Dissecting novel virulent determinants in the \textit{Burkholderia cepacia} complex

George P. Tegos,1,2,3,* Mark K. Haynes2,4 and Herbert P. Schweizer5

1Wellman Center for Photomedicine; Massachusetts General Hospital; Boston, MA USA; 2Department of Dermatology; Harvard Medical School; Boston, MA USA; 3Center for Molecular Discovery; University of New Mexico; Albuquerque, NM USA; 4Department of Pathology; University of New Mexico School of Medicine; Albuquerque, NM USA; 5Department of Microbiology, Immunology & Pathology; Colorado State University; Fort Collins, CO USA

Keywords: \textit{Burkholderia cepacia} complex, toxins, hemolysis, non-ribosomal peptide synthetase, virulence factors, non-vertebrate hosts

Prevention and control of infectious diseases remains a major public health challenge and a number of highly virulent pathogens are emerging both in and beyond the hospital setting. Despite beneficial aspects such as use in biocontrol and bioremediation exhibited by members of the \textit{Burkholderia cepacia} complex (Bcc) some members of this group have recently gained attention as significant bacterial pathogens due to their high levels of intrinsic antibiotic resistance, transmissibility in nosocomial settings, persistence in the presence of antimicrobials and intracellular survival capabilities. The Bcc are opportunistic pathogens and their arsenal of virulence factors includes proteases, lipases and other secreted exoproducts, including secretion system-associated effectors. Deciphering the function of virulence factors and assessment of novel therapeutic strategies has been facilitated by use of diverse non-vertebrate hosts (the fly \textit{Drosophila melanogaster}, the microscopic nematode \textit{Caenorhabditis elegans}, the zebrafish and the greater \textit{Galleria mellonella} wax moth caterpillar larvae). Researchers are now employing sophisticated approaches to dissect the virulence determinants of Bcc with the ultimate goal being the development of novel anti-infective countermeasures. This editorial will highlight selected recent research endeavors aimed at dissecting adaptive responses and the virulence factor portfolio of \textit{Burkholderia} species.

\textbf{Burkholderia cepacia Complex (Bcc)}

The \textit{Burkholderia cepacia} complex (Bcc) currently consists of 17 closely related Gram-negative species that occupy different environmental niches.1-3 Most Bcc species are also opportunistic mammalian pathogens, being particularly problematic for cystic fibrosis (CF) patients and immune-compromised individuals. The two most clinically relevant species are \textit{B. cenocepacia} and \textit{B. multivorans}, accounting for > 85% of all Bcc infections in CF patients.5 Bcc species are metabolically diverse which allows them to thrive in many, even adversarial environments. They also have been shown to produce antifungal agents and were therefore previously used as biocontrol agents for plant protection,6 a practice that has been discontinued due to the risk of opportunistic infection of compromised individuals. Metabolic diversity and survival in diverse ecological niches has been, in part, attributed to the large (7–9 Mb) genomes found in Bcc bacteria. The genomes of all Bcc species sequenced to date have multiple replicons, consisting of three assigned chromosomes. Some strains also contain plasmids that can be quite large.7,8

Some Bcc members have gained attention as significant bacterial pathogens due to their high levels of intrinsic antibiotic resistance,9,10 transmissibility in nosocomial settings, persistence in the presence of antimicrobials11,12 and intracellular survival capabilities.4,13 The Bcc are opportunistic pathogens and their arsenal of virulence factors includes proteases, lipases and other secreted exoproducts, including secretion system associated effectors. Understanding mechanisms of Bcc pathogenesis parallels and supports development of novel therapeutic approaches aimed at disarming the pathogens in the host.14,15

Because BCC bacteria are widely antibiotic resistant, phage therapy is currently being investigated as a possible alternative treatment for these infections.16,17

\textbf{Adaptive Responses}

Development of chronic \textit{B. cenocepacia} lung infections in CF patients requires successful colonization and long-term survival, which necessarily includes adaptation to cope with stressing selection pressures within the CF lung. These include host immune defenses, antimicrobial therapy, nutrient availability and oxygen limitation. Several transcriptomic studies based on DNA microarrays gave mechanistic insights into these adaptive strategies.18,19

One study compared gene expression levels in two clonal variants isolated during long-term colonization of a CF patient who died from cepacia syndrome. The isolates studied represented the first \textit{B. cenocepacia} isolate retrieved from the patient and another isolate, obtained three
years later, which was characterized by increased resistance to different classes of antimicrobials. No fewer than 1,000 genes were found to be differently expressed between the two variants indicating a marked reprogramming of gene expression. Notable upregulated genes included those encoding factors involved in translation, ornithinase biosynthesis (iron acquisition), drug efflux and in adhesion to epithelial lung tissue and mucin. Other genes differentially expressed suggested adaptation to the nutritional and oxygen-limited environments of the CF lung. This transcriptional reprogramming reflects events occurring during long-term colonization, antibiotic therapy and disease progression.18 An independent analysis of B. cenocepacia grown in cystic fibrosis sputum found similar changes in expression of genes linked to antimicrobial resistance, oxidative stress, iron metabolism and motility.19

**Dissecting the Bcc Virulence Portfolio**

Several recent studies were aimed at dissecting key aspects of Bcc virulence. One study was designed to explore the role of the second messenger cyclic diguanosine monophosphate (c-di-GMP) in the regulation of biofilm formation and virulence of B. cenocepacia. c-di-GMP is known to control a wide range of functions in bacteria.20,21 In B. cenocepacia, elevated intracellular levels of c-di-GMP promote wrinkly colony, pellicle and biofilm formation. The bam1349 gene was identified in a screen for transposon mutants rendered unable to respond to elevated levels of c-di-GMP. This gene is predicted to encode a transcriptional regulator of the CRP/FNR super family. Purified Bcm1349 protein was shown to bind c-di-GMP in vitro and to enhance Bam1349 binding to target promoter regions. A bam1349 mutant showed reduced virulence in the Galleria mellonella larvae infection model. Summarily, Bam1349 was shown to be a transcriptional regulator that binds c-di-GMP and regulates biofilm formation and virulence in B. cenocepacia in response to c-di-GMP levels.22

Another study employing transposon mutagenesis identified mutants that were no longer pathogenic in a Caenorhabditis elegans infection model. Surprisingly, the observed attenuation of virulence in these mutants was due to loss of chromosome 3. This prompted a follow-up study that, employing an elegant genetic approach, demonstrated that chromosome 3 was indeed not an essential chromosome but rather a megaplasmid.23 Phenotypic characterization of mutant derivatives missing chromosome 3 revealed that the megaplasmid previously annotated as chromosome 3 encodes traits required for virulence in multiple hosts (rat, zebrafish, C. elegans, G. mellonella and Drosophila melanogaster), enzymes for secondary metabolism (e.g., production of compounds with antifungal activity), metabolic traits (e.g., D-xylose, fatty acid and pyrimidine utilization) and other accessory functions (e.g., exopolysaccharide production and proteolytic activity) in members of the Bcc complex.23

Several studies have investigated the molecular basis for emergence of phenotypic variants during chronic, long-term Bcc infection of CF patients’ airways. One variation is the transition from the mucoid morphology prevalent in Bcc bacteria to a non-mucoid morphotype. Using RNA microarray and proteomic isobaric tagging, so called relative and absolute quantitation technologies, one study examined a pair of mucoid and non-mucoid isolates of B. cenocepacia obtained from a chronically infected CF patient.24 During chronic infection, the mucoid isolate lost the B. cepacia epidemic strain marker and acquired a mutation in the cepR gene, encoding a LuxR homolog quorum sensing regulatory protein. The non-mucoid isolate overexpressed several putative virulence factors, including a nematocidal protein, AidA, and the oxidative stress response protein AhpC, a key microbial determinant for resistance against phagocytic cell killing, presumably as an adaptation to oxidative stress in the non-mucoid isolate. The results support the notion that chronic B. cenocepacia infection produces both genetically and phenotypically distinct variants in the CF lung.24

Trait development during chronic CF lung infection was also studied using two morphologically distinct B. multivorans clonal isolates.25 Expression profiling of mucoid and non-mucoid isolates revealed decreased expression of genes encoding products related to virulence-associated traits and metabolism in the non-mucoid isolate. In comparison to its mucoid predecessor, the non-mucoid variant lacked exopolysaccharide and exhibited lower motility, reduced chemotaxis and increased biofilm formation, particularly under microaerophilic conditions. These traits were paralleled by decreased survival rate of the non-mucoid strain in an acute G. mellonella infection model. The overall conclusions of these studies were that Bcc adaptation during chronic lung infection can result in genotypic and phenotypic variation that likely contributes to their fitness while maintaining their capacity for survival in the opportunistic mammalian niche.25

**Emergence of the G. mellonella Larvae Infection Model for Virulence and Therapeutic Efficacy Studies**

The non-vertebrate hosts (C. elegans, D. melanogaster and G. mellonella) have been used extensively to model pathogenesis with a variety of microorganisms and evaluate the efficacy of novel antimicrobial modalities.26-30 The G. mellonella larvae infection model has recently gained popularity in Burkholderia research. For example the G. mellonella-B. cenocepacia infection model was used to evaluate the therapeutic potential of B. cenocepacia-specific phase16 and small molecule compounds, including fatty acids.31

Bcc infections are difficult to eradicate because of widespread intrinsic and acquired resistance.32-34 Unfortunately, antimicrobial susceptibility in vitro has been a poor predictor of therapeutic efficacy in vivo. The efficacy of phage therapy was therefore assessed in G. mellonella larvae infected with two epidemic CF Bcc strains. The results indicated that in this in vivo model Bcc phage therapy was highly effective under certain conditions and may be a viable alternative therapeutic strategy to antibiotic treatment.16

While exploring the therapeutic efficacy of fatty acid derivatives, the omega-3 fatty acid docosahexaenoic acid (DHA) was found to be the most active compound
in vitro against B. cenocepacia K56-2, a CF epidemic strain, and against one representative member of all 17 Bcc species. The results showed that DHA has in vitro activity against Bcc bacteria. Depending on the concentration used, its mode of action was either bacteriostatic or bactericidal. DHA also showed some in vivo therapeutic efficacy in the G. mellonella-B. cenocepacia infection model when given in a single dose, albeit at very high concentrations (50 mM). The authors concluded that DHA may be a useful nutraceutical for treating CF patients infected with Bcc.31

Lastly, the wax moth larvae infection model has also been employed to compare virulence among different Burkholderia spp, including B. pseudomallei and its close relatives B. thailandensis and B. oklahomensis.35 B. pseudomallei is the causative agent of melioidosis, a difficult-to-treat disease of animals and humans increasingly recognized in tropical and subtropical regions of the globe with a variable and often fatal outcome.36,37 In murine models of infection, different B. pseudomallei strains exhibit varying degrees of virulence, whereas B. thailandensis and B. oklahomensis are highly attenuated in mice. This variability of infection also appears dependent on mouse strain and route of infection.38 Alternative infection models, including G. mellonella, are able to distinguish between strains of B. pseudomallei, B. thailandensis and B. oklahomensis, with B. oklahomensis consistently being the least pathogenic species. These differences reflect, for the most part, virulence patterns observed in murine infection models.39 There are, however, notable exceptions. B. pseudomallei strain 708a, which in the intranasal acute BALB/c mouse model is fully virulent,39 was avirulent in G. mellonella larvae infections.

Concluding Remarks

Burkholderia spp comprise metabolically diverse and apt bacteria whose full virulence potential remains to be elucidated. It is therefore not too surprising that new virulence factors are being discovered on a fairly regular basis. Recent examples include a Bcc toxin that is hemolytic and required for full virulence40 and a B. pseudomallei toxin, named Burkholderia lethal factor 1.41

Using transposon mutagenesis, Thomson et al.40 identified a Bcc gene cluster capable of expressing a toxin that is a broad-specificity hemolysin required for full Bcc virulence. Functionally related to the previously identified antifungal compound burkholdine or occidiofungin, the Bcc toxin is synthesized via a nonribosomal peptide synthetase (NRPS) mechanism, and mutations in this gene cluster cause a significant reduction in both hemolysis and G. mellonella mortality. Molecular screening by PCR of 54 Bcc isolates revealed that not all Bcc species contain this NRPS gene cluster and of those that do, only select strains produce hemolytic activity. Toxic activity by this occidofungin/burkholdine-like compound appeared limited to B. ambifaria, B. contaminans, B. pyroccinia and B. vietnamensis. Of particular interest is that the NRPS cluster responsible for this toxin’s synthesis is not expressed by two of the most clinically important species, B. cenocepacia and B. multivorans. The authors speculate that its identification in Bcc species better adapted to soil environments suggests that this gene cluster and its associated toxin evolved to protect the respective Bcc bacteria from ecological niche predators such as fungi and amoeba.

Burkholderia lethal factor 1 (BLF1) was identified in B. pseudomallei.41 It is a potent cytoxin against eukaryotic cells and lethal when administered to mice via the intraperitoneal route. The toxin acts on translation initiation factor eIF4A and abolishes its helicase activity, thereby inhibiting translation. Unlike other similar cytotoxic factors, for example Escherichia coli cytotoxic necrotizing factor 1 (CNF1-C), BLF1 lacks receptor binding and necrotizing domains, which are essential for cytoplasmic delivery of CNF1-C. Despite lacking these domains, BLF1 is toxic to some eukaryotic cells, for example J774 macrophages. However, other cells such as 3T3 cells were insensitive to BLF1 unless cytoplasmic delivery was assisted with a protein-delivery reagent. It has been argued that the intracellular lifestyle of B. pseudomallei alleviates the need for eukaryotic cell delivery, but lack of an obvious prokaryotic secretion signal does not explain how the toxin is secreted from the bacterial cell for intoxication of its host cell.

Acknowledgments

Burkholderia research in the H.P.S. laboratory is supported by grants from the National Institute of Allergy and Infectious Diseases and the US Defense Threat Reduction Agency.

References

1. Mahenthiralingam E, Urban TA, Goldberg JB. The multifarious, multireplicon Burkholderia cepacia complex. Nat Rev Microbiol 2005; 3:144-56; PMID:15643431; http://dx.doi.org/10.1038/nrmicro1085
2. Vanlaere E, Baldwin A, Gevers D, Henry D, De Brandt E, LiPuma JJ, et al. Taxon K, a complex within the Burkholderia cepacia complex. Nat Rev Microbiol 2009; 7:671-8; PMID:19126732; http://dx.doi.org/10.1038/nrmicro2112-3
3. Vandamme P, Dawyndt P. Classification and identification of the Burkholderia cepacia complex: Past, present and future. Syst Appl Microbiol 2011; 34:87-95; PMID:21257278; http://dx.doi.org/10.1016/j.syapm.2010.10.002
4. Sajjan SU, Carmody LA, Gonzalez CF, LiPuma JJ. A type IV secretion system contributes to intracellular survival and replication of Burkholderia cepacia. Infect Immun 2008; 76:5447-55; PMID:18824538; http://dx.doi.org/10.1128/IAI.00451-08
5. Drevinek P, Mahenthiralingam E. Burkholderia cenocepacia in cystic fibrosis: epidemiology and molecular mechanisms of virulence. Clin Microbiol Infect 2010; 16:821-30; PMID:20880411; http://dx.doi.org/10.1111/j.1469-0012.2010.03257.x
6. Kang Y, Carlson R, Tharpe W, Schell MA. Characterization of genes involved in biosynthesis of a novel antibiotic from Burkholderia cepacia BC11 and their role in biological control of Rhizoctonia solani. Appl Environ Microbiol 1998; 64:3939-47; PMID:9758823
7. Holden MT, Seth-Smith HM, Grossman LC, Sebaiah M, Bentley SD, Cerdeño-Tárraga AM, et al. The genome of Burkholderia cepacia J2315, an epidemic pathogen of cystic fibrosis patients. J Bacteriol 2009; 191:261-77; PMID:18931103; http://dx.doi.org/10.1128/JB.01230-08
8. Agnoli K, Schwager S, Uehlinger S, Vergaust A, Viteri DF, Nguyen DT, et al. Exposing the third chromosome of Burkholderia cepacia complex strains as a virulence plasmid. Mol Microbiol 2012; 83:362-78; PMID:22171913; http://dx.doi.org/10.1111/j.1365-2958.2011.07937.x
9. Jasem AN, Zlotnik JE, Henry DA, Hancock RE, Ernst RK, Speert DP. In vitro susceptibility of Burkholderia viennensis to aminoglycosides. Antimicrob Agents Chemother 2011; 55:2256-64; PMID:21321142; http://dx.doi.org/10.1128/AAC.01434-10

10. Rajendran R, Quinn RF, Murray C, McCalloch E, Williams C, Ramage G. Efflux pumps may play a role in tigecycline resistance in Burkholderia species. Int J Antimicrob Agents 2010; 36:151-4; PMID:20399621; http://dx.doi.org/10.1016/j.ijantimicag.2010.03.009

11. Peeters E, Nelis HJ, Coenye T. In vitro activity of carbapenems against clinical isolates of Burkholderia cenocepacia to adapt to cystic fibrosis patients. Eur J Clin Microbiol Infect Dis 2004; 23:798-800; PMID:15065189; http://dx.doi.org/10.1007/s10096-004-1216-3

12. Colini G, Fava F, Marchetti F, Fontana R. Bacteriostatic and bactericidal activity of levofloxacin against clinical isolates from cystic fibrosis patients. Eur J Clin Microbiol 2004; 23:798-800; PMID:15065189; http://dx.doi.org/10.1007/s10096-004-1216-3

13. Malott RJ, Steen-Kinnaird BR, Lee TD, Speert DP. Identification of hooeareol biosynthesis genes involved in polymyxin resistance in Burkholderia multivorans. Antimicrob Agents Chemother 2012; 56:466-71; PMID:22060609; http://dx.doi.org/10.1128/AAC.00602-12

14. Lee YM, Almquist F, Hultgren SJ. Targeting virulence for antimicrobial chemotherapy. Curr Opin Pharmacol 2003; 3:513-9; PMID:14559097; http://dx.doi.org/10.1016/j.coph.2003.04.001

15. Lebev SL, Kalman D. Aligning antimicrobial drug discovery with complex and redundant host-pathogen interactions. Cell Host Microbe 2009; 5:114-22; PMID:19218083; http://dx.doi.org/10.1016/j.chom.2009.01.008

16. Seed KD, Dennis JJ. Experimental bacteriophage therapy increases survival of Galleria mellonella larvae infected with clinically relevant strains of the Burkholderia cepacia complex. Antimicrob Agents Chemother 2009; 53:2001-8; PMID:19223640; http://dx.doi.org/10.1128/AAC.01166-08

17. Lynch KH, Storch P, Dennis JJ. Genomic analysis and relatedness of P2-like phages of the Burkholderia cepacia complex. BMC Genomics 2010; 11:599; PMID:20973964; http://dx.doi.org/10.1186/1471-2164-11-599

18. Mira NP, Madeira A, Moreia AS, Coutinho CP, Sá-Correia I. Genomic expression analysis reveals strategies of Burkholderia cenocepacia to adapt to cystic fibrosis patients' airways and antimicrobial therapy. PLoS One 2011; 6:e28831; PMID:22216120; http://dx.doi.org/10.1371/journal.pone.0028831

19. Drevinek P, Holden MT, Ge Z, Jones AM, Kerchell I, Gill RT, et al. Gene expression changes linked to antimicrobial resistance, oxidative stress, iron depletion and retained motility are observed when Burkholderia cenocepacia grows in cystic fibrosis sputum. BMC Infect Dis 2008; 8:121; PMID:18801206; http://dx.doi.org/10.1186/1471-2334-8-121

20. Christen M, Kulasekara HD, Christen B, Kulasekara BR, Hoffman LR, Miller SI. Asymmetrical distribution of the second messenger c-di-GMP upon bacterial cell division. Science 2010; 328:1295-7; PMID:20522779; http://dx.doi.org/10.1126/science.1188658

21. Christen M, Christen B, Allan MG, Folker M, Jeni P, Grießeking S, et al. DpaA is a member of a new family of cyclic diguanosine monophosphate receptors and controls flagellar motor function in Caulobacter crescentus. Proc Natl Acad Sci U S A 2007; 104:4112-7; PMID:17360486; http://dx.doi.org/10.1073/pnas.0607738104

22. Fadl M, O’Connell A, Nilsson M, Nieuwhaus K, Dow JM, Girskov M, et al. The CRP/FNR family protein Bcmi349 is a c-di-GMP effector that regulates biofilm formation in the respiratory pathogen Burkholderia cenocepacia. Mol Microbiol 2011; 82:327-41; PMID:21885527; http://dx.doi.org/10.1111/j.1365-2958.2011.07814.x

23. Agouli K, Schwager S, Uehlinger S, Vergaust A, Viteri DF, Nguyen DT, et al. Exposing the third chromosome of Burkholderia cenocepacia complex strains as a virulence plasmid. Mol Microbiol 2012; 83:302-78; PMID:22177193; http://dx.doi.org/10.1111/j.1365-2958.2011.07937.x

24. Zlotnik JE, Speert DP. The role of mucoidy in virulence of bacteria from the Burkholderia cepacia complex: a systemic proctonomic and transcriptional analysis. J Infect Dis 2010; 202:770-81; PMID:20670172; http://dx.doi.org/10.1086/655663

25. Silva IN, Ferreira AS, Becker JD, Zlotnik JE, Speert DP, He J, et al. Mucoidy morphotype variation of Burkholderia multivorans during chronic cystic fibrosis lung infection is correlated with changes in metabolism, motility, biofilm formation and virulence. Microbiology 2011; 157:3124-37; PMID:21835880; http://dx.doi.org/10.1099/m.0.050980-9

26. Fallon JP, Troy N, Kavanagh K. Pre-exposure of Galleria mellonella larvae to different doses of Aspergillus fumigatus conidia causes differential activation of cellular and humoral immune responses. Virulence 2011; 2:413-21; PMID:21921688; http://dx.doi.org/10.4161/viru.2.5.17811

27. Lioniak MS. Drosophila and Galleria insect model hosts: new tools for the study of fungal virulence, pharmacology and immunology. Virulence 2011; 2:521-7; PMID:22186764; http://dx.doi.org/10.4161/viru.2.6.18520

28. Abebe E, Abebe-Akele F, Morrison J, Cooper V, Thomas WK. An insect pathogenic symbiosis between a Caenorhabditis and Serratia. Virulence 2011; 2:158-61; PMID:21380770; http://dx.doi.org/10.4161/viru.2.2.15337

29. Olsen RJ, Winkes ME, Cantu CC, Beres SB, Musser PA, Bokori-Brown M, Chang CT, et al. A Burkholderia pseudomallei toxin inhibits helicase activity of translational factor eIF4A. Science 2011; 334:821-4; PMID:22076380; http://dx.doi.org/10.1126/science.12111915