Variation Among Strains of *Xanthomonas campestris* Causing Citrus Bacterial Spot

JOHN S. HARTUNG and E. L. CIVEROLO, USDA-ARS, Plant Sciences Institute, Fruit Laboratory, BARC-West, Beltsville, MD 20705-2350

ABSTRACT

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A wide range of variation in restriction fragment length polymorphism (RFLP) and aggressiveness was observed among 45 strains of *Xanthomonas campestris* isolated from 20 outbreaks of citrus bacterial spot disease in Florida citrus nurseries. The most aggressive strains, based on an in vitro assay, belonged to a single RFLP type. Although apparently homogeneous in RFLP analyses, members of this group varied in carbon source oxidation. The less aggressive strains comprised a continuum of RFLP types and usually could be separated from the more aggressive group by carbon source oxidation profiles. Carbon source oxidation provided a convenient means for presumptive identification of strains isolated from citrus. Data from the in vitro aggressiveness assays, genomic DNA RFLP analyses, and carbon source oxidation profiles confirmed the separation of these bacteria into two distinct groups designated CBS-A and CBS-B. Based on carbon source oxidation, these strains may be related to some strains of *X. campestris* pv. *dieffenbachiae*.

A previously unknown disease of citrus was discovered in Florida citrus nurseries in 1984 (9,22). The new disease was characterized by flat, water-soaked or necrotic lesions of various sizes on leaves and stems of nursery plants and was caused by strains of *Xanthomonas campestris*. The disease was tentatively identified as a new form of citrus bacterial canker, and the bacteria were identified as novel strains of *X. c.* pv. *citri* (Hasse) Dye. However, neither the raised, hyperplastic lesions typical of citrus bacterial canker (CBC) (23) nor infection of mature trees in groves was observed. In spite of the massive eradication campaign that ensued, new outbreaks of the disease occurred in each succeeding season in different nurseries (9,22).

In genomic fingerprinting (13) and restriction fragment length polymorphism (RFLP) analyses (5,14), strains of *X. campestris* isolated from Florida citrus nurseries were not closely related to known strains of *X. c. citri* and were genetically heterogeneous. The nursery strains could also be distinguished from strains of *X. c. citri* by serology, phage typing (E. L. Civerolo, *unpublished*), and multilocus isozyme analysis (16) as well as by differences in host-pathogen interactions (4,17). For example, the nursery strains do not induce the hyperplasia typical of lesions caused by *X. c. citri*. These differences led us and others to propose that the strains of *X. campestris* isolated from Florida citrus nurseries are not strains of *X. c. citri* and to refer to the disease that they cause as citrus bacterial spot (CBS) (9,10,14). A proposal to revise the taxonomy of all strains of *X. campestris* associated with diseases of citrus and to name the strains causing CBS *X. c.* pv. *citrumelo* Gabriel et al (6) has been severely criticized (2). These nursery strains of *X. campestris* were previously referred to as *X. c. citri* groups E, F, and G (16) or *X. c. citrumelo* groups E1 and E2 (6). Although the taxonomic status of these strains is in dispute (2,6), it is clearly inappropriate to refer to them as strains of *X. c. citri*. We will refer to them simply as strains of *X. campestris* associated with CBS disease or *X. campestris* CBS-A or CBS-B.

A wound-inoculated detached leaf assay was developed to screen hundreds of cultures of *X. campestris* isolated from Florida citrus nurseries for pathogenicity. This assay was similar to one used previously to study strains of *X. c. pruni* (3) and to assays used by others (6,10,15). The CBS strains varied in aggressiveness on the detached leaves, suggesting that strains could be grouped by aggressiveness. Although these responses were reproducible, their biological significance was unclear because of the unnatural conditions of the assay. In genomic fingerprinting (13) and RFLP analy-

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ses of some CBS strains, two (5) or three (14) subgroups were detected. A rapid technique for simultaneously testing a strain's ability to oxidize 95 carbon sources recently became available (1). The technique is based on the reduction of a tetrazolium dye by electrons released when a carbon source is oxidized by the test bacteria.

The present study was initiated to ascertain whether the subgroups of *X. campestris* associated with CBS that were determined by detached leaf inoculations, RFLP analysis, and carbon source oxidation profiles were consistent, and thus reinforcing. We also hoped to identify oxidizable carbon sources that would serve as markers to distinguish subgroups of these strains of *X. campestris*.

MATERIALS AND METHODS

Bacterial strains and RFLP analysis. Genomic DNA from the strains of *X. campestris* (Tables 1 and 2) was isolated as described previously (13). Digestion with restriction endonuclease *Eco*RI, Southern blotting, and hybridizations with biotin-11-dUTP-labeled DNA probes were done as described previously (14). Electrophoresis was in 0.8% agarose gels in TPE (0.08 M Tris-phosphate, 0.002 M EDTA) at 1.5 V/cm for 16 hr. Strain F5 was included within each Southern blot as an internal standard to which all strains were compared by coefficients of similarity (19) using a standard panel of seven randomly selected cosmid clones as hybridization probes (14). RFLP analyses of coded strains were carried out independently of the pathogenicity assays.

Wound inoculation of detached leaves. In two separate tests, young, actively growing terminal leaves were collected from greenhouse-grown seedlings of Swingle citrumelo (*Poncirus trifoliata* (L.) Raf. × *Citrus* × *paradisi* Macfady.), a common citrus rootstock cultivar, or Duncan grapefruit (*C.* × *paradisi* Macfady.) and prepared substantially as described previously (10). The adaxial surface of each leaf was aseptically punctured with a sterile needle five times on each side of the midrib. Two leaves were placed adaxial side up on the surface of 1% water agar in a 100 × 15 mm disposable petri dish. Bacterial strains were grown 2–3 days on Wakimoto's potato semisynthetic medium (7), aseptically suspended in sterile deionized water, and adjusted to contain approximately 1×10^8 cfu/ml. Ten microliters of the bacterial suspension was placed on each wound site. The petri dishes were sealed and incubated with constant lighting for 10 days at 27 C. On control leaves, 10 µl of sterile deionized water was placed on each wound site.

Each wound was rated with a numerical score of 0–3 as follows: 0 = no visible reaction; 1 = indistinct and very limited (less than 1 mm) tissue discoloration (dark brown) irregularly and incompletely distributed around the wound site; 2 = distinct but limited (1–2 mm) tissue discoloration (light brown or rusty orange to dark brown) completely around the wound, with little or no persistent water-soaking; and 3 = extensive (greater than 2 mm), persistent water-soaking around the inoculation wound site, with brown (necrotic) tissue discoloration developing in the center of the lesion immediately around the periphery of the wound. The infection severity index (ISI) for each strain was the average rating from 20 wounds. All inoculations and wound ratings were done

Table 1. Strains of *Xanthomonas campestris* used in this study

Strain[a]	Year of isolation and host	Source nursery code[b]	Coefficient of similarity (*F*) to strain F5[c]	Infection severity index[d]	
				Citrumelo	Grapefruit
RFLP group A					
F1	1984 Carrizo	1	1.0	2.9	2.5
F2	1984 Carrizo	1	1.0	2.7	2.1
F3	1984 Grapefruit	1	1.0	2.9	2.8
F4	1984 Grapefruit	1	1.0	2.5	2.7
F5	1984 Unknown	2	1.0	1.1	1.3
F54	1985 *Citrus sinensis*	8	1.0	2.4	2.9
F224	...[e]	1	1.0	2.5	3.0
F225	...[e]	1	1.0	2.4	2.9
F226	...[e]	1	1.0	2.5	2.8
F228	1984 Grapefruit	3	1.0	2.0	0.8
F229	1984 Grapefruit	3	1.0	2.0	1.8
F269	1987 Swingle	18	1.0	2.4	2.4
F270	1987 Swingle	18	1.0	2.4	1.9
F271	1987 Swingle	18	1.0	2.5	2.4
F272	1987 Swingle	18	1.0	2.4	2.8
F273	1987 Swingle	18	1.0	2.7	2.4
F275	1987 Swingle	18	1.0	2.4	2.6
F276	1987 Swingle	18	1.0	2.8	2.2
F359	1988 Swingle	19	1.0	2.5	2.5
F360	1988 Swingle	19	1.0	2.9	2.6
F361	1988 Swingle	19	1.0	2.8	2.4
F363	1988 Swingle	19	1.0	3.0	2.8
F365	1988 Swingle	19	1.0	2.5	2.6
F367	1988 Swingle	19	1.0	2.5	2.7
RFLP group B					
F6	1984 Unknown	3	0.80	1.9	NT
F11	1984 Murcott	4	0.66	1.3	0.9
F19	1985 Swingle	5	0.74	1.7	0.6
F21	1985 Grapefruit	6	0.70	1.0	0.1
F29	1985 Swingle	7	0.80	1.0	0.8
F49	1985 Swingle	7	0.84	1.8	1.4
F56	1985 Swingle	9	0.70	0.0	0.0
F96[f]	1985 Swingle	10	0.37	2.0	0.3
F100	1985 Swingle	11	0.78	2.0	0.3
F230	1984 Grapefruit	3	0.84	1.8	1.4
F231	1984 Grapefruit	3	0.84	2.0	0.8
F254	1987 Grapefruit	12	0.69	2.0	0.6
F255	1987 Grapefruit	12	0.76	1.5	NT
F257	1987 Grapefruit	12	0.76	1.3	0.3
F258	1987 Navel orange	13	0.68	1.4	1.4
F285	1987 Swingle	20	0.68	1.9	0.7
F294	1987 *Citrus* sp.	14	0.69	1.0	0.4
F299	1987 Grapefruit	15	0.81	1.9	0.0
F300	1987 Swingle	15	0.72	1.5	0.3
F303	1987 Swingle	16	0.75	1.9	0.4
F311	1987 Grapefruit	17	0.73	2.0	0.8

[a] Restriction fragment length polymorphism (RFLP) groups were based on similarity to strain F5.
[b] Source nurseries are coded to protect the identities of the nurserymen.
[c] Coefficients of similarity to strain F5 were calculated as in references 14 and 19.
[d] Mean of 20 wound inoculations on two leaves of Swingle citrumelo or Duncan grapefruit. NT = not tested.
[e] Strains from R. E. Stall, from tissue frozen in 1984.
[f] RFLP group undetermined.

Table 2. Strains of *Xanthomonas campestris* pv. *dieffenbachiae* used in this study

Strain	Year of isolation	Host
X10	1979	Unknown
X11	1984	*Anthurium* sp.
X12	1984	*Philodendron* sp.
X13	1984	*Philodendron*
X14	1984	Unknown
X15	1984	Unknown

using coded bacterial strain designations to avoid potential problems from biased observations.

Carbon source oxidation. The ability of the CBS strains to oxidize an array of carbon sources was determined using 96-well microtiter plates (GN MicroPlates) and proprietary software (MicroLog 2N, Biolog Inc., Hayward, CA). Tryptic soy agar (Difco, Detroit, MI) cultures were inoculated from frozen (−70 C) stocks and incubated at 30 C for 24 hr. A sterile cotton swab was used to harvest the bacteria, which were resuspended in sterile saline (0.85% NaCl) to a final optical density of 0.30 at 590 nm. Aliquots of 150 μl were added to each microtiter plate well and the plates were incubated for 24 hr at 30 C. The optical density at 570 nm for each well, measured with a microtiter plate reader (model MR700, Dynatech Inc., Chantilly, VA), was evaluated with the Microlog 2N software package to identify positive and negative wells using the data-dependent variable threshold algorithm. Each plate was read twice within 60 min, and the entire experiment was repeated, for a total of four determinations per strain. The data from 43 CBS strains were used to construct two entries to supplement the Microlog 2N data base. Strains were assigned to CBS-A or CBS-B based on RFLP data (Table 1). The individual carbon source oxidation profiles were then submitted to the Microlog 2N program for identification.

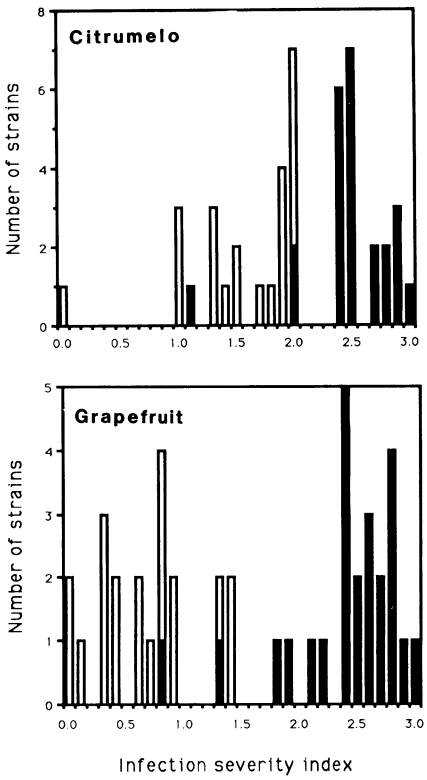

Fig. 1. Infection severity indices for strains of *Xanthomonas campestris* associated with citrus bacterial spot evaluated on Swingle citrumelo and Duncan grapefruit. Solid bars denote strains belonging to CBS-A ($n = 24$) and open bars denote strains belonging to CBS-B ($n = 19$).

RESULTS

Aggressiveness and RFLP profiles. Substantial variation in aggressiveness (as estimated by the ISI) was detected in this collection of 45 strains of *X. campestris* CBS (Table 1). Strains that were indistinguishable by RFLP analysis and highly aggressive were independently isolated at several locations in 1984 and 1985 (F1–F4, F54, F224–F226, F228, and F229), in 1987 (F269–F276), and in 1988 (F360–F367). These strains were indistinguishable from strain F5 (the internal standard) by RFLP analysis and represent CBS-A. Strains that differed from each other both by RFLP analysis and by their aggressiveness were isolated and distinguished in a single disease outbreak in 1984 (F6, F29, F49, and F228–F231). Substantial variation in the coefficient of similarity to strain F5 (Table 1) was observed for the remainder of the strains, designated *X. campestris* CBS-B, which also were less aggressive on both hosts than the CBS-A strains (Table 1, Fig. 1). As determined by RFLP analysis, strain F96 was quite unrelated to the other bacterial strains. The CBS-B strains were more aggressive on Swingle citrumelo than on Duncan grapefruit, consistent with their greater compatibility with Swingle citrumelo (11). Strain F5, the internal standard for the RFLP analyses, was aggressive when used in experiments elsewhere (strain 3162 in reference 6). The aggressiveness of the strain apparently became attenuated prior to the series of experiments reported here.

Carbon source oxidation profiles. Profiles in the Biolog assay were generally, though not entirely, reproducible. However, the profiles varied in at least one carbon source between replicate assays of the same strain performed on different days (41/43 strains, 95%) and in duplicate readings of the same plate taken within 1 hr (16/86, 19%). This variation was primarily found in carbon sources that were only weakly oxidized.

Carbon sources that were oxidized by more than 90% of the strains tested included dextrin, glycogen, *N*-acetyl-D-glucosamine, cellobiose, D-fructose, L-fucose, gentiobiose, α-D-glucose, lactulose, maltose, D-mannose, psicose, sucrose, D-trehalose, methyl pyruvate, monomethyl succinate, *cis*-aconitic acid, succinic acid, bromosuccinic acid, alaninamide, D-alanine, L-alanine, L-alanylglycine, glycyl-L-glutamic acid, L-serine, L-threonine, glycerol, Tween 40, D-galactose, D-melibiose, acetic acid, α-hydroxybutyric acid, α-ketobutyric acid, α-ketoglutaric acid, malonic acid, propionic acid, succinamic acid, L-glutamic acid, and L-proline.

Carbon sources that were oxidized by less than 10% of the strains tested included *N*-acetyl-D-galactosamine, adonitol, L-arabinose, D-arabitol, *m*-inositol, D-mannitol, β-methylglucoside, L-rhamnose, xylitol, formic acid, D-galactonic acid γ-lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, γ-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α-ketovaleric acid, quinic acid,

Table 3. Identification of strains of *Xanthomonas campestris* responsible for citrus bacterial spot based on carbon source oxidation profiles

Profile type[a] submitted	Best identification[b]											
	CBS-A			CBS-B			*X. c. dieffenbachiae* B[d]			Other pathovars[e]		
	No.	%	Sim[c]	No.	%	Sim	No.	%	Sim	No.	%	Sim
CBS-A ($n = 96$)[f]	64	67	0.721	18	19	0.633	12	13	0.478	2	2	NA[g]
CBS-B ($n = 76$)	14	18	0.480	46	61	0.757	3	4	NA	13	17	0.581
X. c. dieffenbachiae ($n = 24$)	0	0	NA	6	25	0.593	16	67	0.649	2	8	NA

[a] Based on restriction fragment length polymorphism data for citrus bacterial spot (CBS) strains (Table 1) or the host from which *X. c. dieffenbachiae* strains were isolated (Table 2).
[b] Identifications were made with Microlog 2N software (Biolog Inc.) using the proprietary data base supplemented with our own profiles of strains associated with CBS. The best identification is the aggregate profile in the combined data base that most closely matched the individual profile submitted for identification.
[c] Average of similarity values for identifications in this cell.
[d] A subgroup within *X. c. dieffenbachiae* identified by Biolog Inc. based on carbon source oxidation.
[e] Pathovars *manihotis*, *vesicatoria*, and *vitians* and strains of *X. campestris* isolated from turfgrass.
[f] Total number of profiles submitted as unknowns.
[g] When three or fewer profiles fell into a given cell, no average similarity value is presented.

D-saccharic acid, sebacic acid, glucuronamide, L-histidine, L-ornithine, L-phenylalanine, L-pyroglutamic acid, DL-carnitine, γ-aminobutyric acid, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, α-cyclodextrin, *i*-erythritol, α-lactose, D-sorbitol, β-hydroxybutyrate, L-asparagine, D-serine, urocanic acid, and inosine.

No carbon source was identified that was exclusively oxidized by members of *X. campestris* groups CBS-A or CBS-B. The carbon sources that were most nearly differential were D-raffinose, glycyl-L-aspartic acid, and DL-α-glycerol phosphate.

The carbon source oxidation profiles of individual CBS strains were compared with profiles of bacteria stored in the commercial data base supplemented with the CBS profiles (Table 3). All such identifications were within *X. campestris*. Profiles that belonged to CBS-A were identified as such in 67% of the tests and those belonging to CBS-B in an additional 19% of the tests. Thus, 85% of the tests resulted in an identification of *X. campestris* CBS, and most of the remainder were identified as *X. c.* pv. *dieffenbachiae* (McCulloch and Pirone) Dye. A similar pattern of correct identifications was observed for the profiles of strains that belonged to the CBS-B group (Table 3). An estimate of the accuracy that the software program assigns to an individual identification is given by the similarity value (Sim). On average, the calculated similarity values for the best identifications were higher for correct than for incorrect identifications (Table 3). The software was generally more "confident" about correct identifications than incorrect ones.

Because of the frequency of the misidentification of CBS strains as *X. c. dieffenbachiae* and evidence that strains of *X. campestris* isolated from the ornamental plant *Strelitzia* were related to CBS strains (12), six strains of *X. c. dieffenbachiae* were profiled by the Biolog system. These profiles were also correctly identified in 67% of the tests. The most common incorrect identification was *X. campestris* CBS-B.

DISCUSSION

A collection of strains that varied in aggressiveness, isolated originally from 20 diseased nurseries over a period of 5 yr, was used in this study. The diversity of these strains compares favorably with that in previous work (6) in which the limited number of strains studied prevented the establishment of a clear relationship between RFLP type and aggressiveness. Aggressiveness tests carried out with detached seedling leaves in vitro are difficult to interpret because of the gross difference between laboratory and field conditions. However, the severity indices data supported the grouping of strains based on RFLP data (Fig. 1). In terms of symptomatology, results from detached leaf assays as described here were positively correlated with results from inoculations of attached leaves in greenhouse and field experiments (10). No RFLP or carbon source oxidation data were presented in the attached leaf study (10). The apparent correlation of high ISI scores with one RFLP pattern and lower ISI scores with all other RFLP patterns was confirmed by comparing the two means with citrumelo as host by a *t* test ($t = 7.0$, df $= 43$, $P < 0.001$) (24). Similar results were obtained with grapefruit as host. Both the aggressiveness assessments and the RFLP analyses were done independently with coded samples. The correlation of RFLP, ISI, and aggressiveness assays on attached leaves provides strong evidence that the detached leaf assay provides a useful measure of aggressiveness for these strains. The CBS-B group of strains, with a continuum of both ISI scores and RFLP types, was considered "moderately aggressive"; CBS-A strains were considered to be "strongly aggressive." Others have concluded from aggressiveness assays of detached and attached leaves that three aggressiveness classes are distinguishable within this group of strains (10). Based on RFLP data taken from 14 strains, we tentatively suggested that three subgroups could be distinguished among strains associated with CBS (14). Although, as described above and by Graham and Gottwald (10), three different disease reactions (and the null reaction) can be observed on inoculated detached leaves, neither the RFLP nor the carbon oxidation data supported the concept of three distinct pathotypes. Instead, from the more complete data now available, it appears that subgroups F and G (14) (E1 in reference 6) form a broad continuum (CBS-B) rather than a more discrete cluster, as do the strains that comprise *X. campestris* CBS-A.

Because RFLP analysis is so laborious, we hoped that a carbon source could be identified for use as a convenient marker for the CBS-A strains. Although no such carbon source was found, screening of additional carbon sources with the Biolog system may yet identify a useful marker. Consistent with the sharp separation of the CBS-A and CBS-B groups of strains based on RFLP and ISI data (Fig. 1), these groups could usually (64%) be separated based on carbon source oxidation data alone (Table 3) despite the lack of a differential carbon source marker.

The carbon source oxidation pattern was more variable in the CBS-B than in the CBS-A group of strains. On average, an individual carbon source oxidation profile differed from the aggregrate profile of its group by 2.2 and 1.7 reactions, respectively. The maximum number of differences between an individual CBS-B or CBS-A profile and its aggregate profile was 8.3 and 7.2, respectively. For comparison, for *X. c. dieffenbachiae* group B, composed of strains originally isolated from several ornamental species and known to be physiologically variable (A. R. Chase, *personal communication*), the average difference between an individual and the aggregate profile was 2.4 reactions and the maximum number was 6.2. The greater diversity in carbon source oxidation profiles within the CBS-B group was also shown by the diversity of pathovars named by the Microlog 2N system when strains belonging to this group were presented as unknowns (Table 3). This is consistent with the RFLP and ISI data (Table 1). The carbon source oxidation profile provides a useful method to use in conjunction with other techniques to distinguish strains of *X. campestris* isolated from citrus.

We suggest that potential users of the Biolog system supplement the commercial data base with their own data prior to attempting identifications. When the Microlog 2N data base was used alone to identify our strains, it made correct identifications in only 33% of the tests (57/172). The most frequent identification was *X. c. dieffenbachiae* B, made in 45% of the tests (78/172). The frequency of correct identifications was doubled by the addition of our own data (Table 3). No *X. campestris* CBS profile was identified as *X. c. citri* (0/172), consistent with other results (5,6,11, 13,14).

The possible relationship of *X. campestris* associated with CBS to *X. c. dieffenbachiae* B is interesting. In previous work, strains of *X. campestris* isolated from ornamental plants (*Strelitzia* spp.) produced lesions on Swingle citrumelo and were very similar to CBS strains as determined by RFLP analysis (12). In the Microlog 2N data base, the strains grouped as *X. c. dieffenbachiae* B were isolated from *Anthurium, Philodendron*, and *Aglaonema*, as well as *Dieffenbachia* (A. R. Chase, *personal communication*). Despite the diverse hosts of origin, this is consistent with the taxonomic concept of *X. c. dieffenbachiae* (18,20,21,25,26). Thus, strains of *X. campestris* that cause leaf-spotting diseases of some ornamental plants may incite similar symptoms in susceptible varieties of citrus grown under favorable conditions. The relationships between strains in this complex deserve further study.

The existence of recognizable groups of strains associated with CBS has several implications. First, the eradication campaign launched in 1984 apparently has failed to eradicate the CBS-A strains, which are strongly aggressive on both Swingle citrumelo and Duncan grapefruit and have been isolated as recently as 1988. Second, the continuum of mod-

erately aggressive CBS-B strains isolated from citrus at numerous locations during the same period is probably of little or no commercial importance, based on the ISI as corroborated by attached leaf inoculation and epidemiological studies (8,10). Regulators may choose to use this information to attempt to control only strains belonging to CBS-A (group E2 in reference 6). Third, the heterogeneous assortment of strains only moderately aggressive to Swingle citrumelo, and perhaps only incidentally associated with citrus, may present further problems for the *X. c. citrumelo* (6) concept, as has been suggested (11,12).

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