Isolation and Characterization of *Burkholderia gladioli* from Orchids in Hawaii

Lisa M. Keith, United States Department of Agriculture–Agricultural Research Service (USDA-ARS), Pacific Basin Agricultural Research Center (PBARC), Tropical Plant Genetic Resource Management Unit (TPGRMU), Hilo, HI 96720; **Kelvin T. Sewake,** University of Hawaii–Cooperative Extension Service, Hilo 96720; and **Francis T. Zee,** USDA-ARS, PBARC, TPGRMU

ABSTRACT

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Bacterial diseases of orchids continue to be serious problems. Bacterial strains were isolated from orchid plants exhibiting disease symptoms in Hawaii. Small to large leaf spots with or without water-soaking or soft rots were observed on various orchid genera, including *Dendrobium*, *Oncidium*, and *Miltonia* spp. and hybrids. Bacteria isolated and cultured from the lesions were tentatively identified using analytical profile index (API) strips and standard physiological and biochemical tests, and confirmed by species-specific polymerase chain reaction and sequencing of the 16S rRNA gene. The variation in pathogenic, morphological, cultural, and molecular characteristics of the orchid isolates also was evaluated. In our studies, a gram-negative, aerobic, rod-shaped bacterium that produced pale yellow, opaque, round colonies with entire margins on nutrient broth yeast extract agar (NBY) was isolated consistently from diseased orchid plants. On yeast dextrose calcium carbonate agar, the isolates produced brownish-yellow, nonmucoid colonies, with the majority of the strains secreting a diffusible yellow or tan pigment into the media. The bacterium was identified as *Burkholderia gladioli*. Molecular analysis indicated very little diversity in the 16S rDNA gene. Testing *B. gladioli* isolates using media containing copper or streptomycin indicated varying levels of resistance (copper resistant = Cu^r; streptomycin resistant, Sm^r), with approximately 75% of the strains resistant to copper and 94% of the strains resistant to streptomycin. The minimum inhibitory concentration (MIC) of cupric sulfate among Cu^r strains ranged from 50 to 1,000 µg/ml and the MIC of streptomycin was 50 to 100 µg/ml for all Sm^r *B. gladioli* strains tested. Field and laboratory data suggest the frequent use of these chemicals in nurseries may have inadvertently resulted in the development of copper and streptomycin resistance in *B. gladioli* from orchids.

Additional keyword: *Orchidaceae*

In 2003, orchid crops in Hawaii, which included potted plants and cut-flower production, had a commercial value of $24 million (2). Although disease symptoms have been found in all orchid production areas of Hawaii, difficulty exists in determining whether the pathogen is viral, fungal, or bacterial in origin based on visual observations alone (30). Diseases affecting the family Orchidaceae include root diseases, stem and pseudobulb decays, leaf spots, and flower blights. Worldwide, the most common and important bacterial disease problems include soft rot caused by *Erwinia carotovora* subsp. *carotovora* and *E. chrysanthemi*, brown rot caused by *E. cypripedii*, and corm and basal leaf rot caused by *Acidovorax cattleya* (25). In Nongkham, Thailand, the losses of *Dendrobium* orchids due to bacterial diseases were estimated at 50% or more (6), and the primary pathogen was *Burkholderia gladioli* (formerly *Pseudomonas gladioli* and *P. marginata*). *B. gladioli* was described originally as a phytopathogen on *Gladiolus* spp., causing rot of gladiolus corms (18); other hosts include onion, iris, and freesia (29). *B. gladioli* has been reported as a pathogen of *Dendrobium* orchids in Hawaii (30), but the occurrence, the genetic diversity, and the extent of the bacterium's ability to cause severe rot on various orchid genera remains unknown. The objectives of this research were to determine the extent of *B. gladioli* as a causal agent of orchid disease in Hawaii, to determine the effectiveness of current molecular techniques in identifying and distinguishing *B. gladioli* from other bacteria, and to determine the levels of copper and streptomycin resistance present in the population. To our knowledge, this is the first study conducted to evaluate the importance of *B. gladioli* as a pathogen on orchids in Hawaii and the effectiveness of chemicals used by growers for bacterial disease control.

Corresponding author: L. M. Keith
E-mail: lkeith@pbarc.ars.usda.gov

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MATERIALS AND METHODS

Field survey and sampling. From May 2002 to February 2004, leaves were collected from various orchid genera with leaf spot or rot symptoms from 18 farms located on the windward side of the island of Hawaii. Samples were collected during farm visits and from disease samples submitted by growers. Digital photographs of leaf symptoms were recorded using a Nikon Coolpix 995 model digital camera. Typical symptoms were noted and described.

Isolation and identification of the pathogen. Diseased samples were processed within 24 h of the collection. Small pieces of leaf tissue were cut from the edge of the advancing portion of a lesion, surface sterilized in 10% bleach for 2 min, rinsed in sterile distilled water, and macerated in a sterile mortar or petri dish with approximately 2 ml of sterile water. A 20-µl sample was streaked onto plates containing nutrient broth yeast extract agar (NBY; 31) supplemented with a filter-sterilized (0.22-µm pore size, nitrocellulose filter) solution of cycloheximide at 50 µg/ml. Plates were incubated at 28ºC. Individual bacterial colonies from plates were purified and stored at –80ºC in 15% aqueous glycerol, and working cultures were kept on NBY agar. For all isolates, gram reactions were determined by the lysis of bacteria in 3% KOH (9), cytochrome C oxidase was tested by using filter paper impregnated with NNN′N′-tetramethyl-p-phenylenediamine dihydrochloride (17), oxidative or fermentation metabolism was evaluated (12), pyocyanin production was tested on King's Medium B (KMB; 15), and growth on selective media was compared (yeast dextrose calcium carbonate [YDC; 34] and Miller Schroth [MS; 20]). *Burkholderia* spp. initially were identified using analytical profile index (API) 20NE test strips (bioMerieux, Inc., Durham, NC) incubated at 30ºC for 24 to 48 h. The API 20NE test strips combine conventional and assimilation tests for the identification of non-enteric gram-negative rods. *B. gladioli* strains were characterized and compared with the results found in the API (bioMerieux, Inc.). Information from Ber-

gey's Manual (16) also was used for comparative purposes.

Pathogenicity tests. Bacteria isolated were grown on NBY agar for 48 h. Healthy orchid plants of the genera *Phalaenopsis*, *Dendrobium*, *Miltonia*, *Oncidium* (approximately 2.5 years old in 10-cm pots), and *Cattleya* (approximately 3 years old in 10-cm pots) were used for the inoculation tests. To fulfill Koch's postulates, individual orchid leaves were inoculated (multiple inoculations on two leaves) by wounding with a sterile toothpick smeared with *B. gladioli*. Sterile water was used for control inoculation. The inoculated plants were covered with plastic bags or placed in a dew chamber in the laboratory at 24ºC with 70% relative humidity for 48 to 72 h. Disease development was evaluated beginning at 2 to 3 days after inoculation by measuring the length of the water-soaked lesion that formed at the inoculation site. Lesion development was monitored for 14 days before bacteria were reisolated.

The ability of the *B. gladioli* orchid strains to cause rot on alternate hosts also was determined. Slices of carrot and onion approximately 1.25 cm thick were surface disinfested by immersion in 10% bleach for 2 min, air dried in a laminar flow hood, and placed in petri dishes containing moistened filter paper. The center, outer surfaces of the carrot and onion slices were inoculated with the *B. gladioli* strains as indicated above and incubated at 28ºC in the dark. A necrotic zone or soft rot lesion at least 2 mm beyond the inoculation site was considered evidence of infection for all pathogenicity tests. Bacteria were reisolated on NBY from tissues near the infected areas to confirm the identity of the pathogen. All inoculation experiments were conducted twice.

Cloning and sequencing of 16S rRNA. Molecular methods were used to confirm identification of the strains and allow for phylogenetic analysis. Template DNA was prepared by picking individual bacterial colonies from agar plates using a sterile toothpick and resuspending in 50 µl of sterile distilled water. The suspension was boiled for 5 min and the cellular debris were pelleted by centrifugation. A 10-µl aliquot of the supernatant was used in the polymerase chain reaction (PCR). The universal bacterial primers used in this study were forward primer Y1 (TGGCTCAAACGAACGCTGGCCCG), which corresponds to position 20 to 43 in the *Escherichia coli* 16S rRNA sequence, and the reverse primer Y2 (CCCACTGCTGCCTCCCGTA), which corresponds to *E. coli* position 361 to 338 (35). Primer concentrations used in PCR reactions were 0.5 µM. The $MgCl_2$ concentration was 2 mM. All reactions used 200 µM dNTPs (New England Biolabs, Beverly, MA) and 1 U of *Taq* polymerase (Promega Corp., Madison, WI). The final volume of the PCR reaction was 50 µl. PCR reactions were performed in an MJ Scientific PTC-100 machine. PCR consisted of an initial denaturation at 94ºC for 3 min; followed by 30 cycles of 94ºC for 45 s, 62ºC for 40 s, and 72ºC for 2 min; with a final extension at 72ºC for 10 min. A 10-µl aliquot of the reaction was analyzed by electrophoresis on a 1.5% agarose gel (Invitrogen Co., San Diego, CA) at 70 V for 2 h in Tris-acetate buffer (24). Gels were stained with ethidium bromide. A 1-kb ladder (Invitrogen Co.) was used as a size marker. PCR products were cloned into the TOPO TA vector (Invitrogen Co.) according to the manufacturer's recommendations. Plasmid DNA for sequencing was prepared with the Qiagen plasmid miniprep kit according to the recommendations of the manufacturer (Qiagen, Inc., Chatsworth, CA). DNA sequencing was performed at MWG Biotech, Inc. (High Point, NC). Sequence data was aligned and homology was determined using the National Center for Biotechnology Information Blast Network Server (1). A multiple sequence alignment was constructed using CLUSTALX (28) and BOXSHADE 3.21 (K. Hofmann, ISREC Bioinformatics group, Lausanne, Switzerland, and M. Baron, Institute for Animal Health, Surrey, U.K.). A phylogenetic tree was constructed by the neighbor-joining method (23) derived from the 16S rDNA sequences.

Species-specific PCR. The identification of *B. gladioli* was confirmed using the primer pair LP1 (GGGGGGTCCATTGCG, target site 872–886) and LP4 (AGAAGCTCGCGCCACG, target site 1,523–1,508), directed toward a species-specific region of the 23S rRNA gene (32). PCR had an initial denaturation of 95ºC for 5 min with a subsequent 30-cycle amplification, and the PCR mixture contained 1 µM each primer, 10 ng of genomic DNA, 200 µM each dNTP, and 1.25 U of *Taq* DNA polymerase, in a total volume of 50 µl. PCR parameters included annealing at 60ºC for 10 s, denaturation at 95ºC for 10 s, and extension at 72ºC for 60 s. All PCR products were electrophoresed, stained, and visualized as mentioned above. The amplified band was 700 bp in length.

Phenotypic characterization of copper and streptomycin resistance. Resistance to copper and streptomycin was tested on 16 strains of *B. gladioli*. Cultures were grown for 48 h on mannitol-glutamate medium (MG; 14) containing 0.025% yeast extract (MGY) at 28ºC, and subsequently were streaked onto MGY plates containing cupric sulfate ($CuSO_4$; MGYCu; Sigma-Aldrich, St. Louis, MO) or streptomycin (MGYSm; Sigma-Aldrich). Minimum inhibitory concentrations (MICs) of cupric sulfate and streptomycin were determined for the *B. gladioli* strains by inoculating them to MGY agar amended with $CuSO_4$ at 375, 500, 750, or 1,000 µg/ml or streptomycin at 50, 75, 100, or 500 µg/ml. Strains that exhibited growth on MGY containing $CuSO_4$ or streptomycin equivalent to that on MGY alone after 72 h at 28ºC were considered resistant. The experiments were conducted twice.

RESULTS

Field survey and sampling. Over a period of a year and a half, samples were collected from orchids showing symptoms of bacterial disease. The size of the orchid farms from which the samples were gathered ranged from small, half-acre farms to large, commercial nurseries. Samples included the commercially cultivated orchid genera *Cattleya*, *Dendrobium*, *Miltonia*, *Oncidium,* and *Phalaenopsis*. The majority of the samples were from potted plants that were maintained under shade cloth and cover. Early removal and sanitation practices were observed in the farm to limit the spread of bacteria. Occasionally, samples with advanced disease symptoms were collected from plants that were exposed to the environment or to excessive overhead irrigation.

Natural symptoms. The most common symptoms observed were infected leaves containing dark-green to brown lesions surrounded by water soaking (Fig. 1A). When plants were exposed to environmental conditions which included rain and high humidity, the wet rot-looking area expanded rapidly to cause soft decay over the entire leaf, sometimes causing the leaf to drop off. Symptoms also were similar on pseudobulbs (Fig. 1B) and flower spikes (Fig. 1D). Symptoms appeared to spread more rapidly on younger plants (Fig. 1C).

Isolation and identification of the pathogens. The most frequently recognized symptom as described above was the focus of this study. Bacterial isolations from this type of diseased plant tissue consistently revealed the presence of a single type of bacterial colony on NBY from the various lesions. The bacterial isolates were gram-negative, aerobic rods. On NBY, they produced pale yellow, opaque, round colonies with entire margins. On YDC agar, the isolates produced brownish-yellow, nonmucoid colonies, with the majority of the strains secreting a diffusible yellow or tan pigment into the media. On MS media, all strains remained greenish-blue, indicating a nonenteric nature, and on KMB the strains produced a diffusible, green pigment that was nonfluorescent. The majority of the strains were oxidase positive (83%). All 18 strains utilized glucose, mannitol, N-acetylglucosamine, gluconate, caprate, and malate. Most of the strains utilized arabinose (96%), mannose (96%), adipate (83%), citrate (83%), and phenyl acetate (96%), and none of the strains produced indole or acidified glucose. On the basis of their morphological, physiological, and biochemical characteristics, the 18 isolates were identified as *B. gladioli* (Table 1).

Results from API 20NE strips and descriptions in Bergey's Manual of Systematic Bacteriology (16) provided further evidence in support of the identification of the isolates.

Pathogenicity tests. Plants artificially inoculated with *B. gladioli* developed symptoms similar to the commonly observed field symptoms, indicating that *B. gladioli* strains are pathogenic to multiple orchid species. All *B. gladioli* strains tested caused lesions around the inoculation sites with necrosis around the wound. On *Cattleya* spp. and hybrids, *Dendrobium* spp., *Miltonia* spp. and hybrids, *Oncidium* spp. and hybrids, and *Phalaenopsis* spp. and hybrids, the water-soaked lesions appeared 1 to 2 days after inoculation, which expanded rapidly to resemble natural infection within 3 to 4 days after inoculation (Fig. 1E). On *Miltonia* and *Oncidium* spp., the brown lesions expanded rapidly and were surrounded by a water-soaked margin. Certain strains of *B. gladioli* (1BB1, 2, and OW4) were noticeably more virulent on certain orchid genera. For example, deep tissue-penetrating lesions were recorded in inoculated *Cattleya* samples. The inoculated site developed 6-mm dark-brown, sunken lesions that appeared to be superficial; however, when the leaf was dissected crosswise, brown discoloration of the tissue underneath the epidermal layer, measuring up to 29 mm from the inoculation site, was recorded. Reisolations from the artificially infected plants yielded pure cultures of *B. gladioli*. Bacterial identification was confirmed with API 20NE strips or PCR using *B. gladioli* species-specific primers, thus fulfilling Koch's postulates. Control plants inoculated with a sterile toothpick never showed symptoms. When inoculation experiments were repeated, lesion sizes were virtually identical in size and shape for all *B. gladioli* strains and orchid genera tested. Lesions appeared within 1 to 2 days and continued to expand. Lesions on *Miltonia* and *Oncidium* spp. were surrounded by water soaking. For all experiments, high humidity after bacterial inoculation was critical for symptom development.

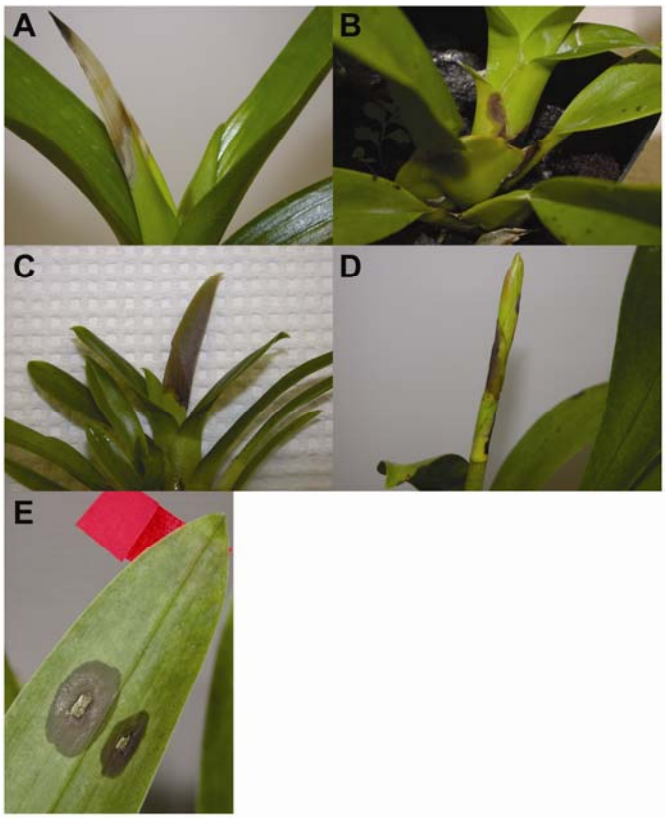

Fig. 1. Natural symptoms of bacterial leaf rot caused by *Burkholderia gladioli*. **A,** *Dendrobium* sp. leaf. **B,** *Oncidium* sp. pseudobulb. **C,** *Oncidium* sp. plantlet. **D,** *Oncidium* sp. spike. **E,** Symptoms of an artificially inoculated *Miltonia* sp. sample.

Table 1. Sources of 18 strains of *Burkholderia gladioli* recovered from diseased orchid plants in Hawaii and used in this study

Strain	Host	Tissue source[a]	Location collected
1BB1	*Dendrobium* sp.	Leaf, S	Pahoa
5-1	*Oncidium* sp.	Leaf, S	Pahoa
6-1	*Oncidium* sp.	Leaf, S	Pahoa
Dean4-2	*Oncidium* hybrid	Leaf, S	Orchidland Estates
F	*Miltonia* sp.	Leaf, S	Mountain View
06023	*Oncidium* sp.	Leaf, S	Kurtistown
1	*Oncidium* sp.	Leaf, P	Hilo
2	*Oncidium* sp.	Leaf, P	Hilo
3	*Oncidium* sp.	Leaf, P	Hilo
4	*Oncidium* sp.	Leaf, P	Hilo
OW2	*Oncidium* hybrid	Leaf, O	Hakalau
OW3	*Oncidium* hybrid	Leaf, O	Hakalau
OW3A2	*Oncidium* hybrid	Leaf, O	Hakalau
OW4	*Oncidium* hybrid	Leaf, O	Hakalau
OW5	*Oncidium* hybrid	Leaf, O	Hakalau
OW61	*Oncidium* hybrid	Leaf, O	Hakalau
OW62	*Oncidium* hybrid	Leaf, O	Hakalau
OW7	*Oncidium* hybrid	Leaf, O	Hakalau

[a] Tissue source: S = small potted plant, 4-in. square; P = potted plant, 5-in. round; O = orchid plant on an Oasis cube.

Cross-inoculation studies. The development of a necrotic zone or lesion of at least 2.0 mm beyond the inoculation site was considered positive for the sliced carrots and onions. The majority of the *B. gladioli* isolates produced a creamy white or light-brown lesion or soft rot when they were administered to the surface of the slices of carrot or onion (Fig. 2). Symptoms usually were visible within 3 days after inoculation and progressed as time elapsed. Some differentiation was observed on the slices between strains of *B. gladioli* from different orchid hosts. For example, on carrot, *B. gladioli* strain OW4 caused a soft rot that covered the width of the slice (*data not shown*), whereas strain OW5 caused a soft rot with visible necrosis (Fig. 2A). On onion slices, *B. gladioli* strain 5-1 caused a spreading, soft rot area (Fig. 2B), whereas strains OW61 and OW3A2 caused more localized lesions (*data not shown*). Control slices inoculated with a sterile toothpick did not show symptoms. When cross-inoculation studies were repeated, all *B. gladioli* strains re-

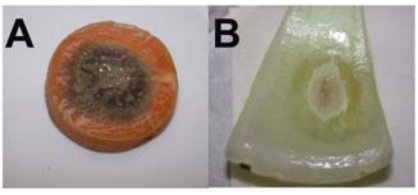

Fig. 2. Symptoms of cross-inoculation studies of *Burkholderia gladioli* on alternative hosts. **A,** *B. gladioli* 5-1 on carrot. **B,** *B. gladioli* OW5 on onion. Pictures were taken 9 days after inoculation.

acted in a similar manner on carrot and onion slices. Symptoms of soft rot with or without necrosis took about 3 days to become visible. Results indicate that carrot and onion can serve as alternative hosts for more than 90% of the *B. gladioli* orchid strains.

16S rRNA sequence analysis. When subjected to PCR with primer pair Y1/Y2, which amplifies the variable region located at the 5′ end of the 16S rRNA gene, all suspected *B. gladioli* isolates yielded an approximately 300-bp product (*data not shown*). Partial 16S rDNA sequences of the 18 suspected *B. gladioli* strains were obtained and deposited into the GenBank database with accession numbers DQ090070 through DQ090087. Both DNA strands were sequenced for verification. The partial 16S rRNA gene sequences of the *B. gladioli* strains were compared with gene sequences of known strains in GenBank. A BLAST search of the 16S rDNA sequences supported the morphological, physiological, and biochemical results that the isolates were *B. gladioli*. A multiple sequence alignment of the gene products was constructed using CLUSTALX. The 18 strains, except for a few nucleotide differences, were virtually identical. Phylogenetic trees were produced from the analysis of the aligned sequences of the 16S rDNAs generated in this study (Fig. 3). The tree indicated that, although overall genetic diversity is low, diversity does exist even among populations isolated from a single location (i.e., OW strains).

Species-specific PCR. The identity of the isolated bacterial strains also was confirmed using *B. gladioli*-specific PCR (32). An approximately 700-bp amplified fragment of the 23S rRNA gene was obtained for all *B. gladioli* strains tested (Fig. 4). No amplified fragment was observed with the other organisms tested. Thus, the species-specific-PCR test with LP1-LP4 could be used to accurately identify the *B. gladioli* isolates.

Copper and streptomycin sensitivity determination. A summary of the bactericide-resistant *B. gladioli* strains is presented in Table 2. Growth of bacteria on MGYCu (50 µg/ml) or MGYSm (25 µg/ml) was sufficient to identify strains with resistance to copper or streptomycin because copper- or streptomycin-sensitive strains of *B. gladioli* did not grow on these media. Resistance to streptomycin was widespread among strains of *B. gladioli* isolated from orchids in this study. Of 16 strains tested, 94% grew on MGY amended with streptomycin sulfate at 50 µg/ml. Copper-resistant (Cu^r) strains were not as prevalent as antibiotic-resistant strains, but a high percentage of resistance was evident (75% of the strains) and resistance to copper occurred over a much greater range. The MIC of cupric sulfate varied among Cu^r strains from 50 to 1,000 µg/ml, indicating a broad range of resistance to the copper salt. The MIC of streptomycin was 50 to 100 µg/ml for all strains tested. Interestingly, those strains that were resistant to copper also were resistant to streptomycin sulfate. The MICs remained constant for all *B. gladioli* strains when bactericide-sensitivity experiments were repeated.

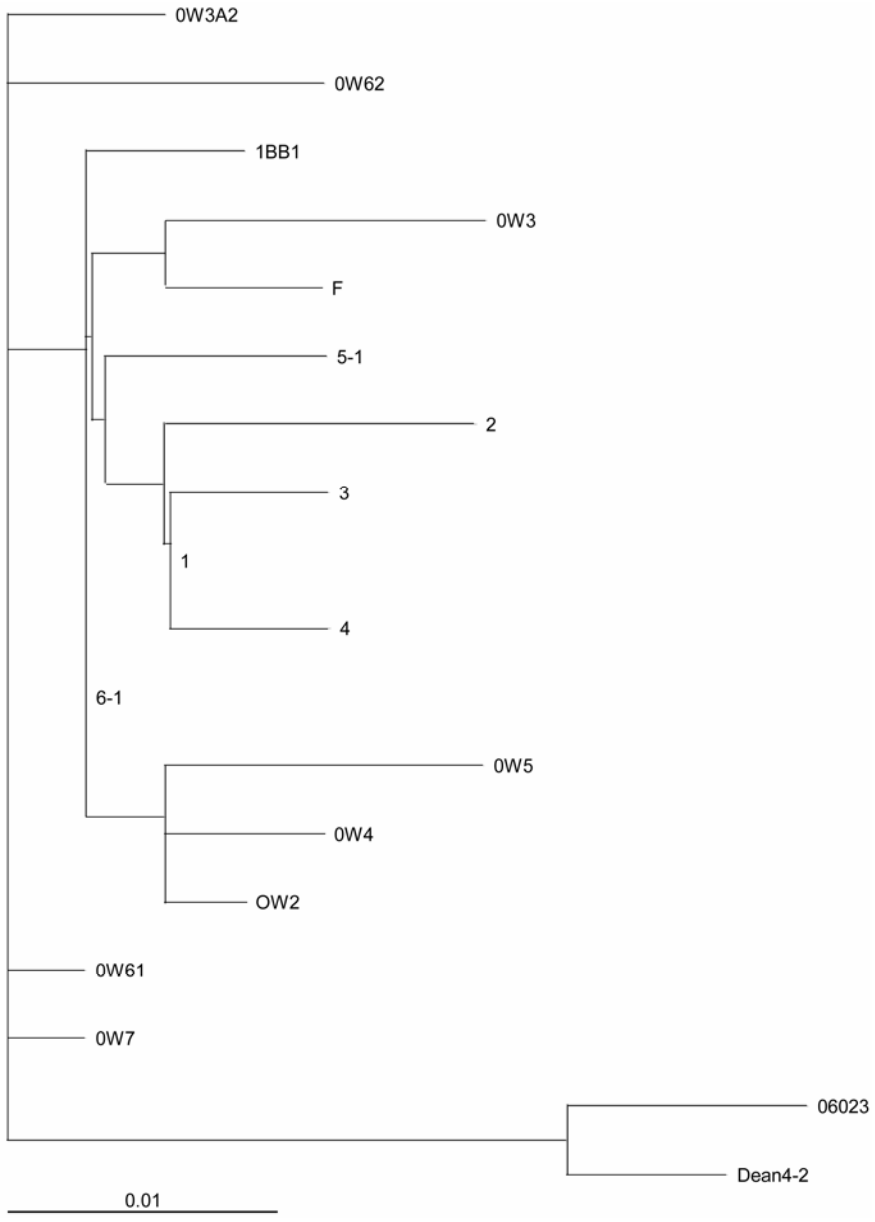

Fig. 3. Phylogenetic tree showing the relationship between *Burkholderia gladioli* strains isolated from orchids. The tree was constructed based on the fragment amplified of the 16S rDNA, using the neighbor-joining algorithm (24). A bootstrap analysis was performed with 100 repetitions.

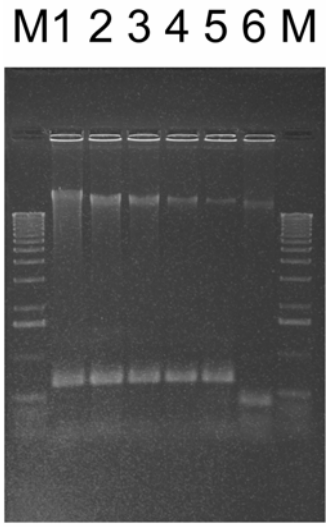

Fig. 4. Ethidium-bromide-stained 0.8% agarose gel displaying the 700-bp amplification product of the 23S rRNA gene from *Burkholderia gladioli* using species-specific polymerase chain reaction primers. Lanes 1 to 5 are five different strains of *B. gladioli* isolated from orchids. Lane 1, strain 1; lane 2, strain 2; lane 3, strain 3; lane 4, strain 4; lane 5, 1BB1. Lane 6 is a *B. cepacia* strain which served as a negative control; M = Invitrogen 1-kb DNA ladder.

DISCUSSION

In the United States, more than 130 plant diseases are reported to affect orchids. In Hawaii, orchid diseases caused by bacteria are not well characterized; however, they can be considered as persistent, with the potential to cause large economic losses if not controlled early. The objectives of this study were to identify the common symptoms, determine the predominant disease-causing agent, evaluate the potential host range of the strains, and determine whether bactericide resistance exists in these strains. Throughout the study, *B. gladioli* was isolated consistently from surface-sterilized plant tissue showing water-soaked lesions, indicating that the bacteria colonized the inside of their hosts (33). *B. gladioli* also was recovered from healthy-looking plants with no signs of infection (*data not shown*), indicating that the pathogen can survive on the leaf surface as an epiphyte. Over a period of 18 months, *B. gladioli* was detected in more than half of the diseased samples tested using traditional and molecular techniques. Strains of *B. gladioli* isolated from orchids were similar to each other in most biochemical reactions evaluated (oxidase test, assimilation of glucose, arabinose, mannose, mannitol, gluconate, caprate, and malate), and were similar to descriptions of the bacterium in Bergey's Manual of Systematic Bacteriology (16) and the type-strain used in the API 20NE test from bioMerieux. All strains of *B. gladioli* produced similar-looking colonies on NBY, YDC, MS, and KMB; however, one strain (OW61) produced a faint purple pigment on NBY. Whitby et al. (32) successfully developed a procedure for the identification of *B. gladioli* using species-specific PCR primers. This primer pair was used in this study and was able to differentiate *B. gladioli* from other bacteria, including the closely related species *B. cepacia*, which was included as a negative control.

Koch's postulates were fulfilled for all 18 strains of *B. gladioli*, and symptoms produced on artificially inoculated samples were virtually identical to those seen in collected field samples (Fig. 1C and E). The host range of the pathogen also was determined and more than 90% of the strains produced symptoms on five different orchid genera (*Phalaenopsis*, *Miltonia*, *Cattleya*, *Dendrobium*, and *Oncidium*) as well as on onion and carrot (Fig. 1E). These results indicate the potentially damaging nature of this organism.

In the 16S rRNA gene sequencing, the primer pair Y1/Y2 amplified a hypervariable region of the 16S rRNA gene which allows for phylogenetic comparison of close relatives. This study identified the genotypic diversity of *B. gladioli* strains that cause disease on orchids in Hawaii. Cluster analysis showed that the *B. gladioli* groups converged to the same origin and were not derived from each other, suggesting the divergent evolution from a common ancestor. The homogeneity of the strains based on genetic, biochemical, and physiological evidence also strongly supports a clonal origin of the strains tested. In spite of the relatively short length of the sequences, the phylogeny could be constructed to show diversity that was not based on host or location (Table 1; Fig. 3).

Jensen et al. (13) developed universal primers and conditions for amplifying the intergenic transcribed spacer (ITS) regions from all prokaryotes, and these primers have been used to identify both human (11,19) and plant (10) pathogens. The multicopy 16S-23S ITS, which separates the rRNA genes, exhibits a greater sequence and length variation and will be used for additional analysis.

Other factors may contribute to the onset of bacterial diseases in Hawaii, including insect and snail vectors causing wounds for bacterial entry and colonization (*data not shown*); a warm, humid environment; low light levels; contaminated water, media, and supplies; and contact between plants (30). Weeds within or near farms also could serve as alternative hosts. In a study conducted by Norman et al. (21) over a period of 1 year, irrigation and storm water runoff (using recycled water) were identified as a potential source of introduction and concentration of soft rot *Erwinia* populations to tropical foliage crops such as *Dieffenbachia*, *Syngonium*, and *Aglaonema* spp., resulting in rapid spread and extensive crop loss. *Burkholderia* spp. can occupy diverse ecological niches, from the soil, water, and the rhizosphere of plants, to humans, animals, and even the hospital environment (7).

The results from this study indicate that copper and streptomycin resistance is widespread among strains of *B. gladioli* causing rot on orchids in Hawaii. The MIC was designated as the concentration of copper sulfate or streptomycin which prevented growth of the culture after a 72-h incubation at 28ºC. Levels of resistance were in the normal range commonly recommended for use in disease control. Even though little streptomycin has been used over the past 10 years to control bacterial diseases in ornamentals in Hawaii (K. T. Sewake, *unpublished information*), resistant strains still are readily isolated. This is similar to findings for *Pseudomonas cichorii* isolated from celery seedbeds in Florida (22). Even though little streptomycin has been used in the past 25 years to control bacterial blight of celery in the Everglades Agricultural Area of Florida, resistant strains still are readily isolated (22). This phenomenon also was seen in studies by Sundin and Bender (27). Results in their studies indicated that resistance to copper and streptomycin by *P. syringae* pv. *syringae* associated with pear trees remained even though the bactericide selection was withdrawn (27).

Copper sometimes is used as a fungicide for disease control on orchids. This practice can contribute to the resistance found in phytopathogenic bacteria. Some of the copper-resistant strains of *B. gladioli* that were isolated formed bright blue colonies when grown on media containing high concentrations of copper (*data not shown*). The blue colonies of resistant bacteria suggest that cellular copper sequestration may play a role as a mechanism of copper resistance (8). In *Xanthomonas campestris* pv. *vesicatoria* and *P. syringae* pv. *tomato*, copper tolerance frequently is encoded on a gene cluster located on a self-transmissible plasmid (3,4,26) that could be transferred to other phytopathogenic bacteria. The plasmids also could harbor other genetic determinants that are beneficial to the bacteria, making them harder to control (27). To our knowledge, this is the first report of copper and streptomycin resistance among populations of *B. gladioli* in Hawaii. The inadvertent selection of copper-resistant strains from continuous application of copper-based bactericides by growers could be a major reason for the failures in controlling the bacterial disease in many plant-production areas (4). Our results show similarities to the copper-resistant strains of *P. syringae* isolated from mango orchards after repeated treatments with Bordeaux mixture (5).

The results of this study indicate that *B. gladioli* is a commonly found pathogen on orchids in Hawaii, with the potential to cause serious damage if not monitored and controlled. Its widespread nature, as indicated by the consistent isolation from different farms over a period of more than a year, may contribute to its ability to cause problems not only on orchids but also on unknown alternate hosts under the right environmental conditions. Because of limited available bactericides and the presence of genetic elements which confer copper and antibiotic resistance, prevention of plant disease caused by *B. gladioli* using consistent practices such as sanitation and rouging is a much better option than chemical control once the problem has presented itself. The molecular and physiological background and adaptability of *Burkholderia* spp. are largely unknown (7).

Table 2. Copper- and streptomycin-resistant strains of *Burkholderia gladioli* recovered from orchid plants in Hawaii

	No. of strains with growth at indicated concentration (Conc.)[a]	
Conc. (µg/ml)	**Cupric sulfate**	**Streptomycin**
0	16	16
50	12	15
100	12	6
500	12	0
750	7	nd
1,000	1	nd

[a] nd = Not determined.

The correct microbial identification and the determination of virulence factors of the bacterium will provide essential information for risk assessment and infection control (7).

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LITERATURE CITED

1. Altschul S. F., Gish W., Miller W., Myers E. W., and Lipman D. J. 1990. Basic local alignment search tool. J. Mol. Biol. 215(3):403-410.
2. Anonymous. 2004. Statistics of Hawaiian Agriculture, 2003. Hawaii Agricultural Statistics Service, Honolulu.
3. Bender, C. L., and Cooksey, D. A. 1986. Indigenous plasmids in *Pseudomonas syringae* pv. *tomato*: conjugative transfer and role in copper resistance. J. Bacteriol. 165:534-541.
4. Bender, C. L., Malvick, D. K., Conway, K. E., George, S., and Pratt, P. 1990. Characterization of pXV10A, a copper resistance plasmid in *Xanthomonas campestris* pv. *vesicatoria*. Appl. Environ. Microbiol. 56:170-175.
5. Cazorla, F. M., Arrebola, E., Sesma, A., Perez-Garcia, A., Codina, J. C., Murillo, J., and de Vicente, A. 2002. Copper resistance in *Pseudomonas syringae* strains isolated from mango is encoded mainly by plasmids. Phytopathology 92:909-916.
6. Chuenchitt, S., Dhirabhava, W., Karnjanarat, S., Buangsuwon, D., and Uematsu, T. 1983. A new bacterial disease on orchids Dendrobium sp. caused by *Pseudomonas gladioli*. Kasetsart J. 17:26-36.
7. Coenye, T., and Vandamme, P. 2003. Diversity and significance of *Burkholderia* species occupying diverse ecological niches. Environ. Microbiol. 5:719-729.
8. Cooksey, D. A. 1990. Bactericide resistance in phytopathogenic bacteria. Annu. Rev. Phytopathol. 28:201-219.
9. Gregersen, T. 1978. Rapid method for distinction of gram-negative from gram-positive bacteria. Eur. J. Appl. Microbiol. Biotechnol. 5:123-127.
10. Guasp, C., Moore, E. R. B., Lalucat, L., and Bennasar, A. 2000. Utility of internally transcribed 16S-23S rDNA spacer regions for the definition of *Pseudomonas stutzeri* genomovars and other *Pseudomonas* species. Int. J. Syst. Evol. Microbiol. 50:1629-1639.
11. Gurtler, V., and Barrie, H. D. 1995. Typing of *Staphylococcus aureus* strains by PCR-amplification of variable-length 16S-23S rDNA spacer regions: characterization of spacer sequences. Microbiology 141:12551265.
12. Hugh, R., and Leifson, E. 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram negative bacteria. J. Bacteriol. 66:24-26.
13. Jensen, M. A., Webster, J. A., and Straus, N. 1993. Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphism. Appl. Environ. Microbiol. 59:945-952.
14. Keane, P. J., Kerr, A., and New, P. B. 1970. Crown gall of stone fruit. Identification and nomenclature of *Agrobacterium* isolates. Aust. J. Biol. Sci. 23:585-595.
15. King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. 44:301-307.
16. Krieg, N. R., and Holt, J. G., eds. 1984. Bergey's Manual of Systematic Bacteriology. Vol. 1. Williams and Wilkins, Baltimore.
17. Kovacs, N. 1956. Identification of *Pseudomonas pyocyanea* by the oxidase reaction. Nature 178:703.
18. McCulloch, L. 1921. A bacterial disease of gladiolus. Science 54:115-116.
19. Mendoza, M., Meugnier, H., Bes, M., Etienne, J., and Freney, J. 1998. Identification of *Staphylococcus species* by 16S-23S rDNA spacer PCR analysis. Int. J. Syst. Bacteriol. 48:1049-1055.
20. Miller, T. D., and Schroth, M. D. 1972. Monitoring the epiphytic population of *Erwinia amylovora* on pear with a selective medium. Phytopathology 62:1175-1182.
21. Norman, D. J., Yuen, J. M. F., Resendiz, R., and Boswell, J. 2003. Characterization of *Erwinia* populations from nursery retention ponds and lakes infecting ornamental plants in Florida. Plant Dis. 87:193-196.
22. Pohronezny, K., Sommerfeld, M. L., and Raid, R. N. 1994. Streptomycin resistance and copper tolerance among strains of *Pseudomonas cichorii* in celery seedbeds. Plant Dis. 78:150-153.
23. Saitou, N., and Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406-425.
24. Sambrook, J., Maniatis, T., and Fritsch, E. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
25. Simone, G. W., and Burnett, H. C. 2002. Diseases caused by bacteria and fungi. Pages 50-73 in: Orchid Pests and Diseases. American Orchid Society, Delray Beach, FL.
26. Stall, R. E., Loschke, D. C., and Jones, J. B. 1986. Linkage of copper resistance and avirulence loci on a self-transmissible plasmid in *Xanthomonas campestris* pv. *vesicatoria*. Phytopathology 76:240-243.
27. Sundin, G. W., and Bender, C. L. 1994. Relative fitness in vitro and in planta of *Pseudomonas syringae* strains containing copper and streptomycin resistance plasmids. Can. J. Microbiol. 40:279-285.
28. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24:4876-4882.
29. Tsuchiya, Y., and Muko, H. 1963. Occurrence of neck rot disease of *Freesia* sp. in Japan. Chukan Hokoku Plant Pathol. Sect. NIAS. No. 16:82-84.
30. Uchida, J. 1995. Bacterial diseases of *Dendrobium*. Hawaii Inst. Trop. Agric. Hum. Resour. Brief 158.
31. Vidaver, A. K. 1967. Synthetic and complex media for rapid detection of fluorescence of phytopathogenic pseudomonads: effect of the carbon source. Appl. Microbiol. 15:1523-1524.
32. Whitby, P. W., Pope, L. C., Carter, K. B., LiPuma, J. J., and Stull, T. L. 2000. Species-specific PCR as a tool for the identification of *Burkholderia gladioli*. J. Clin. Microbiol. 38:282-285.
33. Wilkinson, K. G., Sivasithamparam, K., Dixon, K. W., Fahy, P. C., and Bradley, J. K. 1994. Identification and characterization of bacteria associated with Western Australian orchids. Soil Biol. Biochem. 26:137-142.
34. Wilson, E. E., Zeitoun, F. M., and Fredrickson, D. L. 1967. Bacterial phloem canker, a new disease of Persian walnut trees. Phytopathology 57:618-621.
35. Young, J. P. W., Downer, H. L., and Eardly, B. D. 1991. Phylogeny of the phototrophic *Rhizobium* strain BTAi1 by polymerase chain reaction-based sequencing of a 16S rRNA gene segment. J. Bacteriol. 173:2271-2277.