Comparative Genomics and an Insect Model Rapidly Identify Novel Virulence Genes of *Burkholderia mallei*<sup>SN</sup>†

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*Burkholderia pseudomallei* and its host-adapted deletion clone *Burkholderia mallei* cause the potentially fatal human diseases melioidosis and glanders, respectively. The antibiotic resistance profile and ability to infect via aerosol of these organisms and the absence of protective vaccines have led to their classification as major biothreats and select agents. Although documented infections by these bacteria date back over 100 years, relatively little is known about their virulence and pathogenicity mechanisms. We used in silico genomic subtraction to generate their virulome, a set of 650 putative virulence-related genes shared by *B. pseudomallei* and *B. mallei* but not present in five closely related nonpathogenic *Burkholderia* species. Although most of these genes are clustered in putative operons, the number of targets for mutant construction and verification of reduced virulence in animal models is formidable. Therefore, *Galleria mellonella* (wax moth) larvae were evaluated as a surrogate host; we found that *B. pseudomallei* and *B. mallei*, but not other phylogenetically related bacteria, were highly pathogenic for this insect. More importantly, four previously characterized *B. mallei* mutants with reduced virulence in hamsters or mice had similarly reduced virulence in *G. mellonella* larvae. Site-specific inactivation of selected genes in the computationally derived virulome identified three new potential virulence genes, each of which was required for rapid and efficient killing of larvae. Thus, this approach may provide a means to quickly identify high-probability virulence genes in *B. pseudomallei*, *B. mallei*, and other pathogens.

*Burkholderiaceae* is a family of the betaproteobacteria that colonize a variety of ecological niches. Some species are plant or animal pathogens, while others are important environmental bacteria, including nitrogen-fixing symbionts, plant growth-promoting rhizobacteria, chemolithoautotrophs, and bioremediation agents. Nearly all members of the *Burkholderiaceae* have large (5- to 9-Mb), multireplicon genomes (http://www.genomesonline.org/) comprised of a large core of orthologous genes and smaller subsets of species- or isolate-specific genes for ecological specialization. *Burkholderia pseudomallei* and *Burkholderia mallei* are aggressive human pathogens (7–9, 13) categorized as select agent biothreats with a high potential for misuse (3, 55, 67). The genome sequences of several strains of *B. mallei* and *B. pseudomallei* have revealed that *B. mallei* is a deletion clone of *B. pseudomallei* (27) which lost >1,000 genes. Many of the gene deletions appear to have been caused by insertion sequence-mediated events (49) and likely explain the many physiological differences that led to the classification of these two pathogens as separate species (54). Nearly all genes retained by *B. mallei* share ~99.5% DNA-DNA sequence identity with their *B. pseudomallei* homologs.

*B. pseudomallei*, which causes melioidosis, is an endemic and opportunistic pathogen that inhabits the tropical soils and waters of Southeast Asia and Northern Australia (2, 8, 58, 70), whereas *B. mallei* appears to be a zoonotic pathogen that infects a variety of animals, including equines and humans. The disease caused by *B. mallei*, glanders, is not well studied as there have been few well-documented cases since 1950. For both pathogens, infection usually occurs through wounds, aspiration, and possibly inhalation. Studies of intraperitoneally infected animals have shown that *B. pseudomallei* and *B. mallei* can rapidly migrate to the spleen and liver, where they multiply extensively within membrane-bound phagosomes and form abscesses (25, 37). Untreated infections are often fatal.

Small-animal models are available for studying the pathogenesis of *B. pseudomallei* and *B. mallei* (15, 34, 40, 42, 68). These models include a sensitive (50% lethal dose, <10 cells) Syrian hamster model utilizing intraperitoneal injection and two mouse models utilizing C57BL/6 or BALB/c mice and aerosol, intranasal, or intraperitoneal injection (40, 42). However, these models are expensive, cumbersome, and hazardous due to biosafety and biosecurity issues. A *Caenorhabditis elegans* nematode infection model for *B. pseudomallei* has been described (24, 50, 59), although it may have limited sensitivity due to the extremely high doses required for killing.

Compared to other bacterial pathogens, little is known about the virulence factors of *B. mallei* and *B. pseudomallei* or the molecular basis of the pathogenicity of these organisms. The only verified virulence factors for these pathogens are those that are well known and shared by most, if not all, animal and plant pathogens: exopolysaccharide capsule (CAP) (14, 53), type III secretion systems (T3SS) (19, 20, 23, 60, 64, 66), and lipopolysaccharide O antigen (LPS) (12). Type II secretion, type IV pili, and flagella (10, 11, 18) have been implicated in *B. pseudomallei* pathogenesis. Recently, a type VI secretion

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**ABSTRACT**

*Burkholderia pseudomallei* and its host-adapted deletion clone *Burkholderia mallei*, cause the potentially fatal human diseases melioidosis and glanders, respectively. Their antibiotic resistance profile, ability to infect via aerosol, and the absence of protective vaccines has led to their classification as major biothreats and select agents. Although documented infections by these bacteria date back over 100 years, relatively little is known about their virulence and pathogenicity mechanisms. We used in silico genomic subtraction to generate their virulome, a set of 650 putative virulence-related genes shared by *B. pseudomallei* and *B. mallei*, but absent from five closely related nonpathogenic *Burkholderia* species. Although most of these genes are clustered in putative operons, the number of targets for mutant construction and verification of reduced virulence in animal models is formidable. Therefore, *Galleria mellonella* (wax moth) larvae were evaluated as a surrogate host; we found that *B. pseudomallei* and *B. mallei*, but not other related bacteria, were highly pathogenic in this insect. More importantly, four previously characterized *B. mallei* mutants with reduced virulence in hamsters or mice were similarly reduced in virulence on *G. mellonella* larvae. Site-specific inactivation of select genes from the computationally derived virulome identified three potentially new virulence genes, each of which were required for rapid and efficient killing of larvae. Thus, this approach may provide a means to quickly identify high-probability virulence genes in *B. pseudomallei*, *B. mallei*, and other pathogens.

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**SUBJECT TERMS**

*Burkholderia mallei*, *pseudomallei*, glanders, virulence genes, insect model, *Galleria mellonella*, comparative genomics
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(T6S) gene cluster was shown to be required for the virulence of *B. mallei* (51, 56). Several putative virulence factors, including two proteases (10, 41), a hemolysin (1), a rhamnolipid (28), predicted transporter proteins, and three hypothetical proteins, have been identified using either nematode killing assays (24) or biochemical analysis. However, these factors are yet to be verified by animal experiments. Recently, Tuanyok et al. (62) identified a phospholipase C and a two-component regulatory system mutant that we adapted and verified as correlating with the hamster model commonly used to test *B. mallei* virulence. Our results show that this is a powerful and easily applicable approach that can accelerate research on these important pathogens and serve as an adjunct to much more expensive and problematic animal testing.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** Strains and plasmids used are listed in Table 1. Bacteria were grown at 37°C in LB (45) medium supplemented with 2% glycerol (LBG) or in brain heart infusion (BHI) medium. For some experiments, *B. mallei* was also grown on minimal medium plates (4) with 5% sucrose as the sole carbon source. When necessary, antibiotics were used at the following concentrations: kanamycin, 50 μg/ml; gentamicin, 20 μg/ml; and streptomycin, 10 μg/ml.

**Preparation of electrocompetent *B. mallei*.** An overnight culture was diluted to an *A*$_{600}$ of 0.1 in BHI medium and shaken for ~3 h until the *A*$_{600}$ was 0.8. Cells were harvested by centrifugation at 6°C at 4,000 $g$ and washed with 0.5 volume of 10% sucrose.

**Construction of a sacB deletion strain of *B. mallei* ATCC 23344.** Four PCR primers (see Table S1 in the supplemental material) were used to generate DNA fragments of the 5' and 3' ends of the sacB gene (BMAA0466) from *B. mallei* ATCC 23344, and the fragments were cloned into separate pCR2.1-TOPO vectors. The Sac7/Sac8 product was cut out of pCR2.1-TOPO with EcoRI, and the sacB gene deletion allele was cloned into the corresponding sites of pGRV2 (63), resulting in

### TABLE 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description*</th>
<th>Reference or source</th>
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<tr>
<td><em>B. mallei</em> ATCC 23344</td>
<td>Wild-type pathogen, Pm$^r$ Km$^r$ Gm$^r$ Zeo$^r$</td>
<td>49</td>
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<tr>
<td><em>E. coli</em> TOP10</td>
<td>General cloning strain</td>
<td>Invitrogen</td>
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<td><em>E. coli</em> S17-1</td>
<td>Capsule-deficient mutant</td>
<td>14</td>
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<tr>
<td><em>B. mallei</em> DD3008</td>
<td>T3SS$_{AP}$-deficient mutant</td>
<td>64</td>
</tr>
<tr>
<td><em>B. mallei</em> RDO1</td>
<td>System mutant</td>
<td></td>
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<tr>
<td><em>E. coli</em> S17-1</td>
<td>Capsule-deficient mutant</td>
<td>14</td>
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**Plasmids**

- **pCR-XL-TOPO** Cloning vector, Km$^r$ Zeo$^r$ Invitrogen
- **pCR2.1-TOPO** Cloning vector, Km$^r$ Amp$^r$ Invitrogen
- **pGRV2** Gene replacement suicide vector, Gm$^r$ 63
- **pDD159** SacB gene deletion allele This study
- **pCRXL1517SOE** Spliced flanking regions of BMAA1517 This study
- **pCRXL1621SOE** Spliced flanking regions of BMAA1621 This study
- **pGRV1517SOE** BamHI-XbaI fragment from pCRXL1517SOE This study
- **pGRV1621SOE** SacI-BamHI fragment from pCRXL1621SOE This study

* Pm, polymyxin B; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Zeo, zeocin; Amp, ampicillin.

* A gene was inactivated by insertion of a pCR-XL-TOPO vector containing a 400- to 900-bp PCR-amplified internal fragment of the gene.
RESULTS AND DISCUSSION

Derivation of a virulome for B. mallei and B. pseudomallei.

Despite the extensive ecological diversity of the organisms, genome comparisons of members of the Burkholderiaceae have revealed a core genome comprised of long syntenic regions containing thousands of shared “housekeeping” genes. Of greater interest are the hundreds of species- or isolate-specific genes that confer on each isolate its ability to colonize and survive in a specific niche or host. To find genes that are specific to B. mallei and B. pseudomallei and also have increased probability of major involvement in virulence, we used an in silico genomic subtraction method in which the amino acid sequences encoded by all B. mallei CDSs were individually compared to the amino acid sequences encoded by all the CDSs of four closely related nonpathogenic Burkholderia strains, and all CDS products found in “orthologous” pairs were removed. This yielded a set of 650 CDSs designated the Bm-Bp virulome (see Table S2 in the supplemental material), all of which are present in both B. mallei and B. pseudomallei but whose products have <45% amino acid sequence identity with the product of any other CDS in the genomes of the nonpathogens. In contrast, the average amino acid identity observed for most orthologous CDS pairs is >65%. This virulome comprises ~12% of the genome of B. mallei and appears to be highly enriched in CDSs/genes encoding probable virulence and pathogenicity factors since it contains most of the genes in the animal pathogenic-like T3SS (T3SS$_{vp}$), CAP, T6S, and LPS biosynthetic gene clusters, the only published loci experimentally proven to be critical for causing disease in the hamster model (14, 56, 64).

When we attempted to use the total CDSs of Burkholderia thailandensis E264, a strain previously considered a nonpathogen (26, 47), in a subsequent in silico subtraction experiment to further reduce and refine the virulome, we found orthologs for 70% of the virulome CDSs in the nonpathogen, including the majority of the CDSs in the three large gene clusters (T3SS, T6S, and CAP) that are essential for B. mallei virulence. In contrast, orthologs for <10% of the virulome CDSs were found in the genome of Burkholderia cenocepacia J2315 or B. cenocepacia AU1054, two pathogenic strains isolated from cystic fibrosis patients (5, 44, 65). This is perhaps not surprising considering that B. thailandensis E264 is very closely related to B. pseudomallei and that at relatively high doses it has the ability to kill nematodes (50) and hamsters (2) and has the ability to survive in macrophages (F. Gherardini, personal communication).

Most of the 650 virulome CDSs occur in ~40 operon-like clusters of four or more contiguous genes (only 100 genes are singletons). Not surprisingly, most clusters are on the smaller replicon, which carries the majority of nonhousekeeping genes (31, 49). Particularly high concentrations of the CDSs are found between BMAA0345 and BMAA0410, between BMAA0729 and BMAA0758, and between BMAA1517 and BMAA1565. Very few of the CDSs in clusters have Karlin score deviations (38), but whose products have <45% amino acid sequence identity with the product of any other CDS in the genomes of the nonpathogens. In contrast, the average amino acid identity observed for most orthologous CDS pairs is >65%. This virulome comprises ~12% of the genome of B. mallei and appears to be highly enriched in CDSs/genes encoding probable virulence and pathogenicity factors since it contains most of the genes in the animal pathogenic-like T3SS (T3SS$_{vp}$), CAP, T6S, and LPS biosynthetic gene clusters, the only published loci experimentally proven to be critical for causing disease in the hamster model (14, 56, 64).

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metabolite-producing clusters encoding nonribosomal peptide or polyketide synthetases possibly involved in the cytotoxic activities ascribed to \textit{B. pseudomallei} (6, 29, 30). Also present are CDSs encoding major parts of both the CAP and LPS biosynthesis clusters and two fimbria/pilus biogenesis pathways, in addition to >100 CDSs encoding predicted membrane proteins. The presence of CDSs encoding proteins with predicted enzymatic functions in other clusters suggests that these CDSs may encode parts of metabolic pathways. However, many of the clustered genes have no predicted function; >200 CDSs lack any functional description, and ~100 do not have PFAM domains.

\textit{G. mellonella} larvae are a valid surrogate host for \textit{B. mallei}. The ultimate goal of deriving the virulome was to extract a comprehensive set of potentially novel virulence genes that contained few enough genes that the majority could be individually knocked out and mutants evaluated to determine whether the knockouts reduced virulence in animal models. Uncertainty about the pathogenic potential of \textit{B. thailandensis} precluded using in silico subtraction to reduce the virulome to genes selected from the Bm-Bp virulome. Assays were performed at least three independent times by inoculating 10 larvae with each mutant. The actual number of cells (CFU) injected into the larvae was determined by plating duplicate aliquots of the injectant on LBG plates. The percentages of larvae killed are the averages of three assays; the standard deviations are <15%. Wild-type \textit{B. mallei} and water-inoculated controls were included with each set of assays.

When ~10 wild-type \textit{B. mallei} ATCC 23344 (or \textit{B. mallei} GRS 23344) cells were injected into the hemocoel of larvae, the majority of the larvae were killed within 4 days. In ~20 experiments we inoculated more than 300 larvae with anywhere from 3 to 200 cells of wild-type \textit{B. mallei} and always observed >90% killing within 6 days. Injecting 10 \textit{B. pseudomallei} K96243 cells into the larvae killed them nearly twice as fast (>80% mortality by 2 days). Similar to hamsters infected with \textit{B. mallei} or \textit{B. pseudomallei}, infected larvae showed extensive paralysis 12 h before death. At the onset of paralysis, typically >10^7 \textit{B. mallei} cells/ml were present in the hemolymph; just prior to death >10^6 \textit{B. mallei} or \textit{B. pseudomallei} cells/ml were found. These results indicate that \textit{B. mallei} and \textit{B. pseudomallei} can multiply to very high numbers in the hemocoel of wax moth larvae and are highly pathogenic for this insect. Injection of up to 10^5 cells of the pathogenic cystic fibrosis epidemic strain \textit{B. cenocepacia} J2315, \textit{Pseudomonas putida}, \textit{B. xenovorans}, or \textit{E. coli} W3110 killed <15% of the larvae by day 6; using an inoculum containing 10^3 cells reduced the percentage of dead larvae at day 6 to <10%, which is identical to the level observed if water was injected. Injection of >10^5 \textit{B. thailandensis} cells killed ~35% of the larvae after 7 days. Although this is somewhat inconsistent with the “nonpathogenic” label for \textit{B. thailandensis}, as mentioned above, this species harbors ~70% of the genes in the Bm-Bp virulome and at relatively high doses (>10^5 cells) it also can kill hamsters and nematodes. Moreover, at least one opportunistic infection of a human by \textit{B. thailandensis} has been reported (26).

To show correspondence between the wax moth larva model and mammalian models, we first tested \textit{B. mallei} RD01, which has a polar insertion in the T3SS\_AP gene cluster (64). This T3SS is essential for the virulence of \textit{B. mallei} and \textit{B. pseudomallei} in mice and hamsters, respectively (64, 66). Compared to the wild type, the ability of \textit{B. mallei} RD01 to kill wax moth larvae was dramatically reduced (Fig. 1A, compare WT and T3SS); at lower infection levels (<20 cells/larva), RD01 was largely avirulent (data not shown). When \textit{B. mallei} strain
DD3008, a CPS-deficient mutant with dramatically reduced virulence in hamsters (10), was used, the killing of wax moth larvae was delayed and reduced (Fig. 1A, compare WT and CAP). Next, we constructed an isoleucine-valine auxotroph of B. mallei by insertional inactivation of ilvI (BMA1848) encoding a subunit of acetolactate synthase III. The resultant mutant was not able to grow on minimal glucose medium and, similar to what has been observed with mice (63), showed attenuated virulence in larvae (Fig. 1A, compare WT and IlvI). Thus, the behavior of the three mutants whose virulence in animal models is attenuated correlated well with their virulence in larvae. Inactivation of type II secretion affects the virulence of P. aeruginosa (17, 69), some plant pathogens (36, 43), and B. pseudomallei (10). Therefore, we insertional inactivated gspD (BMA2786), a key component of type II secretion, and found that the killing of wax moth larvae by the resultant GspD mutant was reduced and delayed (Fig. 1A), but not to the extent caused by the T3SS mutation. These data suggest that the wax moth model of infection is an efficient predictor of potential virulence genes in B. mallei.

To verify the stability of the mutant genotype in the wax moth larvae, bacterial cells were recovered from the hemolymph at days 2 and 5 by plating on nonselective LBG agar. All of the 250 resultant colonies tested retained the antibiotic resistance encoded by the plasmid insertion. When auxotrophic ilvI mutant cells were recovered from larval hemolymph and plated on both LBG and minimal media, we found that <1 in 10^5 of the ex vivo cells had reverted to prototrophy, further demonstrating the high level of stability of the cis-merodiploid mutants.

**Use of the wax moth larva model to screen putative virulence mutants.** Based on a manual review of bioinformatic data for virulome CDSs, 10 CDSs were evaluated to determine whether they have a role in virulence (Table 2). Some of these CDSs were chosen because their best BLAST hits were in phylogenetically distant pathogens or in a eukaryote, some were chosen because they appeared to be potential virulence gene regulators, and some were chosen because they appeared to encode polyketide or peptide synthetases that may be involved in cytotoxin production. Knockout mutants for each CDS were constructed and verified by PCR, and their virulence in wax moth larvae was tested as described above. B. mallei strains with mutations in BMA0847, BMA1111, BMA1123, BMA1013, and BMA1621 had marginal or no differences in virulence compared with the wild type (Fig. 1A and data not shown), whereas with strains harboring insertions in BMA0235, BMA1204, BMA1517, BMA1785, and BMA1900 there was reduced and/or delayed killing of the larvae (Fig. 1B).

Of all the mutants tested, inactivation of BMA1517, encoding an AraC-type transcriptional regulator, resulted in the most dramatic reduction in virulence in the larvae. At 7 days postinfection 80% of the larvae infected with ∼200 CFU of the BMA1517 mutant remained asymptomatic, similar to the behavior of the T3SS_Ap mutant. Whole-genome expression profiling of a B. mallei strain overexpressing BMA1517 has shown that a cluster of genes found in the Bm-Bp virulome (BMA0727 to BMA0744) is exclusively upexpressed on average 4-fold; the levels of some genes are elevated >10-fold (56). This previous study also showed that these genes encode components of a T6S system which, when inactivated, rendered B. mallei avirulent in hamsters. When we tested a mutant lacking a critical component of the T6S system (BMAA0739), its virulence in larvae was reduced to the same extent as that of BMA1517 mutants (not shown). These results further support the hypothesis that the wax moth larva model is an accurate predictor of B. mallei virulence genes.

Inactivation of BMA0235 in B. mallei dramatically delayed and reduced killing of larvae in the wax moth infection model. BMA0235 is in an operonlike cluster of six genes that is conserved in all sequenced B. pseudomallei and B. mallei strains but is not present in any other Burkholderia genome or in the 600 microbial genomes at Integrated Microbial Genomes (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi), with the exception of the genome of Photobacterium luminiscens. In fact, P. luminiscens, which can be an insect pathogen or nematode symbiont (21), has similarly organized orthologs of the five genes flanking BMA0235 (Fig. 2). The average amino acid identity between the products of the six CDS pairs from these phylogenetically distant bacteria is 66%, which is quite remarkable considering that the G+C content of the P. luminiscens CDSs is ∼38%, while the average G+C content of the B. mallei orthologs is 68%. The BMA0237 protein has a domain

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<th>B. mallei CDS</th>
<th>Product</th>
<th>Rationale for targeting</th>
<th>Virulence of mutant in larvae</th>
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<tr>
<td>BMA0235</td>
<td>3-Phosphoshikimate 1-carboxyvinyltransferase</td>
<td>Best BLASTP hit in phylogenetically distant pathogen</td>
<td>Reduced</td>
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<tr>
<td>BMA0847</td>
<td>Galactose oxidase-related protein</td>
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<td>BMAA1013</td>
<td>Hypothetical protein</td>
<td>Potential virulence gene regulator</td>
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<td>BMAA1111</td>
<td>Hypothetical protein</td>
<td>Best BLASTP hit in eukaryote</td>
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<td>BMAA1204</td>
<td>Putative PKS</td>
<td>Possible role in cytotoxin production</td>
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<td>Pentapeptide repeat family protein</td>
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See text and Fig. 1B.
similar to a domain in chorismate mutase (PFAM1042), while the BMA0236a and BMA0235 proteins are both assigned to the enoylpyruvate carboxyltransferase family (PFAM0275) and are related to 3-phosphoshikimate 1-carboxyvinyl transferases. Both of the latter proteins were originally annotated as AroA (EC 2.5.1.19), which is involved in aromatic amino acid biosynthesis. However, these proteins show <25% identity to each other and to E. coli AroA. The AroA function of B. mallei more likely resides in the BMA0430 protein, annotated as a bifunctional prephenate dehydrogenase/3-phosphoshikimate 1-carboxyvinyl transferase enzyme whose C-terminal and N-terminal halves show ~50% amino acid identity to full-length E. coli AroA and TyrA, respectively. Thus, the BMA0235 and BMA0236a proteins are more likely novel carboxyvinyl transferases that transfer a vinyl group from phosphoenolpyruvate to an unknown substrate. The BMA0234 protein is annotated as a putative asparagine synthase B (EC 6.3.5.4), as are the products of two other CDSs in the B. mallei genome (BMAA1158 and BMAA1921). The products of these three CDSs do not show >30% amino acid identity to E. coli AsnB. The BMA0234 protein is likely involved in an AsnB-like reaction, transfer of the amido group of glutamine to an aspartate-like substrate. The BMA0233a protein may be a carboxylase, while the BMA0232 protein is a putative drug/metabolite transporter (PFAM 05297). It is plausible that the gene clusters shown in Fig. 2 synthesize a secondary metabolite or toxin that plays a role in insect pathogenesis in P. luminescens and perhaps in B. mallei. This metabolite could be derived from carboxyvinylidene 3,4-dihydroxy-2-butanone 4-phosphate made by the adjacent ribA gene, followed by amidation of the carboxyvinyl group. P. luminescens and its close relatives are well known for production of insect toxins.

BMAA1204 mutants took twice as long as the wild type to start killing larvae; after 7 days only 60% of the mutant-infected larvae were dead, compared to the 100% mortality caused by the wild type. BMAA1204 is a 4,200-residue CDS annotated as encoding a putative polyketide synthase (PKS) in COG family 0332. PKSs are giant multidomain proteins ubiquitous in the prokaryotes. In a manner analogous to fatty acid biosynthesis, they condense or “polymerize” organic acids (e.g., acetate and malonate) into linear polyketides. The resultant polyketides are often cyclized and modified, yielding 300- to 1,500-Da molecules that exhibit diverse antibiotic, antitumor, or even immunosuppressive activities. BMAA1204 is in a large gene cluster along with genes encoding two other putative PKSs and several CDSs whose products are in protein families associated with polyketide synthesis and/or modification. In fact, most CDSs in this region (BMAA1201 to BMAA1222) are in the virulome. It is possible that BMAA 1204 and/or the adjacent PKS genes encode production of one of the “cytotoxin” activities found previously in culture supernatants of B. pseudomallei (16, 41, 42), whose characteristics (e.g., heat stability and molecular weight) are consistent with a polyketide origin. PKSs have been implicated in pathogenesis by several other bacteria (e.g., Mycobacterium ulcerans [35]). B. mallei has seven putative PKS genes; three of these genes (BMAA1021, BMAA1204, and BMAA1451) are present in the virulome but are not present in B. thailandensis.

Inactivation of BMAA1785, whose product is annotated as a “chitin binding domain protein,” reduced virulence quantitatively to an extent very similar to the extent observed after inactivation of the PKS gene BMAA1204. Although nonpathogenic Burkholderia spp. lack orthologs of the BMAA1785 protein, Yersinia pestis and Yersinia pseudotuberculosis have orthologs with 55% amino acid sequence identity. The actual function of the BMAA1785 protein is not clear, but its so-called chitin binding domain 3 (PFAM03067) is related to baculoviral spheroidin and spindolin capsid proteins. It has a predicted signal sequence and is present at moderately high levels in culture supernatants of B. pseudomallei and B. mallei (52; data not shown). We speculate that it binds to some type of chitin-like oligosaccharide moiety and has an as-yet-unknown function.

Inactivation of BMAA1900 caused only a moderate delay (1 day) in the onset of killing and a minor reduction in B. mallei-related mortality of wax moth larvae. This gene is in a cluster (BMAA1897 to BMAA1915) that is predicted to encode one of the four predicted T6S systems in B. mallei (56). BMAA1900 encodes a putative pentapeptide repeat protein (COG family 1357) that is 38% identical to a putative exported protein of Bordetella bronchiseptica, which is located in an analogous T6S system cluster. While one can speculate that BMAA1900 is involved in T6S system-mediated secretion of a virulence factor(s), it seems that this T6S system plays a relatively minor role in virulence for wax moth larvae.

**Conclusion.** To date, relatively few virulence factors of B. mallei have been identified, and most of the factors that have been found are widespread in diverse pathogens (e.g., T3SS and capsule). Some of the methods used to identify these virulence factors, such as subtractive hybridization and transposon mutagenesis, are time-consuming and especially cumbersome with BSL3-requiring select agent pathogens. With the availability of hundreds of bacterial genome sequences, it makes sense to exploit these databases to use new bioinformatic methods to direct functional genomic studies. In silico genomic subtraction provided a set of B. mallei genes that contained genes encoding all known virulence factors and
therefore was very valuable as a starting point for functional analyses to discover new genes with a role in pathogenesis.

*G. mellonella* larvae have successfully been used as a model for infection by several pathogenic bacteria (39), as well as in screening for bacterial mutants with reduced virulence and/or altered host colonization ability (33, 46). We have shown that *G. mellonella* also is a good model for *B. mallei* pathogenesis, because four *B. mallei* strains with mutations in different characterized virulence gene clusters (T3SS, CAP, type II secretion system, and T6S system) showed reduced virulence that correlated well with their behavior in the hamster model of infection. The larva model is a facile and inexpensive method that can be used to test potential virulence mutants of *B. mallei* that may be used as a model for mammalian infection. The larva model is a facile and inexpensive method that can be used to test potential virulence mutants of *B. mallei*.


