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Dissecting novel virulent determinants in the *Burkholderia cepacia* complex

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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 *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 *Drosophila melanogaster*, the microscopic nematode *Caenorhabditis elegans*, the zebrafish and the greater *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 *Burkholderia* species.

Burkholderia cepacia Complex (Bcc)

The *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[4] (CF) patients and immune-compromised individuals. The two most clinically relevant species are *B. cenocepacia* and *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 antimicrobials[11,12] 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]

Adaptive Responses

Development of chronic *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 *B. cenocepacia* isolate retrieved from the patient and another isolate, obtained three

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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, ornibactin 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 in *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 *bcam1349* 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 Bcam1349 protein was shown to bind c-di-GMP in vitro and to enhance Bcam1349 binding to target promoter regions. A *bcam1349* mutant showed reduced virulence in the *Galleria mellonella* larvae infection model. Summarily, Bcam1349 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 phage[16] 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.[35] There are, however, notable exceptions. *B. pseudomallei* strain 708a, which in the intranasal acute BALB/c melioidosis 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 virulence[40] 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 non-ribosomal 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. pyrrocinia* 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 cytotoxin against eukaryotic cells and lethal when administered to mice via the intraperitoneal route. The toxin acts on translation initiation factor eI4A 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.

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