INFECTION AND IMMUNITY, Mar. 2008, p. 1267–1275 Vol. 76, No. 3
0019-9567/08/$08.00+0 doi:10.1128/IAI.01249-07
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Development of *Galleria mellonella* as an Alternative Infection Model for the *Burkholderia cepacia* Complex[▽]

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Received 11 September 2007/Returned for modification 28 November 2007/Accepted 24 December 2007

Burkholderia **is an important bacterial genus with a complex taxonomy that contains species of both ecological and pathogenic importance, including nine closely related species collectively termed the *Burkholderia cepacia* complex (BCC). In order to more thoroughly investigate the virulence of this bacterial complex of microorganisms, alternative infection models would be useful. To this end, we have adapted and developed the use of the *Galleria mellonella* wax moth larvae as a host for examining BCC infections. The experimental conditions affecting the BCC killing of the "wax worm" were optimized. BCC virulence levels were determined using 50% lethal doses, and differences were observed between both species and strains of the BCC. The BCC pathogenicity trends obtained compare favorably with results acquired using other published alternative infection models, as well as mammalian infection models. In addition, BCC killing activity was determined by directly measuring relative bacterial loads in three different BCC strains, thus demonstrating innate differences in BCC strain virulence. Finally, genetically mutated BCC strains were compared to a wild-type BCC strain in order to show concomitant reduction of BCC virulence and increased wax worm survival. For experimentation examining the virulent properties of the BCC, the wax worm has proven to be a useful alternative infection model.**

Members of the *Burkholderia cepacia* complex (BCC) are closely related bacterial species that can act as opportunistic pathogens primarily affecting patients with cystic fibrosis (CF) (17, 33). While *Pseudomonas aeruginosa* may be considered the most prominent chronic respiratory pathogen for these patients (32), BCC infections have had a major impact on the quality of life and mortality of CF patients. Most troublesome is the fact that lung infections with BCC can culminate in a rapidly fatal condition in which patients develop a progressive, necrotizing pneumonia and sepsis commonly referred to as "cepacia syndrome" (23). More typically, CF patients infected with the BCC develop a chronic infection that leads to a slow decline in pulmonary function that is associated with increased mortality and morbidity (31). Because patient-to-patient spread of BCC bacteria is of concern to the CF community (30), social limitations have been placed upon infected individuals, thereby resulting in adverse isolation effects (42). Unfortunately, treatment of the BCC is problematic. Most of the BCC organisms are highly resistant to all major classes of antibiotics, including aminoglycosides (1, 29).

The BCC currently consists of a total of nine *Burkholderia* species: *B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. stabilis*, *B. vietnamiensis*, *B. dolosa*, *B. ambifaria*, *B. anthina*, and *B. pyrrocinia* (8, 9, 10, 44–47). The ability of the BCC to cause infections in the CF population is not species dependent, as members of all species have been recovered from infected individuals (42). However, the vast majority of clinical isolates in North America are *B. cenocepacia* and *B. multivorans*; in Canada, *B. cenocepacia* strains cause approximately 80% of BCC infections in CF patients, although this prevalence varies by region (42). Infections with *B. cenocepacia* are generally associated with the poorest clinical prognosis and the highest rates of transmissibility and mortality. Infections with *B. multivorans*, although quite common among CF patients, are associated with a better prognosis, having reportedly lower transmission and mortality rates (42). The differences in pathogenicity between all BCC species, however, are not well understood (3, 7, 18).

It is probable that opportunistic bacterial pathogens like the BCC use common virulence factors to infect different organisms. In order to better understand the factors that are important to disease causation by the BCC, we have developed an alternative infection model using the "wax worm." Larvae of the Greater wax moth *Galleria mellonella* have been used previously as an infection model for the study of other bacterial human pathogens, including *P. aeruginosa* (19, 24, 38), *Bacillus cereus* (16), *Proteus mirabilis* (39) and, more recently, *Francisella tularensis* (2), as well as fungal pathogens *Cryptococcus neoformans* (40), *Aspergillus* spp. (41, 43), and *Candida albicans* (12). The innate immune systems of insects such as *G. mellonella* share a high degree of structural and functional homology to the innate immune systems of mammals (21). Although the immune systems of insects do not display memory or clonal selection mechanisms, they do offer powerful resistance to microbial infections (48). This defense against microorganisms involves both cellular and humoral defenses (21). The humoral immune response of insects consists of the processes of melanization, hemolymph clotting, and the production of a number of potent antimicrobial peptides. The cellular reactions include phagocytosis, nodulization, and large-scale encapsulation. Therefore, *G. mellonella* is an attractive alternative infection model for a number of reasons. Analysis of insect responses to pathogens can provide an accurate indication of the mammalian response to that pathogen (21, 25). Furthermore, substan-

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▽ Published ahead of print on 14 January 2008.

tial correlation between the virulence of certain microbes in mice and the *G. mellonella* model has been established (4, 24). While the use of higher animals, such as mice (7) and rats (3, 6), for the study of the BCC has provided invaluable information, alternative infection models that could provide comparable data but that are more cost-effective, less labor-intensive, and more ethically acceptable would be highly useful. Other alternative infection models have previously been tested with the BCC, including alfalfa seedlings (3), *Caenorhabditis elegans* (26, 35, 22), and *Acanthamoeba* species (27, 36). Unfortunately, all of these models exhibit at least one deficiency in reproducing the virulence observed with BCC in mice and rats. We have found that the use of *G. mellonella* as an alternative infection model for describing BCC virulence is in some cases more quantitative, more accurate, and more robust than other available alternative infection models.

MATERIALS AND METHODS

Strains and media. The bacterial strains of the BCC used in this study were collected strains from the *Burkholderia cepacia* complex experimental strain panel (34) and the updated experimental strain panel (11). BCC strains were grown in one-half-strength Luria-Bertani (1/2 LB) medium at 30°C. ATCC 29424 mutants were grown in 1/2 LB supplemented with 100-μg/ml trimethoprim at 30°C. Overnight cultures were pelleted and resuspended in 10 mM $MgSO_4$ supplemented with 1.2 mg/ml ampicillin. The presence of ampicillin in the inoculum was to prevent infection with bacteria naturally present on the surface of the larvae. Bacteria collected from the hemolymph of infected larvae were serially diluted in 10 mM $MgSO_4$ and plated onto *B. cepacia* selective agar (20).

***G. mellonella* killing assays.** Larvae were stored in wood chips at 4°C. A 10-μl Hamilton syringe was used to inject 5-μl aliquots into *G. mellonella* via the hindmost left proleg. Following injection, larvae were placed in a static incubator in the dark at 30°C, the optimum temperature for insect growth and development (4). For 50% lethal dose (LD_{50}) experiments, a series of 10-fold serial dilutions containing from 10^6 to 0 bacteria in 10 mM $MgSO_4$ plus 1.2 mg/ml ampicillin were injected into *G. mellonella* larvae. Control larvae were injected with 5 μl of only 10 mM $MgSO_4$ plus 1.2 mg/ml ampicillin in order to measure any potentially lethal effects of the physical injection process. Ten larvae were injected at each dilution, and larvae were scored as dead or alive 48 and 72 h postinfection (p.i.) at 30°C. Larvae were considered dead when they displayed no movement in response to shaking of the petri dish or touch with a pipette tip. For each strain, data from three independent experiments were combined, and LD_{50}s were calculated using the Systat computer program as previously described (24). Briefly, Systat was used to fit a curve to the infection data of the following form: $Y = [A + (1 - A)]/[1 + \exp(B - G \times \ln X)]$, where Y is the fraction of larvae killed by the infection, A is the number of larvae killed by control injections, X is the number of bacteria injected, and B and G are Systat-generated variable parameters designed to best fit the curve to the data points. For linear relationships between X and Y, we used a linear regression model using the Systat computer program to determine the LD_{50}.

For time-to-death experiments, live versus dead larvae were monitored every 24 h postinfection. *Galleria mellonella* larvae were injected with serially diluted bacteria (from 1×10^6 to 0 CFU) and monitored for their survival over a 72-h period. Three independent trials were conducted consisting of 10 worms per bacterial concentration for each specified BCC strain. No more than one control larva died in any given trial. In instances where greater than one control larva died, the data from infected larvae were not used. Results are shown for inoculum concentrations in which the differences between species could be most easily observed (1×10^6 or 1×10^3 CFU).

In order to monitor bacterial loads in larval hemolymph over time, larvae were injected with between 500 and 800 CFU. For the zero time point, larvae were infected and allowed to sit for 20 min before having their hemolymph collected. Equal volumes of hemolymph were collected from five living worms at each time point and combined into a microcentrifuge tube, serially diluted, and plated onto *B. cepacia* selective agar for quantification. Three groups of five worms were used for each time point in order to quantify bacterial loads.

BCC mutants in *G. mellonella* assays. Mutations were isolated or constructed in *B. vietnamiensis* ATCC 29424 (49). Mutations were introduced with random Tn*Mod*-OTp′ plasposon mutagenesis, using a procedure described previously (14). In each example, the mutation was isolated by plasposon rescue, cloned in *Escherichia coli*, and identified by DNA sequence analysis. The resulting mutants were tested to ensure that none exhibited growth defects in 1/2 LB medium. Many different plasposon mutants were tested in the *G. mellonella* infection model, although only the mutants with the most significant virulence defects are shown. Alternatively, site-directed insertion mutagenesis was used to create double mutations in the genes for the transport/structural proteins ExbB1 and TolQ (CS1/BG1) or ExbB2 and TolQ (CH1/BG1). Briefly, genes were amplified using PCR primers designed to BCC database sequences for *B. cenocepacia* J2315 (http://www.sanger.ac.uk/Projects/B_cenocepacia/) and cloned into the *E. coli* plasmid pUC19, which is unable to replicate in BCC. The individual genes were interrupted with an appropriate selectable antibiotic resistance cassette, such as one of those encoding trimethoprim or tetracycline resistance (15), and the plasmids were electrotransformed into ATCC 29424 using standard protocols (13). Loss of the cloning vector and replacement of the wild-type gene by homologous recombination was confirmed by PCR and DNA sequence analysis. Prior to larval injection, all mutants and wild-type BCC ATCC 29424 cells were grown overnight at 30°C to similar optical density at 600 nm values. Larvae were injected with between 5×10^6 and 9×10^6 CFU of bacteria. Three trials were performed in which 10 larvae were injected for each bacterial strain.

RESULTS

LD_{50}s of BCC strains. We determined the LD_{50}s of 23 strains within the *Burkholderia cepacia* complex. As shown in Table 1, *B. cepacia* and *B. cenocepacia* strains had the lowest LD_{50}s, while *B. multivorans* and *B. stabilis* strains had the highest LD_{50}s. The most virulent strain tested, *B. cepacia* ATCC 17759, had an LD_{50} of one bacterium. Some strains within the complex did not exhibit enough larval killing to calculate an LD_{50}, even at the highest dosage tested. Six strains within the complex also exhibited slower killing, and therefore LD_{50}s for those strains could only be calculated after 72 h. Members of *B. cenocepacia* and *B. cepacia* were shown to have lower LD_{50}s than *B. stabilis* and *B. multivorans* using an independent pairs t test ($P < 0.00000001$).

Although some strain variability was noted within BCC species, in general, the pathogenicity displayed toward *G. mellonella* as measured by LD_{50} values (Table 1) was similar between BCC strains of the same species. This similarity between the virulence of strains within a BCC species was particularly true for *B. cepacia* (very low LD_{50}s), *B. cenocepacia* (moderate LD_{50}s), and *B. multivorans* and *B. stabilis* (very high LD_{50}s). Although fewer numbers of the more recently classified species were available for testing, members of *B. vietnamiensis* and *B. dolosa* showed greater variation in virulence between strains. In contrast, there were large differences in virulence toward *G. mellonella* between BCC species. In 48 h, the two *B. cepacia* strains tested killed on average 50% of *G. mellonella* larvae at bacterial numbers ranging from 1 to 30 CFU, while the eight *B. cenocepacia* strains tested killed on average 50% of *G. mellonella* larvae at numbers ranging from 900 to 200,000 CFU. In contrast, the four *B. multivorans* strains tested killed on average 50% of *G. mellonella* larvae under the same conditions, with numbers ranging from 1.0×10^6 CFU or higher, and the two *B. stabilis* strains tested were only lethal at 2.0×10^6 CFU or higher. A chart showing these trends is displayed in Fig. 1.

Table 2 illustrates a comparison between BCC virulence in the *G. mellonella* model with the previously published alfalfa seedling and rat lung agar bead infection models (3). Although identical strains were not used for every species tested, good correlation was observed between the *G. mellonella* and the alfalfa seedling models. *B. multivorans* and *B. stabilis* strains

TABLE 1. LD_{50}s of BCC strains in *G. mellonella* larvae 48 h postinfection

Species and strain	Alternative collection name	Strain source[a]	LD_{50} (CFU) in *G. mellonella*	Reference or strain source
B. cepacia strains				
Cep509	LMG 18821	CF, Australia	30	34
ATCC 17759	LMG 2161	Soil, Trinidad	1	34
B. multivorans strains				
C5393	LMG 18822	CF, Canada	>3,000,000[b]	34
C3430		CF, Canada	>3,000,000[b]	CRRR[d]
C5274		CF, Canada	1,000,000	CRRR[d]
C5568		CF, Canada	>3,000,000[b]	CRRR[d]
B. cenocepacia strains				
PC715j		CF, Canada	4,000	37
J2315	LMG 16656	CF-e, United Kingdom	100,000[c]	34
K56-2	LMG 18863	CF-e, Canada	900	34
C1257		CF, Canada	40,000[c]	CRRR[d]
C4455		CF, Canada	100,000[c]	CRRR[d]
C5424	LMG 18827	CF, Canada	200,000	34
C6433	LMG 18828	CF, Canada	30,000	34
Cep511	LMG 18830	CF, Austrailia	80,000	34
B. stabilis strains				
ATCC BA-67	LMG 14294	CF, Belgium	2,000,000[c]	34
C7322	LMG 18870	CF, Canada	>2,000,000[b]	34
B. vietnamiensis strains				
DBO1	ATCC 29424	Soil, United States	200,000	49
PC259	LMG 18835	CF, United States	>3,000,000[b]	34
B. dolosa strains				
AU0645	LMG 18943	CF, United States	>4,000,000[b]	8
STM1441	LMG 21443	Rhizosphere (Senegal)	40,000	11
B. ambifaria strain				
Cep0996	LMG 19467	CF, Australia	800,000	11
B. anthina strain				
J2552	LMG 16670	Rhizosphere, United Kingdom	300,000	11
B. pyrrocinia strain				
ATCC 15958	LMG 14191	Soil, Japan	300	11

[a] Abbreviations: CF, cystic fibrosis infection; CF-e, strain that has spread epidemically among patients with CF.
[b] Mortality never reached 50%, even at the highest dosage.
[c] The LD_{50} was calculated after 72 h.
[d] CRRR, from the Canadian *Burkholderia cepacia* complex Research and Referral Repository.

were relatively avirulent in both models, producing disease symptoms in 0 to 13% of alfalfa seedlings, whereas *B. cepacia* and *B. cenocepacia* strains were much more virulent in both models, producing disease symptoms in 65 to 100% of the alfalfa seedlings (3). The rat lung agar bead model results similarly compared favorably with the results observed with the BCC in *G. mellonella*; the *B. cepacia* and *B. cenocepacia* strains that resulted in the lowest LD_{50}s in *G. mellonella* (30 to 10^5) had the highest amounts of rat lung pathology after 7 days (22.8 to 43.0%). In comparison, *B. multivorans*, *B. stabilis*, and *B. vietnamiensis* strains, with LD_{50}s of at least 10^6, exhibited the lowest amounts of rat lung pathology (13.3 to 19.7%). An exception was *B. cenocepacia* strain Cep511, which resulted in a moderate LD_{50} in *G. mellonella* (8×10^4), a very high percentage of alfalfa seedlings with disease symptoms (98%), and one of the lowest percentages of pathology observed in the rat lung agar bead model (12.7%) (3). In comparison to these models, the BCC-*C. elegans* infection model did not demonstrate a general virulence trend within species (5) and therefore did not correlate well with the general results observed for the *G. mellonella* model. Instead, the BCC-*C. elegans* infection model displayed significant strain-to-strain variation within species. However, similar to the results obtained for BCC in *G. mellonella*, *B. multivorans* was relatively avirulent, and the more recently classified BCC members, such as *B. dolosa*, *B. ambifaria*, *B. anthina*, and *B pyrrocinia* (11), exhibited significant toxicity in *C. elegans*.

Representative time-to-death curves for four different BCC species at different bacterial doses are shown in Fig. 2. *B. cepacia* LMG 18821 kills all *G. mellonella* at 10^6 CFU within 24 h and 10^3 CFU within 48 h, faster than a 10^6 CFU dose of all other BCC species tested. *B. cenocepacia* C4455 and *B. ambifaria* LMG 19467 exhibit similar pathogenicities toward *G. mellonella* at both 10^6 CFU and 10^3 CFU over 72 h, intermediate to *B. cepacia* LMG 18821and *B. stabilis* LMG 18870. *B. stabilis* LMG 18870 was not toxic toward *G. mellonella* at a

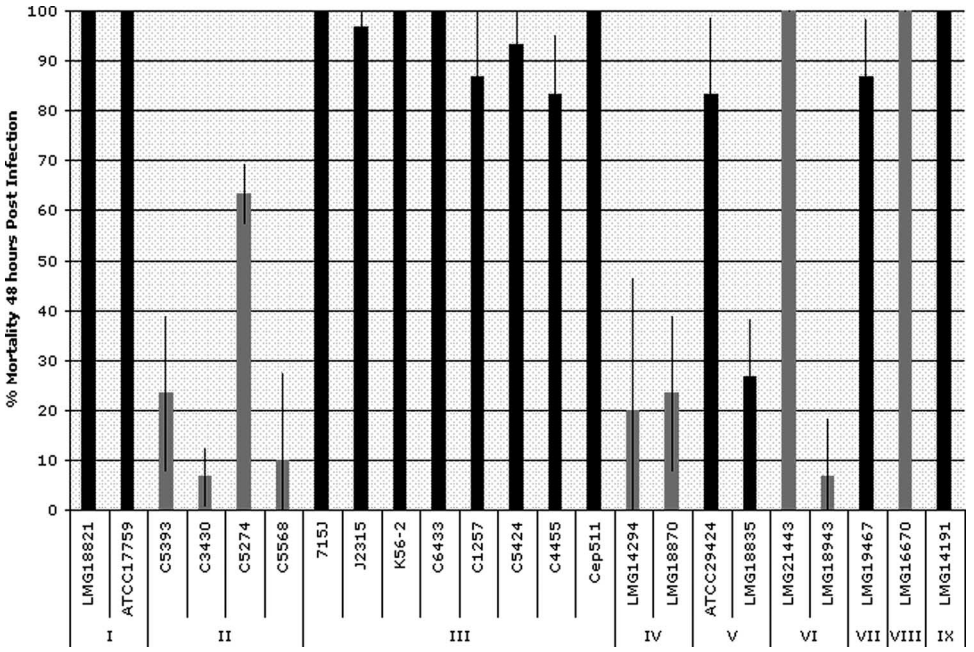

FIG. 1. Differential virulence of *Burkholderia cepacia* complex strains in the *Galleria mellonella* infection model. Larvae were injected with approximately ~10^6 bacteria and monitored for survival 48 h postinfection. Each bar is representative of three independent trials ($n = 10$), and standard deviations are shown. Groups according to BCC genomovar: I, *B. cepacia*; II, *B. multivorans*; III, *B. cenocepacia*; IV, *B. stabilis*; V, *B. vietnamiensis*; VI, *B. dolosa*; VII, *B. ambifaria*; VIII, *B. anthina*; IX, *B. pyrrocinia*.

dose of 10^3 CFU and only able to kill 10% of the larvae at a dose of 10^6 CFU, although at higher doses more killing was observed (data not shown). These results were similar to those observed for *B. multivorans*, as well as some strains of *B. vietnamiensis* and *B. dolosa*, in which case LD_{50} values were difficult to calculate.

Survival and relative bacterial loads in infected *G. mellonella* larvae. Larvae were monitored following infection with three different BCC strains (Fig. 3). The majority of larvae killed following infection with 500 to 800 CFU of *B. cepacia* LMG18821 or *B. cenocepacia* K56-2 died after 36 h p.i. As shown in Fig. 3A and C, the bacterial load for larvae infected with strain *B. cepacia* LMG18821 was approximately 10-fold less than for *B. cenocepacia* K56-2 at 36 h p.i. (1.6×10^4 compared to 1.3×10^5 CFU recovered/10 μl hemolymph), while infection with LMG 18821 resulted in more larval death at 48 h. Accordingly, the LD_{50} for *B. cepacia* LMG 18821 is 30 times lower than the LD_{50} of *B. cenocepacia* K56-2 (Table 1). Due to our ability to collect hemolymph only from living larvae, we anticipate that the relative bacterial loads taken at 48 h p.i. from *B. cepacia* LMG18821 and *B. cenocepacia* K56-2 are likely gross underestimates. *B. multivorans* C3430 was unable to establish significant bacterial loads in the hemolymph of infected wax worms at any of the time points following infection (Fig. 3B). These results indicate that this strain is relatively avirulent, and the results correspond with its relatively high LD_{50} value ($>3 \times 10^6$ CFU/larvae).

Pathogenicity of BCC mutants toward *G. mellonella*. To determine whether the *G. mellonella* model would be useful for identifying BCC mutants with attenuated virulence, we examined the ability of mutants constructed in *B. vietnamiensis* strain ATCC 29424 to affect the survival of infected wax worm larvae. As shown in Fig. 4, five mutants constructed in different genes exhibited differing degrees of decreased virulence compared to the wild-type BCC strain. Plasposon mutants with decreased virulence in vivo contained mutations in the BCC orthologous genes encoding BtuB (the receptor for vitamin B_{12}), Fep (a protein involved in ferri-siderophore uptake), or PurG (an enzyme involved in purine biosynthesis). Site-directed mutants with decreased virulence in vivo contained mutations in the genes encoding transport/structural proteins ExbB1 and TolQ (CS1/BG1) or ExbB2 and TolQ (CH1/BG1). The decreased virulence observed was not due to changes in growth or the presence of an antibiotic resistance cassette in the genome, since other similarly constructed mutants grown under identical conditions did not display decreased virulence (data not shown). The results obtained with these constructed mutants indicate that the *G. mellonella* model will be a valuable experimental system for determining the virulence properties of BCC genetic mutants.

DISCUSSION

In this study, we compare our results for a *G. mellonella* infection model with previously published BCC data from other infection models, taking care to use many of the identical BCC strains previously tested in order to ensure valid comparison. We observed that *G. mellonella*, as an infection model for the BCC, possessed several advantages over other infection models. Using *G. mellonella*, we obtained similar results to both mouse and other alternative infection models with identical BCC strains. In our experience, no other alternative infection models as tested were as sensitive, accurate (with respect to mice and rat lung model results), or as robust or

TABLE 2. Comparison of BCC virulence in different infection models

Species and strain	LD_{50} in *G. mellonella*	% Alfalfa seedlings with symptoms[a]	Virulence in rats (% with pathology on day 7 p.i.)[a]	*C. elegans* pathogenicity score[b]
B. cepacia strains				
Cep509	30	85 ± 18[c]	43.0 ± 2.0[c]	0
ATCC 17759	1	73 ± 13	ND[d]	ND
B. multivorans strains				
C5393	>3,000,000	0	13.3 ± 5.9	0
C3430	>3,000,000	ND	ND	0
C5274	1,000,000	ND	ND	0
C5568	>3,000,000	ND	ND	1
B. cenocepacia strains				
PC715j	4,000	80 ± 18	22.8 ± 10.8	ND
J2315	100,000	65 ± 10	38.0 ± 21.0	1
K56–2	900	100 ± 0	40.5 ± 4.2	3
C1257	40,000	ND	ND	ND
C4455	100,000	ND	ND	1
C5424	200,000	ND	ND	0
C6433	30,000	ND	ND	ND
Cep511	80,000	98 ± 3	12.7 ± 1.53	0
B. stabilis strains				
ATCC BA-67	2,000,000	13 ± 12	16.0 ± 5.6	0
C7322	>2,000,000	10 ± 17	ND	0
B. vietnamiensis strains				
DBO1	200,000	ND	ND	ND
PC259	>3,000,000	7 ± 6	19.7 ± 0.6	1
B. dolosa strains				
AU0645	>4,000,000	25 ± 0	ND	1
STM1441	40,000	ND	ND	2
B. ambifaria strain				
Cep0996	800,000	53 ± 16	ND	3
B. anthina strain				
J2552	300,000	ND	ND	3
B. pyrrocinia strain				
ATCC 15958	300	ND	ND	3

[a] Data are from Bernier et al. (3).
[b] Data are from Cardona et al. (5). Score is indicated by the number of disease symptoms from 0 for no signs of disease to 3 for three disease symptoms.
[c] Values are means ± standard deviations.
[d] ND, not determined.

simple to use as the *G. mellonella* model. This was despite the fact *G. mellonella* possesses a relatively complex innate immune response, which is lacking in plant infection models. Furthermore, due to ethical and regulatory issues, it is now difficult to perform LD_{50} experiments with mice. In addition, amoebae and *C. elegans* are somewhat more difficult to work with, and the BCC do not appear to give consistent results in *C. elegans*. All of these problems are eliminated or resolved by using the *G. mellonella* infection model. In a direct comparison of several BCC strains infecting *G. mellonella*, alfalfa seedlings, or *C. elegans*, we observed that the *G. mellonella* model was the most sensitive and consistent infection model with respect to the BCC, as well as being easy to use and relatively rapid (data not shown). We therefore proceeded to characterize this BCC infection model further.

BCC species have previously been characterized as having differing pathogenicity levels (3, 7). *B. cenocepacia* strains have been observed to cause a greater degree of illness than *B. multivorans* in a murine model of pulmonary infection (7). Similarly, Bernier et al. (3) was able to demonstrate that most BCC species exhibit similar levels of pathogenicity in an alfalfa infection model as well as a rat agar bead infection model and that *B. multivorans* strains did not produce severe symptoms of lung pathology in the rat agar bead model or disease symptoms in the alfalfa model. These findings are consistent with the results we obtained for the BCC with the *G. mellonella* infection model (Fig. 1; Tables 1 and 2). We have demonstrated that *B. multivorans* strains are relatively avirulent in *G. mellonella*. In contrast, the most virulent BCC strains tested in *G. mellonella* belonged to the BCC species *B. cepacia* and *B. cenocepacia*, and these were also the most virulent strains tested in the rat agar bead model and the alfalfa model (3). In the leukopenic mouse model (7), *B. cenocepacia* was more damaging to lung tissue but persisted less well than *B. multivorans*, which in general persisted longer but without toxicity. Two of the most virulent BCC strains tested in the rat agar

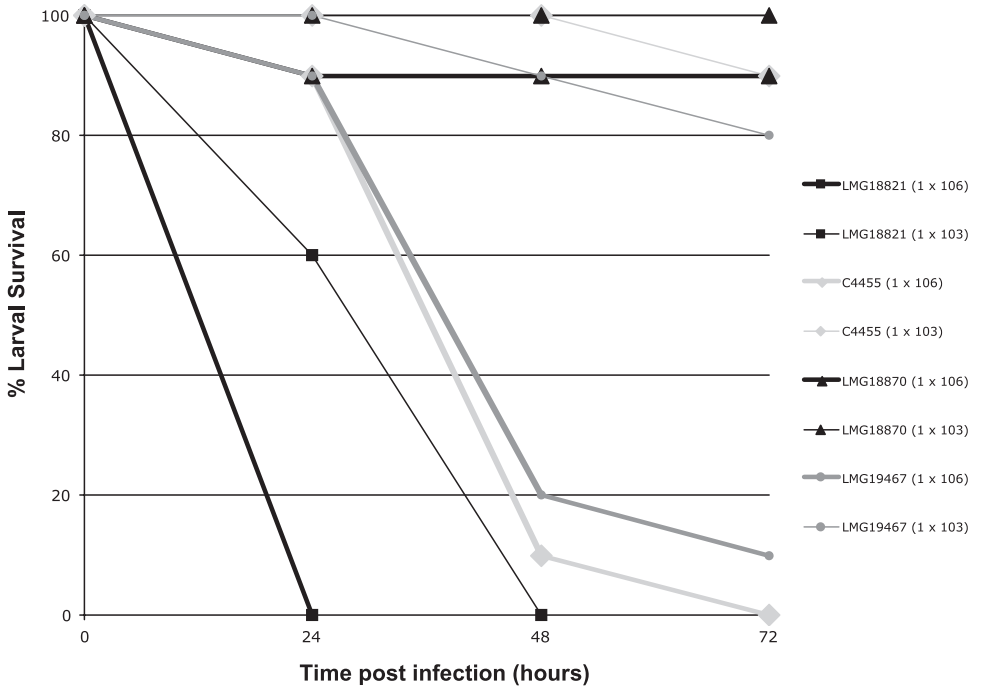

FIG. 2. *G. mellonella* larvae survival over time when infected with strains of the *Burkolderia cepacia* complex. *Galleria mellonella* larvae were injected with bacteria (1×10^6 or 1×10^3) and monitored for their survival over a 72-h period. Each data set is representative of a single trial with the specified strain ($n = 10$). No more than one uninfected control larvae died in any given trial.

bead model (*B. cepacia* Cep509 and *B. cenocepacia* K56-2) were also two of the most virulent BCC strains in the *G. mellonella* model, with LD_{50}s of 30 and 900, respectively. *B. stabilis* strains tested in these models were found to be rapidly cleared or relatively avirulent (3, 7), which is consistent with what we have observed in the *G. mellonella* infection model. Therefore, in general, there is a good correlation between the pathogenicity of BCC species infecting *G. mellonella* and that observed in higher mammalian models.

In support of these findings suggesting *G. mellonella* is an ideal infection model for the BCC, a significant positive correlation was previously observed between the virulence of *P. aeruginosa* PA14 mutants in mice and *G. mellonella* (24). However, no significant correlation was observed between the virulence of these bacterial strains in mice compared to that observed in plants or nematodes, suggesting that *G. mellonella* may be a more predictive alternative model system for studying the infection process in mammals (24). Similarly, a good correlation was observed between the virulence of *C. albicans* mutants in the *G. mellonella* model and the virulence measured in a mouse model of infection (4).

In comparison to other BCC alternative infection models, *G. mellonella* may prove to be the most useful infection model currently available. For example, no special equipment is required for either the alfalfa or *G. mellonella* models; however, results can be obtained from the *G. mellonella* model within 72 h versus 9 days for the alfalfa model (3). Bacterial virulence in the alfalfa model is quantified based on the visual assessment of symptoms, such as yellow leaves, brown necrotic regions, and stunted roots, whereas the *G. mellonella* model uses larvae death as a finite assay end point. Furthermore, bacteria are exposed to and must overcome a relatively sophisticated innate immune system in the *G. mellonella* larvae versus that found in an alfalfa seedling.

With respect to nematode models of infection, BCC pathogenicity results are particularly inconsistent. For example, Markey et al. (35) reported that *B. cenocepacia* strain C5424 effectively kills *C. elegans*, while Cardona et al. (5) reported that the same strain was nonpathogenic toward *C. elegans*. Furthermore, Cardona et al. indicated that *B. cenocepacia* strains that are considered to be clonal exhibit considerably different pathogenicity phenotypes in *C. elegans* (5). These discrepancies suggest that results obtained with BCC using this infection host are not necessarily reliable. One potential reason for this inconsistency is that *C. elegans* can experience either fast or slow killing when exposed to bacterial pathogens (26). Furthermore, *C. elegans* is unable to survive temperatures similar to those observed in animal models, its optimal growth temperature being 20 to 23°C (28). Temperature-associated changes in bacterial virulence can be addressed in the *G. mellonella* infection model because wax worm larvae can be maintained at temperatures up to 37°C. In addition, both nematode age and the growth medium noticeably impact the ability of the BCC to kill *C. elegans* (26, 28), whereas *G. mellonella* larvae do not require feeding during the course of the infection. Finally, in the fast killing model, bacterial pathogenicity is based on subjective indices of *C. elegans* appearance, such as reduced locomotive capacity.

Free-living amoebae have been proposed as a reservoir for the acquisition and transmission of the BCC, and therefore the BCC have been investigated for their ability to cause infection in *Acanthamoeba* species (27, 36). Approximately one-third of

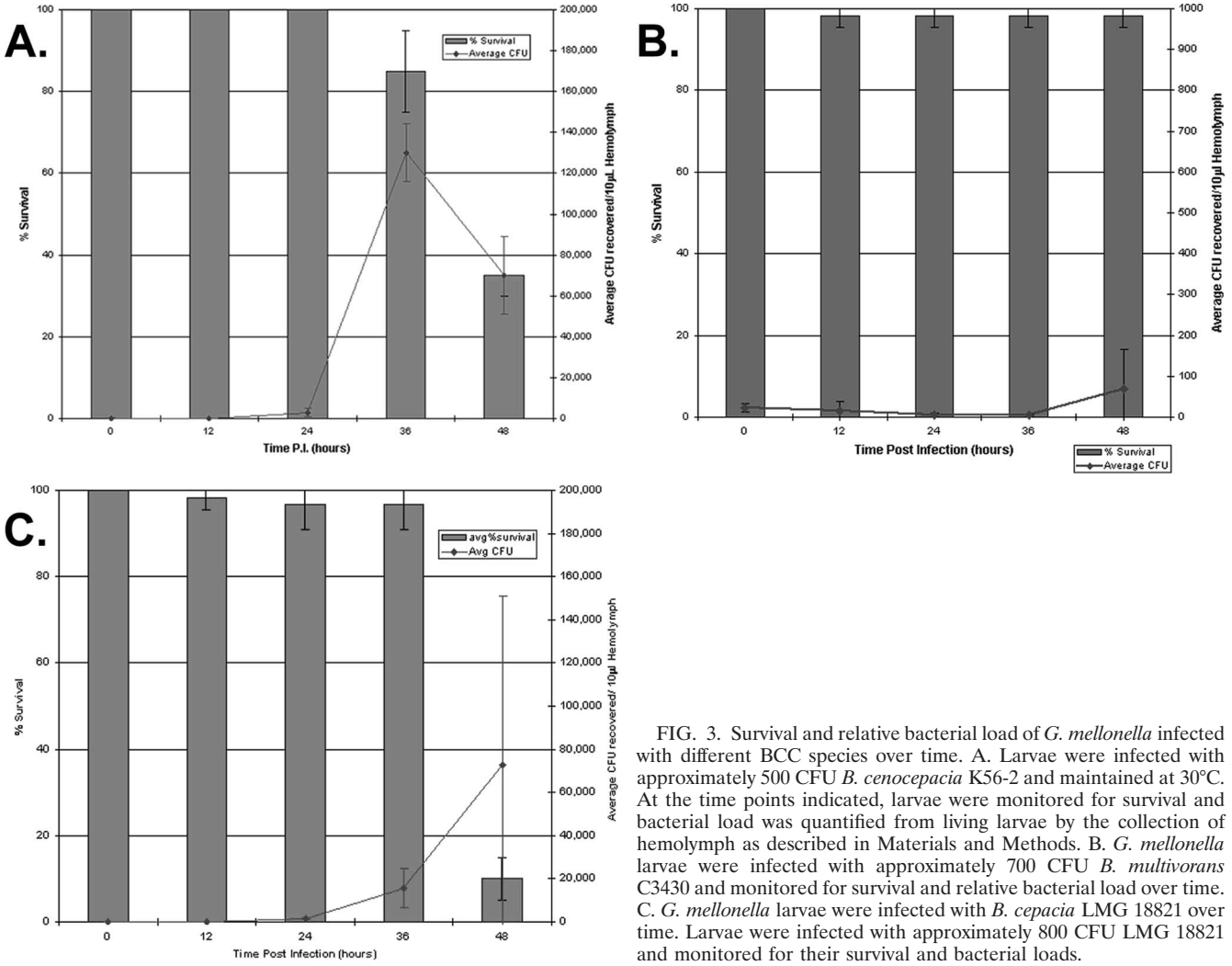

FIG. 3. Survival and relative bacterial load of *G. mellonella* infected with different BCC species over time. A. Larvae were infected with approximately 500 CFU *B. cenocepacia* K56-2 and maintained at 30°C. At the time points indicated, larvae were monitored for survival and bacterial load was quantified from living larvae by the collection of hemolymph as described in Materials and Methods. B. *G. mellonella* larvae were infected with approximately 700 CFU *B. multivorans* C3430 and monitored for survival and relative bacterial load over time. C. *G. mellonella* larvae were infected with *B. cepacia* LMG 18821 over time. Larvae were infected with approximately 800 CFU LMG 18821 and monitored for their survival and bacterial loads.

B. cenocepacia strains tested in this model were found to infect *Acanthamoeba*, although most of the strains that tested positive for infection did so at the lower levels of scored infectivity, compared to *P. aeruginosa* PAO1 (36). Somewhat surprisingly, many strains of *B. cepacia* and *B. vietnamiensis* were able to infect *Acanthamoeba* species, while almost all *B. multivorans* and *B. cenocepacia* strains were noninfective (36). Therefore, this model may have limited use as a BCC infection model, given that the vast majority of clinical isolates are *B. multivorans* and *B. cenocepacia* (42). However, these results do support the idea that there are general differences in virulence between the BCC species, even though strain-to-strain variation does occur.

In order to examine the sensitivity of *G. mellonella* to the BCC in more detail, we observed the rate at which killing occurred in four different BCC species (Fig. 2). These results showed that in this model, as in other animal models, *B. cepacia* is exquisitely toxic, even at lower bacterial challenge concentrations. The nature of this toxicity is unknown; however, it is interesting that one strain of *B. cepacia* tested (ATCC 17759), originally isolated from soil, had an LD_{50} of 1 in *G. mellonella*. This is equivalent to the LD_{50} previously determined for *P. aeruginosa* in the *G. mellonella* infection model (LD_{50} of 1 to 10) (24). *B. cenocepacia* and *B. ambifaria* were less virulent but still able to kill 90% and 80% of the larvae, respectively, within 48 h at 10^6 CFU. However, this killing appeared to be dependent upon the bacterial concentration, since 10^3 CFU of either BCC strain did not result in more than 20% larval death, even at 72 h p.i. This suggests that either the mechanism of killing is significantly different in *B. cenocepacia* and *B. ambifaria* versus *B. cepacia* infections or that there is a similar mechanism of killing for all BCC strains but differences in virulence factor expression levels. Multiple mechanisms of killing have been reported for *P. aeruginosa* (19). In contrast, *B. stabilis* was avirulent in *G. mellonella* at 10^3 CFU and killed only 10% of the infected larvae at 10^6 CFU. This indicates that *B. stabilis* is relatively nonpathogenic in vivo, at least in this *G. mellonella* model, and this correlates well with its relative nonprevalence as a human pathogen (42).

In order to better understand what was occurring during BCC infection in vivo, we examined the bacterial loads of infected *G. mellonella* larvae over time, in three different BCC strains (Fig. 3). Prior to the majority of larval death at 36 h p.i., the level for *B. cenocepacia* K56-2 was significantly higher ($P =$

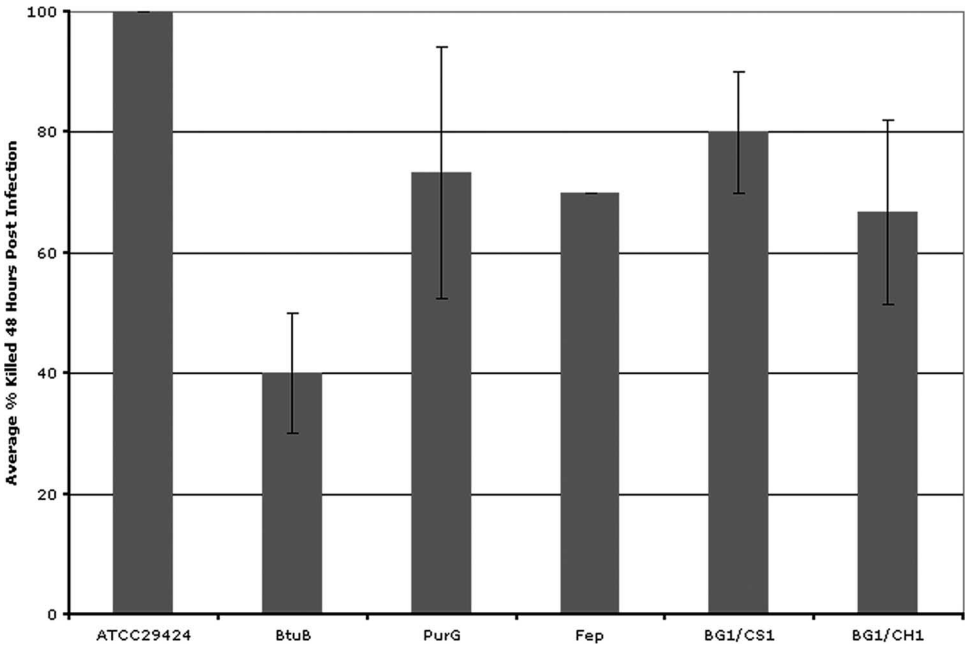

FIG. 4. Ability of *B. vietnamiensis* ATCC 29424 mutants to cause disease in the *G. mellonella* model. Larvae were injected with between 5×10^6 and 9×10^6 CFU. Three trials were performed in which 10 larvae were injected for each strain. Larvae were scored as dead or alive 48 h p.i. at 30°C.

<0.001) than the level for *B. cepacia* LMG 18821 in infected larvae. This suggests that the increased virulence of *B. cepacia* LMG 18821 compared to *B. cenocepacia* K56-2 (as also indicated by their LD_{50} values) is not mediated by high levels of bacterial growth within the hemolymph. Instead, these data suggest that *B. cepacia* LMG 18821 exerts its toxic effect on wax worm larvae by another means. In comparison, *B. multivorans* C3430 exhibited poor growth in vivo as determined by viable bacterial counts, even at 48 h p.i., and subsequently showed little toxicity toward the *G. mellonella* larvae. The bacterial loads at 48 h p.i. could not be determined as accurately in *G. mellonella* infected with the more virulent BCC strains, because bacteria could not be easily recovered from coagulated and melanized dead or nearly dead larvae. This unfortunately resulted in substantial amounts of error for bacterial numbers of *B. cepacia* LMG 18821 collected at 48 h p.i. (Fig. 3C).

As the pathogenic differences between BCC species are not well understood, the use of this *G. mellonella* model may provide a high-throughput, cost-effective screen with which to better elucidate the virulence mechanisms underlying these differences. As shown in Fig. 4, we tested several BCC genetic mutants in the *G. mellonella* model to demonstrate the usefulness of this approach. Compared to wild-type *B. vietnamiensis* ATCC 29424, all of the mutants displayed significantly less virulence toward *G. mellonella* larvae. We anticipate that some of these mutant strains are defective in a system important for in vivo growth. Proteins such as those involved in iron uptake (Fep and ExbB) have been previously been shown to be virulence factors, though not necessarily in the BCC. Under some growth conditions, purine pathway mutations will reduce physiological fitness, and this appears to be the case for the PurG mutant. However, we did not predict that a BCC mutation to *btuB*, the gene encoding the outer membrane receptor for vitamin B_{12}, would produce such a significant reduction to BCC virulence. The results suggest that the BtuB mutation in *B. vietnamiensis* ATCC 29424 is significantly less virulent than the other mutants examined with virulence defects, and it is approximately 40% less virulent than the parental strain. Because this protein is as yet uncharacterized in BCC, it is impossible to know whether its function is similar to the BtuB protein in *E. coli*. However, this result clearly demonstrates the value of in vivo screening for virulence factors.

In conclusion, the differences in virulence toward *G. mellonella* observed between species of the BCC coincide with observations made using other BCC infection models. The *G. mellonella* infection model can be used to detect pathogenicity differences between both BCC species as well as different strains within a BCC species. It is likely that bacterial pathogens like the BCC use common virulence factors to infect different hosts. We have demonstrated that insect model systems can be useful for the identification and characterization of BCC virulence factors involved in causing disease in vivo. This model should provide a cost-effective, practical, ethically acceptable, user-friendly alternative for the study of the BCC which will further our understanding of this diverse group of opportunistic bacterial pathogens.

ACKNOWLEDGMENTS

This study was funded by an operating grant (to J.J.D.) from the Canadian Cystic Fibrosis Foundation. K.D.S. was supported by a PGS-D award from the Natural Sciences and Engineering Research Council of Canada.

We thank D. Henry, P. Sokol, and G. Zylstra for strains used in this study. We thank M. R. Clark for assistance with statistical analysis, and B. Gee, C. Handford, and C. Shipp for technical assistance in the construction of bacterial mutants.

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Editor: V. J. DiRita