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Role of phages in the pathogenesis of *Burkholderia* or “Where are the toxin genes in *Burkholderia* phages?”

Elizabeth J. Summer¹, Jason J. Gill¹, Chris Upton², Carlos F. Gonzalez³, and Ry Young^{1,*}

¹ Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77843-2128: elizsum@tamu.edu, jason.gill@tamu.edu

² Department of Biochemistry and Microbiology, University of Victoria, 150 Petch Building, PO Box 3055, V8W 3P6, Victoria, BC, Canada: cupton@uvic.ca

³ Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX 77843-2132: cf-gonzalez@tamu.edu

Summary

Most bacteria of the genus *Burkholderia* are soil- and rhizosphere- associated, noted for their metabolic plasticity in the utilization of a wide range of organic compounds as carbon sources. Many *Burkholderia* species are also opportunistic human and plant pathogens and the distinction between environmental, plant, and human pathogens is not always clear. *Burkholderia* phages are not uncommon and multiple cryptic prophages are identifiable in the sequenced *Burkholderia* genomes. Phages have played a crucial role in the transmission of virulence factors among many important pathogens, however, the data does not yet support a significant correlation between phages and pathogenicity in the *Burkholderia*. This may be due to the role of *Burkholderia* as a “versaphile” such that selection is occurring in several niches, including roles as a pathogen and in the context of environmental survival.

Introduction: *Burkholderia*: rhizosphere associated and human pathogens

Early hopes of exploiting the metabolic plasticity of many *Burkholderia* species for use in bioremediation and biocontrol have been subdued by the increased detection of typically environmental or plant-pathogenic species in the context of human infections [1,2]. This is epitomized by the clinical isolation of typically rice pathogenic *B. glumae* [3] and conversely the isolation from agricultural soils of *B. cenocepacia* strains closely related to clinical isolates [4,5]. Most *Burkholderia* infections in humans are derived from two groups, or clades, in terms of pathology, taxonomy, and global distribution [1]. Members of the notorious *B. pseudomallei* clade are endemic in tropical countries, especially in Southeast Asia, and include *B. mallei* and *B. pseudomallei*, the causative agents of glanders and melioidosis, respectively [6]. The second clade of *Burkholderia* pathogens includes the nine species originally defined as the *B. cepacia* complex (Bcc) [7]. Bcc species, most of which are temperate soil saprophytes and plant pathogens, are associated with severe, often fatal, pulmonary infections in persons with cystic fibrosis (CF). All Bcc species have been isolated from sputum cultures of CF patients [1]. The traits that allow *Burkholderia* species to exploit such diverse niches, including that of a human pathogen, are poorly understood.

* Corresponding author: ryland@tamu.edu

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In many pathogens, phages have been instrumental in the acquisition of virulence traits [8,9]. Moreover, in many bacteria, prophages are largely responsible for strain-specific differences in toxin and effector gene content [10-14]. With the recent completion of several *Burkholderia* species and *Burkholderia* phage genomes, it can be asked if phages have been instrumental in the development of pathogenicity among this group of versatile pathogens. There are no reviews on *Burkholderia* phages. This review will cover the majority of recent literature on *Burkholderia* phages and the role they might play in the transmission of fitness traits, especially those related to virulence, to their versatile hosts. The traits being considered are not limited specifically to toxins but instead includes any phage-encoded protein that might confer a selective advantage to the host.

Pathogenicity traits of *Burkholderia*

It is significant that there have been no experimental results in *Burkholderia* analogous to those obtained from other pathogen systems i.e., no experimentally identified *Burkholderia* pathogenicity factor has been found to be associated with a phage element. This is not due to a lack of understanding of pathogenesis in the *Burkholderia*. There are commonalities between the mechanisms of pathogenesis for the Bcc and *B. pseudomallei*: they are able to elaborate an exopolysaccharide that is required for full virulence; they possess one or more type III secretion systems (TTSS); and they are able to adhere to and invade host cells and persist in the intracellular compartment [6,7]. Two type IV secretion systems have been identified in *B. cenocepacia* belonging to the epidemic ET12 clonal lineage, with the plasmid encoded one related to elaboration of a plant watersoaking phenotype and the chromosomal one bearing homology to the VirB/D4 of *Agrobacterium tumefaciens* [15]. In plant pathogenic *B. cepacia* a plasmid encoded pectate hydrolase is a virulence factor necessary for maceration of onion tissue [16]. Putatively autosecreted BimA is required for actin-mediated intracellular motility in *B. pseudomallei* [17]. Other known *Burkholderia* virulence factors include exotoxins, siderophores, and enzymes like lipases, catalases and proteases [6,7]. *Burkholderia* lipopolysaccharide (LPS) is strongly pro-inflammatory and it has been suggested that the lack of correlation between Bcc strain (species) and serotype might be the result of horizontal transfer of the O-antigen serotype locus [18]. Capsule expression is also implicated in antimicrobial resistance, as increased resistance has also been noted in several Bcc species when grown in biofilms rather than in a planktonic form [19,20].

Phages of *Burkholderia*: any virulence factors?

There are no reports of any empirically characterized *Burkholderia* virulence determinants being associated with a phage element. *Burkholderia* phages, however, are common, especially in high organic content agricultural soils [21-23]. Moreover, functional virions are produced by many lysogenic strains [21,22,24,25]. Specialized transduction by a *Burkholderia* phage of a phenotype that might be significant to host pathology has been demonstrated in only one case. Phage BcP15, produced by *B. cepacia* DR11, appears to transmit co-trimoxazole, trimethoprim and erythromycin resistance not only to *Burkholderia* but also to *Shigella flexneri* KN1925 [26,27]. A classic mechanism by which phage can transmit virulence factors is through generalized transduction. There are a few reports on generalized transduction mediated by *Burkholderia* phages [28,29]. Two phages, NS1 and NS2, were demonstrated to mediate generalized transduction of a ceftazidime resistance marker. NS2, which has not been sequenced, is produced by Bcc strain ATCC17616, the same strain that produces sequenced phage Bcep176 (NC_007497). NS2 and Bcep176 are not identical as NS2 was reported to possess a contractile tail where as Bcep176 possesses a non-contractile tail. Polylysogenic *Burkholderia* strains sometime produce more than one phage type [25,30]. This indicates that in the absence of molecular data, it should not be assumed that descriptions of phages produced by any particular host strain are referring to the same phage [31].

Implications from *Burkholderia* phage genomics

In the absence of experimental data, does phage genomics suggest that virulence factors are encoded by *Burkholderia* phages? Phage-encoded toxin and effector genes are frequently arranged in a transcriptionally self-contained cluster termed a “moron” [9]. Morons are frequently transcribed in the opposite orientation as the surrounding phage genes and are often located near the end of the prophage, as in lambda *bor*, where as others are embedded in an otherwise typical phage operon, as in lambda *lom* [32,33]. Temperate phages produced by several human-pathogenic *Burkholderia* species have been isolated and characterized. These include *B. cenocepacia* J2315 (BcepMu), *B. pseudomallei* strains K96243 and 1026b (phiK96243, and phi1026b respectively), as well as a phage produced by *B. thailandensis* E125 (phiE125) that forms plaques on *B. mallei*. Although these phages have typical mosaic genomes, they can be grouped into three categories based on some protein- and gene- order similarity to classic coliphages. BcepMu, as the name implies, is a Mu-like transposable phage of *B. cenocepacia* [34]. phiE125 phi1026b and Bcep176 have siphophage morphology, with many structural protein homologies to Lambda (Fig. 1) [25,35]. The phiE125 genome was shown to be preferentially methylated by a phage-encoded DNA methylase [36]. PhiK96243 has myophage morphology with structural protein homologies to P2 (Fig. 2). Therefore, among the *Burkholderia*, there exists phages which are extensively related in at least their structural genes to phages that have been demonstrated, in other pathogens, to carry virulence factors.

Lambda-related phages of *Burkholderia*

B. pseudomallei and *B. thailandensis* temperate phages phi1026b and phiE125 encode homologs of RelE (Fig. 1) [25,35]. *E. coli* RelE is part of a toxin-antitoxin (TA) system. In the absence of the inhibitory subunit, RelE functions as an RNase that cleaves mRNAs present in polysomes under conditions of amino acid starvation [37]. Some TA systems are plasmid addiction modules, however, RelE has been proposed to be a component of a stress response pathway that produces persisters, i.e. cells that neither grow nor die in the presence of bactericidal agents [38]. In almost all TA systems, the gene immediately distal to the toxin gene encodes the anti-toxin, which is also a transcription factor [39]. The gene downstream of the relE homologue is a member of the HTH_XRE family, transcriptional activators associated with xenobiotic response, and is thus likely to be the anti-toxin gene. There are no other reported phage-encoded RelE homologues.

In addition to RelE, phi1026b encodes a LysR-like transcription regulator (gp58) adjacent to a major facilitator superfamily (MFS) transporter (gp59). COG0477 members, including gp59, are MFS metabolite:H⁺ symporters responsible for the uptake of a wide variety of substrates. In order to determine if gp59 provided any selective advantage for the lysogen, a strain of *B. pseudomallei* 1026b was constructed with a deletion in gp59 of the prophage [35]. A comprehensive analysis was performed of the growth characteristics of the deletion mutant relative to the wild-type lysogen, including a screen of the ability to utilize 190 carbon sources by phenotype microarrays, however no differences were observed. There was also no difference in virulence between the two strains in a hamster model, leading to the conclusion that this gene is not essential for pathogenicity. Homologs of the LysR/MFS gene pair are found in tandem in several recently completed bacterial genomes, including those of *Ralstonia solanacearum*, *B. fungorum*, and *Pseudomonas syringae*. In phi1026b, these genes have a classic moron organization as they are encoded in the opposite orientation of flanking genes. When Bcep176, phi1026b and phiE125 are aligned, this region is highly variable and shows signs of multiple insertions and deletions (Fig 1). Despite its relationship to phi1026b and phiE125, *B. multivorans* ATCC17616 phage Bcep176 (NC_007497) does not encode recognizable homologs of known virulence- or potential host fitness-factors.

PhiE125 and *B. cenocepacia* phage BcepB1A encode homologs of phosphoadenosine phosphosulphate (PAPS) reductase [23,25]. PAPS-reductase uses thioredoxin as an electron donor for the reduction of PAPS to phospho-adenosine-phosphate (PAP). PAPS reductase functions in the assimilatory sulfate reduction pathway in which environmental inorganic sulfate is reduced to form organic thiols. The only other phage known to encode a PAPS-reductase is coliphage 186, where it was shown to be a non-essential gene [40]. Although PAPS-reductase has not been demonstrated to be a virulence factor, it is reasonable that it could be a fitness factor under conditions of sulfur limitation. In *Burkholderia*, it has been shown that sulfur limitation preferentially affects biosynthesis of the siderophore pyochelin over that of cysteine synthesis [41]. Siderophores are known *Burkholderia* pathogenicity factors and pyochelin is one of a number of endogenous and exogenous siderophores that *Burkholderia* utilizes, contributing to success in the iron-limiting lung environment [42].

Mu and P2- related phages of *Burkholderia*

Burkholderia phage phiK96243 exhibits gene order conservation and primary amino acid sequence similarity across the structural genes with the classic *E. coli* temperate myophage P2 (Fig 2). The phiK96243 sequence was derived from the *B. pseudomallei* K96243 genome entry and corresponds to genomic island 2, shown to produce a UV-inducible prophage [43]. BLAST searches with the predicted proteins of phiK96243 detect clusters of related genes in many sequenced *Burkholderia* genomes indicating that related cryptic P2-like prophages are common (EJ Summer *et al.*, unpublished). PhiK96243 does not encode recognizable homologs of known virulence or host fitness factors. In *B. pseudomallei*, the TTSS effector BopE affects invasion of non-phagocytic cells. However, these *Shigella* SopE homologs are not encoded by cryptic P2-like prophage but rather in the type III secretion system gene cluster [44]. Of course in these, as is the case in all phages, the high percentage of ORFs encoding proteins of unknown function are obvious candidates for virulence- or host fitness- factors. In phiK96243, there are two gene clusters that encode putative transcription factors (Fig. 2). These clusters are transcribed from the opposite strand as surrounding genes, a property suggestive of morons.

BcepMu is a Mu-like prophage present in most *B. cenocepacia* strains of the highly virulent and epidemic ET12 lineage [31,34]. A consequence of the replication and packaging strategy used by Mu-like phages is that about 2 kbp of host DNA is present in every virion, making every particle theoretically capable of generalized transduction. The nearly random integration of the lysogen makes Mu phages mutators, which also has potential effects on pathogenicity. In addition to possessing these general Mu-like phage features, BcepMu additionally encodes a specific potential virulence factor: an acyltransferase homolog encoded by *gene53*. The acyltransferase encoded by *Shigella* phage Sf6 modifies the LPS O-antigen, resulting in serotype conversion and protection from superinfection [45,46]. The *B. cenocepacia* ET12 type strain J2315 is a BcepMu lysogen, whereas the related ET12 strain K56-2 lacks BcepMu [34]. J2315 also has an O-antigen biosynthesis defect compared to K56-2, due to an IS402 element inserted within a glycosyltransferase gene [47]. The J2315 LPS is highly acylated and is also highly endotoxic, more so than strain K56-2 LPS [48]. However, it has not been demonstrated that the LPS is a substrate of the BcepMu putative acyltransferase.

The second proposed virulence trait candidate encoded by BcepMu is controversial. Based solely on primary structure similarity, BcepMu gp8 is related to ExeA of *Aeromonas hydrophila*. The ExeAB complex of *A. hydrophila* is required for the localization and assembly of the oligomeric ring structure of ExeD, shown in other systems to be a secretin involved in secretion of toxins such as aerolysin [49]. However, another model for the function of BcepMu gp8 has been proposed that does not implicate a role in pathogenicity. In the annotation of BcepMu-related *Pseudomonas* phage B3, the authors propose that the BcepMu gp8 equivalent is actually a transposase B (TnpB) subunit [50]. This was due primarily to the proximity of

with the BcepMu gp9 transposase A (TnpA) subunit equivalent. Homologs of BcepMu gp8 and gp9 are usually found as gene pairs in numerous bacteria lending support to the idea that these are interacting proteins. If gp8 is TnpB, the similarity to ExeA is likely due to an embedded ATP-binding domain, which can be found within proteins of unrelated functions [51].

Other *Burkholderia* phages

Many of the phages whose genomic sequences are in Genbank (Bcep1, Bcep43, Bcep781, BcepF1, BcepC6B, BcepB1A, Bcep22, BcepGomr and BcepNazgul) have hosts that are Bcc members isolated from agricultural soils (EJ Summer *et al.*, unpublished). These phages are not closely related to phages known to carry pathogenicity factors. Some of these encode genes that, when found in other phages, have been suggested to be virulence factors. Phage-encoded VirE homologs have been suggested to be potential virulence factors, i.e. *Vibrio* phage VP16T [52]. VirE homologs, first identified on a genomic fragment present in virulent but not avirulent strains of *Dichelobacter nodosus*, the causative agent of bovine foot rot [53], are encoded by Bcep1, Bcep43, Bcep781 and BcepF1 [23]. The Bcep1 VirE homolog, however, was recently connected to the prim-pol primase superfamily of DNA polymerases [54]. A more conservative role for VirE is not in promoting host pathogenicity but rather in phage DNA replication.

BcepGomr (EF523948) shares structural gene order and amino acid similarity with lambdoid phages (Fig. 1). A gene cluster in the unpublished sequence of BcepGomr encodes homologs of proteins involved in the highly interconnected processes of folate, nucleotide and amino acid utilization, biosynthesis and recycling pathways (Fig. 1). BcepGomr gp37 encodes a member of the Radical SAM superfamily, characterized by the capacity for the reductive cleavage of S-adenosylmethionine in order to provide radical species to catalyze diverse “difficult chemistry” reactions [55]. BcepGomr gp34 is related to 6-pyruvoyl-tetrahydropterin synthase (PTPS) which catalyses a step in the synthesis of tetrahydrobiopterin from GTP. BcepGomr gp33 encodes a homolog of FolE, the first enzyme of *de novo* tetrahydrofolate biosynthesis. BcepGomr gp35 is related to ExsB, homologs of which modify stress response phenotypes such as exopolysaccharide levels and aluminum tolerance [56,57]. ExsB, PTPS and FolE homologs are also encoded by Mycobacteriophage Rosebush (gp3, gp4 and gp6). These proteins were speculated to be involved in the biosynthesis of tetrahydrobiopterin, a cofactor for nitric oxide synthase which is a key enzyme in the host defense against mycobacterial infections [58].

Cryptic prophages in the sequenced genomes of *Burkholderia*

Has host genomic analysis illuminated the role of phages in pathogenicity or niche exploitation by *Burkholderia*? In the case of *B. pseudomallei* (**Bp**), the answer appears to be that there are several cryptic prophages and some of these encode potential virulence factors, but these probably do not have a role in virulence comparable to the role played by prophages of *E. coli*, *Streptococcus*, *Shigella* and *Salmonella* are. The 7.3 Mb Bp genome is organized into two circular chromosomes predicted to encode 5,855 proteins [43]. Sixteen genomic islands (GI) composed of mobile genetic elements such as insertion sequences (IS), plasmids and phage make up over 10% of the *B. pseudomallei* genome. Eight of these islands have some prophage-like characteristics and one, GI 2, was shown to produce phiK96243. It is likely that horizontally acquired GIs, including prophages, are responsible for much of the diversity between Bp strains. Even though still large compared to most bacteria, at 5.8 Mb the genome of the obligate mammalian pathogen *B. mallei* (**Bm**) is smaller than that of Bp and shows evidence of genome reduction, analogous to that described for the evolution of other obligate parasites [59]. The most significant mechanism mediating the evolution of Bm appears to be numerous syntenic breaks and large-scale deletions in Bm as compared to Bp due not to phages

but to recombination among the unusually large number of IS elements present in the genome. Adaptation and survival of Bm to a mammalian host, in particular evasion of the immune response, was attributed to variations in cell surface features induced by simple sequence repeats.

The 6.8 Mb genome of the non-pathogenic *B. thailandensis* (Bt) also appears to have been a product of reduction from an ancestral Bp strain [60,61]. Genomic analysis does not implicate a seminal role for phages in the evolution of Bt, similar to Bm. Even though Bt E264 was shown in earlier work to produce two phages, one a clear plaque former and the other a turbid plaque former, these prophages were not identified in the Bt E264 genome [25,61]. A conclusion of the analysis of the Bt ATCC700388 genome was that phage-mediated recombination did not play a relevant role in the evolution of Bt from a Bp-like ancestor [60].

The genome of non-pathogenic, PCB-degrading *B. xenovorans* LB400 has also been published [62]. At 9.7 Mb, this giant is notable for encoding a high percentage (17.6 % of all genes) of paralogs and functionally redundant proteins on its three chromosomes. These most likely arose due to internal duplication and subsequent divergence. Many traits known to be involved in virulence in the Bcc, including adhesins and siderophores, are encoded in the LB400 genome. These are probably also important for survival in the soil [62,63]. Over 20 % of the genome is composed of horizontally-acquired GIs of which at least three appear to be cryptic prophages of unknown functional status.

Conclusion

So where are the virulence genes in *Burkholderia* phages? While there were no identifiable homologs of known toxin or pathogenicity factors among *Burkholderia* phages present in the current data base, some do encode proteins whose putative function suggests they might affect host fitness outside the lytic phage replication cycle. A similar result was observed among 14 mycobacteriophage genomes where no known homologs of toxin genes were identified [58]. However, genes encoding proteins that could modulate host cell fitness in the context of either a saprophyte or a pathogen were identified.

For most *Burkholderia*, pathogenicity appears to be one of several potential lifestyles. In addition to living as soil saprophytes and as plant and animal pathogens, *Burkholderia* versatility extends to colonizing such diverse places as pharmaceuticals and disinfectants [64]. *Burkholderia* spp. can form tenacious biofilms in tubing and contaminate water supplies. The most famous of these is *Burkholderia* SAR-1, sequenced to a very high coverage during the landmark Sargasso Sea metagenomic analysis [65]. It is doubtful this Bcc strain is a normal inhabitant of the oligotrophic Sargasso Sea but was probably present in boat wastewater discharged before the sample was obtained [66].

The genetic basis for this niche plasticity can be described as an “always be prepared” approach to gene content. Genomic analysis has revealed that the *Burkholderia* carry a veritable arsenal of genes on large chromosomes, which are among the largest of the microbial world. Horizontal gene transfer is a major factor in the acquisition of traits that allow for the exploitation of multiple niches. Generalized transduction mediated by phages is undoubtedly as important in horizontal gene transfer for *Burkholderia* as it is for other bacterial genera. In terms of the specialized transmission of specific virulence genes such as toxins, bacterial genomic analysis has corroborated phage genomic analysis which suggests that phages have not played an obvious role in the evolution of these “versaphiles” as pathogens as they have in the evolution of the “professional pathogens” such as enterohemorrhagic *E. coli* and *Streptococcus* [11, 13].

Burkholderia phage-encoded potential host fitness traits related to environmental survival might affect the unusual, often long-term disease progression observed in these bacteria as well as their ability to survive in the environment. The amount of genomic and experimental data for these organisms is, however, limiting. The majority of phage-encoded proteins do not have functional annotations and it is possible that some of these encode uncharacterized pathogenicity factors. A larger scale analysis of *B. pseudomallei*, *B. mallei*, and *B. thailandensis* phages is in progress; this effort should provide new insight into the role of phages in *Burkholderia* pathogenicity (D. DeShazer, personal communication). Additionally, the lack of published analysis of Bcc member genomes makes it difficult to extrapolate to this group of important pathogens. In summary, while *Burkholderia* phages have not yet been demonstrated to encode identifiable toxins or other virulence factors, these phages do appear to encode proteins that can contribute to *Burkholderia* fitness in its role as a versaphile.

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Lambda

phiE125

phi1026b

BcepGomr

Bcep176

Figure 1.

Lambdoid phages of *Burkholderia* encode potential host fitness factors. Genomic maps of coliphage lambda (GI:215104), phiE125 (GI:17484022), phi1026b (GI:38505382), BcepGomr (GI:145321088) and Bcep176 (GI:76885811) are shown to emphasize the conservation in structural gene order among these non-contractile tailed phage. Red boxes indicate genes encoding proteins with demonstrated (lambda Lom and Bor) or potential (*Burkholderia* phages) roles in host virulence. Green boxes in the *Burkholderia* phage genome maps indicated genes encoding proteins with homologs among the lambda-like viruses (taxonomic id:186765). Proteins encoded by select lambda genes are annotated following lambda nomenclature. Transcription orientation on forward and reverse strand is indicated by the position of boxes above and below the center line.

Figure 2.

Burkholderia pseudomallei phage phiK96243 exhibits structural gene similarity and gene order conservation with *Enterobacteria* phage P2. The phiK96243 map corresponds to the prophage encoded by genomic island 2 in NC_006350 (encompassing GI:53717770 to GI:53717819). The P2 map is drawn in the orientation of the prophage using GI:3139086 as a reference. Genes encoding proteins exhibiting primary structural similarity between the two phages are indicated in yellow and are labeled according to P2 nomenclature. The position of Z/Fun, which corresponds to the location of the coding region of the type III secretion effector protein SopE in ϕ SopE, is indicated in turquoise. The clusters of genes indicated in red in phiK96243 each encode a helix-turn-helix (HTH) potential transcription factors and are moron candidates.