Evidence for Acquisition of Copper Resistance Genes from Different Sources in Citrus-Associated *Xanthomonas*

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ABSTRACT

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We determined that multiple and independent introductions of copper resistance genes have taken place for strains of *Xanthomonas citri* subsp. *citri* from Argentina and strains of *X. alfalfae* subsp. *citrumelonis* from Florida. This study compared the partial nucleotide sequences of principal copper resistance genes *copL*, *copA*, and *copB* from *X. citri* subsp. *citri* and *X. alfalfae* subsp. *citrumelonis* to strains of other *Xanthomonas* spp. resistant to copper that were isolated from 12 different countries or territories. The survey confirmed that the *copLAB* gene cluster is present in many species of *Xanthomonas* from different parts of the world. Align-

ment of partial nucleotide sequences of copper resistance genes among the copper-resistant (Cu^R) strains of *Xanthomonas* detected homology of ≥ 92 , ≥ 96 , and $\geq 91\%$ for *copL*, *copA*, and *copB*, respectively. Grouping of strains based on branching patterns of phylogenetic trees was similar for *copL* and *copA* but differed for *copB*. When the three genes were concatenated and analyzed using various phylogenetic methods, it appeared that the plasmid had been horizontally transferred and various populations were mutating based on selection pressure unique to geographic regions. Although high homology of the genes among the strains indicated that the copper resistance in xanthomonads has a common origin, the slight differences in nucleotide sequences within groups of strains indicated that Cu^R genes have been independently exchanged among species of *Xanthomonas* throughout the world.

For many decades, copper bactericides have been used for control of bacterial diseases affecting a wide range of annual and perennial crops. A widespread problem with the repeated application of copper bactericides is the development of copper-resistant (Cu^R) strains of the target pathogen and reduced disease control (15,37,40). Once copper resistance is acquired, the pressure posed by the continual applications of copper sprays gradually increases the frequency of the resistant population of the pathogen and compromises the efficacy of copper (52). Cu^R strains have been identified in many plant-pathogenic bacterial species, including species of *Pseudomonas* (4,12,16,48,52), *Pantoea* and (43), *Erwinia* (2) and several species of *Xanthomonas*, such as *Xanthomonas euvesicatoria* (formerly *X. campestris* pv. *vesicatoria*), *X. axonopodis* pv. *vesicatoria* (40,50), *X. arboricola* pv. *juglandis* (37), *X. alfalfae* subsp. *citrumelonis*, and *X. citri* subsp. *citri* (10).

Chemical control of citrus canker, caused by *X. citri* subsp. *citri*, in endemic areas is based exclusively on sprays of copper bactericides (8,38). Copper bactericides have no curative or systemic activity and, therefore, are applied preventively for management of citrus canker. Such bactericides reduce inoculum build-up on susceptible leaf flushes and protect expanding fruit surfaces from infections, which may cause premature fruit drop when infections occur in the early stage of fruit development (7,27,29,32,41,55). Although copper bactericides reduce citrus

canker incidence on leaves and fruit, they do not completely suppress the inoculum in orchards. Hence, repeated copper sprays over several seasons and years are necessary to minimize crop loss (8,28) which, in turn, may lead to development of copper resistance in *X. citri* subsp. *citri* populations (9,11).

Cu^R strains of *X. citri* subsp. *citri* have been detected only in Argentina (15). Characterization of the major molecular copper resistance determinants for these strains revealed that the resistance is due mainly to *copL*, *copA*, and *copB* genes, which are also present in Cu^R strains of *X. alfalfae* subsp. *citrumelonis* (10), the citrus-associated xanthomonads that cause citrus bacterial spot (CBS). Moreover, it has been demonstrated that homologs of the *cop* genes (*coh* genes) are present on the chromosome of xanthomonads. The role of the chromosomal copies of *cop* genes remains unknown because distinction of *coh* and *cop* genes had not been done until recently (10,46). Differently from what has been annotated, *coh* genes are not responsible for copper resistance but likely are necessary for homeostasis or tolerance (10).

CBS has quite distinct symptoms compared with citrus canker. The importance of this disease has been related to outdoor nurseries in Florida. Hence, new regulations in Florida that mandate the propagation of certified, pathogen-free citrus trees in enclosed nurseries has kept CBS under control in that state (25). Thus, because CBS is restricted to nursery environments, the disease poses no threat to citrus production (31). However, for several years, *X. alfalfae* subsp. *citrumelonis* populations in nurseries were exposed to frequent sprays of copper as a protective measure against CBS-induced bud failure (30), leading to the development of Cu^R strains of *X. alfalfae* subsp. *citrumelonis* (10).

Considering that *X. alfalfae* subsp. *citrumelonis* and *X. citri* subsp. *citri* thrive on the same host and that the horizontal transfer

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of Cu^R genes between these two species is possible, as demonstrated previously (9), the concern is that Cu^R strains of *X. alfalfae* subsp. *citrumelonis* may serve as a reservoir of copper resistance for movement to *X. citri* subsp. *citri*. In fact, not only *X. alfalfae* subsp. *citrumelonis* poses a risk for development of copper resistance in *X. citri* subsp. *citri* because copper resistance genes from several species of *Xanthomonas* may move intra- and inter-specifically (9,50). In addition, homologs of *copL*, *copA*, and *copB* were demonstrated to be present in *X. euvesicatoria* (58) and strains of *Stenotrophomonas maltophilia* isolated from an immunosuppressed patient (20) and from the citrus phyllosphere (9). Moreover, Behlau et al. (9) demonstrated that the homologs from *S. maltophilia* FB03P were able to confer copper resistance to copper-sensitive (Cu^S) strains of *Xanthomonas*.

The copper resistance mechanism and role of the *copLAB* gene cluster in *Xanthomonas* remain to be fully elucidated. Previous findings indicate that cellular copper sequestration and accumulation is the main mechanism for copper resistance in *Xanthomonas* spp. and that *copL* regulates the expression of *copA* and *copB*, which are presumed to encode for copper-binding proteins (10,18,58). Previous reports indicate that copper resistance determinants in some bacteria such as species of *Pseudomonas* (39) and *Xanthomonas* (5,37) may also be located on the chromosome. However, genes associated with copper resistance in plant-pathogenic bacteria are located predominantly on plasmids (9,13,17,18,50), which facilitates their movement among different bacterial species and transfer to sensitive strains. In *Pseudomonas syringae* pv. *tomato*, the copper resistance genes reside on a 35-kb plasmid in strains isolated in California (12,17). In *X. campestris* pv. *vesicatoria*, the copper resistance determinant resides on large plasmids in strains isolated in Florida and Oklahoma (13,42). Likewise, in *X. citri* subsp. *citri* from Argentina, the copper resistance genes are located on a ≈300-kb plasmid (9).

Horizontal transfer of copper resistance determinants through conjugation is the main mechanism for acquisition of copper resistance by bacteria (9,18,50,57). It is unlikely that bacteria become resistant to copper through spontaneous mutations because copper resistance is regulated by several genes in these organisms (18). The possibility for plasmid transfer between *X. campestris* pathovars exists in both host and non-host plants (13). Timmer et al. (56) demonstrated that *X. axonopodis* pv. *vesicatoria* was able to multiply on leaves of non-host plants such as plum and peach. Once the right conditions are provided and bacteria are present, interpathovar transfer of the copper resistance plasmid may occur in nature (13). A highly conjugative copper resistance plasmid from *X. campestris* pv. *campestris* hybridized strongly with the cloned copper resistance genes from *X. axonopodis* pv. *vesicatoria* (57). This observation points out the probability that copper resistance plasmids have been exchanged among pathovars of related plant pathogens, as suggested for *P. syringae* (12,18). In *P. syringae* pv. *syringae* Cu^S strains isolated from cherry trees were able to acquire the 61-kb plasmid containing genes which confer copper resistance from all donors tested, and the transfer frequency was highest between isolates from the same orchard (52).

Considering the risk for development of Cu^R strains of *X. citri* subsp. *citri* where the disease is endemic and is being managed with frequent sprays of copper-based bactericides (9,11), as observed in Argentina (14), the aim of the present study is to compare the copper resistance genes from *X. citri* subsp. *citri* and *X. alfalfae* subsp. *citrumelonis* with different species of *Xanthomonas* and establish the phylogenetic relationship of these sequences among strains from different crops and geographic localities. This information will help to better predict the risk for development of resistance in *X. citri* subsp. *citri* by understanding the origin of such resistance genes and to what extent the copper resistance genes are related phylogenetically in different species and pathovars of *Xanthomonas*.

Bacterial strains and scope of study. *Xanthomonas* strains used throughout this study are listed in Table 1, which include Cu^R strains of *X. citri* subsp. *citri*, *X. alfalfae* subsp. *citrumelonis*, *X. arboricola* pv. *juglandis*, *X. vesicatoria*, *X. euvesicatoria*, *X. perforans*, *X. gardneri*, and *Xanthomonas* sp. from the United States (Florida, Michigan, and Ohio), Argentina, Barbados, Brazil, Costa Rica, Guadeloupe, Mexico, New Zealand, Puerto Rico, Spain, and the U.S. Virgin Islands; and Cu^S strains of *X. citri* subsp. *citri*, *X. alfalfae* subsp. *citrumelonis*, *X. vesicatoria*, *X. euvesicatoria*, and *X. perforans*, used as negative controls. The inclusion of strains from other *Xanthomonas* spp. nonpathogenic to citrus was necessary to show the relatedness of the *copLAB* genes among species. It was also important to show geographical distribution of copper resistance and its potential relevance as a source of resistance.

The strains were tested for the presence of *X. citri* subsp. *citri*-like copper resistance genes by polymerase chain reaction (PCR) analysis using the primer sets designed in the present study to specifically amplify partial sequences of the copper resistance genes *copL*, *copA*, and *copB*. Partial nucleotide sequences of PCR products were aligned and compared phylogenetically to determine the relatedness of the copper resistance genes among different strains of *X. citri* subsp. *citri* and *X. alfalfae* subsp. *citrumelonis* and with other *Xanthomonas* spp. Furthermore, the level of copper resistance for selected strains was measured on solid medium.

Characterization of level of copper resistance in strains of *Xanthomonas*. Strains were grown overnight on nutrient agar (NA) amended with 80 μM copper sulfate pentahydrate (CSP) for induction of resistance, suspended in sterile tap water at approximately 10⁸ CFU ml⁻¹, and then spotted (10 μl) in triplicate on mannitol-glutamate yeast (MGY) agar supplemented with 0, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1.2, 1.6, 2.4, and 3.2 mM CSP. MGY agar is a standard, low-complexing medium utilized to assess copper resistance in vitro (19). Plates were incubated for 96 h prior to assessing bacterial growth. The level of copper resistance was determined by comparing bacterial growth on MGY amended with various concentrations of copper and on MGY alone. For each concentration tested, bacterial growth of the spotted area was rated as confluent (+), with uniform, full growth; weak (±), with irregular, incomplete growth; or negative (–), with no growth. Previously characterized Cu^R strains *X. citri* subsp. *citri* A44 and *X. alfalfae* subsp. *citrumelonis* 1381 (10) were used as positive controls. Cu^S strains of *X. citri* subsp. *citri*, *X. alfalfae* subsp. *citrumelonis*, *X. vesicatoria*, and *X. perforans* were used as negative controls.

Design of primers for copper resistance genes *copLAB* from *X. citri* subsp. *citri*. Primers were designed to specifically amplify partial nucleotide sequences of copper resistance genes *copL*, *copA*, and *copB* from *X. citri* subsp. *citri* (10) using PCR analysis. The *copLAB* gene cluster, which is also present in Cu^R *X. alfalfae* subsp. *citrumelonis*, has been demonstrated to be the main determinant of copper resistance in *X. citri* subsp. *citri* (10). Due to the presence of copper resistance homolog genes on the chromosome of *X. citri* subsp. *citri* and other *Xanthomonas* spp. known to be Cu^S (10,22,46,54), nucleotide sequences of *copL*, *copA*, and *copB* from *X. citri* subsp. *citri* A44 were individually aligned with chromosomal homologous open reading frames (ORFs) of these genes (XAC3629, XAC3630, and XAC3631, respectively) from Cu^S *X. citri* subsp. *citri* 306 (22) using Clustal W. The chromosomal genes, which have been renamed *cohL*, *cohA*, and *cohB* (10), are thought to be housekeeping genes present in Cu^R and Cu^S strains important for copper homeostasis at low concentrations of this element.

Primer sets were separately designed for the *cop* genes *copL*, *copA*, and *copB*, based on the regions of low or no similarity be-

tween *cop* and *coh* genes to specifically amplify genes responsible for copper resistance in resistant strains.

PCR analysis. The methodology described here is applied for the primer sets designed for each of the three *cop* genes that gave the best results in the preliminary primer screening tests. Primers were synthesized by Sigma-Aldrich (St. Louis). Amplification of target genes from the strains tested was performed using a DNA thermal cycler (PTC 100; MJ Research, Cambridge, MA) and a Taq polymerase kit (Promega Corp., Madison, WI). For extraction of template DNA, strains were individually grown overnight on NA, suspended in sterile deionized water, boiled for 15 min, cooled on ice for 5 min, centrifuged at 15,000 rpm for 5 min, and then kept on ice. The supernatant was used for PCR reactions. Each PCR reaction mixture consisted of 25 µl of total volume, which included 10.3 µl of sterile water, 5 µl of 5× PCR buffer,

1.5 µl of 25 mM MgCl₂, 4 µl of deoxyribonucleoside triphosphates (0.8 mM each dATP, dTTP, dGTP, and dCTP), 0.5 µl of each primer (stock concentration, 25 pmol µl⁻¹), 3 µl of template, and 0.2 µl (5 U/µl) of Taq DNA polymerase. PCR reactions were initially incubated at 95°C for 5 min and then 30 PCR cycles were run under the following conditions: denaturation at 95°C for 30 s, primer annealing at 60°C for all set of primers for the three *cop* genes for 30 s, and DNA extension at 72°C for 45 s in each cycle. After the last cycle, PCR tubes were incubated for 10 min at 72°C and then at 4°C. Cu^R *X. citri* subsp. *citri* A44 and Cu^S *X. citri* subsp. *citri* 306 were used as positive and negative controls, respectively. PCR reaction mixtures were analyzed by 2% agarose gel electrophoresis (Bio-Rad Laboratories, Hercules, CA) with a Tris-acetate-EDTA buffer system. A 50-bp DNA ladder (Promega Corp.) was used as the standard molecular size marker for sizing

TABLE 1. Bacterial strains tested for the presence of copper resistance genes through polymerase chain reaction (PCR) analysis using primers designed based on *copL*, *copA*, and *copB* genes from the copper-resistant strain of *Xanthomonas citri* subsp. *citri* A44

Organism ^a	Strain	Origin	Year ^b	Source ^c	Copper resistance ^d	Level of resistance (mM CSP) ^e	PCR product size (bp) ^f		
							<i>copL</i>	<i>copA</i>	<i>copB</i>
Xcc	306	Brazil	1997	(20)	–	±0.3	n/a	n/a	n/a
Xcc	FB06C	Florida	2007	This study	–	+0.2	n/a	n/a	n/a
Xcc	AR78	Argentina	2008	Canteros, B. I.	–	+0.2	n/a	n/a	n/a
Xcc	AR81	Argentina	2008	Canteros, B. I.	–	+0.2	n/a	n/a	n/a
Xcc	AR63	Argentina	2008	Canteros, B. I.	+	+1.6	356	870	531
Xcc	AR72	Argentina	2008	Canteros, B. I.	+	+1.6	356	870	531
Xcc	AR77	Argentina	2008	Canteros, B. I.	+	+1.6	356	870	531
Xcc	AR79	Argentina	2008	Canteros, B. I.	+	+1.6	356	870	531
Xac	1390	Florida	2000	(8)	–	±0.3	n/a	n/a	n/a
Xac	1382	Florida	2000	DPI	+	+1.6	356	870	531
Xac	1383	Florida	2000	DPI	+	+1.6	356	870	531
Xac	7589	Florida	2007	DPI	+	+1.2	356	870	384
Xac	9226	Florida	2007	DPI	+	+1.6	356	870	531
Xac	29354	Florida	2009	DPI	+	+1.6	356	870	531
Xv	82-8	Florida	1982	Stall, R. E.	–	+0.2	n/a	n/a	n/a
Xv	1111	New Zealand	1955	(39)	+	+1.6	356	870	531
Xv	BV5-4	Argentina	1987	Canteros, B. I.	+	+1.6	356	870	531
Xv	Xv56	Brazil	nd	nd	+	+1.6	356	870	531
Xv	Xv446	Costa Rica	1991	Jones, J. B.	+	+1.6	356	870	531
Xv	Xv1288	Michigan	nd	Jones, J. B.	+	+1.6	356	870	531
Xe	81-23	Florida	1981	Stall, R. E.	+	+1.6	356	870	384
Xe	E-3	Florida	1960	Stall, R. E.	+	+1.6	356	870	384
Xe	75-3	Florida	1975	Stall, R. E.	+	+1.6	356	870	384
Xe	Xv221	Guadeloupe	1990	Jones, J. B.	+	+1.6	356	870	531
Xe	Xv264	Guadeloupe	1990	Jones, J. B.	+	+1.6	356	870	531
Xe	Xv304	Barbados	1990	Jones, J. B.	+	+1.6	356	870	384
Xe	Xv477	Costa Rica	1991	Jones, J. B.	+	+1.6	356	870	531
Xe	Xv669	Puerto Rico	1991	Jones, J. B.	+	+1.6	356	870	384
Xe	Xv718	Puerto Rico	1991	Jones, J. B.	+	+1.6	356	870	384
Xe	Xv787	U.S. Virgin Islands	1991	Jones, J. B.	+	+1.6	356	870	531
Xe	Xv800	U.S. Virgin Islands	1991	Jones, J. B.	+	+1.6	356	870	531
Xe	Xv818	Spain	nd	Jones, J. B.	+	+1.6	356	870	384
Xe	Xv881	Mexico	1992	Jones, J. B.	+	+1.6	356	870	384
Xe	Xv1025	Mexico	1992	Jones, J. B.	+	+1.6	356	870	384
Xp	91-118	Florida	1991	(39)	–	+0.2	n/a	n/a	n/a
Xp	1-7	Florida	2006	Stall, R. E.	+	+1.2	356	870	531
Xg	Xv444	Costa Rica	1991	Jones, J. B.	+	+1.2	356	870	531
Xaj	Xj71	Italy	2008	Dallai, D.	+	+1.6	356	870	531
Xaj	Xj79	Italy	2008	Dallai, D.	+	+1.6	356	870	531
Xsp	1219	Ohio	1995	Jones, J. B.	+	+1.6	356	870	684
Xsp	INA69	Florida	1984	(8)	+	+1.2	356	870	531

^a Xcc, *X. citri* subsp. *citri*; Xac, *X. alfalfae* subsp. *citrumelonis*; Xv, *X. vesicatoria*; Xe, *X. euvesicatoria*; Xp, *X. perforans*; Xg, *X. gardneri*; Xaj, *X. arboricola* pv. *juglandis*; and Xsp, *Xanthomonas* sp.(1219, pathogenic; INA69, citrus epiphytic).

^b Year of isolation; nd = not determined.

^c DPI, Division of Plant Industry, Gainesville, FL; Canteros, B. I., Instituto Nacional de Tecnología Agropecuaria, Bella Vista, Argentina; Dallai, D., Università degli Studi di Modena e Reggio Emilia, Reggio Emilia, Italy; Jones, J. B., and Stall, R. E., University of Florida, Gainesville.

^d Copper-sensitive (–) or -resistant (+) strain. For reference, copper-resistant strains were those that produced confluent growth on mannitol-glutamate yeast (MGY) agar amended with at least 0.8 mM copper sulfate pentahydrate.

^e Highest concentration of copper sulfate pentahydrate (CSP) amended to MGY agar with confluent (+) or weak (±) bacterial growth. The maximum concentration of confluent growth for strains that produced weak growth is the one immediately below the one indicated with ±.

^f For *copL* and *copA*, PCR product size is estimated based on the complete sequence of these genes in *Xanthomonas citri* subsp. *citri* A44; for *copB*, the three different sizes, from the smaller to the larger, are estimated based on the complete sequence of this gene in *X. euvesicatoria* 81-23, *X. citri* subsp. *citri* A44, and *Xanthomonas* sp. 1219, respectively; n/a = not applicable, because no PCR bands were observed.

the PCR product. Reaction products were visualized by staining the gel with ethidium bromide (0.5 µg ml⁻¹) for 20 min and then photographed using a UV transilluminator and Quantity One software (Universal Hood II; Bio-Rad Laboratories).

PCR analysis using the copBF/copBR primer set revealed three different product sizes among the strains tested. Complete sequences of *copB* from one representative *Xanthomonas* strain for each product size were PCR amplified using primers copBBF (5'-AGGTAGCCGACGCACGTATC-3') and copBBR (5'-CCACCGC AACCAATGCCACG-3'), which were designed based on the pXccCu2 sequence (10). These PCR reactions were performed as previously described (10) and analyzed by 1% agarose gel electrophoresis. Lambda DNA digested with *Hind*III and *Eco*RI (Promega Corp.) was used as molecular size marker.

DNA sequencing. DNA sequencing was performed by the DNA Sequencing Core Laboratory of the Interdisciplinary Center for Biotechnology Research, University of Florida, Gainesville. Sequencing of PCR products of *copL*, *copA*, and *copB* was performed with primers designed in the present study and used for PCR analysis. For complete sequences of *copB*, sequencing was initiated using the primer set used for PCR analysis (copBBF and copBBR). Primers designed based on the partial sequences obtained with copBBF and copBBR primers were used to complete the sequencing. Sequencing was performed twice using products of independent PCR reactions for each targeted DNA and was repeated one more time in case of conflict between sequences in the first two attempts. Sequences were submitted to GenBank and assigned GenBank accession numbers (GANs).

Comparison of copper resistance genes. Partial nucleotide sequences of copper resistance genes *copL*, *copA*, and *copB* from Cu^R strains (Table 1) were compared through alignment and phylogenetic analysis. Other bacterial strains that had the equivalent region of the *cop* genes previously sequenced and that were detected to have high homology (>90%) with the *cop* genes from *X. citri* subsp. *citri* A44 (10), such as *X. alfalfae* subsp. *citrumelonis* 1381 (10), *X. euvesicatoria* 7882 (syn. *X. axonopodis* pv. *vesicatoria* 7882) (58), *S. maltophilia* K279a (20), and FB03P (9), were included in the phylogenetic analysis.

Alignment of sequences and determination of similarities among sequences were performed using the Basic Alignment Search Tool (BLAST) network service (3). Phylogenetic trees were constructed by using the partial nucleotide sequence of each gene separately and by using multilocus sequence analysis (MLSA) (1) of the concatenated sequences of the three genes featured in this study for several Cu^R strains and species of *Xanthomonas* (Table 1). All trees were drawn to the same scale. The purpose of this analysis was to investigate the relatedness of these strains and the existence of a relevant subset of strains based on the copper resistance gene cluster *copLAB*. Trees based on the individual *cop* genes were built using the Molecular Evolutionary Genetics Analysis (MEGA) software (version 5.05; Arizona State University, Phoenix). Chromosomal homologues of *copL*, *copA*, and *copB* (ORFs XAC3629, XAC3630, and XAC3631, respectively) from Cu^S *X. citri* subsp. *citri* 306 (22) were used as outgroups. Sequences were aligned separately for each of the three genes and

for MLSA using the default settings of Clustal W algorithm implemented in MEGA 5.05. The alignments were assembled into maximum-likelihood (ML) trees using the Tamura-Nei substitution model, uniform rates among sites and the nearest-neighbor-interchange heuristic method. Branch support for the ML trees was estimated using nonparametric bootstrap analysis with 1,000 replicates (24). The percentage of replicate trees in which the associated strains clustered together in the bootstrap test is shown next to the branches (26). Moreover, the concatenated *copL*, *copA*, and *copB* genes were phylogenetically analyzed using ML and Bayesian methods. The concatenated genes were aligned as previously mentioned, and the chromosomal homologues of the *cop* genes were concatenated and served as the outgroup. jModelTest 2.1.1 (21,33) was used to select for the best nucleotide model that fit this data set. Using the Akaike's information criterion, the best model was TIM2+G and the settings to run ML and Bayesian analysis were adjusted to fit this substitution model. PAUP* version 4.0b10 was used for the ML analysis (53). Trees were created by performing heuristic searches with random stepwise addition and tree bisection/reconnection branch swapping (TBR). Nonparametric bootstrapping at *n* = 100 replicates by TBR swapping at *n* = 100 replicates provided branch support. Bayesian analysis was performed as outlined by Hong et al. (34). Burn-in was set at 7,500. Splits Tree 4.12.6 was used to construct the phylogenetic network analysis and to run the ϕ -statistics (35). The splits network was constructed using the neighbor-net setting and the uncorrected *P* character transformation. Bootstrap values were derived from 1,000 bootstrap replicates. Sequences for the species tree based on 16S rRNA were obtained from GenBank: *Xylella fastidiosa* (GAN NR_041783.1), *Xanthomonas arboricola* pv. *juglandis* (GAN NR_027606.1), *X. axonopodis* pv. *citri* (GAN AF442744.1), *X. axonopodis* pv. *citrumelo* (GAN CP002914.1), *X. gardeneri* (GAN AF123093.2), *X. vesicatoria* (GAN AF123088.2), *X. euvesicatoria* (GAN FR733718.1), and *S. maltophilia* (GAN AM743169.1). Sequences were aligned using Muscle (23) and manually manipulated using Se-Al 2.0a11 (47). The 16S rRNA sequences were analyzed in the same manner as were the concatenated *cop* genes, with TrN+I settings adjusted in the ML and Bayesian analyses. The burn-in was set at 1,500 for the Bayesian analysis.

RESULTS

Characterization of level of copper resistance in strains of *Xanthomonas*. Regardless of the species, all Cu^R strains of *Xanthomonas*, including the positive controls, were able to grow on MGY agar amended with 1.2 to 1.6 mM CSP (Table 1). A few Cu^R strains grew only at 1.2 mM CSP, whereas most of them developed confluent growth at 1.6 mM. No growth of Cu^R strains was observed at 2.4 mM. Conversely, Cu^S negative controls strains did not grow on MGY agar amended with more than 0.2 to 0.3 mM CSP (Table 1).

***copLAB* primers and PCR analysis.** The primer sets copLF/copLR, copAF/copAR, and copBF/copBR, designed in this study based on nucleotide sequences of *copL*, *copA*, and *copB* from

TABLE 2. Oligonucleotide primer sets designed for copper resistance genes *copL*, *copA*, and *copB* from *Xanthomonas citri* subsp. *citri*

Gene	Primer name	Sequence (5'–3')	Length (bp)	GC (%) ^a	Tm (°C) ^b	Product size (bp) ^c
<i>copL</i>	copLF	CCGTGTCAGCCTCCTCACTTCTAC	24	58	64	356
	copLR	CAGCGGCATGACATCCAGGCC	21	67	63	...
<i>copA</i>	copAF	CCTCCATGGCAGGACACTTCCATC	25	60	65	870
	copAR	CCAGACATATCCATCGACCCATGATCCA	28	50	63	...
<i>copB</i>	copBF	CTCAGGATCACTCTGCACATCAG	23	52	60	531
	copBR	GCACGTAGCTCTTAATCGAGTTGTC	25	48	60	...

^a Guanine (G) and cytosine (C) content.

^b Calculated melting temperature.

^c Expected polymerase chain reaction product size is based on the *copL*, *copA*, and *copB* genes from *X. citri* subsp. *citri* strain A44.

X. citri subsp. *citri* A44 (Table 1), respectively, were effective for distinguishing all Cu^R and Cu^S strains of different species of *Xanthomonas*. No DNA amplifications were observed for Cu^S strains. General characteristics and the region of the gene covered by the newly developed primers are described in Table 2 and Figure 1A, respectively. The PCR analysis revealed that the three copper resistance genes are conserved in Cu^R strains of *X. citri* subsp. *citri*, *X. alfalfae* subsp. *citrumelonis*, *X. arboricola* pv. *juglandis*, *X. euvesicatoria*, *X. gardneri*, *X. vesicatoria*, *X. perforans*, and *Xanthomonas* sp.

PCR product sizes of *copL* and *copA* for all Cu^R strains were similar to *X. citri* subsp. *citri* A44. Conversely, three different sizes for *copB* were observed among the strains tested for the partial region covered by the copBF/copBR primer set. Most of the strains analyzed produced an intermediate *copB* size. Of the 37 Cu^R strains included in this study, 1 (3%), 10 (27%), and 26 (70%) strains had a larger, smaller, and intermediate size sequence, respectively, for *copB* (Table 1). Based on the copper assays on solid medium, the differences in size of *copB* had no effect on copper resistance levels (Table 1).

Comparison of nucleotide sequences of *copLAB* gene cluster.

Alignment of partial nucleotide sequences of copper resistance genes among the strains of *Xanthomonas* revealed homology of ≥92, ≥96, and ≥91% for *copL*, *copA*, and *copB*, respectively. GANs assigned for these sequences are listed in Supplemental Table 1.

Sequencing of PCR products confirmed that sequence sizes were identical for *copL* and *copA* from all strains tested (data not shown). It also confirmed that the partial sequence sizes of *copB* were identical for strains within the same PCR product size group (Table 1). Analysis of the complete nucleotide sequence of *copB* from *X. citri* subsp. *citri* A44, *X. euvesicatoria* 81-23, and a pathogenic *Xanthomonas* with no defined host (*Xanthomonas* sp. 1219), selected as representative strains for intermediate-, small-, and large-size *copB*, respectively, revealed that *copB* sizes were 1,269, 1,158, and 1,425 bp in these strains (Fig. 1B).

The alignment of nucleotide sequences of the three *copB* sizes demonstrates that the shorter sequence length in *X. citri* subsp. *citri* A44 and *X. euvesicatoria* 81-23 is due to a large nucleotide gap present in the same region of the gene in comparison with *Xanthomonas* sp. 1219 (Fig. 1B). In *X. citri* subsp. *citri* A44, there is a 153-bp gap from position 414 to 566 compared with *Xanthomonas* sp. 1219. In *X. euvesicatoria* 81-23, there is a 300-bp gap in the same region of *copB* from nucleotide position 375 to 674 compared with *Xanthomonas* sp. 1219 (Fig. 1B).

These gaps in *X. citri* subsp. *citri* A44 and *X. euvesicatoria* 81-23 are located within the region of *copB* amplified by the primer set designed in this study. Other differences include a 36-bp gap in *X. citri* subsp. *citri* A44 and *Xanthomonas* sp. 1219 compared with *X. euvesicatoria* 81-23 and an extra codon in the beginning of the N-terminal of *copB* in *Xanthomonas* sp. 1219 in comparison with the other two strains (Fig. 1B). These sequence differences are located outside the region amplified by the copBF/copBR primer set. All gaps observed when comparing the nucleotide sequence of *copB* from these strains were in frame. Nucleotide similarities among strains in the regions of *copB* common to *X. citri* subsp. *citri* A44, *X. euvesicatoria* 81-23, and *Xanthomonas* sp. 1219, excluding the aforementioned gaps, were ≥98%.

Phylogenetic analysis of copper resistance genes. Partial nucleotide sequences of *copL*, *copA*, and *copB* obtained from different Cu^R strains of *Xanthomonas* (Table 1) were used for phylogenetic analysis. The results previously described for the alignment of nucleotide sequences revealed high homology (>90%) of the three copper resistance genes among all Cu^R strains of *Xanthomonas* tested. Slight differences were discerned through phylogenetic analysis, which separated or grouped the strains based on the conservation of nucleotide sequences of these genes.

Overall, placement of strains within the trees was not based on the species of origin of copper resistance; however, it appears that strains grouped based on geographic origin. Phylogenetic trees created by analysis of the individual and concatenated genes displayed four major clades, three of which contained strains predominantly from one geographic location: Florida, Virgin Islands, and Argentina (Figs. 2 to 4). The fourth clade will be considered the worldwide clade, which includes strains originating from various locations, including Florida and Argentina.

The five strains of *X. citri* subsp. *citri* which were isolated from different citrus hosts and different citrus-growing areas in Argentina, including the previously sequenced *X. citri* subsp. *citri* A44 (10), grouped with the Argentina clade (Figs. 3 and 4). This clade also contained *X. euvesicatoria*, *X. gardneri*, and *X. vesicatoria* strains isolated from Costa Rica and Guadeloupe (Figs. 3 and 4). The remaining Argentina strain, *X. citri* subsp. *citri* AR79, was grouped in the worldwide clade (Figs. 3 and 4). Alignment of partial nucleotide sequences of the *copLAB* genes from *X. citri* subsp. *citri* with the phylogenetically more closely related strains from other species revealed 100% similarity among the strains of *X. citri* subsp. *citri* A44, AR63, AR72, and AR77 from Argentina; *X. euvesicatoria* Xv264 and Xv221 from Guadeloupe; and *X.*

Fig. 1. Copper resistance genes from *Xanthomonas* spp. **A**, Regions of *copL*, *copA*, and *copB* from *Xanthomonas citri* subsp. *citri* A44 amplified by the copLF/copLR, copAF/copAR, and copBF/copBR primers, respectively. Numbers between parentheses indicate total gene size in base pairs and numbers below the arrows indicate the position of the first nucleotides of the forward and reverse primers within the gene. **B**, Comparison of complete nucleotide sequences of *copB* from *X. citri* subsp. *citri* A44 (Xcc A44), *Xanthomonas* sp. 1219 (Xsp 1219), and *X. euvesicatoria* 81-23 (Xe 81-23). Dashed shapes and black arrows indicate sequence gap in comparison with other strains and total sequence length in base pairs, respectively. Numbers within the shapes and after the arrows indicate gap and gene sizes in base pairs, respectively.

Fig. 2. Phylogenetic trees of copper resistance genes from xanthomonads. Trees were constructed from individual alignments of partial nucleotide sequences of copper resistance genes **A**, *copL*; **B**, *copA*; and **C**, *copB* using the maximum likelihood method. Bootstrap values, as percentage out of 1,000 replicates, are shown at each node. Taxon information indicates organism, strain, and geographical origin, respectively. Xcc, *Xanthomonas citri* subsp. *citri* (dark circles); Xac, *X. alfalfae* subsp. *citrumelonis* (dark triangles); Xv, *X. vesicatoria*; Xe, *X. euvesicatoria*; Xp, *X. perforans*; Xg, *X. gardneri*; Xj, *X. arboricola* pv. *juglandis*; Xsp, *Xanthomonas* sp. (1219, pathogenic); INA69, citrus epiphytic; Sm, *Stenotrophomonas maltophilia*. The sequence of the corresponding regions of homolog genes *cohL*, *cohA*, *cohB*, and *cohLAB* from *X. citri* subsp. *citri* 306, present on the chromosome of copper-resistant and -sensitive *Xanthomonas* strains, were used as outgroups for phylogenetic analysis. All trees are drawn to the same scale.

euvesicatoria Xv477, *X. vesicatoria* Xv446, and *X. gardneri* Xv444 from Costa Rica. In the other group, *X. vesicatoria* BV5-4 from Argentina was the only strain that had the partial sequence of the three *cop* genes identical to *X. citri* subsp. *citri* AR79 (Supplemental Figure 1).

The *copL* and *copA* trees produced the greatest and least divergences among the Cu^R strains, respectively (Fig. 2A and B). Additionally, these two genes were the ones that showed the greatest and least distances for the copper homolog outgroups *cohL* and *cohA*, respectively (Fig. 2A and B). Strain grouping and branching patterns of trees were similar for *copL* and *copA*, which differed from *copB* (Fig. 2A to C). When the three genes were concatenated and analyzed, the *copL/copA* tree shape prevailed (Fig. 3). Conversely, the 16S rRNA tree had a different topology than the aforementioned trees (Supplemental Figure 2). Based on the 16S rRNA tree, *S. maltophilia* was distantly related to the other strains, whereas it grouped with *X. vesicatoria* BV5-4 and *X. citri* subsp. *citri* AR79 in the concatenated tree. The four major clades could be observed when the concatenated dataset was analyzed by eburst (Fig. 4). The long branches of the Florida, Argentina, and Virgin Islands clades indicate that these genes

Fig. 3. Phylogenetic tree of copper resistance genes from xanthomonads. Trees were constructed by concatenating *copL*, *copA*, and *copB* using the maximum likelihood and Bayesian methods. Posterior probability values and weighted lines indicated a bootstrap value of $\leq 75\%$. The sequence of the corresponding regions of homolog genes *cohL*, *cohA*, and *cohB* from *Xanthomonas citri* subsp. *citri* 306, present on the chromosome of copper-resistant and -sensitive *Xanthomonas* strains, were concatenated and used as the outgroup. The solid line on the right indicates the Argentine clade, the small dashed line indicates the Virgin Island clade, and the large dashed line indicates the Florida clade; all other strains, except for *X. citri* subsp. *citri* 306 *cohA*, belong in the worldwide clade. Taxon information indicates organism, strain, and geographical origin, respectively. Xcc, *X. citri* subsp. *citri*; Xac, *X. alfalfae* subsp. *citrumelonis*; Xv, *X. vesicatoria*; Xe, *X. euvesicatoria*; Xp, *X. perforans*; Xg, *X. gardneri*; Xj, *X. arboricola* pv. *juglandis*; Xsp, *Xanthomonas* sp. (1219, pathogenic); INA69, citrus epiphytic; Sm, *Stenotrophomonas maltophilia*.

have been adapting to selection pressure unique to these geographic locations. The boxes and links observed with the remaining strains, those included in the worldwide clade, indicated exchange of the Cu^R plasmid or that the plasmid may have been recently acquired. The ϕ -statistic found significant evidence for recombination with $P = 6.532^{-12}$.

In all constructed trees, six strains of the citrus pathogen *X. alfalfae* subsp. *citrumelonis*, a weak pathogen reported only in Florida, were placed into the Florida or worldwide clades. Four of them, including *X. alfalfae* subsp. *citrumelonis* 1382, 1383, 29354, and the previously sequenced strain 1381 (10), were closely related to a strain of *X. perforans* and a citrus epiphytic *Xanthomonas* sp., both from Florida. Strain 7589 grouped with strains of *X. vesicatoria* Xv669 and Xv818 from Puerto Rico and Spain, respectively. The remaining strain, 9226, clustered differently from other strains according to the genes studied but always separated from the other strains of *X. alfalfae* subsp. *citrumelonis* (Figs. 2 to 4).

DISCUSSION

In *Xanthomonas* spp., copper resistance has been characterized as a qualitative trait, and the threshold usually adopted as reference for resistance determination is 0.8 mM CSP amended to a solid medium such as NA (5,50), glucose-nutrient agar (45), or MGY agar (10,11). Based on this assay, copper resistance was confirmed for all presumed Cu^R strains of *Xanthomonas* included in the study, because all strains were able to grow over the threshold concentration. A qualitative separation of Cu^R and Cu^S strains was also observed as the ability to grow on medium amended with copper. Moreover, media and PCR assays showed 100% correspondence. Furthermore, the primer sets designed for the detection of copper resistance genes were highly specific not only for Cu^R strains of *X. citri* subsp. *citri* but also for several other xanthomonads.

Cu^R strains have previously been reported in several species of plant-pathogenic xanthomonads such as *X. euvesicatoria* (40,50), *X. arboricola* pv. *juglandis* (29), *X. citri* subsp. *citri*, and *X. alfalfae* subsp. *citrumelonis* (10). However, this is the first time that copper resistance has been compared in the three *X. axonopodis* pv. *vesicatoria*-derived species (*X. vesicatoria*, *X. euvesicatoria*, and *X. perforans*) and in *X. gardneri* following reclassification (36). Moreover, the copper resistance determinants identified in the strains *X. arboricola* pv. *juglandis* 71 and 79 included in this study are different from those studied by Lee et al. (37). For *X. arboricola* pv. *juglandis* C5 in the referenced work, the copper resistance determinants were located on the chromosome and organized in four ORFs similar to the *copABCD* structure of the copper resistance genes from *P. syringae* pv. *tomato* (42), with some minor differences in DNA sequence and gene size. Conversely, PCR analysis revealed that strains 71 and 79 contain a *copLAB* gene cluster like *X. citri* subsp. *citri* A44, with nucleotide homology >98% between the two species. Sequence alignment of partial *cop* sequences of strains 71 and 79, which are identical, to *copABCD* from strain C5 demonstrated homology only at the *copA* region, with a similarity of 78%. Partial *copL* and *copB* sequences from strains 71 and 79 produced no homology with C5.

Sequence alignments of *copLAB* genes from different strains or species of *Xanthomonas* and *Stenotrophomonas* indicated that the resistance genes are conserved among the Cu^R strains with an identity of nucleotide sequences >90%. Although the high homology of the genes among the strains studied indicates that the copper resistance in xanthomonads has a common origin, slight differences in nucleotide sequences within groups of strains, as revealed by the phylogenetic analysis, demonstrates that Cu^R genes have been independently exchanged among different species of xanthomonads throughout the world. This exchange into *X. citri* subsp. *citri* and *X. alfalfae* subsp. *citrumelonis* very likely occurred by horizontal transfer, as previously discussed (9,11).

Fig. 4. Phylogenetic network based on the concatenated *copL*, *copA*, and *copB* genes. Numbers displayed on the branches of the tree represent the bootstrap values of 1,000 replications. Taxon information indicates organism, strain, and geographical origin, respectively. Xcc, *Xanthomonas citri* subsp. *citri*; Xac, *X. alfalfae* subsp. *citrumelonis*; Xv, *X. vesicatoria*; Xe, *X. euvesicatoria*; Xp, *X. perforans*; Xg, *X. gardneri*; Xj, *X. arboricola* pv. *juglandis*; Xsp, *Xanthomonas* sp. (1219, pathogenic; INA69, citrus epiphytic); Stm, *Stenotrophomonas maltophilia*.

copA is the most conserved copper resistance gene among the Cu^R *Xanthomonas* strains studied. This gene produced the highest nucleotide homology (≥96%) and the shortest distance among strains in the phylogenetic analysis. Comparison of the partial *copA* consensus sequence from *Xanthomonas* spp. obtained in this study to previously sequenced organisms revealed that, in addition to *Xanthomonas* spp., high homology was also observed for *S. maltophilia* strains K279a and FB03P, in which the resistance to copper is either putative (20) or confirmed (9), respectively. Furthermore, of the three *cop* genes studied, *copA* produced the highest nucleotide similarity (≈75%) with chromosomal homologs present in several species of *Xanthomonas*, such as *X. citri* subsp. *citri* (GAN: AE008923), *X. alfalfae* subsp. *citrumelonis* (GAN: P002914), *X. axonopodis* pv. *vesicatoria* (GAN: AM039952), *X. campestris* pv. *raphani* (GAN: CP002789) and pv. *campestris* (GAN: AE008922, AM920689, and CP000050), and *X. oryzae* pv. *oryzicola* (GAN: CP003057) and pv. *oryzae* (GAN: AP008229, AE013598, and CP000967). The homolog gene has been recently renamed *cohA* and is not responsible for copper resistance but possibly for copper homeostasis in Cu^R and Cu^S strains of *Xanthomonas* at low level of copper (10). Sequence alignments also revealed that *copA* is the most conserved *cop* gene in other bacterial species. A partial nucleotide sequence of this gene produced ≈75% similarity with homologs from Cu^R strains of *Pseudomonas* (GAN: M19930) and noncharacterized strains of *Stenotrophomonas* (GAN: HE798556, CP002986, CP001111, and FJ514243) *Pseudoxanthomonas* (GAN: CP003093 and CP002446), *Pseudomonas* (GAB: CP002870, AE015451, CP000094, AP012280, CP000438, and AE004091) and *Xylella* (GAB: CP000941 and AE003849).

Partial sequences of *copL* and *copB* were less similar among the Cu^R *Xanthomonas* strains. The *copL* sequences produced the greatest distances among the strain clusters in the phylogenetic trees and to the chromosomal homolog *cohL*. Overall, *copL* is the least conserved *cop* gene in previously sequenced bacteria. As for *copA* and *copB*, partial *copL* is also present in Cu^R strains of *X. citri* subsp. *citri*, *X. alfalfae* subsp. *citrumelonis*, *X. euvesicatoria*, and *S. maltophilia* previously characterized (9,10,47), with homology >90% among strains. Nonetheless, no *copL* or *copB* nucleotide homology is observed for other bacterial species using the partial sequence generated in this study, as observed for *copA*. Some homology can be detected when the translated amino acid sequence is used for comparison, as previously demonstrated (10).

The topology of the phylogenetic trees and phylogenetic network analyses indicates that this plasmid has been laterally transferred, and that transfer may not be species specific. This can be observed by comparison of the placement of *S. maltophilia* in the concatenated tree and the 16S rRNA tree (Fig. 3). *S. maltophilia* was once placed to the genus *Xanthomonas*, however, due to unique characteristics, *S. maltophilia* was transferred to its own genus (44). Based on the topology of the individual gene trees, it appears that greater selection pressure has been exhibited predominantly on *copL* and *copA* due to their similar topology compared with *copB*, and the geographic grouping of the analyzed strains. The difference of *copB* was further observed with the different partial product sizes that amplified with the primer set designed for this gene. These results corroborate previous work that shows that horizontal gene transfer is likely the main mechanism by which the copper resistance genes are exchanged intra- and interspecifically in plant-pathogenic bacteria (6,9,12, 13,18,50,51,52). Likewise, some of the Cu^R strains used in the present study were previously shown to be able to transfer the copper resistance determinants by conjugation within and among different *Xanthomonas* spp., including *X. citri* subsp. *citri*, *X. alfalfae* subsp. *citrumelonis*, *X. euvesicatoria*, and *X. perforans* (9,50).

The long branches of the Florida, Virgin Island, and Argentina clades imply that these strains acquired this plasmid and, due to

selective pressure unique to the geographic regions, these strains are adapting by having similar mutations. Strains in these clades were collected at different time periods and these mutations have persisted. If a plasmid, especially a large plasmid that is costly to maintain, is essential for bacterial survival, the plasmid will persist over time (49). It is possible for these mutations to have reverted to the original state; thus, these strains could be included in the worldwide clade. It is also possible that strains isolated in Florida or Argentina that grouped within the worldwide clade could have acquired the plasmid more recently and, thus, they were not subjected to these pressures. It is interesting to note that the majority of the *X. euvesicatoria* strains grouped together, possibly indicating that there is pressure from the chromosome placed on the plasmid to appear more like the chromosome (Fig. 4). It has previously been noted that the more homogeneous a genome is, the more similar were the corresponding plasmids, with plasmid similarity being related to plasmid size (14).

Our findings indicate that multiple and independent introductions of copper resistance genes have taken place for Cu^R strains of *X. citri* subsp. *citri* and *X. alfalfae* subsp. *citrumelonis*. The results also revealed that the *copLAB* gene cluster, which is responsible for copper resistance in xanthomonads (9,10,58), is disseminated in *Xanthomonas* populations throughout the world. The presence of homologs of *copLAB* in epiphytic bacteria such as *Xanthomonas* and *Stenotrophomonas*, as demonstrated in this study and elsewhere (9,10,20,58), and the incessant movement of plant material, especially seed, among countries may account for such wide dissemination of these genes into different *Xanthomonas* populations in different parts of the world. These findings corroborate previous studies (9,11) that indicate a risk for copper resistance development in *Xanthomonas* pathogens of citrus under constant exposure to copper.

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