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Comparative Analysis of Plant and Animal Models for Characterization of *Burkholderia cepacia* Virulence

Steve P. Bernier,[1] Laura Silo-Suh,[2]† Donald E. Woods,[1] Dennis E. Ohman,[2,3] and Pamela A. Sokol[1]*

Department of Microbiology and Infectious Diseases, University of Calgary Health Sciences Center, Calgary, Alberta T2N 4N1, Canada[1]; Department of Microbiology and Immunology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298-0678[2]; and McGuire Veteran Affairs Medical Center, Richmond, Virginia 23249[3]

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A simple alfalfa model was developed as an alternative infection model for virulence studies of the *Burkholderia cepacia* complex. Symptoms of disease were observed in wounded alfalfa seedlings within 7 days following inoculation of 10^1 to 10^5 CFU of most strains of the *B. cepacia* complex. Strains from seven genomovars of the *B. cepacia* complex were tested for virulence in the alfalfa model, and the degree of virulence was generally similar in strains belonging to the same genomovar. Strains of *Burkholderia multivorans* and some strains of *Burkholderia stabilis* did not cause symptoms of disease in alfalfa seedlings. Representative strains were also tested for virulence using the rat agar bead model. Most of the strains tested were able to establish chronic lung infections; *B. stabilis* strains were the exception. Most of the strains that were virulent in the alfalfa infection model were also virulent in the lung infection model. The *B. cepacia* genomovar III mutants K56*pvdA*::tp and K56-H15 were significantly less virulent in the alfalfa infection model than their parent strain. Therefore, this alfalfa infection model may be a useful tool for assessing virulence of strains of the *B. cepacia* complex and identifying new virulence-associated genes.

Burkholderia cepacia is a gram-negative bacillus commonly found in soil, vegetation, and water (10, 13). *B. cepacia* was originally described as a phytopathogen causing soft rot in onions by Burkholder in 1950 (2). Individual strains of *B. cepacia* may have the ability to protect plants from disease, cause plant disease, degrade environmental pollutants, or cause nosocomial infections (23). *B. cepacia* has emerged as an important opportunistic pathogen in immunocompromised patients including those with cystic fibrosis (CF) and chronic granulomatous disease (8, 10). Furthermore, patient-to-patient transmission of virulent strains (16) and multiple antibiotic resistance of *B. cepacia* (31) are significant concerns within the CF research community. Pulmonary infection with *B. cepacia* can lead to three different outcomes: long-term asymptomatic colonization, chronic colonization with a progressive pulmonary decline, and a rapid pulmonary decline with necrotizing pneumonia, fever, and occasional septicemia often referred to as cepacia syndrome (23).

Recently, *B. cepacia* has been classified into nine genotypically distinct but phenotypically similar species (genomovars) forming the *B. cepacia* complex, all of which may infect CF patients. The nine species of the *B. cepacia* complex are as follows: *B. cepacia* genomovar I, *Burkholderia multivorans* (formerly genomovar II), *B. cepacia* genomovar III, *Burkholderia stabilis* (formerly genomovar IV), *Burkholderia vietnamiensis* (formerly genomovar V) (41), *B. cepacia* genomovar VI (6), *Burkholderia ambifaria* (formerly genomovar VII) (7), *Burkholderia anthina* (40), and *Burkholderia pyrrocinia* (8). In Canada, approximately 80% of the *B. cepacia* complex strains that colonize CF patients belong to genomovar III, which has generally been associated with a high rate of mortality and morbidity (36). In the United States, approximately 50% of CF isolates belong to genomovar III, and 38% belong to *B. multivorans* (15).

B. cepacia secretes extracellular virulence factors in vitro, including hemolysin, proteases, lipase, pili, and siderophores (10). The roles of only a few of these virulence factors have been examined in animal infection models (28, 32–24). Studying virulence in in vivo animal models is often complex, time-consuming, and expensive. Because of these limitations, alternative host models have been developed for some important human pathogens. For example, a multihost pathogenesis system has been used to identify virulence-associated genes in *Pseudomonas aeruginosa*. Alternative host models used include *Arabidopsis thaliana* (24–26), *Caenorhabditis elegans* (38, 39), *Drosophila melanogaster* (9), and *Galleria mellonella* (14). By using this multihost pathogenesis system, some virulence-associated genes, such as *gacA*, *lasR*, *ptsP*, and *phzB,* have been shown to be important both in nonmammalian hosts and in the mouse burn infection model (18). Recently, a new, simple, and inexpensive plant model has been developed in alfalfa for *P. aeruginosa* (30).

Although appropriate animal models are currently used for studying *B. cepacia* complex chronic respiratory infections (5, 27, 32, 33), alternative infection models for preliminary analysis of virulence of the *B. cepacia* complex have not yet been described. The objectives of this study were to determine

* Corresponding author. Mailing address: Department of Microbiology and Infectious Diseases, University of Calgary Health Sciences Center, 3330 Hospital Dr., N.W., Calgary, Alberta, Canada T2N 4N1. Phone: (403) 220-6037. Fax: (403) 270-2772. E-mail: psokol@ucalgary.ca.

† Present address: Department of Biological Sciences, Auburn University, Auburn, AL 36849.

TABLE 1. Comparison of the virulence of strains of the *B. cepacia* complex in an alfalfa infection model

Strain	Source and location[a]	% Seedlings with symptoms[b]	No. of CFU (10^7) recovered/seedling[c]	Reference
B. cepacia genomovar I				
ATCC 25416	Onion, US	73 ± 14	5.5 ± 3.6	19
ATCC 17759	Soil, Trinidad	73 ± 13	15.9 ± 14	19
Cep509	CF, Australia	85 ± 18	8.9 ± 8.1	19
B. multivorans				
ATCC 17616	Soil, US	0	1.1 ± 0.6	19
C5393	CF, Canada	0	1.3 ± 0.2	19
C1576	CF-e, UK	0	1.3 ± 0.8	19
LMG 13010	CF, Belgium	0	0.5 ± 0.4	19
CF-A1-1	CF-e, UK	0	0.8 ± 0.6	19
JTC	CGD, US	0	1.0 ± 0.7	19
C1962	Clinical, UK	0	1.2 ± 0.8	19
B. cepacia genomovar III				
K56-2	CF-e, Canada	100 ± 0	8.9 ± 3.6	19
ATCC 17765	UTI, UK	32 ± 13	8.1 ± 6.6	19
PC715j	CF, Canada	80 ± 18	8.8 ± 3.5	21
J2315	CF-e, UK	65 ± 10	3.9 ± 5.1	19
BC7	CF-e, Canada	65 ± 13	1.8 ± 2.6	19
Cep511	CF-e, Australia	98 ± 3	12 ± 12	19
PC184	CF, US	22 ± 13	3.4 ± 2.5	19
J415	CF, UK	53 ± 3	4.0 ± 4.3	19
B. stabilis				
LMG 14086	Respirator, UK	0	0.7 ± 0.4	19
LMG 18888	Clinical, Belgium	0	0.7 ± 0.5	19
LMG 14294	CF, Belgium	13 ± 12	0.9 ± 0.5	19
C7322	CF, Canada	10 ± 17	0.3 ± 0.2	19
B. vietnamiensis				
PC259	CF, US	7 ± 6	0.8 ± 0.7	19
LMG 16232	CF, Sweden	33 ± 10	12 ± 12	19
FC441	CGD, Canada	68 ± 8	23 ± 10	19
LMG 10929	Rice, Vietnam	42 ± 16	10 ± 6	19
B. cepacia genomovar VI				
LO6	CF	53 ± 24	13 ± 11	6
LMG 18943	CF, US	25 ± 0	1.7 ± 1.1	6
B. ambifaria				
ATCC 53266	Corn roots, biocontrol, US	63 ± 20	4.7 ± 1.9	7
AMMD	Pea rhizosphere, biocontrol, US	65 ± 20	7.2 ± 2.5	7
Cep996	CF, Australia	53 ± 16	4.3 ± 3.3	7

[a] Abbreviations: CF, infection of a CF patient; CF-e, strain that spread epidemically among patients with CF; CGD, infection of a patient with chronic granulomatous disease; UTI, patient with a urinary tract infection; US, United States; UK, United Kingdom.

[b] Values are means ± standard deviations of three assays (20 seedlings/assay). The starting inoculum was between 1×10^5 and 3×10^5 CFU for all experiments. Any seedlings with visible symptoms including yellow leaves, stunted root, and brown necrotic regions were considered positive for symptoms of disease.

[c] Values are means ± standard deviations of two assays (three seedlings/assay).

whether the *B. cepacia* complex was able to cause infection in alfalfa and to determine whether an alfalfa model could be used to differentiate genomovars of the *B. cepacia* complex. We also compared virulence in the alfalfa model and the well-established rat agar bead chronic infection model (3).

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains of the *B. cepacia* complex used in this study are described in Table 1. Most of the strains are from the experimental panel of the *B. cepacia* complex (19). All strains in this study were stored in 10% skim milk (BBL, Sparks, Md.) at −70°C. The strains were streaked from frozen stocks onto Luria-Bertani (LB) (Invitrogen, Burlington, Ontario, Canada) agar plates and incubated at 37°C for 24 to 48 h. For plant inoculation, cultures grown in LB medium overnight were diluted (1:25) with 12-ml portions of fresh LB medium in 125-ml Erlenmeyer flasks and incubated at 37°C with shaking to an approximate optical density at 600 nm of 2.0. For animal experiments, cultures were grown in dialyzed and chelated Trypticase soy broth (22) medium overnight.

Alfalfa infection assay by strains of the *B. cepacia* complex. Alfalfa seeds (variety 57Q77) were provided by Pioneer Hi-Bred International, Inc. (Johnston, Iowa). Alfalfa seeds were prepared as previously described (30). To disinfect and accelerate germination, they were immersed in concentrated sulfuric acid (approximately 10 ml for 300 seeds) for 20 min and then washed with 500 ml of distilled water (dH_2O) four times. The seeds were covered with 60 ml of sterile dH_2O in a 125-ml Erlenmeyer flask and incubated at 32°C with shaking for 6 to 8 h to encourage uniform imbibition and germination (11). The seeds were rinsed twice with 60 ml of sterile dH_2O and incubated overnight in 60 ml of sterile dH_2O at 32°C with shaking. The following day, the seedlings (10 per plate) were placed with sterile forceps onto the surface of water agar (deionized water solubilized with 1% Difco Bacto Agar and 1% Difco Noble agar).

Within 1 h, one leaf of each seedling was wounded by piercing the leaf with a 20-gauge needle. Immediately after the leaves were wounded, the seedlings were

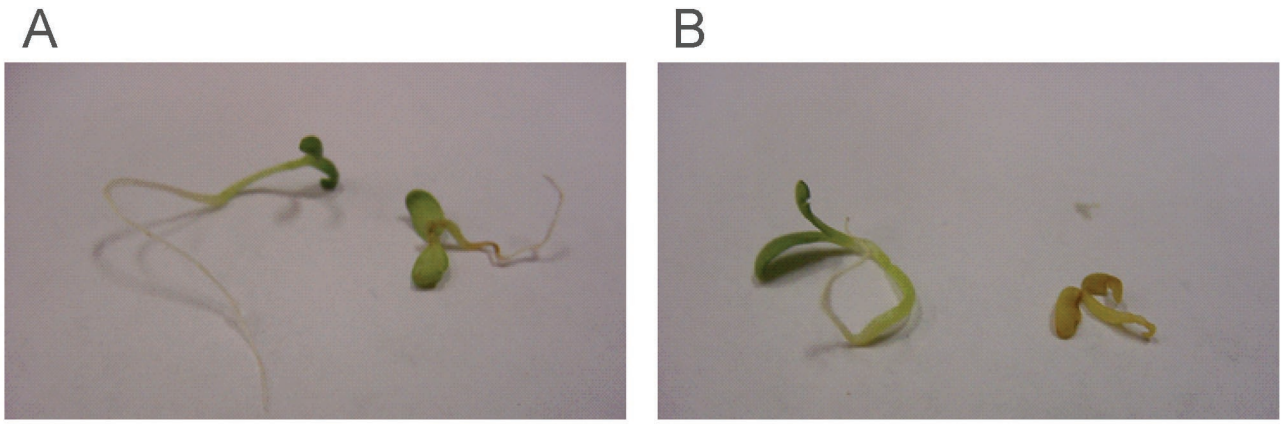

FIG. 1. Effect of temperature on visual symptoms of pathology on alfalfa. Photographs of wounded alfalfa seedlings after inoculation with 10^5 CFU of *B. cepacia* K56-2 are shown. Seedlings were incubated at 30°C (A) and 37°C (B). In each panel, the seedling on the left is the negative control (inoculated with saline), and the seedling on the right was inoculated with K56-2.

surface inoculated with 10-μl aliquots of diluted bacterial cells. The cultures used for the inoculum were serially diluted in 0.85% NaCl, and aliquots were plated onto LB agar plates for quantitation. Controls included seedlings wounded and inoculated with 10 μl of 0.85% NaCl and untreated seedlings. The petri plates containing seedlings were sealed with parafilm in order to maintain a high level of humidity and incubated in a 37°C warm room under a desk lamp with an average of 8 to 12 h of artificial light per day at an intensity of 640 lx. The seedlings were visually monitored for disease symptoms at 7 days postinfection (p.i.). Symptoms, including yellow leaves, stunted roots, and brown necrotic regions on the seedlings, were considered disease symptoms. At least 20 seedlings were used per bacterial inoculum.

Recovery of bacteria from infected alfalfa seedlings. For each strain, six seedlings (three seedlings/assay) were homogenized in a Kontes tissue grinder in 1 ml of 0.85% NaCl. The resulting suspension was serially diluted in 0.85% NaCl and plated onto LB agar to determine the number of CFU per seedling. Seedlings with disease symptoms were randomly selected for bacterial quantitation except for seedlings inoculated with strains that did not cause symptoms of disease.

Animal studies. Groups of nine male Sprague-Dawley rats (150 to 175 g) (Charles River Canada, Inc.) were given tracheostomies under anesthesia and inoculated with approximately 10^4 CFU of the appropriate strain embedded in agar beads as previously described (3). On days 7 and 21 p.i., the lungs from three animals from each group were removed aseptically and homogenized (Polytron homogenizer; Brinkman Instruments, Westbury, N.Y.) in 3 ml of phosphate-buffered saline (PBS) (10 mM sodium phosphate, 150 mM NaCl [pH 7.5]). The homogenates were serially diluted in PBS and plated on Trypticase soy agar and *Burkholderia cepacia* isolation agar (12). On day 7 p.i., the lungs of three additional animals from each group were removed en bloc, fixed in 10% formalin, and examined for qualitative and quantitative pathological changes. Infiltration of the lung with inflammatory cells and exudate was measured using a point counting method as previously described (35) with the following modifications. The lung sections were scanned using an Epson 1650 scanner, and areas of inflammation were digitized with Scion Image software and reported as the percentage of lung inflammation.

Statistical analyses. Analysis of variance (ANOVA) and linear regression were performed with INSTAT software (GraphPad Software, San Diego, Calif.). A P value of <0.05 was considered statistically significant.

RESULTS

Ability of *B. cepacia* strain K56-2 to infect wounded alfalfa. To determine whether *B. cepacia* strain K56-2 could cause infections in alfalfa, wounded plant seedlings were inoculated with doses of bacteria ranging from 10^1 to 10^5 CFU and were examined for symptoms. Seedlings infected with strain K56-2 had symptoms of disease that were clearly visible 7 days p.i. (Fig. 1B). At higher inoculations, 90% of the infected seedlings displayed symptoms of disease, including yellowing of the leaves and root necrosis when incubated at room temperature. Infection with as few as 10^1 CFU resulted in 40% of seedlings displaying symptoms (Fig. 2).

Effects of incubation temperature and inoculum dose on disease symptoms. To determine whether the incubation temperature of the seedlings affected disease symptoms, plant seedlings were infected with 10^1 to 10^5 CFU of *B. cepacia* strain K56-2 and incubated at room temperature, 30°C, and 37°C and examined for symptoms 7 days p.i. Both the inoculum dose and incubation temperature affected the percentage of seedlings developing symptoms of disease (Fig. 2). Incubation of the seedlings at 37°C resulted in a greater percentage of seedlings with disease at most inoculum doses (Fig. 2). These infected seedlings were smaller, with smaller yellow leaves, and stunted roots than those incubated at 30°C. There was also evidence of brown necrotic regions on the seedlings (Fig. 1). In subsequent experiments, seedlings were inoculated with 10^5 CFU and incubated at 37°C.

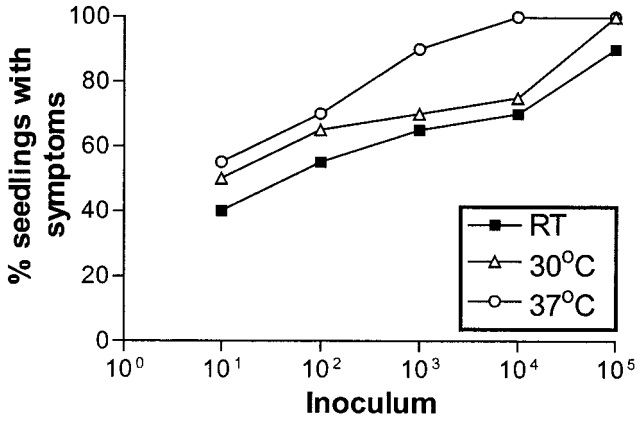

FIG. 2. Effects of temperature and inoculum on alfalfa infections. Groups of 20 seedlings were inoculated with doses of *B. cepacia* K56-2 ranging from 10^1 to 10^5 CFU and incubated at three different temperatures: room temperature (RT), 30°C, and 37°C. The results shown are the percentages of seedlings with disease symptoms visible on day 7 p.i.

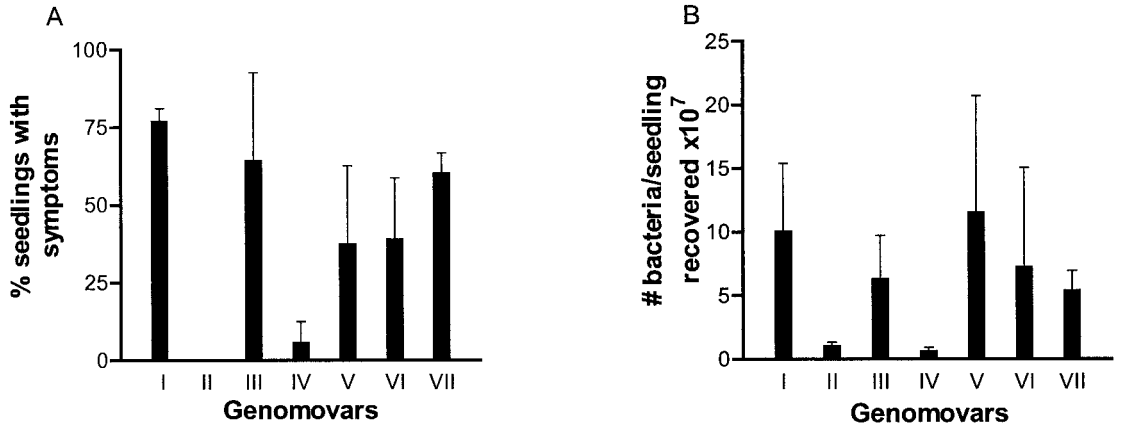

FIG. 3. Comparison of the ability of *B. cepacia* complex genomovars to cause infections in alfalfa. Seedlings were inoculated with 10^5 CFU of the strains shown in Table 1 and incubated at 37°C for 7 days. Data from Table 1 for the percentage of seedlings with symptoms of disease for each genomovar (A) and for the number of bacteria recovered (10^7) from individual homogenized alfalfa sprouts for each genomovar (B) are summarized. The means ± standard deviations (error bars) of the values in Table 1 are shown.

Ability of strains and genomovars of the *B. cepacia* complex to cause disease symptoms in alfalfa. Thirty-one strains from seven different genomovars of the *B. cepacia* complex were tested for their ability to cause disease in alfalfa. The percentages of seedlings with disease symptoms visible on day 7 p.i. for the strains are shown in Table 1. Although strain variability was noted within genomovars, the ability to cause disease symptoms in alfalfa generally was similar in the strains of a genomovar. *B. cepacia* genomovars I and III appeared to be the most virulent in the alfalfa infection model, whereas *B. multivorans* (genomovar II) and some strains of *B. stabilis* (genomovar IV) were avirulent (Table 1 and Fig. 3A). As shown in Fig. 3A, the mean percentage of seedlings with symptoms for all strains in *B. cepacia* genomovar I was 77% compared to 0% for *B. multivorans* ($P < 0.001$ by ANOVA) and 8% for all *B. stabilis* strains ($P < 0.001$ by ANOVA). *B. cepacia* genomovar III strains caused symptoms in 64% of seedlings and were also significantly more virulent than *B. multivorans* and *B. stabilis* ($P < 0.001$ by ANOVA). *B. ambifaria* (genomovar VII), which caused symptoms in 60% of alfalfa sprouts, was also more virulent than *B. multivorans* ($P < 0.01$ by ANOVA) and *B. stabilis* ($P < 0.05$ by ANOVA). *B. vietnamiensis* and *B. cepacia* genomovar VI were able to cause disease symptoms in alfalfa.

Interestingly, strains of *B. multivorans* and *B. stabilis*, with the exception of LMG 14294 and C7322, did not cause disease in the alfalfa model. There was no difference between seedlings infected with the other strains of these two genomovars and the negative-control seedlings.

Ability of strains of the *B. cepacia* complex to grow on wounded alfalfa seedlings. To determine whether the difference in the ability to cause disease in alfalfa in strains of the different genomovars was due to differences in the ability of the strains to grow on the alfalfa seedlings, the number of bacteria present on infected seedlings was determined on day 7 p.i. (Table 1). For all strains tested, the number of bacteria recovered was at least 10-fold higher than the inoculum, indicating that all strains were able to grow on alfalfa. *B. cepacia* complex was able to survive on the water agar medium without seedlings but was not able to grow (data not shown). Strains of *B. multivorans* and *B. stabilis* grew poorly, generally increasing by only 10- to 100-fold, whereas strains of the other genomovars increased by 100- to 1,000-fold (Table 1 and Fig. 3B). The reduced growth of *B. stabilis* and *B. multivorans* could account for their inability to cause disease symptoms, since there was a correlation between the number of bacteria recovered and the percentage of seedlings with symptoms in all of the strains examined ($r = 0.666$ by linear regression; $P < 0.0001$).

Comparison of *B. cepacia* complex strain virulence in the alfalfa infection model versus the rat agar bead infection model. Rats were infected with agar beads containing selected *B. cepacia* complex strains based on differences in their virulence properties in alfalfa. Quantitative bacteriology was performed on days 7 and 21 p.i. on lungs removed from infected animals, and quantitative histopathological analysis was performed on day 7 p.i. (Table 2). With the exception of *B. stabilis*, all the strains tested were able to establish a chronic infection (Table 2). Quantitative bacteriological analysis demonstrated that strains of *B. cepacia* genomovar I, *B. multivorans*, *B. cepacia* genomovar III, and *B. vietnamiensis* persisted in the lungs for at least 21 days. Genomovar III strains K56-2 and Pc715j have been previously shown to persist for at least 28 days (32, 34) and therefore were not tested in this study. *B. stabilis* strain LMG 14086 cleared within 21 days and strain LMG 14294 cleared by 7 days p.i., suggesting that these strains are not able to establish persistent infections in the agar bead infection model.

Most strains of *B. cepacia* genomovars I and III and *B. vietnamiensis* tested were virulent in this animal model, as determined by the percentage of the lungs infiltrated with inflammatory exudates. *B. cepacia* strains Cep509, K56-2, and J2315 were the most virulent strains tested in this animal model (Table 2). Two rats infected with strain J2315 died within 48 h p.i. Generally, infections with strains of *B. multivorans* and *B. stabilis* resulted in less lung pathology than infections with strains belonging to the other genomovars tested (Table 2). A correlation between virulence in the alfalfa infection model and virulence in the rat agar bead model was demonstrated, as measured by changes in lung histopathology

TABLE 2. Comparison of the virulence of strains of the *B. cepacia* complex in a chronic respiratory infection model

Strain	Virulence (CFU/ml/lung)[a]		% Pathology on day 7 p.i.[a]
	Day 7 p.i.	Day 21 p.i.	
B. cepacia genomovar I			
Cep509	$8.1 \times 10^5 \pm 6.4 \times 10^5$	$1.2 \times 10^5 \pm 1.4 \times 10^5$[b]	43.0 ± 2.0
B. multivorans			
C5393	$1.3 \times 10^6 \pm 1.4 \times 10^6$	$3.7 \times 10^5 \pm 1.5 \times 10^5$	13.3 ± 5.9
C1576	$1.2 \times 10^5 \pm 1.7 \times 10^5$	$4.5 \times 10^4 \pm 4.6 \times 10^4$	9.0 ± 4.6
B. cepacia genomovar III			
K56-2	$1.5 \times 10^6 \pm 2.4 \times 10^6$[e]	ND	40.5 ± 4.2[e]
PC715j	$3.5 \times 10^5 \pm 3.4 \times 10^5$[f]	ND	22.8 ± 10.8[f]
J2315	$4.6 \times 10^3 \pm 7.8 \times 10^3$	5.0×10^2[c]	38.0 ± 21.0
Cep511	$2.7 \times 10^5 \pm 2.2 \times 10^5$	$3.3 \times 10^3 \pm 5.1 \times 10^3$	12.7 ± 1.53
J415	$3.1 \times 10^5 \pm 5.2 \times 10^5$	$6.5 \times 10^3 \pm 1.2 \times 10^4$	13.0 ± 1.73
B. stabilis			
LMG 14086	$3.3 \times 10^2 \pm 2.9 \times 10^2$	0	20.0 ± 1.7
LMG 14294	0	0	16.0 ± 5.6
B. vietnamiensis			
PC259	$1.2 \times 10^4 \pm 2.0 \times 10^4$	$2.0 \times 10^2 \pm 1.7 \times 10^2$	19.7 ± 0.6
FC441	$8.5 \times 10^2 \pm 6.6 \times 10^2$	$7.1 \times 10^3 \pm 9.3 \times 10^3$[d]	29.0 ± 1.0

[a] Values are means ± standard deviations for three animals unless noted otherwise. ND, not determine
[b] Values are mean ± standard deviation for four animals.
[c] Values are for one animal (one animal died on day 1 p.i., and one animal died on day 2 p.i.).
[d] Values are mean ± standard deviation for five animals.
[e] Data previously reported by Sokol et al. (32).
[f] Values are means ± standard deviations for eight animals in the experiment previously described by Sokol et al. (32).

($r = 0.5913$ by linear regression; $P < 0.05$). Genomovar III strain Cep511 was an exception, since it was one of the less virulent strains in terms of lung histopathology yet caused disease symptoms in 98% of the alfalfa seedlings.

Ability of *B. cepacia* complex strain K56-2 mutants to cause disease in the alfalfa model. To determine whether the alfalfa model might be useful for assessing the virulence of *B. cepacia* complex mutants, the ability of selected mutants of strain K56-2 to cause disease symptoms in this model was examined. As shown in Table 3, K56*pvdA*::tp, which contains a deletion in a biosynthetic gene for production of the siderophore ornibactin (33), was significantly less virulent than K56-2. This mutant was able to grow on the wounded alfalfa but caused symptoms of disease in fewer plants. Interestingly, K56*orbA*::tp, which contains a mutation in the ornibactin receptor gene (32) was as virulent as K56-2 in this model. K56-2-9, which has a mutation in an extracellular zinc metalloprotease gene (8a), was also virulent in this model. K56-H15, a Tn*5*-OT182 insertion mutant, did not cause symptoms of disease in the alfalfa model and also did not grow as well as the parent strain on the wounded seedlings. Results with these mutant strains suggest that the alfalfa model may also be a useful tool in assessing the virulence properties of genetic mutants.

DISCUSSION

It has been known for decades that *B. cepacia* is a natural phytopathogen (2) and an opportunistic pathogen for CF patients (8, 10). Although several animal infection models have been used to study the virulence of strains of the *B. cepacia* complex (4, 5, 28, 32–24), this study describes a simple plant infection model that may have applications as an alternative model.

Silo-Suh et al. have used the wounded alfalfa model for

TABLE 3. Virulence of *B. cepacia* K56-2 mutants in the alfalfa model

Strain	Gene mutated	% Seedlings with symptoms[a]	No. of CFU (10^7) recovered/seedling[b]	Reference
K56-2 (wild type)		100	16 ± 6.4[c]	19
K56*pvdA*::tp	Ornibactin biosynthesis	30 ± 5[d]	1.5	33
K56*orbA*::tp	Ornibactin receptor	98 ± 3	8.3	32
K56-2-9	Zinc metalloprotease	100	6.5	8a
K56-H15	Tn*5*-OT182 insertion	3 ± 6[d]	0.29 ± 0.2[c,e]	Unpublished data

[a] Values are means ± standard deviations of three assays (20 seedlings/assay). The starting inoculum was between 1×10^5 and 3×10^5 CFU. Any seedlings with visible symptoms including yellow leaves, stunted root, and brown necrotic regions were considered positive for symptoms of disease.
[b] Values are means of one assay (two seedlings/assay) unless noted otherwise.
[c] Values are means ± standard deviations of two assays (two seedlings/assay).
[d] Significantly different from the value for K56-2 ($P < 0.001$ by ANOVA).
[e] Significantly different from the value for K56-2 ($P < 0.05$ by ANOVA).

P. aeruginosa. *P. aeruginosa* caused chlorosis symptoms, water-soaked lesions, and complete maceration of tissue at room temperature (30). The symptoms observed with *B. cepacia* complex infections were slightly different than those observed in *P. aeruginosa* infections. *B. cepacia* caused necrosis, evident by brown areas on the seedling, lack of root hair, stunting of root growth, and chlorosis, which is a yellowing of leaf tissue due to a lack of chlorophyll. *P. aeruginosa* infections appeared to remain localized to the leaves and resulted in a greater maceration of tissue. *B. cepacia* complex strain K56-2 exhibited similar symptoms of disease, whether or not the leaves were wounded prior to infection (data not shown). *B. cepacia* complex strain K56-2 and other *B. cepacia* complex strains were determined to be more virulent in seedlings incubated at 37°C than at room temperature. Previously, it was shown that the sour skin disease on onions caused by *B. cepacia* was most damaging at temperatures above 30°C (23). These studies suggest that there may be a difference in *B. cepacia* complex virulence factor expression between 37°C and lower temperatures.

The *Arabidopsis* infection model has been a successful tool to find virulence-associated genes in *P. aeruginosa* (18, 24–26). Although the virulence of the *B. cepacia* complex was not tested in the *Arabidopsis* model in this study, the simplicity of the alfalfa model offers several advantages over the *Arabidopsis* model. Studies with *Arabidopsis* are routinely performed using 3- to 8-week-old plants (24) and require special incubators and considerable space for plant growth. Results can be obtained from the alfalfa model within 9 days, and no special equipment is necessary. Most strains of *B. cepacia* complex were able to establish an infection in alfalfa, whereas only a few strains of *P. aeruginosa*, UCBPP-PA14 and UCBPP-PA29, were able to infect *Arabidopsis* leaves (25). Many strains of *P. aeruginosa* can also infect alfalfa (30); therefore, the alfalfa model applications are not limited to only a few strains. Although the *Arabidopsis* model has been very useful for assessing virulence factors in some strains of *P. aeruginosa*, the alfalfa model may be useful in studying the virulence of strains of several genomovars in the *B. cepacia* complex, with the possible exceptions of *B. multivorans* and *B. stabilis*. One advantage of the *Arabidopsis* model over the alfalfa model is that the *Arabidopsis* genome sequence is available, making it possible to investigate the effects of host genes on infection (1, 37). Because alfalfa cannot be easily manipulated genetically, it would be interesting to determine whether *B. cepacia* complex caused similar infections in seedlings of the related model legume *Medicago truncatula* (the *M. truncatula* genome sequence is being determined [15]).

The ability of bacteria to grow on alfalfa seedlings correlated with their ability to cause disease symptoms (Fig. 3). Less bacteria were recovered from seedlings infected with *B. multivorans* or *B. stabilis* strains than from seedlings infected with the other genomovars, and infection with these strains rarely resulted in disease symptoms. There were differences in the percentage of seedlings displaying symptoms in strains within a genomovar, suggesting that there is variability among strains of the same species.

For strains tested in both the alfalfa and rat agar bead models, there was a general correlation in virulence for the two infection models. *B. stabilis* was generally avirulent in both models. *B. multivorans* was able to persist in the lung but did not result in severe symptoms of lung pathology and also did not cause disease symptoms in alfalfa. Previously, the agar bead model has primarily been used for *B. cepacia* genomovar III strains, but this study shows that strains from five genomovars (genomovars I to V) can establish lung infections in this model, although *B. stabilis* strains appear to be cleared rapidly (Table 2). These observations correlate with those reported by LiPuma et al. (17), which demonstrate that all *B. cepacia* complex species (genomovars I to VII) have been found to infect CF patients. These results suggest that the agar bead model may be useful for virulence analysis in many genomovars of the *B. cepacia* complex.

The most virulent strains tested in either the alfalfa or rat agar bead infection model belonged to *B. cepacia* genomovar I or III (Fig. 3A and Table 1 and 2). Interestingly, *B. cepacia* genomovar III strains were also found to cause greater lung pathology than *B. multivorans* in a leukopenic mouse model (4). In the leukopenic mouse model, however, all strains of *B. cepacia* genomovar III cleared within 16 days, whereas genomovar III strains persisted in the agar bead lung infection model for at least 21 days p.i. The establishment of a persistent chronic infection by genomovar III strains in the agar bead model is more consistent with the clinical profile of patients infected with these strains. BC7, a genomovar III CF isolate, was shown to persist in the lungs of $Cftr^{-/-}$ mice for at least 9 days and result in lung histopathological changes (27). Genomovar III strains were also shown to be invasive in a mouse agar bead model and an A549 cell invasion assay (4). Preliminary data suggest an association between poorer clinical outcome and infection with strains of *B. cepacia* genomovar III versus *B. multivorans* (20).

Strains of *B. multivorans*, two strains of *B. stabilis*, and *B. vietnamiensis* PC259 were avirulent in the alfalfa model. *B. stabilis* strains cleared from the lungs in both the rat agar bead model and the leukopenic mouse model (4), suggesting that in contrast to genomovar III strains, a correlation exists between all three models for *B. stabilis*. Using well-differentiated airway epithelial cell cultures, Schwab et al. (29) demonstrated that unlike other species of the *B. cepacia* complex, *B. stabilis* does not produce biofilms. A *B. stabilis* strain was able to penetrate the epithelium, but the majority of bacteria were located between epithelial cells (29). In contrast, genomovar III strains formed biofilms, which subsequently invaded and destroyed epithelial cells (29). The inability to form biofilms or the reduced ability to disrupt the actin cytoskeleton may account for the reduced virulence of *B. stabilis* in lung infection models.

Although *B. cepacia* genomovar I strains are not isolated often from CF patients (17, 20), their virulence in both the alfalfa and agar bead models was comparable to that of genomovar III strains (Fig. 3A and Table 2). Genomovar I strains were not examined in the leukopenic mouse model or the human airway epithelial cell invasion assays (4, 29).

The *B. cepacia* mutants K56*pvdA*::tp, K56*orbA*::tp, and K56-2-9 were previously reported to be less virulent than K56-2 in the agar bead model, as measured by decreased persistence of bacteria in the lung and decreased lung histopathological changes (8a, 32, 33). In the alfalfa infection host model, only K56*pvdA*::tp and K56-H15 were less virulent than the wild-type strain (Table 3). The difference in the virulence of the

K56*pvdA*::tp and K56*orbA*::tp mutants on alfalfa may be explained by their different capabilities to utilize siderophore-mediated iron uptake systems. Both ferric-salicylic acid uptake and ferric-ornibactin uptake are defective in K56*pvdA*::tp, whereas only ferric-ornibactin uptake is defective in K56*orbA*::tp (32, 33). It is also possible that ornibactin has a direct effect on virulence in addition to its role in iron acquisition. The mutation responsible for the decrease in virulence in K56-H15 is currently under investigation. It will be interesting to determine the nature of this mutation and whether this mutant also has reduced virulence in the lung infection model.

In summary, we have developed an alfalfa infection model for *B. cepacia* complex strains. Of 31 strains tested, 21 strains belonging to seven different genomovars caused disease symptoms in alfalfa. Strains that were virulent in the alfalfa model were generally virulent in the rat agar bead model. Therefore, this alternative model may potentially be used to assess the virulence of strains, to identify new virulence-associated genes in *B. cepacia* complex strains, and to identify common virulence factors in both plant and animal infections.

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