

Identification and Onion Pathogenicity of *Burkholderia cepacia* Complex Isolates from the Onion Rhizosphere and Onion Field Soil[∇]

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Burkholderia cepacia complex strains are genetically related but phenotypically diverse organisms that are important opportunistic pathogens in patients with cystic fibrosis (CF), as well as pathogens of onion and banana, colonizers of the rhizospheres of many plant species, and common inhabitants of bulk soil. Genotypic identification and pathogenicity characterization were performed on *B. cepacia* complex isolates from the rhizosphere of onion and organic soils in Michigan. A total of 3,798 putative *B. cepacia* complex isolates were recovered on *Pseudomonas cepacia* azelaic acid tryptamine and trypan blue tetracycline semiselective media during the 2004 growing season from six commercial onion fields located in two counties in Michigan. Putative *B. cepacia* complex isolates were identified by hybridization to a 16S rRNA gene probe, followed by duplex PCR using primers targeted to the 16S rRNA gene and *recA* sequences and restriction fragment length polymorphism analysis of the *recA* sequence. A total of 1,290 isolates, 980 rhizosphere and 310 soil isolates, were assigned to the species *B. cepacia* (160), *B. cenocepacia* (480), *B. ambifaria* (623), and *B. pyrrocinia* (27). The majority of isolates identified as *B. cepacia* (85%), *B. cenocepacia* (90%), and *B. ambifaria* (76%) were pathogenic in a detached onion bulb scale assay and caused symptoms of water soaking, maceration, and/or necrosis. A phylogenetic analysis of *recA* sequences from representative *B. cepacia* complex type and panel strains, along with isolates collected in this study, revealed that the *B. cenocepacia* isolates associated with onion grouped within the III-B lineage and that some strains were closely related to strain AU1054, which was isolated from a CF patient. This study revealed that multiple *B. cepacia* complex species colonize the onion rhizosphere and have the potential to cause sour skin rot disease of onion. In addition, the onion rhizosphere is a natural habitat and a potential environmental source of *B. cenocepacia*.

Burkholderia (formerly *Pseudomonas*) *cepacia* was first described by W. H. Burkholder in 1950 as the phytopathogen responsible for causing sour skin, a bacterial soft-rotting disease of onion in New York (8). *B. cepacia* and two isolates of the related species *B. cenocepacia* have since been isolated from diseased onion bulbs in Japan (46), and *B. cenocepacia* has been isolated as the causal agent of fingertip rot disease of banana (26). Similarly, *B. cepacia* emerged as an important opportunistic human pathogen in the 1970s and 1980s following isolation from the respiratory tracts of patients with cystic fibrosis (CF) or chronic granulomatous disease (23, 32, 44). In addition, a variety of *Burkholderia* spp. have been characterized as important environmental strains, with phenotypes that include biological control of plant root-infecting fungi, plant growth promotion, nitrogen fixation, and biodegradation of recalcitrant compounds in soil (36).

During the last decade, advances in genetic techniques have facilitated a reassessment of the *B. cepacia* species framed against the background that organisms known as *B. cepacia*

included plant pathogens, human pathogens, biological-control strains, and strains active in biodegradation. The results from these genetic analyses revealed that bacteria formerly identified as *B. cepacia* represent a complex of genetically related yet phenotypically diverse strains collectively referred to as the *B. cepacia* complex (3). The *B. cepacia* complex currently encompasses at least nine distinct species, *B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. stabilis*, *B. vietnamiensis*, *B. dolosa*, *B. ambifaria*, *B. anthina*, and *B. pyrrocinia* (11, 12, 31, 37, 50). Strains from each of these species have been isolated from human CF patients, and all except *B. dolosa* have been isolated from the plant rhizosphere or soil (10). Currently, the species most commonly associated with clinical infection are *B. cenocepacia* and *B. multivorans* (10, 32). The species associated with natural occurrences of plant disease are *B. cepacia* and *B. cenocepacia* on onion and *B. cenocepacia* on banana (20, 24).

Since members of most species of the *B. cepacia* complex occur naturally in the environment and are opportunistic human pathogens, the source of strains infecting humans has been a subject of intense interest (2, 5, 22). The environmental distribution of *B. cepacia* complex strains includes the rhizospheres of several agricultural-crop plants, soil in woodland and urban habitats, and river water (1, 13, 15, 16, 34, 39, 41, 55). These results may implicate environmental sources as a natural niche for potentially human-pathogenic strains. This hypothesis was reinforced by Lipuma et al. (27) with genetic

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data linking the identities of CF patient isolates to isolates recovered from the environment. Concomitant with these results are epidemiological studies indicating that patient-to-patient acquisition of *B. cepacia* complex strains decreased dramatically after the introduction of infection control measures (32, 51). Thus, new acquisitions could be due to strains originating from the environment.

The risk posed to humans by naturally occurring strains of the *B. cepacia* complex requires further study. For example, the prevalence of each of the nine species in natural environmental settings remains to be determined by systematic study. Rhizosphere distribution studies have been conducted on maize and rice (15, 38, 55), both plant species that are not subject to infection by *B. cepacia* complex strains. In contrast, onion is a host of *B. cepacia*; however, only one study has examined the occurrence of *B. cepacia* populations associated with decayed onions and onion field soil (54), and this work was done prior to the current changes in the taxonomy of the species. Thus, the occurrence and potential pathogenicity of other species of the *B. cepacia* complex in the onion rhizosphere have not been fully evaluated. We hypothesized that because onion is a host for plant-pathogenic strains of *B. cepacia*, the onion rhizosphere would harbor populations of *B. cepacia* and possibly other *B. cepacia* complex species that were distinct from those previously found in the rhizospheres of maize and rice. In addition, we wondered if isolates of any other *B. cepacia* complex species that colonized the onion rhizosphere would also be pathogenic to onion. The aim of this work was to isolate, identify, and determine the pathogenicity of *B. cepacia* complex strains from onion field soil and the rhizosphere of onion and to genetically compare these strains with reference strains from humans and the rhizospheres of other plants.

MATERIALS AND METHODS

Sample collection, bacterial isolation, and culture conditions. Bulk soil and rhizosphere (soil loosely associated with onion roots) samples were collected on four and three sampling dates, respectively, from each of six commercial onion fields in Michigan during the 2004 onion-growing season. According to the U.S. Department of Agriculture Natural Resources Conservation Service Websoil Survey (<http://websoilsurvey.nrcs.usda.gov>), the soil type from each field sampled was a combination of Houghton, Adrian, and/or Palms muck high-organic-matter soil. Bulk soil samples were taken on 6 June (populations 1 to 3, located in Eaton county) and 14 June (populations 4 to 6, located in Allegan county). In subsequent samplings, both bulk soil and rhizosphere samples were taken. Populations 1 to 3 were sampled on 21 June (onion phenology, two-leaf seedling stage), 14 July (immature-bulb stage), and 31 August (mature-bulb stage). Populations 4 to 6 were sampled on 13 July (immature-bulb stage), 2 August (immature-bulb stage), and 26 August (mature-bulb stage). Global positioning satellite (GPSMAP 76S; Garmin International, Olathe, KS) coordinates were utilized so that subsamples (five per field) could be taken from the same locations at each of the sampling dates. Soil was collected using a standard soil probe that was surface sterilized between samplings. The onion roots were collected by gently removing whole onion plants from the soil. Each sample was placed into a sterile plastic bag and transported to the laboratory on ice for processing.

Bacterial isolation and selection of putative *B. cepacia* complex isolates. Soil and root samples were weighed (2-g fresh weight) and separately placed in 20 ml prechilled buffer (0.1 M potassium phosphate [pH 7.0], 0.1% peptone), and bacterial cells were dislodged by sonication for 7 min in an ultrasonic bath (model 250T; VWR Scientific, Houston, TX). Samples (0.1 ml) from appropriate serial dilutions of the sonicate were plated on trypan blue tetracycline (TB-T) (21) and *P. cepacia* azelaic acid tryptamine (PCAT) (7) semiselective media. The plates were incubated at 29°C for 96 h, following which 50 putative *B. cepacia* complex colonies were chosen (if present) for further analyses from each of the sampling substrates (soil and rhizosphere) and medium type (TB-T and PCAT) isolation combinations from the four sampling dates. The method of selecting

bacterial isolates involved placing the isolation plates on a 50-square numbered grid; a single colony from each of the 50 grids (if present) was chosen randomly for further testing. The total number of subpopulation samples was 84 (six populations times seven sampling substrates times two media). Thus, a potential maximum of 4,200 (84×50) putative *B. cepacia* complex colonies could have been isolated if at least 1 colony was present in each grid on each sampling plate. A final collection of 3,794 isolates was maintained for characterization. The bacterial isolates were maintained as stock cultures in 20% glycerol at -80°C .

Identification of isolates. A multistep hybridization and PCR method was used for identification of isolates to the individual *B. cepacia* complex species level. The isolates were grown on King's B (KB) agar medium (25) for 48 h at 29°C. Cells from a single colony were removed from the plates using a sterile toothpick and placed into 100 μl of lysis buffer (0.05 M KCl, 0.01 M Tris-HCl, 1% Tween 20, pH 8.3) in a sterile microcentrifuge tube. Genomic DNA for PCRs was prepared by lysing the cells at 100°C for 15 min. *B. cepacia* complex isolates were identified using hybridization with a 16S probe followed by a *recA* and 16S rRNA gene-targeted multiplex PCR assay as previously described (41, 42). Final identification to the species level was done using a restriction fragment length polymorphism analysis of *recA*-amplified *recA* as described previously (31, 37). The sequences of the *recA* primers (primers BCR1 and BCR2) and 16S rRNA gene primers (primers CeMV and Burk3) were as described previously (41). For DNA-DNA dot blot hybridization, 2 μl of cell lysate was spotted onto Hybond-N+ membranes (Amersham, Piscataway, NJ), and the DNA was cross-linked to the membranes using a Stratilinker (Stratagene, La Jolla, CA). Probe labeling, hybridizations, posthybridization washes, and detection of hybridizing sequences were conducted as described previously (41). The positive control strains used in hybridizations were gifts from C. F. Gonzalez and included *B. cepacia* ATCC 25416, *B. cenocepacia* K56-2, *B. multivorans* ATCC 17616, and *B. stabilis* DB01. The negative control strains used in hybridizations were *Escherichia coli* DH10B, *Pseudomonas aeruginosa* PAO1, and *Pseudomonas syringae* 7B12 and DC3000.

Onion tissue maceration model. Yellow globe onions (*Allium cepa*) grown in Michigan were used throughout this study in pathogenicity assays. The thin, papery covering, along with the outer bulb scale, of each onion was removed prior to quartering the bulbs with a sterile knife. The quartered individual onion scales were placed in a sterile aluminum pan (22.9 cm by 33.0 cm) containing two sheets of sterile Whatman no. 1 filter paper premoistened with 90 ml of sterile distilled water. The bacterial isolates used in the assays were grown overnight in KB broth. Individual onion scales were wounded on the inner surface with a sterile pipette tip (1- to 200- μl volume), and 5 μl of bacterial culture (10^7 CFU/ml) was inoculated into the wound. The onion scales were incubated at 30°C for 48 h. The degree of maceration was estimated by probing with a toothpick. A rating scale of 0 to 3 was used to indicate the degree of tissue maceration. A rating of 0 indicated no maceration, 1 indicated 1 to 33% macerated tissue area, 2 indicated 34% to 66% macerated tissue area, and 3 indicated 67% to 100% macerated tissue area. Inoculations with each isolate were replicated three times, and an average rating was tabulated. Negative controls consisted of no wounding and inoculation with *B. cepacia* strain ATCC 25416, and wounding and inoculation with KB broth. The positive control consisted of wounding and inoculation with *B. cepacia* strain ATCC 25416.

Phylogenetic analysis. The *recA* gene was chosen for phylogenetic analyses of *B. cepacia* complex species, as that sequence had been used previously for the same purpose by other research groups (37, 41). A representative sample of 26 *B. cepacia* complex isolates from onion was chosen for analysis. We initially focused our isolate characterization on population 6 because that population contained the largest number of *B. cepacia* complex isolates and isolates from all four species. We later included randomly selected isolates representative of each population and sampling date so that each population and sampling date was represented in the final collection. In addition, *recA* gene sequences of 17 reference *B. cepacia* complex strains were obtained from GenBank. Descriptions of all of these strains are given in Table 1. The oligonucleotide primers BCR1 and BCR2 (39) were used to amplify the 1,041-bp *recA* coding sequence. The PCR methodology and DNA fragment purification were as described previously (29); all DNA sequencing was done at the Research Technology Support Facility at Michigan State University.

Nucleotide sequences were aligned by eye using MacClade (30). Phylogenetic analyses were performed using PAUP*4.0b10 (47) via neighbor joining (NJ) and maximum parsimony (MP). NJ trees were generated using the algorithm of Saitou and Nei (45) with Kimura two-parameter distances. MP analysis employed heuristic searches with 10 sequences of random taxon addition and tree bisection and reconnection branch swapping. Support for groups obtained in phylogenetic analyses was assessed by bootstrap analysis (1,000 pseudoreplicates) using PAUP*4.0. Sequences from 17 reference strains obtained from

TABLE 1. Onion rhizosphere populations of *B. cepacia* complex species recovered on PCAT and TB-T media on three sampling dates in 2004

Species ^a	No. recovered on sampling date ^b					
	Day 172	Day 194	Day 195	Day 214	Day 243	Day 238
Population 1						
<i>B. cepacia</i>	0		0		16	
<i>B. cenocepacia</i> IIIB	0		57		38	
<i>B. ambifaria</i>	6		21		12	
<i>B. pyrrocinia</i>	0		0		0	
Total <i>B. cepacia</i> complex	6		78		66	
Population 2						
<i>B. cepacia</i>	0		0		0	
<i>B. cenocepacia</i> IIIB	0		44		33	
<i>B. ambifaria</i>	82		35		26	
<i>B. pyrrocinia</i>	0		0		0	
Total <i>B. cepacia</i> complex	82		79		59	
Population 3						
<i>B. cepacia</i>	0		0		0	
<i>B. cenocepacia</i> IIIB	13		90		30	
<i>B. ambifaria</i>	28		4		35	
<i>B. pyrrocinia</i>	0		0		0	
Total <i>B. cepacia</i> complex	41		94		65	
Population 4						
<i>B. cepacia</i>		0		0		13
<i>B. cenocepacia</i> IIIB		0		0		0
<i>B. ambifaria</i>		0		0		1
<i>B. pyrrocinia</i>		0		0		0
Total <i>B. cepacia</i> complex		0		0		14
Population 5						
<i>B. cepacia</i>		1		18		17
<i>B. cenocepacia</i> IIIB		0		14		7
<i>B. ambifaria</i>		67		25		16
<i>B. pyrrocinia</i>		0		0		0
Total <i>B. cepacia</i> complex		68		57		40
Population 6						
<i>B. cepacia</i>		18		30		16
<i>B. cenocepacia</i> IIIB		0		53		60
<i>B. ambifaria</i>		36		10		27
<i>B. pyrrocinia</i>		16		0		0
Total <i>B. cepacia</i> complex		70		93		109

^a Identification of isolates to the putative *B. cepacia* complex species level was done using a restriction fragment length polymorphism analysis of PCR-amplified *recA* as described previously (31, 34).

^b Day of the year 2004 when samples were taken.

GenBank were also utilized in the analysis, and the *recA* sequence from *B. pseudomallei* strain K96243T was used as the outgroup.

Statistical analyses. Fisher's exact test for count data, analyses of variance (ANOVA), Tukey's honestly significant difference test, and multivariate linear regressions were performed with the statistical package R (39a; <http://www.R-project.org/>). In multiple regressions, isolate count data (Tables 1 and 2) were found to deviate from normal distributions and were then square-root transformed prior to performing statistical calculations (40).

RESULTS

Bacterial isolation and isolate identification. Totals of 310 and 980 presumptive *B. cepacia* complex isolates were recovered from onion soil and the onion rhizosphere, respectively. The ratio of *B. cepacia* complex isolates identified to total bacterial isolates selected from the PCAT and TB-T plates was

TABLE 2. Number of isolates, identification by species, and recovery on each of two selective media of *B. cepacia* complex isolates from soil and the rhizosphere of onion from six populations in Michigan

Species	Population	No. of isolates ^a from:		No. of isolates on:	
		Soil	Rhizosphere	PCAT	TB-T
<i>B. cepacia</i>					
	1	1	16	2	15
	2	4	0	1	3
	3	2	0	0	2
	4	26	13	4	35
	5	13	36	15	34
	6	6	43	11	38
Total		52	108	33	127
<i>B. cenocepacia</i>					
	1	24	95	70	49
	2	0	77	52	25
	3	25	128	74	79
	4	0	0	0	0
	5	8	8	10	6
	6	2	113	90	25
Total		59	421	296	184
<i>B. ambifaria</i>					
	1	4	39	7	36
	2	28	143	44	127
	3	35	73	18	90
	4	1	1	0	2
	5	72	106	32	146
	6	48	73	24	97
Total		188	435	125	498
<i>B. pyrrocinia</i>					
	1	0	0	0	0
	2	0	0	0	0
	3	0	0	0	0
	4	0	0	0	0
	5	0	0	0	0
	6	11	16	9	18
Total		11	16	9	18

^a Identification of isolates to the putative *B. cepacia* complex species level was done using a restriction fragment length polymorphism analysis of PCR-amplified *recA* as described previously (31, 37).

1,290/3,798 (34%). Isolate identification to the species level was done by comparing restriction fragment patterns of the *recA* gene fragment (data not shown), and the *Burkholderia* species identified in this study were *B. cepacia*, *B. cenocepacia*, *B. ambifaria*, and *B. pyrrocinia*. Specific *recA* restriction patterns were also utilized to determine that all of the *B. cenocepacia* isolates recovered belonged to the previously described III-B lineage (31).

Recovery of *B. cepacia* complex isolates from bulk soil and the onion rhizosphere. *B. cepacia* complex isolates were less prevalent in bulk soil samples than in the rhizosphere, as the soil isolates comprised 24% of the 1,290 total *B. cepacia* complex isolates. The predominant species present in bulk soil was *B. ambifaria*, representing 61% of the total soil isolates, with *B. cepacia*, *B. cenocepacia*, and *B. pyrrocinia* representing 17%, 19%, and 3%, respectively (Fig. 1). *B. ambifaria* was also the most common organism recovered from the rhizosphere but was recovered in proportions similar to those of *B. cenocepacia* (44% and 43%, respectively, of the total) (Fig. 1), which dif-

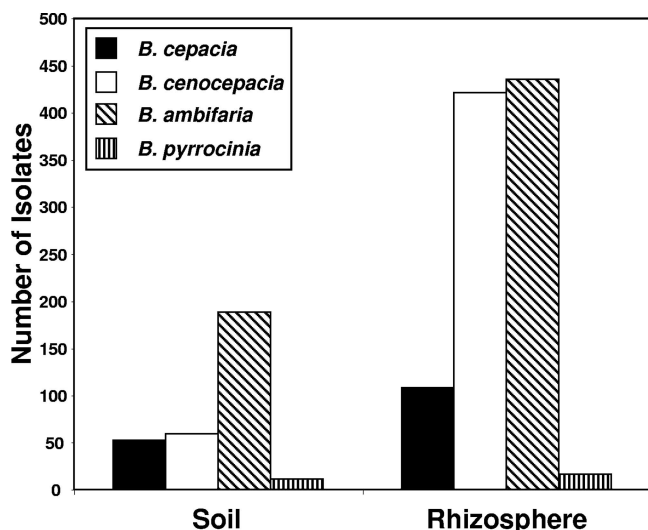


FIG. 1. Total numbers of *B. cepacia* complex isolates (*B. cepacia*, *B. cenocepacia*, *B. ambifaria*, and *B. pyrrocinia*) recovered from field soil and the rhizosphere of onion from six populations in Michigan over three sampling dates in 2004.

ferred from the proportions of these organisms in bulk soil. Isolates of the onion pathogen *B. cepacia* were also recovered from the onion rhizosphere (11% of the total), and *B. pyrrocinia* was recovered from only one site (soil and rhizosphere) and represented 2% of the total isolates (Fig. 1).

A numerical analysis of rhizosphere isolates recovered from each field population indicated differences in the total numbers and frequencies of species per population, as well as differences according to the sampling date. For example, at least 150 *B. cepacia* complex isolates were recovered from five of the six populations, but only 14 isolates were recovered from population 4 (Table 1). *B. ambifaria* was commonly observed at all three sampling times, but only 13 isolates of *B. cenocepacia* (all from population 3) were recovered at the first sampling, when the onion root mass was very small, as the roots were in initial stages of development (Table 1). At the second sampling, when the onion roots were extensively developed, there was a spike in recovery of *B. cenocepacia* in all populations except population 4, while the numbers of *B. ambifaria* isolates decreased in most cases (Table 1).

A summary analysis of the frequency of isolation of each species from bulk soil and the onion rhizosphere from each field population is shown in Table 2. Populations were used as a factor, with six modalities. The effects of populations on the number of isolates recovered from soil or rhizosphere was determined by multiple linear regression analyses, and the statistical significance was determined by using 1,000 permutations of the data. The population origin had no significant effect on the number of isolates recovered from soil ($F = 0.141$; $P = 0.758$) nor on that recovered from the rhizosphere ($F = 0.230$; $P = 0.542$). Interestingly, species had a significant effect on the number of isolates recovered from both soil ($F = 0.661$; $P = 0.015$) and rhizosphere ($F = 0.861$; $P = 0.003$). For each species, a significant difference was found between the number of isolates retrieved from soil and rhizosphere: *B. cepacia* (Fisher's exact test, $P = 7.611 \times 10^{-10}$; *B. cenocepacia*, $P =$

9.114×10^{-12} ; and *B. ambifaria*, $P = 2.885 \times 10^{-8}$). In each case, there were significantly more isolates recovered from the rhizosphere than from soil (Table 2).

Effects of location and sampling time on the composition of *B. cepacia* complex populations. Statistical analyses were performed on populations grouped by field location, with group 1 comprising populations 1 to 3 and group 2 comprising populations 4 to 6. There was a significant association of population with sampling date in the number of recovered *B. cepacia* complex isolates in group 1 (Fisher's exact test, $P = 1.926 \times 10^{-13}$). The detailed analysis revealed that only isolate counts at day 195 and day 243 were not significantly different from each other across the three populations ($P = 0.693$), whereas all other pairwise comparisons were highly significant ($P \ll 0.05$). A significant association of population with sampling date in the number of recovered *B. cepacia* complex isolates was also observed in group 2 (Fisher's exact test, $P = 2.349 \times 10^{-8}$). All pairwise comparisons among sampling dates produced significant differences among populations ($P \ll 0.05$), and all pairwise comparisons of populations were significant, except between populations 4 and 5 ($P = 0.058$).

To test the effects of the sampling time on *B. cepacia* complex species recovery, each population was treated as a replicate for statistical analyses. *B. pyrrocinia* was removed from the subsequent analyses because there were data only on day 194 in population 6. ANOVA were used to determine if the factor time had an influence on the isolate recovery rate for each separate species. The sampling date was significant only to explain *B. cenocepacia* isolate counts (ANOVA, 5 df; $F = 4.272$; $P = 0.0183$), marginally significant for *B. cepacia* isolate counts ($F = 2.552$; $P = 0.085$), and not significant to explain *B. ambifaria* isolate counts ($F = 0.4309$; $P = 0.819$). Tukey's honestly significant tests were further applied to the *B. cenocepacia* data to determine which dates provided significant differences in the mean counts. All pairwise comparisons of mean differences were not significantly different from each other, except between dates 172 and 214 (mean difference = 63.7; $P = 0.022$) and between dates 194 and 214 (mean difference = 59.3; $P = 0.034$).

Frequencies of recovery of *B. cepacia* complex isolates on PCAT and TB-T selective media. A numerical analysis of the isolation frequencies for the four *B. cepacia* complex species indicated a differential recovery of each species on PCAT and TB-T selective media (Table 2). Both *B. cepacia* and *B. ambifaria* were preferentially recovered on TB-T medium, with 79% and 80% of the total isolates, respectively, of the two species obtained from TB-T (Table 2). Similar results were observed for *B. pyrrocinia*, although only 27 total isolates were obtained in this study (Table 2). In contrast, *B. cenocepacia* was recovered more efficiently on PCAT medium, with 62% of the total isolates obtained on this medium (Table 2).

Onion pathogenesis. Each of the 1,290 *B. cepacia* complex isolates identified in this study was assayed for pathogenicity on detached onion bulb scales (Fig. 2). After inoculation and incubation at 30°C for 48 h, the percent area of macerated tissue was estimated, and a rating of 0 to 3 was given. Isolates of each of the species examined (*B. cepacia*, *B. cenocepacia*, *B. ambifaria*, and *B. pyrrocinia*) were determined to be pathogenic on onion bulb scales, and only 14 of 1,290 total isolates were nonpathogenic (Table 3). The large

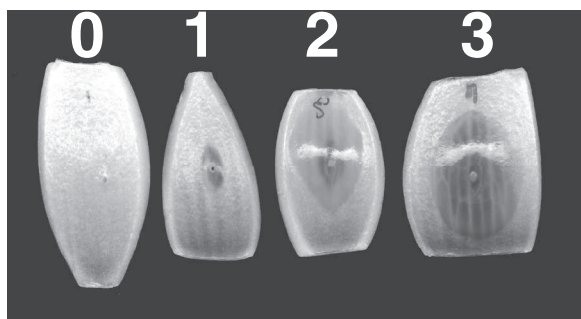


FIG. 2. Rating system for the onion pathogenesis assay used to type *B. cepacia* complex isolates. Onion slices were wounded, inoculated with 5 μ l of bacterial culture (10^7 CFU/ml), and incubated at 30°C for 48 h. A rating scale of 0 to 3 was used to indicate the degree of tissue maceration. A rating of 0 indicated no maceration, 1 indicated 1 to 33% macerated tissue area, 2 indicated 34% to 66% macerated tissue area, and 3 indicated 67% to 100% macerated tissue area.

majority of isolates tested were highly virulent, with pathogenicity ratings of 2 or 3 indicating maceration of 34 to 100% of the onion bulb tissue (Table 3). In addition to water-soaking and maceration symptoms, necrosis was observed with a small percentage of the *B. cepacia* isolates. A total of 86% of isolates of *B. cepacia* (137/160) were highly virulent (rating, 2 to 3) (Fig. 2) in this model; this frequency was actually surpassed by *B. cenocepacia*, where 90% (434/480) of the isolates were highly virulent (Table 3). A slightly reduced percentage of *B. ambifaria* isolates (76%; 476/623) and only 15% (4/27) of *B. pyrrocinia* isolates were highly virulent (Table 3).

Phylogenetic analysis. The *recA* genes from 26 *B. cepacia* complex isolates collected in this study were amplified by PCR and sequenced. Phylogenetic analysis of 1,041 aligned nucleotide positions of the *recA* gene was carried out using NJ and MP (Table 4). The two techniques yielded essentially identical results, and the NJ tree is shown in Fig. 3. The NJ and MP trees were characterized by the presence of five well-supported major lineages, corresponding to the four recognized species (*B. cenocepacia* III-B, *B. cepacia*, *B. pyrrocinia*, and *B. ambifaria*) plus a fifth lineage comprised of three additional *B. cenocepacia* (III-A) strains. The relationships of these five major groups to each other in our analyses are the same as that observed by Payne et al. (37), with the exception that in our analysis, the clade containing *B. cenocepacia* J2315 was basal to the *B. cepacia*, the *B. pyrrocinia*, and the other *B. cenocepacia* clades (instead of being a sister to *B. ambifaria*).

In total, four to six polymorphic *recA* alleles were identified from the onion isolates in this study (Fig. 3). The onion isolates recovered in the study each fell within one of the major clades. For example, 1RP102, 6RT131, 6RP111, 6RT130, 6RT111, and 3RP82 all fell within the well-supported *B. cenocepacia* (III-B) clade, which includes the human pathogens *B. cenocepacia* AU1054 and PC184 (Fig. 3). The onion isolates of *B. cepacia*, *B. pyrrocinia*, and *B. ambifaria* were all grouped similarly with the reference strains and, in some cases, shared identical DNA sequences (Fig. 3).

DISCUSSION

Three *B. cepacia* complex species, *B. cepacia*, *B. cenocepacia*, and *B. ambifaria*, were regularly isolated and a fourth, *B. pyrrocinia*, was sporadically isolated from the onion rhizospheres and onion field soils from several locations in Michigan. *B. cenocepacia* and *B. ambifaria* were the most prevalent species recovered in this study, representing 85.5% of the total (Table 1). This result is somewhat surprising considering that *B. cepacia* has historically been the species most often associated with onion rot diseases (53) and the association of *B. cenocepacia* with the onion rhizosphere had not been previously documented. In this study, only 11.2% of the onion rhizosphere isolates were *B. cepacia*, which is close to the 20.4% recovery of *B. cepacia* reported from the maize rhizosphere (41). Infection of onion occurs as bacteria invade wounds in young leaves and move downward through leaves into the corresponding scales in the bulb (24). Splashing water from rain or overhead irrigation is thought to disseminate bacterial inoculum from the soil to the necks of onion plants and plays a key role in the level of disease incidence (49). The linkage between rhizosphere populations and onion disease has not been fully elucidated, but presumably the increased populations of pathogenic *B. cepacia* complex bacteria around developing onion leaves and bulbs provide bacteria that can be disseminated to wounds by splashing water.

The plant rhizosphere appears to represent an important ecological niche for *B. cepacia* complex bacteria, as these organisms have been isolated from or shown to colonize the rhizospheres of diverse hosts, including perennial ryegrass, pea, cotton, and coffee (48). The high frequency of *B. ambifaria* in onion field soil and in the onion rhizosphere adds to a body of knowledge that this species comprises strains that are adapted to the soil environment and are effective colonizers of the rhizospheres of many plant species (16, 42). Likewise, *B. cenocepacia* strains have also been isolated from soil and the rhizospheres of plants, including maize, wheat, and lupine (1). The environmental prevalence of *B. cenocepacia* is potentially clinically significant in that an epidemic CF strain has also been isolated from onion field soil from New York (27).

In this study, significantly more *B. cenocepacia* bacteria were recovered from rhizosphere than from soil samples, and analyses of the temporal distribution also indicated a significant association between the sampling time and recovery of *B. cenocepacia*. This species was most commonly isolated after the development of the onion rhizosphere and was extremely rare at the first sampling time, when onion roots were in an initial

TABLE 3. Pathogenicities of *B. cepacia* complex isolates from soil and the rhizosphere of onion in Michigan

Species	No. of isolates	No. of isolates with an onion pathogenicity rating of ^a :			
		0	1	2	3
<i>B. cepacia</i>	160	0	23	52	85
<i>B. cenocepacia</i>	480	0	46	174	260
<i>B. ambifaria</i>	623	4	143	223	253
<i>B. pyrrocinia</i>	27	10	13	3	1

^a The positive control strain *B. cepacia* ATCC 25416 was included in each pathogenicity assay and consistently produced a pathogenicity rating of 3.

TABLE 4. Strains of *B. cepacia*, *B. cenocepacia*, *B. ambifaria*, and *B. pyrrocinia* utilized for a phylogenetic analysis of *recA* sequences

Species and strain ^a	Source or relevant information	GenBank accession no. of <i>recA</i> sequence	Source or reference
<i>B. cepacia</i>			
3ST4	Onion field soil	EU079008	This study
4ST9	Onion field soil	EU079004	This study
5RT136	Onion rhizosphere	EU079007	This study
5RT161	Onion rhizosphere	EU079011	This study
6RP83	Onion rhizosphere	EU079006	This study
6RT68	Onion rhizosphere	EU079005	This study
6RT141	Onion rhizosphere	EU079009	This study
6SP128	Onion field soil	EU079010	This study
KBC-3	Maize rhizosphere	AY769897	40
ATCC 25416	Onion	AF143786	31
<i>B. cenocepacia</i>			
1RP102	Onion rhizosphere	EU079000	This study
3RP82	Onion rhizosphere	EU078998	This study
6RP111	Onion rhizosphere	EU079003	This study
6RT111	Onion rhizosphere	EU078999	This study
6RT130	Onion rhizosphere	EU079001	This study
6RT131	Onion rhizosphere	EU079002	This study
AU1054	Human pathogen	NC_008060.1	27
B9	Banana, Taiwan	AY598028	26
CA2	Banana, Taiwan	DQ664181	26
GQ52	Banana, Taiwan	DQ664182	26
HI2424	Soil	NC_008542	27
J2315	Human pathogen, United Kingdom	<i>_b_</i>	31
KBC-4	Maize rhizosphere	AY769898	40
K56-2	Human pathogen, Canada	AF143779	31
MC0-3	Maize rhizosphere	NZ_AAVA01000001 in silico	
MQ41	Banana, Taiwan	DQ664183	26
PC184	Human pathogen, United States	AF143784	31
<i>B. ambifaria</i>			
2SP165	Onion field soil	EU079012	This study
5RT130	Onion rhizosphere	EU079013	This study
5SP7	Onion field soil	EU079017	This study
6RT55	Onion rhizosphere	EU079016	This study
6SP146	Onion field soil	EU079018	This study
6ST151	Onion field soil	EU079014	This study
6ST196	Onion field soil	EU079015	This study
AMMD	Pea rhizosphere	NC_008390	36
AU0212	Human isolate	AF456005	37
KBC-7	Maize rhizosphere	AY769901	40
<i>B. pyrrocinia</i>			
6RP78	Onion rhizosphere	EU079020	This study
6RT51	Onion rhizosphere	EU079019	This study
6RT94	Onion rhizosphere	EU079021	This study
6ST17	Onion field soil	EU079023	This study
6ST128	Onion field soil	EU079022	This study
ATCC 39277	Soil, United States	AF143801	14
BC011	Water, United States	AF456045	14

^a Codes for identification of *B. cepacia* complex strains from this study are as follows. The first number represents the populations, R or S refers to recovery from rhizosphere or soil, P or T refers to recovery on PCAT or TB-T medium, and the last number refers to the sampling date (1 to 50, first sampling date; 51 to 100, second sampling date; 101 to 150, third sampling date; 151 to 200, fourth sampling date).

^b Draft sequence information for *B. cenocepacia* J2315 *recA* is available from http://www.sanger.ac.uk/Projects/B_cenocepacia/.

stage of growth (Table 1). The temporal results suggest that *B. cenocepacia* is efficiently selected from soil by the onion rhizosphere and that the organism can then readily colonize this habitat. A comparison of distribution analyses from other studies indicated that *B. cenocepacia* was widely prevalent in the rhizospheres of wheat and lupine (1) but represented only a small fraction of the recovered *B. cepacia* complex strains from the maize rhizosphere (3.2% of the total) (41) and was not recovered from the rice rhizosphere (55). However, in another

study, *B. cenocepacia* represented 70.5% of the isolates from the maize rhizosphere from field sites in Italy (17). It should be noted that a follow-up analysis of some of these isolates using multilocus sequence typing indicated that only 7% were *B. cenocepacia*, with another 39% belonging to potentially new *B. cepacia* complex groups that were closely related to but distinct from *B. cenocepacia* (15). It is unknown how plant species and environmental factors influence the rhizosphere colonization of *B. cenocepacia*. The prevalence of the species in the onion

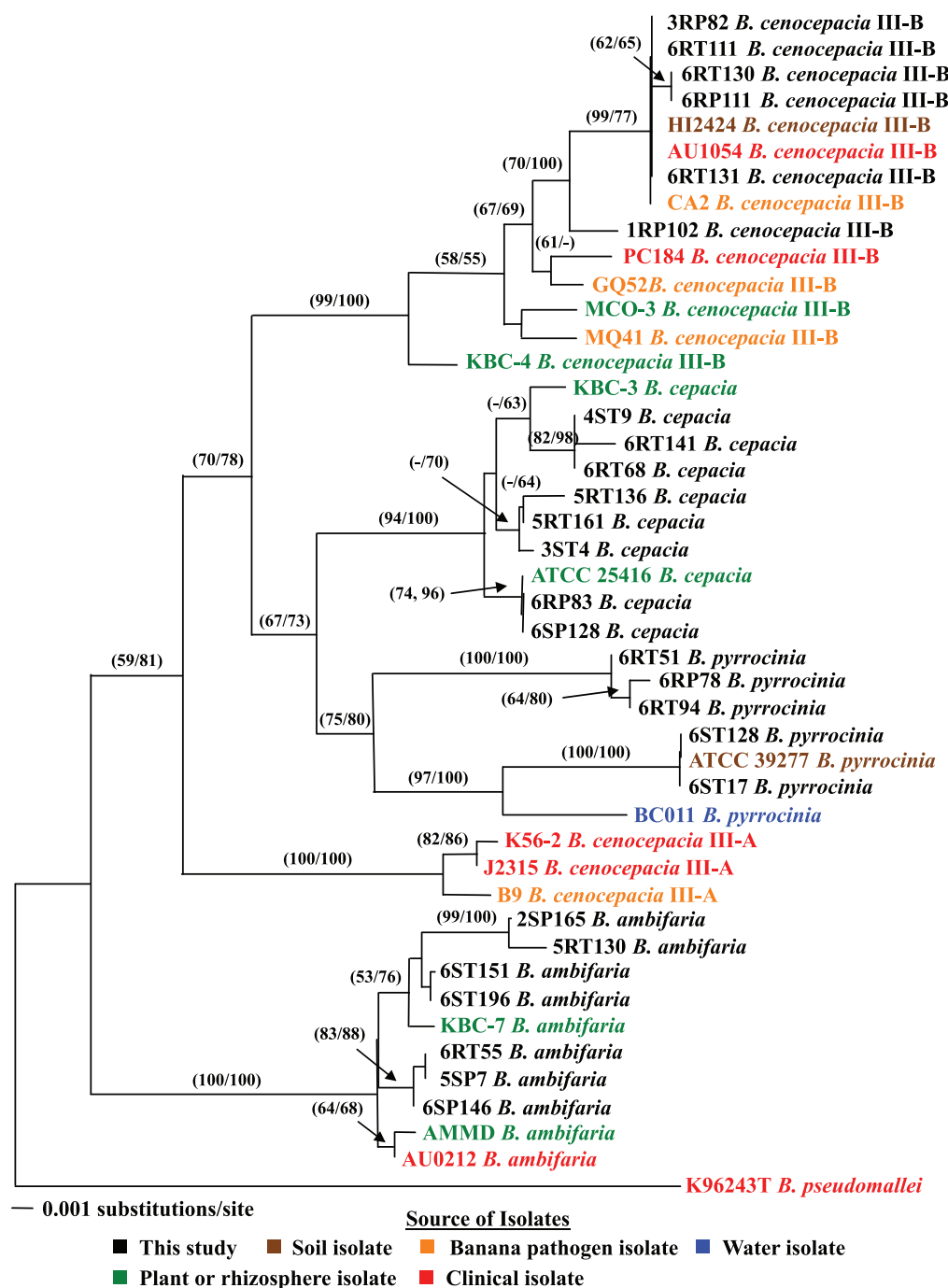


FIG. 3. Phylogenetic analysis of the *recA* data set from 45 *B. cepacia* complex strains with *B. pseudomallei* K96243T as the outgroup (descriptions of isolates and reference strains are in Table 4). A color key is used to designate the source of isolates used in this analysis. The naming convention for the isolates from this study (e.g., 3RP82) is defined in a footnote to Table 4. The tree shown was obtained by NJ using Kimura two-parameter distances. The numbers in parentheses (MP/NJ) on the branches show the MP and NJ bootstrap values (>50).

rhizosphere, coupled with the knowledge that isolates of *B. cenocepacia* from diseased onions were shown to be pathogenic on onion in a prior study (46), implies that host factors unique to the onion plant may play an important role in the colonization of onion by *B. cenocepacia*.

The numbers of *B. cepacia* complex isolates from five of six

onion field sampling locations in this study were relatively similar. However, sampling location 4 yielded only 14 total *B. cepacia* complex isolates, all obtained at the third sampling time (Table 1). The factors resulting in this much reduced occurrence are currently unknown. In Michigan, onion crops are sometimes rotated in the following season with crops such

as maize, radish, or soybean. If *B. cepacia* complex bacteria do not colonize the rhizospheres of these hosts as readily as that of onion, the overall soil populations could decrease over time. The factors resulting in the reduced *B. cepacia* complex populations at sampling location 4 could reveal important insights into the ecology of these organisms in managed agricultural habitats.

In this study, we found that various *B. cepacia* complex species were differentially recovered on the PCAT and TB-T selective media. Our results are similar to those of Dalmastri et al. (17) in this regard, with approximately four times more *B. cepacia* and *B. ambifaria* bacteria recovered on TB-T than on PCAT medium and an increased number of *B. cenocepacia* bacteria recovered on PCAT medium. Although neither of these media is sufficiently selective to eliminate other organisms, as shown in this study and in previous studies (21, 34), we recommend that both PCAT and TB-T media be used in combination in population analyses of *B. cepacia* complex from environmental sources.

From a total of 1,290 *B. cepacia* complex isolates examined in an onion pathogenicity assay, only 14 were incapable of causing water-soaking and maceration symptoms, while 1,051 (81.5%) caused significant symptoms with a disease rating of 2 or 3 (Table 3). To the best of our knowledge, this is the first report of *B. ambifaria* and *B. pyrrocinia* causing water-soaking and maceration symptoms in onion bulb scale tissue. The results of many studies have shown that strains of *B. cepacia* and *B. cenocepacia* are capable of causing water soaking and onion tissue maceration in laboratory inoculations (9, 18, 52, 53). Many clinical *B. cenocepacia* strains isolated from CF patients are nonpathogenic on onion (6, 19, 53), although some CF strains, including known epidemic strains, have been shown to cause onion maceration (9). In addition, one strain of *B. vietnamiensis* was reported to produce the plant tissue water-soaking phenotype on onion (18).

Phylogenetic analysis, based on the *recA* sequence, was used to identify four well-supported major groups, which corresponded to recognized species as reported previously (37). Onion isolates of *B. cepacia*, *B. cenocepacia*, *B. ambifaria*, and *B. pyrrocinia* clustered with reference strains in each of the species. A recent phylogenetic analysis using multilocus sequence typing included five sublineages of *B. cenocepacia*, with the human pathogen strains AU1054 and PC184 placed in lineage III-B and J2315 and K56-2 placed in lineage III-A (3). The *recA* sequence from AU1054 was identical or closely similar to those of several of the onion isolates from this study. The determination of whether onion isolates such as 6RT131 are potential human pathogens is a current focus in our laboratory.

The *recA* sequences from onion isolates of *B. ambifaria* analyzed in this study were similar to those of the reference strains KBC-7, isolated from the maize rhizosphere; AMMD, isolated from the pea rhizosphere; and AU0212, a human isolate from a CF patient (12). However, of the *B. ambifaria* isolates examined, only the isolates from onion macerated onion tissue in a pathogenicity assay, while the reference strains AMMD and KBC-7 did not (data not shown). A major virulence factor in onion pathogenicity is the presence of a polygalacturonase enzyme involved in tissue maceration that is encoded by the plasmid-borne *pehA* gene (20). It is possible that virulence

determinants are disseminated among *B. cepacia* complex bacteria via horizontal transfer in the onion rhizosphere.

The results of many gene distribution studies have shown that species such as *B. cenocepacia* are comprised of subpopulations that differ in the carriage of specific virulence genes associated with human or animal pathogenesis (reviewed in reference 32). In some cases, rhizosphere or plant pathogen isolates of *B. cenocepacia* have also been examined for the distribution of markers, such as the *B. cepacia* epidemic strain marker and cable pilin gene (4, 26). The genetic distinction between plant pathogen and human pathogen appears to be blurred in species such as *B. cenocepacia*, and the relatively large genome sizes of these organisms seem to enable the carriage of a diverse gene set conferring fitness in a wide range of ecological niches. Studies of the genetic requirement for plant rhizosphere colonization in *B. cepacia* complex species have been initiated (35), and this topic has been examined in related organisms, such as *P. aeruginosa* and *Pseudomonas putida* (28, 33, 43). The role of the plant host in the selection of rhizosphere-colonizing *B. cepacia* complex strains and the maintenance of specific virulence genes also remain to be elucidated.

In summary, we determined that *B. cepacia*, *B. cenocepacia*, and *B. ambifaria* were the dominant *B. cepacia* complex species present in the onion rhizosphere and onion field soil in Michigan. The vast majority of isolates from each of these species and a few isolates of *B. pyrrocinia* were pathogenic in an onion disease assay, causing water soaking and tissue maceration. While *B. cenocepacia* collectively includes organisms that are opportunistic human pathogens and pathogens of plants, including onion and banana, *B. ambifaria* and *B. pyrrocinia* had not been previously reported as potential plant pathogens. The genetic content, distribution, and mobility of gene sequences involved in virulence are variable within the *B. cepacia* complex. Our current investigations focus on the identification of gene sets important for rhizosphere colonization and plant pathogenesis and the ecological factors that influence the maintenance of these determinants in environmental *B. cepacia* complex populations.

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