Isolation and Characterization of *Burkholderia rinojensis* sp. nov., a Non-*Burkholderia cepacia* Complex Soil Bacterium with Insecticidal and Miticidal Activities

Ana Lucia Cordova-Kreylos, Lorena E. Fernandez, Marja Koivunen, April Yang, Lina Flor-Weiler, Pamela G. Marrone

Marrone Bio Innovations, Inc., Davis, California, USA

Isolate A396, a bacterium isolated from a Japanese soil sample demonstrated strong insecticidal and miticidal activities in laboratory bioassays. The isolate was characterized through biochemical methods, fatty acid methyl ester (FAME) analysis, sequencing of 16S rRNA, multilocus sequence typing and analysis, and DNA-DNA hybridization. FAME analysis matched A396 to *Burkholderia cenocepacia*, but this result was not confirmed by 16S rRNA or DNA-DNA hybridization. 16S rRNA sequencing indicated closest matches with *B. glumae* and *B. plantarii*. DNA-DNA hybridization experiments with *B. plantarii*, *B. glumae*, *B. multivorans*, and *B. cenocepacia* confirmed the low genetic similarity (11.5 to 37.4%) with known members of the genus. PCR-based screening showed that A396 lacks markers associated with members of the *B. cepacia* complex. Bioassay results indicated two mechanisms of action: through ingestion and contact. The isolate effectively controlled beet armyworms (*Spodoptera exigua*; BAW) and two-spotted spider mites (*Tetranychus urticae*; TSSM). In diet overlay bioassays with BAW, 1% to 4% (vol/vol) dilution of the whole-cell broth caused 97% to 100% mortality 4 days postexposure, and leaf disc treatment bioassays attained 75% $\pm$ 22% mortality 3 days postexposure. Contact bioassays led to 50% larval mortality, as well as discoloration, stunting, and failure to molt. TSSM mortality reached 93% in treated leaf discs. Activity was maintained in cell-free supernatants and after heat treatment (60°C for 2 h), indicating that a secondary metabolite or excreted thermostable enzyme might be responsible for the activity. Based on these results, we describe the novel species *Burkholderia rinojensis*, a good candidate for the development of a biocontrol product against insect and mite pests.

The bacterial species in the genus *Burkholderia* are ubiquitous organisms in soil, rhizospheres, insects, fungi, and water (1, 2). The *Burkholderia* genus, beta subdivision of the proteobacteria, comprises more than 60 species that inhabit diverse ecological niches (3). Traditionally, they have been known as plant pathogens, *Burkholderia cepacia* being the first one discovered and identified as the pathogen causing disease in onions (4). Several *Burkholderia* species have developed beneficial interactions with their plant hosts (5, 6) and are able to fix atmospheric nitrogen (7) or nodulate plant roots (6). Additionally, some *Burkholderia* species have been found to have potential as biocontrol products against soilborne (8), foliar (9), and postharvest (10–15) plant pathogens or have been effectively used in bioremediation to treat polluted soil or groundwater (16, 17). Further, some *Burkholderia* species have also been found to secrete a variety of extracellular enzymes with proteolytic, lipolytic, and hemolytic activities, as well as toxins, antibiotics, and siderophores (18). This metabolic diversity makes the genus *Burkholderia* very desirable for biotechnological applications.

On the other hand, several *Burkholderia* species are also opportunistic human pathogens (19–21), the best known of which are the species of the *Burkholderia cepacia* complex as well as *B. gladioli* and *B. fungorum*. *B. pseudomallei* and *B. mallei* are the only other known members of the genus *Burkholderia* that are primary pathogens of humans and animals, causing melioidosis in humans (19) and glanders in horses (21). The *Burkholderia cepacia* complex (Bcc) has emerged as an important group of opportunistic pathogens, particularly for patients with suppressed immune systems and more specifically for cystic fibrosis patients (2). The species of the Bcc are phenotypically almost identical, making their identification and differentiation by common biochemical tests very difficult. The Bcc is composed of 17 officially recognized

strains (22) that have been isolated both from cystic fibrosis patients and from diverse environmental samples. Members of this complex have high homology of the 16S rRNA gene but moderate hybridization values (30 to 60%), adding to the difficulty of unequivocally identifying and differentiating them (23, 24). They are versatile microorganisms with large complex genomes (17), able to metabolize a wide variety of carbon sources (25) and produce diverse secondary metabolites (18).

Increasing environmental concerns and problems caused by some synthetic chemicals have stimulated interest in the development of biopesticides as pest management tools. Application of synthetic chemicals not only can cause environmental hazards but also may affect nontarget organisms, including bees, humans, and other mammals. The use of biopesticides such as fungi, bacteria, baculoviruses, and some botanicals merits attention because they have demonstrated effective commercial pest control with a high degree of safety to nontarget organisms and the environment. A number of economically important pests are successfully controlled by biopesticides. *Bacillus thuringiensis* is a bacterial biopesticide that has been successfully used to control lepidopteran, dipteran, and coleopteran pests (26–29) and popularly applied as a

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Address correspondence to Ana Lucia Cordova-Kreylos, alcordova@marronebio.com.

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foliar agent or in transgenic plants (30). Due to their high efficacy, *B. thuringiensis* products (Bt products) have held a large market share out of all microbial biopesticides (31, 32), which constitutes about 2% of the total market of insecticides (26, 31). Numerous strains of *B. thuringiensis* have been reported to have activity on dipterans (33–35), including immature stable flies (36, 37). *Chromobacterium subtsugae* is another example of a recent product brought to market for insect control (38–40).

We isolated a soil bacterium, A396, that showed efficacy against arthropod pests. Bioassays were conducted on representative chewing (beet armyworms [BAW]; *Spodoptera exigua*) and sucking (two-spotted spider mites [TSSM]; *Tetranychus urticae*) arthropod pest species to determine the insecticidal activity of A396 using a range of whole-cell broth (WCB) dilutions under laboratory conditions. Characterization of the microbe focused on providing an accurate taxonomic position and determining if it belonged to the Bcc group. The information reported in this article provides a basis for future development of isolate A396 as a potential biopesticide for insect pest management.

MATERIALS AND METHODS

Bacterial isolation and preliminary identification. Microbial isolate A396 was recovered during the course of Marrone Bio Innovation's routine discovery screening for new biopesticides. The microorganism was isolated from a soil sample collected in the vicinity of the Rinoji Temple in Nikko, Japan, in 2008. The soil sample was suspended in sterile water, serially diluted, and plated onto agar plates of various compositions. Isolate A396 was recovered from potato dextrose agar (PDA) plates that had been incubated at 25°C in the dark for approximately 1 week. The isolate was initially identified as *B. plantarii* by sequencing of a 500-bp fragment of the 16S rRNA and as *B. multivorans* when a slightly larger fragment (~750 bp) was sequenced.

Bacterial cultivation and production of test substances. The isolate was deposited with the ARS Culture Collection under accession code NRRL B-50319. *B. multivorans* ATCC 17616 and *Pseudomonas fluorescens* CL145A were used as controls for several experiments. Isolate A396 was maintained on PDA (same medium as used for isolation from soil) plates at 25°C and, when needed, was grown on liquid media [Hy-Soy, 15 g/liter; NaCl, 5 g/liter; KH₂PO₄, 5 g/liter; MgSO₄·7H₂O, 0.4 g/liter; (NH₄)₂SO₄, 2 g/liter; glucose, 5 g/liter (pH 6.8)] in 250-ml to 2-liter fermentation flasks at 200 rpm and 25°C for 5 days. When only a supernatant was required for testing, cells were removed by centrifugation and filtration through a 0.22-μm syringe nylon filter to yield a cell-free supernatant. Fermentation material was inactivated for certain bioassays by heating to 60°C and holding at this temperature for 2 h. This treatment ensured that no live cells remained in the fermentation material.

Phylogenetic analysis of *Burkholderia* sp. strain A396. (i) Amplification and sequencing of the 16S rRNA gene. Isolate A396 was grown on PDA plates overnight at 25°C in the dark. Fresh growth was scraped from the plate using a sterile disposable loop. The collected biomass was suspended in extraction buffer, and DNA was extracted using a commercially available kit, MoBio Ultra Clean Microbial DNA (MoBio Laboratories, Inc., CA). DNA extract was checked for quality and quantity by running 5 μl on a 1% agarose gel and comparing bands to a Hi-Lo mass ladder (Bionexus, CA). The 16S rRNA portion of the genome was amplified via PCR. PCRs were set up as follows: 2 μl of DNA extract, 5 μl of PCR buffer, 1 μl of deoxynucleoside triphosphates (dNTPs; 10 mM each), 1.25 μl of forward primer (27F, AGA GTT TGA TCM TGG CTC AG), 1.25 μl of reverse primer (1525R, AGA GTT TGA TCC TGG CTC AG), and 0.25 μl of *Taq* enzyme. The reaction volume was made up to 50 μl using sterile nuclease-free water. The PCR included an initial denaturation step at 95°C for 10 min, followed by 30 cycles of 94°C for 30 s, 57°C for 20 s, and 72°C for 30 s and a final extension step at 72°C for 10 min. The PCR product's approximate concentration and size were calculated by running

a 5-μl volume on a 1% agarose gel and comparing the product band to a Hi-Lo mass ladder (Bionexus). Excess primers, dNTPs, and enzyme were removed from the PCR product with commercially available MoBio UltraClean PCR cleanup kit (MoBio Laboratories, Inc.). The cleaned PCR product was directly sequenced using primers 27F and 1525R. Closely related sequences were obtained using nucleotide BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and EZ-Taxon (41). Obtained sequences were imported into MEGA5 software (<http://www.megasoftware.net/>) and aligned with MUSCLE for phylogenetic analysis.

(ii) Bcc-specific *recA* amplification. Amplification of the *Bcc recA* gene (1,040 bp) was attempted using specific primers BCR1 (TGA CCG CCG AGA AGA GCA A) and BCR2 (CTC TTC TTC GTC CAT CGC CTC) and BCRBM1/BCRBM2 (CGG CGT CAA CGT GCC GGA T/TCC ATC GCC TCG GCT TCG T), as described elsewhere (42). The PCRs were set up as follows: GoTaq Master Mix, 25 μl; forward primer, 1.2 μl; reverse primer, 1.5 μl; and template, 2 μl; the volume was brought up to 50 μl with nuclease-free water. The primer stock solution concentration was 20 μM. Amplification was carried out in a Techne TC-5000 thermal cycler (Bibby Scientific US, NJ) as follows: initial denaturation for 5 min at 94°C, 30 cycles of 30 s at 94°C, 45 s at the proper annealing temperature, and 60 s at 72°C, and final extension for 10 min at 72°C. PCR products (5 μl) were loaded onto a 1.5% agarose gel with EZ-Vision dye and visualized under UV light. The performance of the PCR and primers was tested using *B. multivorans* ATCC 17616 (positive control) and *P. fluorescens* CL145A (negative control).

(iii) MLST and multilocus sequence analysis (MLSA). Amplification and sequencing of seven loci were performed as described by Spilker et al. (43). A phylogenetic tree was constructed from the concatenated sequences of *atpD*, *gltB*, *gyrB*, *lepA*, *phaC*, *recA*, and *trpB* obtained from A396, and representative *Burkholderia* species available from the *Bcc* multilocus sequence typing (MLST) database (<http://pubmlst.org/bcc/>). Neighbor-joining trees were built in MEGA, version 5.05, software, and significance was evaluated by bootstrap analyses. Alleles and sequence type were determined with the sequence query tool available from the *Bcc* MLST database (http://pubmlst.org/perl/bigdb/bigdb.pl?db=pubmlst_bcc_seqdef&page=sequenceQuery).

(iv) DDH. DNA-DNA hybridization (DDH) experiments were performed with isolate A396 and type strains of closely related microorganisms according to 16S rRNA phylogenetic results. DDH experiments were contracted out to DSMZ in Braunschweig, Germany. DNA was isolated using a Thermo Spectronic French pressure cell (Thermo Spectronic, USA) and was purified by column chromatography on hydroxyapatite as described by Cashion et al. (44). DDH was carried out as described by De Ley et al. (45) under consideration of the modifications described by Huss et al. (46) using a model Cary 100 Bio UV-visible (UV-vis) spectrophotometer equipped with a Peltier Thermostatted multicell changer (Agilent Technologies, USA) and a temperature controller with an *in situ* temperature probe (Varian, CA).

Phenotypic and biochemical characterization. Fatty acid composition was determined by Microbial ID, Inc. (Newark, DE), according to well-established standard protocols. The reported fatty acid profile was compared to those of closely related species and to the MIDI Sherlock database for identification. Temperature tolerance was tested by growing the isolate on PDA plates at 16, 25, 30, and 37°C. The antibiotic susceptibility of A396 was tested using antibiotic susceptibility discs (BD, NJ) on Mueller-Hinton medium inoculated with A396 to confluent growth. Results were read after 72 h of incubation at 25°C. Antibiotic susceptibility was evident by a clear halo around the antibiotic-loaded discs. Biochemical characterization was done with Biolog Microbial Identification GENIII (Hayward, CA) plates that were set up and evaluated by following the manufacturer's guidelines. Extensive characterization of biochemical capabilities was performed through a complete Biolog phenotypic microarray panel (see the supplemental material).

Insect colonies. First and third larval instars of the beet armyworm (*Spodoptera exigua* Hübner; BAW) and two-spotted spider mite (*Tet-*

ranychus urticae Koch; TSSM) adults were used in the feeding and contact bioassays. The BAW colony originated from eggs purchased from Bio-Serv (NJ) and then established and maintained on an artificial diet containing standard growth nutrients necessary for insect propagation (see the supplemental material). The BAW colony was kept in an incubator at 26°C, with a 12-h photoperiod. The TSSM colony was established from specimens collected in Davis, CA. TSSM were reared on lima beans, *Phaseolus lunatus*, at 26°C, with a 12-h photoperiod.

Insect bioassays. Insecticidal activity was determined by observing the response of BAW and TSSM to A396 whole-cell broth (WCB; endpoint harvest fermentation material with cells) and heat-treated-WCB exposure in artificial diet overlays and excised-leaf-disc bioassays. The WCB was stored at -80°C until used in these evaluations.

Larval toxicity in artificial-diet overlay. Toxicity via feeding was evaluated in artificial-diet overlay assays using 96-well microtiter plates (ThermoFisher Scientific, Rochester, NY) as described elsewhere (47). Dilutions of 4.0, 2.0, 1.0, and 0.5% (vol/vol) WCB were made in sterile distilled water. Sterile distilled water and Javelin WG (a commercial Bt product) were used as the negative and positive controls, respectively. One hundred fifty microliters of BAW artificial diet was added to each well, followed by 100 µl of the WCB or cell-free supernatant dilutions and negative and positive controls. The plates were dried in a fume hood at room temperature. The WCB dilutions were done in 40 replications (i.e., wells), and one first-instar BAW larva was introduced into each well. Plates were sealed with a clear sheet of adhesive Mylar, and one pin size hole was made in the seal over each well for aeration. The plates of treated insects were incubated at 26°C, with a 12-h photoperiod. Larval mortality was assessed 3 and 4 days after exposure to the treated diet, and average percent mortality of larvae in each treatment was determined.

Larval toxicity in leaf disc bioassay. Leaf disc bioassays have been previously described for the evaluation of insecticidal active ingredients (48). Toxicity via feeding was evaluated using treated broccoli leaf discs on 1% water agar in small petri plates (50-mm diameter). Leaves were excised with a 42-mm-diameter corer and treated with a 3.0% (vol/vol) solution of A396 WCB or heat-treated WCB in sterile distilled water. Treatments of 3% (vol/vol) Xentari (a commercial Bt product) and sterile distilled water were prepared as the positive and negative controls, respectively. Leaf discs were immersed in each treatment solution for 1 min, air dried, and then placed on the agar, abaxial side up. Four newly emerged second-instar BAW larvae were introduced into the agar plates containing the treated leaf discs. The agar plates were then covered with Parafilm punctured with holes for aeration and kept at room temperature, with a 12-h photoperiod. Treatments were replicated six times. Mortality of larvae was evaluated after 72 h of exposure to treated leaf discs.

Contact bioassay. Contact bioassays were performed as described previously (49). Briefly, one newly emerged third-instar BAW larva was placed in a 1.25-oz clear plastic cup (PL1; Solo Cup Company, Highland Park, IL) with a 1-cm² cube of BAW artificial diet. One microliter of A396 WCB was applied to the thorax of each larva using a Hamilton micropipette (PB-600, Reno, NV). One microliter of sterile water applied to the thorax of each larva served as the negative control. Each cup containing a single treated larva became the experimental unit, with 10 larvae per treatment. The whole bioassay was set up twice. The cups and treated larva were covered with Parafilm, punctured for aeration, and incubated at room temperature, with a 12-h photoperiod. Mortality and negative effects, including stunted growth, were recorded 3 days after treatment and then immediately after larvae pupated.

TSSM test *in vivo*. The *in vivo* efficacy of A396 WCB and heat-treated WCB against TSSM was evaluated in a fava bean leaf disc bioassay (50). Twelve-well polystyrene plates (ThermoFisher Scientific, Rochester, NY) were filled with cotton saturated with water. Fava bean leaf discs were made using a 3/4-in.-diameter cork borer. Treatment solutions were prepared in a 0.01% Tween 20 solution: 6% (vol/vol) A396 WCB, 6% (vol/vol) A396 heat-treated WCB, and Avid 0.15 EC (10% [vol/vol]). Water and Avid were the negative and positive controls, respectively. Leaf discs

FIG 1 Neighbor-joining tree inferred from the 16S rRNA gene, showing the phylogenetic relationship between isolate A396 and closely related *Burkholderia* species. The bootstrap consensus tree inferred from 2,000 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (2,000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. There were a total of 1,529 positions in the final data set. Evolutionary analyses were conducted in MEGA5.

were immersed in each treatment solution for 1 min and then air dried. A treated disc was transferred to each well that contained water-saturated cotton. Ten adult female TSSM were introduced onto each treated leaf disc. Treatments were replicated 6 times. The treated TSSM were incubated at room temperature, with a 12-h photoperiod. Mortality of adult mites was evaluated 3 days after introduction onto treated leaf discs. Mortality data were analyzed using one-way analysis of variance (ANOVA), and significant differences among treatment means were then separated using Fisher's least significant difference at a *P* value of <0.05 (least significant difference [LSD]) (PROC GLM; SAS Institute, 2011).

Nucleotide sequence accession numbers. Gene sequences for all loci used in the MLST and 16S rRNA phylogenetic analyses have been deposited in the GenBank nucleotide database under accession numbers KF650989 through KF650996.

RESULTS

Amplification and sequencing of 16S rRNA gene. The 16S rRNA sequence of isolate A396 was compared with those of available type strains of the *Burkholderia* genus in EzTaxon. EzTaxon contains a manually curated database of type strains of prokaryotes and provides identification tools using a similarity-based search (<http://eztaxon-e.ezbiocloud.net/>). According to EzTaxon results, 25 type strains of *Burkholderia* showed 97% or more similarity to A396, and the closest matches were *B. plantarii* LMG 9035^T (98.9% pairwise similarity) and *B. glumae* LMG 2196^T (98.69% pairwise similarity). Isolate A396 was very similar to several members of the Bcc, and no significant similarities (above 97%) to other taxa were found. Isolate A396 was 95.17% similar to *Pandoraea thiooxydans* ATSB16^T. In a comparison to all type strain sequences for *Burkholderia*, the lowest similarity was 94.13%. A neighbor-joining tree was built using MEGA5 with the A396 16S rRNA full sequence and 16S rRNA sequences for all type strains within the genus *Burkholderia* that showed 97% or more similarity to A396. Sequences were aligned by MUSCLE in MEGA5. The phylogenetic tree (Fig. 1) and the estimate of evolutionary divergence for A396 compared to top matches indicate that there is no definitive species level match to A396. However, the closest branches in the tree include *B. plantarii* LMG 9035^T and *B. glumae* LMG 2196^T.

Bcc-specific *recA* gene amplification. Procedures for *recA* gene amplification described by Mahenthalingam et al. (42) were followed in detail. This protocol has been successfully ap-

TABLE 1 Biochemical characteristics for the differentiation of isolate A396 from closely related *Burkholderia* species^a

Parameter	Result for organism				
	1	2	3	4	5
Assimilation					
L-Arabinose	—	+	+	+	+
Cellobiose	—	—	d+	+	+
D-Glucose	+	+	+	+	+
Lactose	—	+	—	+	—
Maltose	—	—	—	+	—
Raffinose	—	—	d+	—	—
D-Xylose	—	+	+	+	+
Dulcitol	—	—	+	+	+
Caprate	+	+	+	+	±
Citrate	—	+	+	+	±
Phenylacetate	—	+	—	+	—
% DNA similarity to type strains	NA	35.7–44.5	11.5–20.6	37.3–37.4	33.4–37.4
Fatty acid content (%)					
C _{16:0}	24.4	19	28.1	22.9	25.9
C _{17:0} cyclo	7.09	5.3	2.3	2.1	3.8
Summed feature 2	5.86				
Summed feature 3	20.18	16.3	22.6	6.5	21.2
Summed feature 8	26.19	37.4	33.4	11.5	29

^a Data were adapted from references 34 and 74. 1, isolate A396; 2, *B. cenocepacia*; 3, *B. glumae*; 4, *B. multivorans*; 5, *B. plantarii*. NA, not applicable. d+, up to 89% of strains are positive.

plied to classification and identification of environmental and clinical *Burkholderia* isolates to determine if they are part of the human-pathogenic *B. cepacia* complex. PCR amplification yielded no products when performed with strain A396. *B. multivorans* ATCC 17665 was used as a positive control and yielded strong bands for both primer sets. *P. fluorescens* CL145A was used as a negative control and yielded no amplification product.

DDH. Based on results from full 16S rRNA sequencing, fatty acid methyl ester (FAME) analysis, and phenotypic characterization, DDH experiments were run with *B. glumae* DSM 9512^T (=LMG 2196^T), *B. plantarii* DSM 9509^T (=LMG 9035^T), and *B. cenocepacia* DSM 16553^T (=LMG 16656^T). All DDH experiments yielded low or intermediate DNA-DNA similarities ranging from 11.5 to 44.5% (Table 1). Based on the recommended threshold value of 70% DNA-DNA similarity for the definition of bacterial species (51), isolate A396 is not identical with any of the species listed above.

Phenotypic and biochemical characterization. Based on fatty acid composition, isolate A396 was identified as *B. cenocepacia* GC subgroup B by Sherlock MIDI database, with a 0.885 similarity index. Other close matches included *B. cepacia* and *B. gladioli*. The most abundant fatty acids in strain A396 were C_{16:0} (24.47%), C_{17:0} cyclo (7.09%), summed feature 2 (might include 12:0 aldehyde, 16:1 isoI, 14:0 3OH, and an unknown peak at 10.95; 5.86%), summed feature 3 (might include 16:1ω7c and ω6c; 20.18%), and summed feature 8 (might include 18:1ω7c and ω6c; 26.19%). The following fatty acids contributed less than 2% each to the total fatty acid composition: 14:1w5c, 17:0, 16:1 2OH, 16:0iso 3OH, 16:0 2OH, summed feature 5, 18:0, 18:1ω7c 11me, 19:0, and 18:1 2OH. Isolate A396 grew at all temperatures tested (16, 25, 30, and 37°C), although growth at 16°C was very slow.

Growth of A396 on Mueller-Hinton agar plates was suppressed by kanamycin (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), piperacillin (100 μg), imipenem (10 μg), and sulfamethoxazole-trimethoprim (23.75/25 μg). Resistance was observed for tetracycline (30 μg), erythromycin (15 μg), streptomycin (10 μg), penicillin (10 μg), ampicillin, oxytetracycline, gentamicin, and cefuroxime (30 μg). Additional information regarding antibiotic resistance was obtained from phenotypic microarray analysis. The antibiotic resistance profile (diverse antibiotics distributed across several plates) indicated that A396 is sensitive to (concentration ranged from 1 to 4 μg/ml) cloxacillin, minocycline, nalidixic acid, oxacillin, novobiocin, sulfadiazine, tylosin, oleandomycin, sulfisoxazole, and vancomycin (see the supplemental material). According to phenotypic microarray results, A396 is capable of using the following substrates as carbon sources for growth: L-proline, D-trehalose, D-mannitol, L-glutamic acid, D-glucose-6-phosphate, α-D-glucose, L-glutamine, D-fructose-6-phosphate, L-malic acid, pyruvic acid, γ-amino-N-butyric acid, butyric acid, capric acid, caproic acid, 5-keto-D-gluconic acid, and dihydroxyacetone. The following compounds can be used by A396 as nitrogen sources for growth: ammonia, nitrite, nitrate, urea, L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-glutamic acid, L-glutamine, glycine, L-histidine, L-isoleucine, L-lysine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, D-alanine, D-asparagine, D-glutamic acid, D-serine, L-homoserine, L-pyroglutamic acid, ethanolamine, putrescine, agmatine, β-phenylethylamine, N-acetyl-D-glucosamine, adenine, adenosine, cytosine, guanosine, thymine, thymidine, uracil, inosine, xanthine, xanthosine, uric acid, allantoin, parabanic acid, γ-amino-N-butyric acid, ε-amino-N-caproic acid, and α-amino-N-valeric acid. Isolate A396 does not grow at or above 2% NaCl, 3% KCl, or 4% urea. No growth was detected at pHs of ≤4 and ≥10.

Beet armyworm toxicity. Second larval instars of BAW were negatively affected by A396 WCB-treated broccoli leaf discs 3 days after exposure (Fig. 2). After larvae fed on A396-treated leaf discs for 3 days, the mortality rate was 75% ± 22%, which was not statistically different from the observed mortality in the Xentari treatment and was significantly higher than observed mortality in the water treatment. Repeated trials provided consistent results, with A396 providing good control of BAW larvae. Similarly, ex-

FIG 2 Average percent mortality for BAW larvae on broccoli leaf discs treated with A396 WCB, 3 days postexposure. Means with the same letter are not statistically different (SAS analysis, one-way ANOVA; *P* value < 0.0001; LSD, α = 0.05).

TABLE 2 Average percent mortality of first-instar beet armyworm larvae, *Spodoptera exigua*, exposed to A396 WCB

Treatment	% mortality			
	Cell-free supernatant		WCB	
	Day 3	Day 4	Day 3	Day 4
WCB (% vol/vol)				
0.5	11.5	20.8	55.6	88.2
1	30.8	37.5	85	97.4
2	28.6	46.4	90	95
4	57.7	88.5	90	100
<i>B. thuringiensis</i> (positive control)	98.2	100	100	100
Water (negative control)	3.6	3.6	1.9	1.9

posure of first-instar BAW larvae to diets treated with different dilutions of A396 WCB and cell-free supernatants resulted in high mortality. Eighty-five percent of the larvae that ingested a diet treated with 1% A396 WCB died within 3 days (Table 2). The mortality rate dropped to 56% after larvae ingested a diet treated with 0.5% A396 WCB. Four days after ingestion, the diet treated with A396 WCB at 0.5 and 1.0% resulted in 88.2 and 97.4% mortality, respectively. The negative control exhibited 2% larva mortality. The efficacy of the cell-free supernatants was lower than that of the WCB. At the maximum dose of 4%, 88.5% mortality was reached by day 4, compared to 100% with WCB. Cell-free supernatants dosed at 2% were only half as efficacious as WCB at the same concentration.

Contact activity against BAW by topical application to the larval thorax was demonstrated, with an average of $50\% \pm 28\%$ larvae negatively affected (i.e., dead or stunted) 3 days after treatment with WCB, and up to 90% when heat-treated WCB was used (Table 3). In the water control most larvae survived, pupated, and eclosed normally. Larvae treated with WCB and larvae treated with heat-treated WCB showed stunted growth at 3 days after treatment and at the pupation stage. Less than half of the treated larvae pupated and eclosed successfully for any of the A396 treatments. Several larvae eclosed as stunted individuals and died. Overall, the heat-treated WCB had a slightly higher mortality rate than that of the regular WCB. For both treatments, treated larvae showed discoloration and stunting and were unable to molt or pupate normally (Fig. 3).

TSSM toxicity. A396 WCB at 6% (vol/vol) showed significant activity against TSSM adults. The mortality rate was 93% 3 days after exposure to treated leaf discs, which was significantly greater than the mortality observed in the water treatment (paired *t* test;

FIG 3 Photographs illustrating the effects of A396 treatments of BAW. (A) Stunted larva compared to normally developing larva; (B) larva with liquefied frass; (C) larva with molting problems; (D) larva with molting problems (left) compared to control larva. (Photos by Sarah Han.)

$P = 0.023$) (Fig. 4). Heat-treated WCB had slightly lower activity, but the difference was not statistically significant. When observed under a microscope, TSSM adults that had died due to A396 treatment were dark in color and soft and easily disintegrated when touched with a paint brush or pin.

DISCUSSION

The taxonomic position of *Burkholderia* sp. A396 was elucidated by following a polyphasic approach. A comparison of the 16S rRNA sequence of strain A396 to known members of the genus *Burkholderia* initially showed a close resemblance to Bcc type strains. Results from pairwise comparisons in EzTaxon yielded over 97% similarity against 25 validly named type strains, including several representatives of the Bcc (see supplemental material). Bootstrap analysis of the phylogenetic tree (Fig. 1) indicated that A396 occupies a different branch in the tree but is closely related to *B. glumae* and *B. plantarii*. Relationships between microorganisms that branch closely in the phylogenetic tree must be verified through DNA-DNA hybridization experiments. Hybridization experiments between isolate A396 and closely related type strains yielded no match at the species level. The percent similarity was well below the accepted threshold of 70% similarity for isolates of the same species (Table 1).

TABLE 3 Effects of topical applications of A396 WCB and heat-treated WCB on first-instar beet armyworm larvae

Treatment	No. of larvae in expt 1/2 3 days after treatment and at pupation stage ^a							
	3 days after treatment			At pupation stage				
	Dead	Stunted	Alive	Dead	Stunted	Pupated	Eclosed	% affected
Water	0/1	0/0	10/9	0/1	0/0	10/9	10/9	0–10
A396 heat-treated WCB	1/4	8/1	1/5	7/7	2/2	1/1	0/1	90–100
A396 WCB	4/1	3/2	3/7	6/4	0/4	4/2	4/2	60–80

^a *n* = 10 for all experiments.

FIG 4 Average percent mortality of TSSM adults 3 days after exposure to fava bean leaf discs treated with A396 WCB. The asterisk indicates a statistically significant difference (SAS analysis, one-way ANOVA; $P < 0.0001$; LSD, $\alpha = 0.05$).

Biochemical characterization in a commercial platform (Biolog GENIII) yielded inconclusive results and a match to the genus level only. However, certain characteristics were identified in the full phenotypic microarray that could be used for initial differentiation of A396 from closely related *Burkholderia* species when combined with 16S rRNA sequencing. Most *Burkholderia* species assimilate cellobiose, arabinose, and citrate, but strain A396 does not.

According to *Bergey's Manual of Systematic Bacteriology* (see Table BXII.β0.1 in reference 52), members of ribosomal DNA group II, which includes *Burkholderia*, have fatty acid compositions containing 14:0 3OH, 16:0 2OH, and 18:1 2OH. Most strains also contain 16:0 2OH and 16:1 2OH. Our FAME analysis results indicate that A396 is most similar to ribosomal DNA group II members, except for 16:1 3OH, which is not present in A396. According to the latest review regarding the classification and identification of the *B. cepacia* complex (6), FAME profiles have been discontinued for identification purposes due to the inability to distinguish isolates with certainty at the species level, to the inability to differentiate *B. gladioli*, and to the high standard deviation that render the profiles unreliable for unequivocal identification of *Burkholderia* isolates. Overall, we were able to confirm that A396 belongs to the genus *Burkholderia*, but more refined identification can be accomplished only by analysis of the genetic data obtained.

A study on the antibiotic susceptibility of selected Bcc strains to an array of antibiotics (53) found that all strains were resistant to polymyxin B, and the majority of strains were resistant to chloramphenicol and trimethoprim. Isolate A396, in comparison, is resistant to polymyxin B (1 to 4 $\mu\text{g}/\text{ml}$) but is suppressed by chloramphenicol (30 μg) and sulfamethoxazole-trimethoprim (23.75/25 μg). *Bergey's Manual of Systematic Bacteriology* (52) indicates that all strains of *B. cepacia* are resistant to novobiocin, but A396 was found to be sensitive to novobiocin (see the supplemental material).

Limitations to the development of *Burkholderia* species for biocontrol are the implications for human and animal health, and therefore, it is important to determine if A396 is a member of the Bcc. Our initial characterization based on 16S rRNA (*rss* gene)

indicated that isolate A396 was phylogenetically situated in the plant-pathogenic *Burkholderia* cluster and close to the *B. mallei* and Bcc clusters (Fig. 1). It has been proposed that the large divergence in 16S rRNA sequence similarity between the different *Burkholderia* lineages is indicative of different species clusters, with one lineage made up of saprophytic species and another made up of the Bcc, pathogens, and opportunistic pathogens of plants, humans, and animals (54). Several research groups have successfully applied Bcc-specific *recA*-based PCR test as a diagnostic tool for the identification of *B. cepacia* complex isolates in clinical (42, 55) and environmental (56–58) settings. Specific *recA* primers have also been used to determine relatedness of potential biocontrol and environmental isolates to Bcc species (59–61). In fact, the PCR-based evaluation and sequencing of the *recA* gene have been identified as a powerful tool for identification and classification of *Burkholderia* isolates. In their most recent review, Vandamme and Dawyndt (22) indicate that *recA* is an indispensable tool. They also report that sequence similarity within Bcc species is 98 to 99%, while interspecies sequences are 94 to 95% similar (42, 62, 63). The most recent literature suggests that for the majority of the *B. cepacia* complex isolates, *recA* and MLST techniques provide the best results for identification (22, 64). For isolate A396 in particular, we were unable to amplify the *recA* gene using Bcc-specific primers, but we were able to obtain a *recA* sequence from MLST primers. A BLAST search yielded matches with several strains of *B. mallei* and *B. pseudomallei* but only 92% similarity, well below the threshold suggested for interspecies sequences. MLST analysis of A396 revealed that all seven MLST loci have unique sequences compared to all available allele types in the MLST database (<http://pubmlst.org/bcc/>). All allele types are unique, and A396 does not share any allele types with any other Bcc or non-Bcc isolates in the database. The sequence type for A396 was submitted to the database and designated ST669. Allele types were as follows: *atpD*, 297; *gltB*, 342; *gyrB*, 500; *recA*, 316; *lepA*, 362; *phaC*, 272; and *trpB*, 345.

Two large clades of *Burkholderia* are recognized. The first includes the “*pseudomallei*” group, Bcc species, plant-pathogenic species, and endosymbionts of plant-pathogenic fungi; the second clade includes species that have been isolated from soils and other environmental samples, nonpathogenic and generally beneficial to plants. The sequences obtained from the sequencing of the seven MLST loci were concatenated and aligned against representatives of the Bcc with locus profiles in the Bcc MLST database. A neighbor-joining tree built from the alignment (Fig. 4) indicated that isolate A396 groups with *B. oklahomensis*, closest to the plant-pathogenic cluster and separate from the Bcc cluster (Fig. 5). Based on the interpretation of all of characterization results, we are able to place isolate A396 near the plant-pathogenic cluster of *Burkholderia* but not related to Bcc, as A396 lacks *recA* and MLST similarity to Bcc species. Additionally, we did not find any clinical *Burkholderia* isolate sequences (16S rRNA, *recA*, or MLST) with significant similarity to those of A396.

Additional work has been done to genetically characterize isolate A396. The genome has been sequenced, and work is ongoing to annotate a draft assembly (data not yet published). In the process of the annotation, additional genes were surveyed, including *acdS* and *rpoB*. The *rpoB* homologous gene displayed >90% similarity to several *Burkholderia* species. A neighbor-joining tree based on *rpoB* sequences (data not shown) placed isolate A396 close to several *B. mallei* and *B. pseudomallei* strains, but on a

FIG 5 Neighbor-joining tree inferred from the alignment of concatenated sequences of *atpD*, *gltB*, *gyrB*, *lepA*, *phaC*, *recA*, and *trpB*. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (2,000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the number-of-differences method and are in the units of the number of base differences per sequence. The analysis involved 57 nucleotide sequences. There were a total of 2,782 positions in the final data set. Evolutionary analyses were conducted in MEGA5.

district branch from Bcc species. This result agrees with our findings based on *recA* sequences, and the tree also corresponded with previous reports indicating that the *Burkholderia* species clusters into four lineages: (i) Bcc; (ii) *B. mallei*, *B. pseudomallei*, and *B. thailandensis* (where isolate A396 clustered); (iii) plant pathogens; and (iv) nonpathogenic environmental isolates (65). In the case of the *acdS* gene, the best matches reached only 91% similarity to several *Burkholderia ambifaria* strains. A neighbor-joining tree was built based on the best matches from BLAST, and isolate A396 occupied a distinct branch, in proximity to several isolates of plant- and rhizosphere-associated *Burkholderia*. Previous studies have shown that *Burkholderia acdS* gene intraspecies similarity ranges between 76 and 99% (66), and that is distributed across pathogenic, opportunistic pathogenic, and nonpathogenic environmental isolates. Both *rpoB* and *acdS* preliminary analyses support our proposal of isolate A396 as a novel species of *Burkholderia*. Data and analysis regarding the genome of isolate A396 will be expanded and published in the future.

The high efficacy of isolate A396 observed against the BAW and TSSM in both contact and feeding bioassays points to the potential for strain A396 to be developed as a biocontrol product. The activity observed translated from *in vitro* testing to semi-*in planta* testing and was maintained even when cells were removed and when material was heat treated, strongly suggesting that an excreted compound or secondary metabolite is involved in the insecticidal and miticidal activities. The reduced efficacy of the cell-free supernatant might be due to the removal of cell-associated active compounds during the filtration process. The activity was only minimally reduced by the heat treatment (60°C for 2 h), indicating that the active compounds are heat stable.

No attempts were made to recover isolate A396 from the dead insects, so an assessment of pathogenicity to the pests is not possible. However, experiments with cell-free supernatants clearly indicated that live cells are not required for activity. In fact, the activity was maintained at similar levels after removing the cells and heating the material. Characteristic for the genus *Burkholderia* is the wide variety of secondary metabolites produced by these ubiquitous bacteria. Many of them secrete a variety of extracellular enzymes with proteolytic, lipolytic, and hemolytic activities, as well as toxins, antibiotics, exopolysaccharides, and siderophores. In a recent review, Vial et al. (18) discuss the great diversity and versatility of extracellular compounds produced by the different species of *Burkholderia*. Some of the known toxins produced by *Burkholderia* spp. include toxoflavin {1,6-dimethylpyrimido[5,4-e]-1,2,4-triazine-5,7(1H, 6H)-dione} and fervenulin (a tautomeric isomer of toxoflavin) with antibacterial, antifungal, and herbicidal activities (67); rhizobitoxin {[2-amino-4-(2-amino-3-hydroxypropoxy)-*trans*-but-3-enoic-acid]}, which, among other phytotoxic effects, induces foliar chlorosis due to inhibition of cystathione- β -lyase (68); and rhizoxin, a macrocyclic polyketide which kills rice seedlings through binding to β -tubulin, resulting in inhibition of the normal cell division cycle (69). This compound also demonstrates broad antitumor activity *in vitro* (70); bongkrekic acid, which inhibits adenine nucleotide translocase as well as cell apoptosis (71); rhizonins A and B, hepatotoxic cyclopeptides that were first discovered from a fungal *Rhizopus* sp. but later on were shown to be produced by a bacterial endosymbiont of the genus *Burkholderia* (72); and tropolone (2-hydroxy-2,4,6-cycloheptatrien-1-one), a nonbenzenoid aromatic compound with both phenolic and acidic moieties and proven antimicrobial, an-

tifungal, and insecticidal properties (73). Tropolone is produced by *B. plantarii*, and insecticidal activity has been reported against *Tyrophagus putrescentiae* (Formosan subterranean termite), *Dermatophagoides farina* (mold mite), and *Coptotermes formosanus* (house dust mite) (73), and repellency has been reported against *Callosobruchus chinensis* (cigarette beetle) (74). We have conducted experiments toward the purification of the active compounds produced by isolate A396 (75) and determined that the activity is associated with at least two amide-type compounds and at least one depsipeptide-type molecule. Tropolone has so far not been detected but would not be unexpected given the insecticidal activity displayed and phylogenetic proximity to *B. plantarii*.

Observations made during the evaluation of insect feeding and contact bioassays may indicate effects on the insect and mite cuticle development and molting. TSSM treated with A396 displayed melanization (i.e., dark color), as well as loss of integrity of the exoskeleton (i.e., insects disintegrated upon light touch). In the case of the BAW, the bioassay observations are indicative of disruption of the molting and cuticle formation processes. BAW larvae presented stunted growth (treated larvae were significantly smaller than control larvae), problems molting (larvae did not shed cuticle or only partially shed cuticle), and liquefied frass. It is interesting that this effect was observed in the larvae that were treated topically by application to the thorax, as well as in those that fed on the A396-treated diet. Both observations can be linked to enzymatic degradation of the insect exoskeletal structures and interference with the molting process. Chitinases hydrolyze the structural polysaccharide chitin, a linear homopolymer of 2-acetamido-2-deoxy-D-glucopyranoside, linked by β -1,4-linkages, which is a component of the exoskeletons and gut linings of insects. Insect cuticles provide a physical barrier to protect the insect from pathogens or other environmental hazards and are composed primarily of chitin (76). The entomopathogenic fungi *Metarhizium anisopliae*, *Beauveria bassiana*, *Beauveria amorpha*, *Verticillium lecanii*, and *Aspergillus flavus* all secrete chitinases to break down the cuticle and enter the insect host (77). The peritrophic membrane, which lines the insect midgut, is another primarily chitin-composed barrier that protects insects from pathogens. Any enzyme that can puncture this membrane has potential as a bioinsecticide (78). Several thermostable chitinases are reported in the literature (79, 80). Proteases with insecticidal activity fall into three general categories: cysteine proteases, metalloproteases, and serine proteases (81). Proteases of these classes target the insect midgut, cuticle, and hemocoel, enhancing the insecticidal activity of agents such as baculovirus (82, 83). The peritrophic matrix of the midgut is an ideal target for insect control because it lines and protects the midgut epithelium from food particles, digestive enzymes, and pathogens in addition to acting as a biochemical barrier (84). Enhancins are zinc metalloproteases expressed by baculoviruses that facilitate nucleopolyhedrovirus infections in lepidopterans. These proteases promote the infection of lepidopteran larvae by digesting the invertebrate intestinal mucin protein of the peritrophic matrix, which, in turn, promotes infection of the midgut epithelium (85). Homologs of enhancin genes found in baculovirus have been identified in the genomes of *Yersinia pestis*, *Bacillus anthracis*, *Bacillus thuringiensis*, and *Bacillus cereus* (86, 87). *Burkholderia* isolates are known producers or chitinases (88–90) and proteases (91, 92), as well as other enzymes and metabolites that could contribute to the degradation of the insect cuticle and midgut (93). *Bacillus* spp. are known to produce

thermostable toxins and proteases with insecticidal activity (94). The slow action of A396 also points to a nonneurotoxic mode of action. Typical symptoms of neurotoxicity, such as tremors and paralysis, were not observed in the treated insects. More detailed work will be needed to fully elucidate the insecticidal mode of action for strain A396 and to determine if there are any peptides or proteins contributing to the insecticidal activity.

Based on results presented here, we conclude that *Burkholderia* sp. A396 is a novel member of the *Burkholderia* genus with insecticidal and miticidal activities and that it lacks the genetic markers commonly associated with members of the *B. cepacia* complex. We propose the name *Burkholderia rinojensis* sp. nov.

Description of *Burkholderia rinojensis* sp. nov. *Burkholderia rinojensis* (ri.no.jen.sis. Fem.L. adj. from Rinoji, referring to the location of the soil from which the isolate was recovered).

Cells are Gram-negative, nonsporulating, oxidase-positive, catalase-positive, urease-positive, small straight rods that grow as cream-colored shiny colonies on potato dextrose agar. Colonies turn into a light shade of pink after 48 h of incubation. Sensitive to cloxacillin, minocycline, nalidixic acid, oxacillin, novobiocin, sulfadiazine, tylosin, oleandomycin, and sulfisoxazole; resistant to lincomycin, vancomycin, troleandomycin, oxytetracycline, polymyxin B, and penicillin G (data from Biolog Phenotypic microarray). Grows well between pHs 5 and 9.5 and is able to grow at pH 4.5 in the presence of L-methionine. When evaluated in the Biolog format, negative for assimilation of L-arabinose, cellobiose, lactose, maltose, raffinose, D-xylose, dulcitol, citrate, and phenylacetate but able to assimilate D-glucose, D-mannitol, and caprate. It can utilize urea and γ -aminobutyric acid for growth and tolerate up to 1% NaCl. Does not grow at 2% NaCl or higher. Fermentation supernatants of A396 display insecticidal and miticidal activities against *Spodoptera exigua* and *Tetranychus urticae*. The only specimen from this new species is isolate A396, which is also the type strain, *Burkholderia rinojensis* A396^T (=NRRL B-50319^T). *Burkholderia rinojensis* effectively controls BAW and TSSM, causing high mortality rates for the pests through ingestion and contact activity.

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