also affected by exopolysaccharide synthesis (41), motility (67), and iron availability (13). There are conflicting reports concerning the increased antibiotic resistance in *B. cenocepacia* growing in biofilms compared to that of planktonic cells, with some studies showing increases in antibiotic resistance in biofilms (27, 45). However, another study showed little difference in antibiotic resistance between bacteria grown planktonically and those grown in biofilms (126). Together, these attributes indicate that biofilm formation is a complex process involving numerous *B. cenocepacia* virulence determinants. This makes the disruption of biofilm growth an attractive target for the development of new antibiotics, and many quorum-sensing inhibitors that are being developed also inhibit biofilm formation (124).

Secretion systems. Many pathogenic bacteria employ specialized systems for the secretion of effector molecules that contribute to cause disease by disrupting host cellular processes (55). Various secretion systems have been disrupted in *B. cenocepacia* to investigate their relevance to virulence. The type III secretion system (T3SS) is required by *B. cenocepacia* for survival during murine agar bead infections (159). In this study, the average number of bacteria in the spleens and lungs of mice infected with a T3SS mutant was reduced by 2 and 3 log units, respectively, compared to the number of bacteria in mice infected with the wild-type strain (159). One of the two predicted type IV secretion systems of *B. cenocepacia* is necessary for causing disease in onions (48) and for intracellular survival in epithelial cells and macrophages (138), while the other is involved in plasmid mobilization (181). The type VI secretion system (T6SS) was recently identified in a number of organisms, including *B. cenocepacia*, where its expression is negatively regulated by the sensor kinase-response regulator AtsR (6). The T6SS is also required for protection from predation by the amoeba *Dictyostelium discoideum* (6). Furthermore, macrophages infected with *B. cenocepacia* cells overexpressing the T6SS make actin-rich cellular projections that can harbor bacterium-containing vacuoles (6). Finally, the T6SS also plays a role in infection, as three independent mutants in the T6SS gene cluster were attenuated in the rat agar bead model (68).

Secreted proteins. *B. cenocepacia* produces two distinct extracellular zinc metalloproteases, named ZmpA and ZmpB (40, 81). Disruption of *zmpA* in one of two strains of *B. cenocepacia* resulted in a mutant that was much less persistent in the rat agar bead model, and many rats cleared the mutant by day 14 after infection (40). However, the second strain of *B. cenocepacia* did not require ZmpA for virulence in this model, indicating strain differences in terms of the requirement for virulence determinants (40). A *B. cenocepacia zmpB* mutant persisted in the rat agar bead model at numbers similar to those of the wild-type strain. However, rats infected with the *zmpB* mutant have decreased lung tissue inflammation, based on the histopathology at 14 days postinfection (81). Purified, recombinant versions of both of these proteins can degrade lactoferrin, type IV collagen, immunoglobulins, and antimicrobial peptides, such as human β-defensin-1 and LL-37 *in vitro* (79–81).

Secreted lipases have also been implicated in the ability of *B. cenocepacia* to cause disease. Mullen et al. (116) showed that Bcc species, including *B. cenocepacia*, secrete lipases and that pretreatment of epithelial cells with commercially available *B. cepacia* lipase resulted in roughly 50% more invasion of epithelial cells by *B. cenocepacia*, while pretreatment of *B. cenocepacia* with a lipase inhibitor decreased the level of invasion.

Colony variants. *B. cenocepacia* can produce different colony morphologies, at least one of which, the shiny colony morphology variant (or shv), results in bacteria that are avirulent in the alfalfa model of infection (14). These isolates can still establish chronic infections in the rat agar bead model, but the average number of viable bacteria per lung in the infected rats decreased by approximately 1 log unit compared to the parental strain number, and infection with the shv isolate resulted in decreased inflammation. Switching to the shv morphology has pleiotropic effects on the bacteria, with decreases in biofilm formation, motility, and production of extracellular matrices and siderophores being found. The extracellular matrix made by the virulent, rough colony morphology isolates is shown in Fig. 1A. Some of the shv isolates arise due to a spontaneous mutation in a LysR-type regulator (ShvR), while others do not have this spontaneous mutation, suggesting that there are multiple pathways that *B. cenocepacia* can take to arrive at the shv phenotype (14).

Resistance to oxidative stress. Phagocytic cells produce reactive oxygen species to help eliminate bacteria, and *B. cenocepacia* has numerous mechanisms to resist oxidative stress. Multiple strains of *B. cenocepacia* possess *in vitro* catalase, peroxidase, and superoxide dismutase activities (92). Two catalase/peroxidases with different functions were identified in *B. cenocepacia*: KatB, the major catalase/peroxidase of *B. cenocepacia*, and KatA, a secondary catalase/peroxidase required for resistance to hydrogen peroxide under conditions of iron limitation and growth in the presence of carbon sources metabolized through the tricarboxylic acid cycle (91). Also, *B. cenocepacia* possesses a periplasmic superoxide dismutase, SodC, required for resistance to extracellular superoxide, and a *B. cenocepacia sodC* mutant is more rapidly killed, in an NADPH oxidase-dependent fashion, by murine macrophages than the wild-type strain (76). Similar results were observed for a melanin-like pigment expressed by strains of *B. cenocepacia* that belong to the IIIA lineage (75). Disruption of *hppD*, a gene encoding an enzyme required for production of the pigment, resulted in a nonpigmented strain with increased sensitivity to extracellular hydrogen peroxide and superoxide and decreased survival in murine macrophages (75). Inhibition of either NAPDH oxidase or inducible nitric oxide synthase resulted in increased survival of the mutant during macrophage infections (75), suggesting that this pigment protects *B. cenocepacia* from both reactive oxygen and reactive nitrogen species.

Iron acquisition. The ability to grow under conditions of iron limitation is an important characteristic for pathogens because the host is extremely limited in the amount of freely available iron that it has (115). Members of the Bcc can synthesize four different siderophores (pyochelin, ornibactin, cepaciachelin, and cepabactin) for iron chelation and uptake (158). The predominant siderophore produced by most strains of *B. cenocepacia* appears to be ornibactin, while some strains also synthesize small amounts of pyochelin (42, 158). The synthesis and uptake pathways of ornibactin and their regulation have been well characterized in *B. cenocepacia* (3, 94, 149, 150), and this siderophore is required for virulence in the rat agar bead, *G. mellonella*, and *C. elegans* infection models (150, 164, 172). *B. cenocepacia* can also use iron obtained from the iron-binding protein ferritin, likely through the proteolytic degradation of ferritin (174). Since ferritin is found at up to 100-fold higher concentrations in the bronchoalveolar lavage fluid of CF patients than in that of healthy individuals (156), it has been suggested that ferritin might serve as an important iron source to *B. cenocepacia* during CF lung infections (174), although this has not yet been tested experimentally. *B. cenocepacia* can also use heme as a source of iron (174). Together, these results demonstrate that like many other bacterial pathogens, *B. cenocepacia* has specialized mechanisms to acquire iron during infections.

Lipopolysaccharide and other cell envelope structures. LPS is a complex glycolipid located in the outer leaflet of the outer membrane of Gram-negative bacteria, which plays a significant role in bacterial pathogenesis (129). *B. cenocepacia* LPS can activate immune cells through Toll-like receptor 4-mediated signaling (11). The O-antigen portion of the LPS molecule is important for resistance to serum-mediated killing (120). It is also prevents both bacterial binding to epithelial cells and bacterial phagocytosis by macrophages (142). However, not all strains of *B. cenocepacia*, including epidemic strain J2315, make polymeric O antigen, so it is not required for virulence (120). Mutant strains of *B. cenocepacia* unable to synthesize the LPS inner core oligosaccharide (deep-rough LPS mutants) have impaired virulence in the rat agar bead, *C. elegans*, and *G. mellonella* infection models and fail to survive in murine macrophages (99, 121, 164). *B. cenocepacia* deep-rough LPS mutants are also more sensitive to cationic antimicrobial peptides, including human neutrophil peptide 1 (99).

Another interesting and unique aspect of the LPS biology of *B. cenocepacia* and other *Burkholderia* spp. is the constitutive presence of 4-amino-4-deoxy-L-arabinose (Ara4N) residues in the lipid A and inner core oligosaccharide. Many Gram-negative bacteria replace lipid A phosphate residues with molecules of Ara4N *in vivo* as a regulated mechanism of cationic antimicrobial peptide resistance, and this pathway is usually dispensable under most growth conditions (49, 117, 166, 175). Remarkably, this pathway is essential for *B. cenocepacia* survival, and depletion of the proteins of this pathway in conditional mutants leads to increased susceptibility to antimicrobial agents and accumulation of membranous material inside the cell (122) in a fashion that is similar to depletion of the machinery responsible for LPS transport to the outer membrane (178).

Other cell envelope structures required for virulence include the flagellum (Fig. 1B), disruption of which results in a nonmotile strain that is avirulent in the mouse agar bead model of infection (165), and cable pili, which are required for binding to cytokeratin 13 on epithelial cells (139) and killing of human airway epithelial cells *ex vivo* (32). Additionally, Sousa et al. (152) have shown that a *B. cenocepacia* mutant strain lacking an acyl carrier protein (ACP) has alterations in fatty acid content and increased cell surface hydrophobicity. This mutant strain has a greater ability to form biofilms *in vitro* but has decreased pathogenicity in the *C. elegans* infection model (152).

MgtC. MgtC is a virulence protein that is required by distantly related bacterial pathogens, such as *Salmonella enterica*, *Mycobacterium tuberculosis*, and *B. cenocepacia*, for intraphagosomal survival in macrophages and growth under conditions of low magnesium but whose function is currently unknown (18, 20, 89, 107). Data suggest that these are two separate roles for MgtC (132). *B. cenocepacia mgtC* mutant strains fail to survive in murine macrophages and in the rat agar bead model of chronic lung infection (68, 107), but MgtC is not required for resistance *in vitro* to conditions that are encountered in macrophages, such as reactive oxygen and nitrogen species, low pH, and cationic antimicrobial peptides (107), so the function of this protein in virulence remains elusive.

Phenylacetic acid catabolic pathway. As noted above, transposon mutants in numerous metabolic pathways were attenuated in the rat agar bead model, including mutants in the phenylacetic acid catabolic pathway (68). This pathway is the point at which the catabolism of many aromatic compounds converges (100). In *B. cenocepacia*, this pathway is also required for virulence in the *C. elegans* model (90) and is upregulated *in vitro* when bacteria are grown in synthetic cystic fibrosis medium (62), a defined medium developed by Palmer et al. (123) that is based on the contents of CF sputum and that

contains significant amounts of aromatic amino acids. Since phenylacetic acid catabolism is linked to the catabolism of aromatic amino acids, it is conceivable that this pathway may be important for nutrient acquisition or the metabolism of infecting bacteria in the host environment.

Exopolysaccharide. Exopolysaccharides (EPSs) are branched, repeating polysaccharide subunits that are secreted by bacteria into the extracellular milieu (173). The synthesis of EPS occurs during CF infections with Bcc bacteria (38, 64), although it is not always seen, and commonly studied *B. cenocepacia* clinical isolates such as J2315 and K56-2 produce little or no exopolysaccharide (182). EPS from EPS-producing *B. cenocepacia* clinical isolates may contribute to the virulence of the organism through inhibiting both neutrophil chemotaxis and neutrophil generation of H_2O_2 and O_2^- (23), and production of EPS results in slower clearance of bacteria from murine lungs (38).

Genomic islands. The genome of *B. cenocepacia* strain J2315 contains more than a dozen genomic islands, most of which are still uncharacterized (65). One genomic island, the *Burkholderia cepacia* epidemic strain marker (BCESM), contains putative virulence-enhancing factors, including an amidase (AmiI), an outer membrane porin (OpcI), and the *cciRI* quorum-sensing system (10). Recently, a genomic island identified in *B. cenocepacia* strain K56-2 that is absent from strain J2315 was shown to contain a gene that encodes a predicted regulatory protein, Pbr (131). Disruption of *pbr* resulted in a strain with a number of defects, including increased susceptibility to oxidative and temperature stress, decreased virulence in *C. elegans*, and an inability to synthesize phenazines, which are antibiotics that are thought to interfere with electron transport and impair respiration (131).

LIMITATIONS

Despite the abundance of studies described above, there are some important limitations to the currently available data on virulence determinants in *B. cenocepacia*. First, for many of the determinants described above, experiments have been limited to only one or two infection models. This is particularly relevant if the infection models chosen are limited to only tissue-cultured cell-based assays, and many promising mutant strains must be characterized in additional infection models in order to determine the scope to which they are required for virulence.

Uehlinger et al. (164) have made a first attempt at addressing this issue. The authors of that study tested over a dozen *B. cenocepacia* mutant strains in up to four different infection models and found that the LPS core oligosaccharide, the siderophore ornibactin, and the cepRI quorum-sensing system were required in at least three of the models tested (Table 2). The remaining virulence determinants tested in that study were required in only one or two of the infection models. To date there is no mutant strain that has been tested and shown to be avirulent in all the different types of infection models (vertebrate, invertebrate, plant, and tissue culture models).

Second, there appears to be significant disparity between results for a given virulence determinant when it is tested across many virulence models. For example, mutants lacking ornibactin or the LPS core oligosaccharide have significant impairments in virulence in multiple animal models but have wild-type levels of virulence in the alfalfa seedling model (Table 2). The infection models used represent different environmental and clinical niches that Bcc bacteria can be found in, none of which accurately represent the environment of the CF lung. The rat agar bead model does replicate some of the characteristics of the CF lung, such as increased cytokine levels and neutrophil influx, and the agar mimics bacterial biofilms. However, instillation of the bacteria into the lungs circumvents innate host defenses and initial steps in bacterial colonization (85). Perhaps new models that appear to more closely replicate human CF disease, such as the *cftr*$^{-/-}$ pig (135), will be helpful in identifying determinants of *B. cenocepacia* virulence that also contribute to disease in CF patients.

Third, there is great strain-to-strain variability within the species *B. cenocepacia*. For example, three well-studied strains, strains H111, K56-2, and J2315 (the last two being clonally related isolates, as described above), all behave quite differently in the *G. mellonella* and *C. elegans* infection models (164). Additionally there are a number of examples of strain-to-strain variation in the production of, requirement for, or even presence of certain virulence determinants. A melanin-like pigment required by *B. cenocepacia* strain C5424 for survival in macrophages is made in much larger amounts in this strain than in clonally related strain J2315 or K56-2 (75). The extracellular protease ZmpA is required for virulence in the rat agar bead model in a strain-dependent fashion (40). The regulatory protein Pbr that contributes to virulence of strain K56-2 in *C. elegans* is found on a genomic island that is absent from strain J2315 (131). Virulence determinants required in a range of infection models should be tested to see if they are required for virulence in a variety of *B. cenocepacia* strains as well as in other Bcc bacteria.

CONCLUDING REMARKS

Since Bcc bacteria were initially recognized as causing severe lung infections in CF patients (69), much progress in the characterization of virulence determinants of *B. cenocepacia* has been made, particularly in the last decade. These include extracellular and cell surface polysaccharides, systems that regulate gene expression, secretion systems, metabolic and nutrient acquisition pathways, molecules required for resistance to host antimicrobial compounds, and proteins whose functions are not well understood. To aid in these studies, a number of genomes have been sequenced, sophisticated tools for genetic manipulation have been developed, and numerous *in vivo* models have been established.

These studies should form the base for the development of novel antimicrobial agents that can target *B. cenocepacia in vivo*. Some of these types of studies are under way, such as the development of molecules to inhibit the addition of Ara4N residues to lipid A (78) or the synthesis of L-glycero-D-manno-heptose sugars required for the LPS inner core oligosaccharide (43), as well as the manipulation of siderophores to act as "Trojan horses" for the delivery of antibiotics to bacteria (115). Furthermore a vaccine that prevents *B. cenocepacia* colonization in CF patients could provide additional benefit, and mucosal vaccines for this purpose are being developed (17, 106).

B. cenocepacia is a member of a highly adaptable genus of bacteria that live under diverse circumstances in nature (36)

and that can rapidly evolve under *in vitro* stress conditions or during infections (46, 125, 128). This adaptability may be key to the pathogenesis of *B. cenocepacia*, especially to the ability of these bacteria to establish opportunistic chronic infections (167). Recently identified factors required for *B. cenocepacia* virulence, such as metabolic pathways, alternative sigma factors and other regulatory molecules, and mechanisms of resistance to oxidative stress and host antimicrobial proteins and peptides, support this idea. Finally, several other bacterial species phenotypically similar to Bcc bacteria and often even misidentified at hospitals as Bcc bacteria have also been isolated from CF lung infections (34, 155), and it is possible that the study of *B. cenocepacia* pathogenesis may serve as a model for these other organisms.

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