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Molecular Characterization of Copper Resistance Genes from *Xanthomonas citri* subsp. *citri* and *Xanthomonas alfalfae* subsp. *citrumelonis*[▽]

Franklin Behlau,[1]‡ Blanca I. Canteros,[2] Gerald V. Minsavage,[1] Jeffrey B. Jones,[1] and James H. Graham[3]*

Department of Plant Pathology, University of Florida, Gainesville, Florida 32611[1]; Instituto Nacional de Tecnología Agropecuaria, Estación Experimental Agropecuaria, Bella Vista, Corrientes, Argentina[2]; and Soil and Water Science Department, University of Florida, Lake Alfred, Florida 33850[3]

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Copper sprays have been widely used for control of endemic citrus canker caused by *Xanthomonas citri* subsp. *citri* in citrus-growing areas for more than 2 decades. *Xanthomonas alfalfae* subsp. *citrumelonis* populations were also exposed to frequent sprays of copper for several years as a protective measure against citrus bacterial spot (CBS) in Florida citrus nurseries. Long-term use of these bactericides has led to the development of copper-resistant (Cu^r) strains in both *X. citri* subsp. *citri* and *X. alfalfae* subsp. *citrumelonis*, resulting in a reduction of disease control. The objectives of this study were to characterize for the first time the genetics of copper resistance in *X. citri* subsp. *citri* and *X. alfalfae* subsp. *citrumelonis* and to compare these organisms to other Cu^r bacteria. Copper resistance determinants from *X. citri* subsp. *citri* strain A44(pXccCu2) from Argentina and *X. alfalfae* subsp. *citrumelonis* strain 1381(pXacCu2) from Florida were cloned and sequenced. Open reading frames (ORFs) related to the genes *copL*, *copA*, *copB*, *copM*, *copG*, *copC*, *copD*, and *copF* were identified in *X. citri* subsp. *citri* A44. The same ORFs, except *copC* and *copD*, were also present in *X. alfalfae* subsp. *citrumelonis* 1381. Transposon mutagenesis of the cloned copper resistance determinants in pXccCu2 revealed that copper resistance in *X. citri* subsp. *citri* strain A44 is mostly due to *copL*, *copA*, and *copB*, which are the genes in the cloned cluster with the highest nucleotide homology (≥92%) among different Cu^r bacteria.

The copious use of copper-based bactericides on vegetable and fruit crops for control of bacterial and fungal pathogens has led to the development and prevalence of copper-resistant (Cu^r) strains of several species of bacteria affecting plants (1, 2, 4, 16, 25, 33, 37, 39). Although most copper resistance genes characterized from plant-pathogenic bacteria have been shown to be plasmid borne (4, 6, 12, 26, 37, 43), chromosomal copper resistance genes have also been identified (3, 22, 23).

Cellular copper sequestration has been suggested as the copper resistance mechanism in resistant strains of *Pseudomonas syringae* (12). In *P. syringae*, the copper resistance operon, *copABCD*, encodes four proteins, CopA, -B, -C, and -D, and is present on plasmid pPT23D (11, 26). This operon is regulated by a copper-inducible promoter that requires the regulatory genes *copR* and *copS*, located downstream of *copD* (27), which suggests that *P. syringae* employs a two-component sensory transduction system to alter gene expression in response to environmental stimuli and regulate copper resistance gene expression. When grown on copper-amended medium, strains harboring plasmid pPT23D accumulate copper, indicating that resistance is due to an uptake mechanism (14). Studies have shown that *P. syringae* containing the *cop* operon accumulates more copper than strains lacking the operon (5, 11, 15) and that this operon confers copper resistance to *P. syringae* at least in part by sequestering and accumulating copper in the periplasm with copper binding proteins, which may prevent toxic levels of copper from entering the cytoplasm (11, 13). According to Rouch et al. (34), genes that confer copper resistance are regulated and induced only by high levels of copper. Copper inducibility of the *pco* genes of *Escherichia coli* showed that the lag phase observed upon addition of copper to the growth medium could be reduced by preinduction with copper sulfate (34).

In *Escherichia coli*, copper resistance is regulated by different systems, including the multicopper oxidase *cueO*, which protects periplasmic enzymes from copper-mediated damage (21), the *cus* determinant, which confers copper and silver resistance (28), and the *pcoABCD* operon (32). The last is known as an efflux mechanism and is responsible for pumping excess copper out of the cytoplasm (13). The *pcoABCD* operon shares homology with the *copABCD* operon from *P. syringae* and, as in *P. syringae*, is followed by two regulatory genes, *pcoR* and *pcoS* (26).

Copper resistance genes have also been cloned from *Xanthomonas vesicatoria* (3, 12), *Xanthomonas arboricola* pv. juglandis (22), and *Xanthomonas axonopodis* pv. vesicatoria (44). Plasmid-borne genes for copper resistance in *X. vesicatoria* have similarities to the *cop* operon from *P. syringae* (43). Nevertheless, on the chromosome, the organization of the copper resistance genes appears to be uncommon, and occurrence of this type of resistance is rare in *X. vesicatoria* (3). The copper resistance genes described by Lee et al. (22) in *X. arboricola* pv.

* Corresponding author. Mailing address: University of Florida, Soil and Water Science Department, Citrus Research and Education Center, 700 Experiment Road, Lake Alfred, FL 33850. Phone: (863) 956-1151. Fax: (863) 956-4631. E-mail: jhg@crec.ifas.ufl.edu.

‡ Present address: Fundecitrus—Fundo de Defesa da Citricultura, Av. Adhemar Pereira de Barros 201, 14807-040 Araraquara, SP, Brazil.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic[a]	Source or reference
Strains		
Escherichia coli		
DH5α	F80d *lacZ15 recA1*	GIBCO-BRL
C2110	Nal^r *polA*	7
Xanthomonas citri subsp. *citri* A44	Cu^r	9
Xanthomonas alfalfae subsp. *citrumelonis* 1381	Cu^r	This study
Xanthomonas perforans 91-118	Kan^r	This study
Plasmids		
pLAFR3	Tet^r *rlx*$^+$, RK2 replicon	38
pBluescript KS+/−	Phagemid, pUC derivative, Amp^r	Stratagene
pRK2073	ColE1 replicon, Tra$^+$ Mob$^+$ Sp^r	19
pXccCu1	Tet^r Cu^r, ~17-kb EcoRI-HindII fragment of *X. citri* subsp. *citri* A44 in pLAFR3	This study
pXccCu2	Tet^r Cu^r, 9.5-kb EcoRI-EcoRI fragment of pXccCu1	This study
pXacCu1	Tet^r Cu^r, ~17-kb EcoRI-HindII fragment of *X. alfalfae* subsp. *citrumelonis* 1381 in pLAFR3	This study
pXacCu2	Tet^r Cu^r, 9.6-kb HindIII-EcoRI fragment of pXacCu1	This study

[a] Nal^r, nalidixic acid resistant; Kan^r, kanamycin resistant; Tet^r, tetracycline resistant; Amp^r, ampicillin resistant; Sp^r, spectinomycin resistant; Cu^r, copper resistant.

juglandis are located on the chromosome and have the same general *copABCD* structure as the genes from *P. syringae*, with some differences in DNA sequence and gene size. In *X. axonopodis* pv. vesicatoria, copper resistance genes are plasmid borne and expression of these genes was demonstrated to be regulated by *copL*, which is the immediate open reading frame (ORF) upstream of *copAB* (44). Homologs of the *copRS* regulatory genes, which are present in *P. syringae* (26), have been found only on the chromosome of a unique strain of *Xanthomonas campestris* pv. vesicatoria (3).

Sprays of copper-based bactericides have been widely used for more than 2 decades in citrus-growing areas to control endemic citrus canker caused by *Xanthomonas citri* subsp. *citri* (synonym, *Xanthomonas axonopodis* pv. *citri*). In Florida, frequent use of copper sprays for control of canker has been adopted just recently, after the citrus canker eradication program was suspended in 2006. In contrast, *Xanthomonas alfalfae* subsp. *citrumelonis* (synonyms, *X. campestris* pv. citrumelo and *X. campestris* pv. citri strain E) has been exposed for years to copper used for control of citrus bacterial spot (CBS), a foliar disease restricted to nursery environments in Florida that no longer poses a threat to citrus production (20).

The development of Cu^r strains of *X. citri* subsp. *citri* has been reported only in Argentina (9). The resistant strains were first isolated in 1994 from a citrus grove located in the province of Corrientes which showed a lack of response to the numerous copper sprays used for control of recurrent outbreaks of citrus canker (9). As with most Cu^r bacteria, copper resistance genes from *X. citri* subsp. *citri* are located on the plasmid (10). Copper resistance has also been recently identified in *X. alfalfae* subsp. *citrumelonis* in Florida (F. Behlau, R. E. Stall, J. B. Jones, and J. H. Graham, unpublished data). However, the genetics of the copper resistance in these two species of *Xanthomonas* remain unknown. Thus, the objectives of this study were to characterize molecularly the copper resistance determinants in *X. citri* subsp. *citri* and *X. alfalfae* subsp. *citrumelonis* and to compare these with other bacteria.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used in molecular studies and their relevant characteristics and sources are listed in Table 1. Cu^r strain A44 from Argentina isolated in 1994 and strain 1381 from Florida isolated in 2000 were used for characterization of the Cu^r genes from *X. citri* subsp. *citri* and *X. alfalfae* subsp. *citrumelonis*, respectively. *Xanthomonas* strains were maintained in nutrient agar (NA) at 28°C, whereas cultures of *E. coli* were grown in Luria-Bertani (LB) broth (24) at 37°C. A pLAFR3 (38) cosmid library of *X. citri* subsp. *citri* A44 and *X. alfalfae* subsp. *citrumelonis* 1381 was maintained in *E. coli* DH5α on LB medium containing tetracycline. All other strains were stored in sterile tap water at room temperature or in 20% glycerol at −70°C or both. Antibiotics were used to maintain selection for resistance markers at the following final concentrations: for ampicillin, 100 mg liter^{-1}, for kanamycin, 50 mg liter^{-1}, for spectinomycin, 100 mg liter^{-1}, for rifamycin, 80 mg liter^{-1}, and for tetracycline, 12.5 mg liter^{-1}. Nutrient broth (NB) and LB broth were used as liquid media to grow *Xanthomonas* and *E. coli*, respectively. Cultures in liquid medium were grown for 24 h at 28°C on a KS10 orbital shaker (BEA-Enprotech Corp., Hyde Park, MA) at 200 rpm. Copper was used as copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) and added to the liquid or solid medium from a 1- or 50-mg-ml^{-1} stock solution, respectively, before being autoclaved.

Construction of genomic libraries and isolation of copper-resistant clones. A pLAFR3 cosmid (38) library of DNA from strains *X. citri* subsp. *citri* A44 and *X. alfalfae* subsp. *citrumelonis* 1381 was created as previously described (24) and maintained in *E. coli* DH5α. Total genomic DNA was extracted using genomicPrep cell and tissue DNA isolation kit (GE Healthcare, Piscataway, NJ) by following the manufacturer's instructions. Constructed plasmids were introduced into Kan^r *Xanthomonas perforans* ME24 from *E. coli* DH5α by triparental matings with pRK2013 as the helper plasmid (19). Matings were carried out by mixing mid-exponential-phase cells of the recipient strain ME24 with cosmid donors and with pRK2073 on NYG agar (42) at a ratio of 2:1:1 (vol/vol/vol) of the recipient, donor, and helper strains, respectively. After 24 h of incubation at 28°C, the mating mixtures were resuspended in 2 ml of mannitol-glutamate-yeast extract (MGY) broth amended with 1 mg liter^{-1} of copper for induction of presumptive copper resistance genes to be screened. Aliquots of 50 μl were spread onto NA plates containing kanamycin and tetracycline for selection of transconjugants. Transconjugants were grown overnight on NA amended with 20 mg liter^{-1} of copper for induction of resistance to copper (3) and suspended in sterile tap water visually to approximately 10^8 CFU ml^{-1}. Suspensions were then spotted on NA amended with 200 mg liter^{-1} of copper to screen for clones carrying copper resistance genes.

General DNA manipulations. Miniscale preparations of *E. coli* plasmid DNA were obtained by alkaline lysis as described by Sambrook et al. (35). Subcloning of the DNA insert from a cosmid carrying the copper resistance gene cluster was

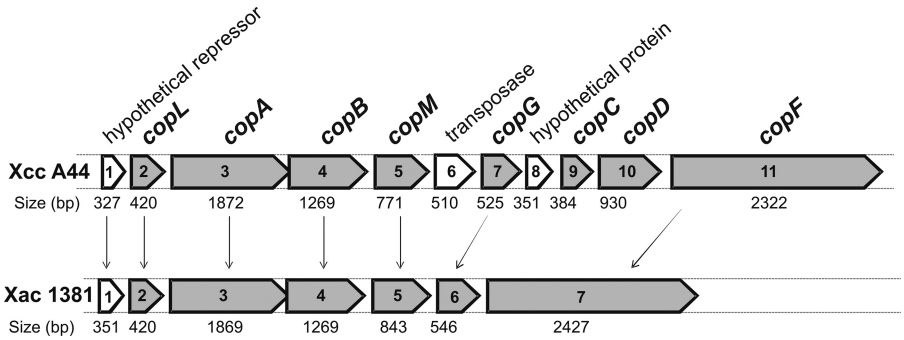

FIG. 1. Copper resistance determinants in *Xanthomonas citri* subsp. *citri* (Xcc) strain A44 and *Xanthomonas alfalfae* subsp. *citrumelonis* (Xac) strain 1381. ORF numbers are indicated inside the shapes.

performed by restriction digestion of the original clone with various enzymes and purification of fragments from an agarose gel by using the Wizard PCR Preps DNA purification system (Promega, Madison, WI). Fragments were ligated into the pBluescript II/KS (Stratagene, La Jolla, CA) and pLAFR3 (38) vectors for nucleotide sequencing and for checking for copper resistance activity in ME24 by triparental mating as aforementioned. Ligation was performed with T4 DNA ligase (Promega, Madison, WI), used according to the manufacturer's instructions. Ligation products were transformed into competent cells of *E. coli* DH5α produced by the calcium chloride procedure as described by Sambrook et al. (35).

Design of primers for *copF* and PCR analysis. Analysis of the nucleotide sequence of pXccCu2 revealed the absence of *copF*, which is present in *X. alfalfae* subsp. *citrumelonis* 1381 and other previously sequenced Cu^r bacteria, such as *Stenotrophomonas maltophilia* K279a (17) and *X. axonopodis* pv. vesicatoria 7882 (44). The presence of *copF* in *X. citri* subsp. *citri* A44 was investigated through PCR analysis using primers copFF (5′-GCCCTGTTCCAGAGCACCT ACGG-3′) and copFR (5′-CCTTGTTGGCATCGAGCTTGGTG-3′), designed based on homologous sequences (95% nucleotide sequence identity) from *S. maltophilia* K279a (17), with primer copFF overlapping the C terminus of pXccCu2.

Primers were synthesized by Sigma-Aldrich (St. Louis, MO). Amplification of the target gene was performed using a DNA thermal cycler (model PTC 100; MJ Research, Cambridge, MA) and the *Taq* polymerase kit (Promega, Madison, WI). For extraction of template DNA, *X. citri* subsp. *citri* strain A44 was grown overnight on NA, suspended in sterile deionized water (DI), boiled for 15 min, cooled on ice for 5 min, centrifuged at 15,000 rpm for 5 min, and kept on ice. The supernatant was used for PCRs. Each PCR mixture consisted of a 25-μl total volume, which included 10.3 μl of sterile water, 5 μl of 5× PCR buffer, 1.5 μl of 25 mM $MgCl_2$, 4 μl 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 the template, and 0.2 μl (5 U/μl) of *Taq* DNA polymerase. PCR mixtures were initially incubated at 95°C for 5 min, followed by 30 PCR cycles, which were run under the following conditions: denaturation at 95°C for 30 s, primer annealing at 60°C for 30 s, and DNA extension at 72°C for 2.5 min in each cycle. After the last cycle, PCR tubes were incubated for 10 min at 72°C and then at 4°C. Copper-sensitive (Cu^s) strain *X. citri* subsp. *citri* 306 was used as the negative control. PCR mixtures were analyzed by 1% agarose gel electrophoresis (Bio-Rad Laboratories, Hercules, CA) with a Tris-acetate-EDTA (TAE) buffer system. Lambda DNA digested with HindIII and EcoRI (Promega, Madison, WI) was used as the molecular size marker. Reaction products were visualized by staining the gel with ethidium bromide (0.5 $\mu g\ ml^{-1}$) for 20 min and then photographed using a UV transilluminator and Quantity One software (Universal Hood II imaging system; Bio-Rad, Hercules, CA).

DNA sequencing. DNA sequencing was performed by the DNA Sequencing Core Laboratory of the Interdisciplinary Center for Biotechnology Research (ICBR), University of Florida, Gainesville, FL. For sequence analysis of the copper resistance determinants, DNA fragments from *X. citri* subsp. *citri* A44 and *X. alfalfae* subsp. *citrumelonis* 1381(pLAFR3) cosmids were cloned into the vector pBluescript II/KS (Stratagene, La Jolla, CA) using appropriate enzymes. Sequencing was initiated using the standard flanking vector F20 and R24 primers. Custom primers designed based on the sequences obtained with F20 and R24 primers were used to complete the sequencing. The exact location of Tn*3-uidA* insertions was determined by sequencing plasmid DNA from insertion derivatives using primer RST92 (5′-GATTTCACGGGTTGGGGTTTCT-3′), which is complementary to the N terminus of the transposon. Sequencing of PCR products of *copF* was performed with primers used for PCR analysis. Additional custom primers designed based on sequences obtained with PCR primers were utilized for the complete sequencing of *copF*.

Transposon mutagenesis of copper resistance genes from *Xanthomonas citri* subsp. *citri*. Mutagenesis was performed by randomly inserting the Tn*3-uidA* transposon as previously described (8) into pXccCu2 from *X. citri* subsp. *citri* A44 to assess genes involved in copper resistance. Individual insertion derivatives were analyzed by extracting plasmid DNA and sequencing for the location of transposon insertion within the 9.5-kb cloned fragment carrying copper resistance genes. Selected pXccCu2 derivatives were transferred to the recipient strain *X. perforans* 91-118, which is resistant to rifamycin and spectinomycin through triparental mating as described previously. To assess for copper resistance, transconjugants were grown overnight on NA amended with 20 mg $liter^{-1}$ of copper for induction of resistance (3), suspended in sterile tap water at approximately 10^8 CFU ml^{-1}, and then spotted (10 μl) on MGY agar (6, 15) amended with 0, 25, 50, 100, 150, 200, 300, 400, 600, and 800 mg $liter^{-1}$ of copper. The growth of transconjugants was assessed after 96 h of incubation at 28°C.

Nucleotide sequence accession numbers. The nucleotide sequences for the copper resistance genes from *X. citri* subsp. *citri* and *X. alfalfae* subsp. *citrumelonis* have been assigned accession numbers HM362782 and HM579937, respectively, by GenBank.

RESULTS

Cloning and subcloning of copper resistance genes from *Xanthomonas citri* subsp. *citri* and *Xanthomonas alfalfae* subsp. *citrumelonis*. Approximately 600 and 1,600 clones were screened for *X. citri* subsp. *citri* A44 and *X. alfalfae* subsp. *citrumelonis* 1381 genomic libraries, respectively. One cosmid clone from each genomic library conferred copper resistance in Cu^s *X. perforans* ME24 transconjugants. Different fragment sizes from the original clones were subcloned into pLAFR3 (38) and checked for copper resistance. A 9.5-kb EcoRI-EcoRI subclone (pXccCu2) obtained from the ~17-kb Sau3AI-Sau3AI cosmid clone from *X. citri* subsp. *citri* A44(pXccCu1) and a 9.6-kb HindIII-EcoRI subclone (pXacCu2) from the ~17-kb Sau3AI-Sau3AI original clone (pXacCu1) from *X. alfalfae* subsp. *citrumelonis* 1381 conferred resistance to copper on media containing 200 mg $liter^{-1}$ of copper sulfate.

Ten ORFs were identified for the sequence of the 9.5-kb DNA insert of pXccCu2 from *X. citri* subsp. *citri* A44 (Fig. 1). These ORFs are located within ~7.9 kb. No ORF was identified in the 1.6 kb positioned upstream of the first ORF. Seven ORFs are closely related to copper resistance genes previously sequenced (17, 44). ORF2, ORF3, ORF4, ORF5, ORF7, ORF9, and ORF10 are ≥96% identical to genes related to

TABLE 2. Comparison of the nucleotide sequences of genes *copL*, *copA*, *copB*, *copM*, *copG*, *copC*, *copD*, and *copF* from different strains

Gene	Organism and strain[a]	% identity[b] to:		
		X. citri subsp. *citri* A44	*X. alfalfae* subsp. *citrumelonis* 1381	*X. axonopodis* pv. vesicatoria 7882
copL	Xcc A44			
	Xac 1381	92 (100)		
	Xav 7882	93 (100)	96 (100)	
	Stm K279a	96 (100)	94 (100)	95 (100)
copA	Xcc A44			
	Xac 1381	95 (100)		
	Xav 7882	95 (100)	97 (100)	
	Stm K279a	97 (100)	95 (100)	95 (100)
copB	Xcc A44			
	Xac 1381	92 (100)		
	Xav 7882	93 (100)	94 (100)	
	Stm K279a	99 (100)	92 (100)	
copM	Xcc A44			
	Xac 1381	91 (75)		
	Xav 7882	89 (75)	94 (100)	
	Stm K279a	99 (100)	91 (69)	89 (75)
copG	Xcc A44			
	Xac 1381	69 (52)		
	Xav 7882	68 (55)	96 (100)	
	Stm K279a	100 (100)	69 (70)	68 (55)
copC	Xcc A44			
	Xac 1381	NC		
	Xav 7882	NC	NC	
	Stm K279a	99 (100)	NC	NC
copD	Xcc A44			
	Xac 1381	NC		
	Xav 7882	NC	NC	
	Stm K279a	98 (100)	NC	NC
copF	Xcc A44			
	Xac 1381	97 (97)		
	Xav 7882	95 (100)	94 (100)	
	Stm K279a	99 (100)	97 (92)	95 (97)

[a] Xcc, *Xanthomonas citri* subsp. *citri*; Xac, *Xanthomonas alfalfae* subsp. *citrumelonis*; Xav, *Xanthomonas axonopodis* pv. vesicatoria; Stm, *Stenotrophomonas maltophilia*.

[b] Numbers in parentheses indicate the sizes of comparable sequences as a percentage of the total gene size. NC, not comparable due to the absence of the gene in one or both strains.

copper resistance, namely, *copL*, *copA*, *copB*, *copM*, *copG*, *copC*, and *copD*, respectively, from *S. maltophilia* K279a isolated from an immunosuppressed human patient (17) (Table 2; Fig. 2A and B). Additionally, *copL*, *copA*, and *copB* from *X. citri* subsp. *citri* A44 are ≥93% identical to the same *cop* genes from *X. axonopodis* pv. vesicatoria 7882 (*X. axonopodis* pv. vesicatoria 7882) (44), which lacks *copC* and *copD* (Table 2; Fig. 2A and B). *copM* and *copG* from *X. citri* subsp. *citri* A44 are not as similar to the homologs in *X. axonopodis* pv. vesicatoria 7882 as to those in *S. maltophilia* K279a (Table 2; Fig. 2B). The percentages of identity of *copM* and *copG* between the *X. citri* subsp. *citri* and *X. axonopodis* pv. vesicatoria strains are lower than 70% and 90%, respectively (Table 2).

Immediately downstream of *copD* and *copG* in *S. maltophilia* K279a and *X. axonopodis* pv. vesicatoria 7882, respectively, there is an ORF named *copF* that is absent in pXccCu2 (Fig. 2A); its sequence, based on that of *S. maltophilia* K279a, ends 44 bp upstream of the last nucleotide of *copD*. PCR analysis of *X. citri* subsp. *citri* A44 using primers designed based on *S. maltophilia* K279a and sequencing of the PCR product revealed the existence of *copF* in *X. citri* subsp. *citri* A44 (ORF11) (Fig. 1), which is highly similar (≥95%) to *copF* from *S. maltophilia* K279a and *X. axonopodis* pv. vesicatoria 7882 (Table 2; Fig. 2B). It also confirmed that the cloned fragment harboring the copper resistance determinants from *X. citri* subsp. *citri* A44 lacks the last 44 nucleotides of *copD*. ORF1, ORF6, and ORF8 from pXccCu2 are also present in *S. maltophilia* K279a and seem to be related to a hypothetical transcriptional repressor, a transposase, and a hypothetical protein, respectively (Fig. 1 and 2A). Part of ORF1 is present in *X. axonopodis* pv. vesicatoria 7882, and ORF6 and ORF8 are absent in that strain (44) (Fig. 2A).

Seven ORFs were identified for the sequence of the 9.6-kb DNA insert of pXacCu2 from *X. alfalfae* subsp. *citrumelonis* 1381 (Fig. 1). These ORFs are located within ~8.1 kb. No significant ORF was identified in the 1.2 kb and 0.3 kb positioned upstream of the first ORF and downstream of the last ORF, respectively. All ORFs except ORF1 are related to copper resistance genes previously described for *S. maltophilia* K279a (17) and *X. axonopodis* pv. vesicatoria 7882 (44). Copper resistance genes from *X. alfalfae* subsp. *citrumelonis* 1381 are more closely related to *X. axonopodis* pv. vesicatoria 7882, whereas *X. citri* subsp. *citri* A44 shows greater similarity with *S. maltophilia* K279a (Table 2; Fig. 2A and B). ORF2, ORF3, ORF4, ORF5, ORF6, and ORF7 from pXacCu2 are ≥94% identical to *copL*, *copA*, *copB*, *copM*, *copG*, and *copF* from *X. axonopodis* pv. vesicatoria 7882 (Table 2; Fig. 2B). There is high sequence identity (≥92%) between *copL*, *copA*, *copB*, and *copF* from *X. citri* subsp. *citrumelonis* 1381, *X. citri* subsp. *citri* A44, *X. axonopodis* pv. vesicatoria 7882, and *S. maltophilia* K279a (Table 2; Fig. 2B). However, in *X. alfalfae* subsp. *citrumelonis* 1381 and *X. axonopodis* pv. vesicatoria 7882, *copC* and *copD* are absent and the nucleotide sequences of *copM* and *copG* are not very similar to their homologs in *X. citri* subsp. *citri* A44 and *S. maltophilia* K279a (Table 2; Fig. 2B). ORFs related to a transposase and a hypothetical protein present in pXccCu2 from *X. citri* subsp. *citri* A44 are absent in pXacCu2 from *X. citri* subsp. *citri* 1381, and as for *X. citri* subsp. *citri* A44, ORF1 from *X. alfalfae* subsp. *citrumelonis* 1381 has high homology to a hypothetical transcriptional repressor (Fig. 1 and 2A).

The cluster *copLAB* is the most conserved region among the strains *X. citri* subsp. *citri* A44, *X. alfalfae* subsp. *citrumelonis* 1381, *X. axonopodis* pv. vesicatoria 7882, and *S. maltophilia* K279a. The nucleotide identity of these genes among the strains ranges from 92 to 99%. As reported by Canteros et al. (10), the *X. citri* subsp. *citri* A44 and *X. alfalfae* subsp. *citrumelonis* 1381 copper resistance genes are located on plasmids (Behlau et al., unpublished). Nonetheless, homologs of these plasmid-borne copper resistance genes are present on the chromosomes of copper-sensitive and -resistant *Xanthomonas* strains and display the same organizational pattern observed for the resistance genes from the Cu^r strains *X. citri* subsp. *citri* A44, *X. alfalfae* subsp. *citrumelonis* 1381, *X. axonopodis* pv.

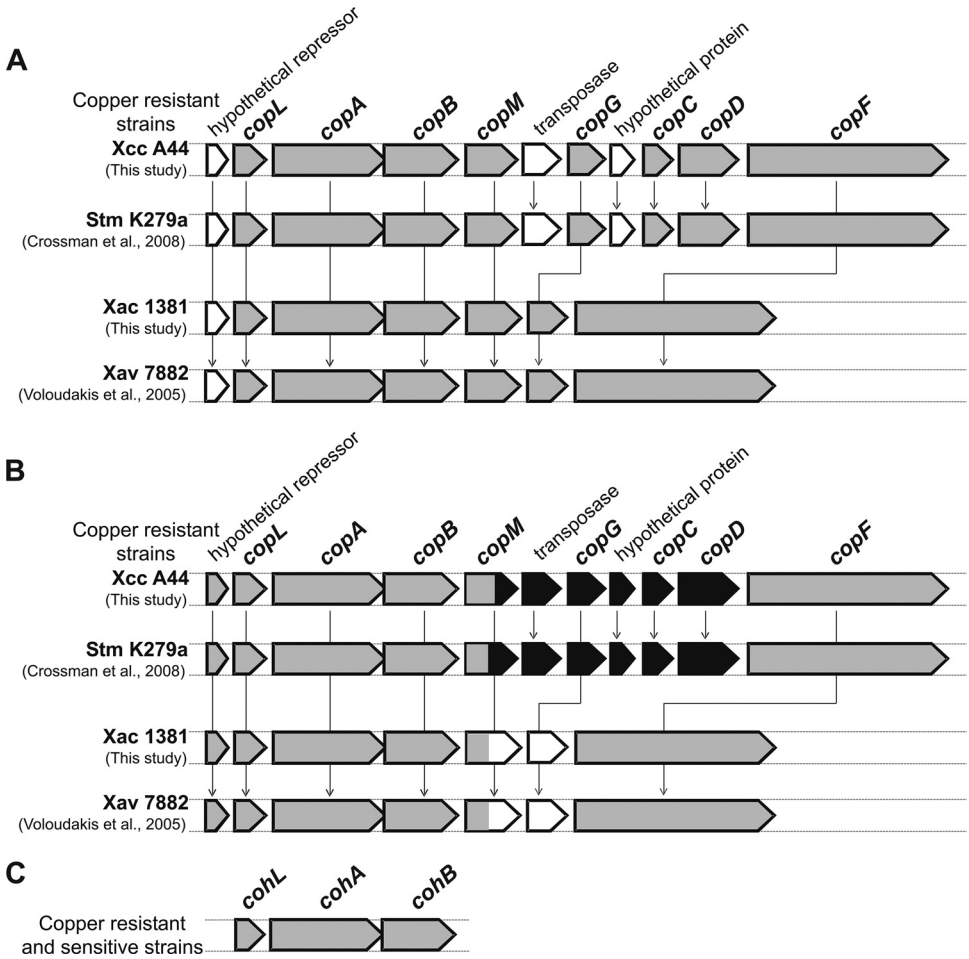

FIG. 2. Comparison of genes involved in copper metabolism. (A) Comparison of different bacterial strains with regard to the composition of the copper resistance gene cluster. (B) Comparison of copper resistance gene clusters with regard to the identity of nucleotide sequences. Areas with the same color indicate conservation of nucleotide sequences among the strains (an identity of ≥92%). The references in the figure refer to reference numbers 17 and 44. (C) The chromosomal genes *cohL*, *cohA*, and *cohB* are homologous to *copL*, *copA*, and *copB*, respectively, which are present in both copper-sensitive and -resistant strains of *Xanthomonas*. Xcc, *Xanthomonas citri* subsp. *citri*; Stm, *Stenotrophomonas maltophilia*; Xac, *Xanthomonas alfalfae* subsp. *citrumelonis*; Xav, *Xanthomonas axonopodis* pv. vesicatoria.

vesicatoria 7882, and *S. maltophilia* K279a (Fig. 2A and C). However, on the chromosome, no other homolog or additional gene is present downstream of *copB* (Fig. 2C). Homology between chromosomal and plasmid-borne genes is higher for *copA* and *copB* than for *copL*. The amino acid sequences of the products of the copper resistance genes *copA* and *copB* from *X. citri* subsp. *citri* A44 are approximately 55 to 77% similar to the products of the chromosomal homologs from strains *X. citri* subsp. *citri* 306 (18) and *X. vesicatoria* 85-10 (41), which are known to be copper-sensitive strains. In contrast, the similarity of the amino acid sequences of the products of plasmid-borne *copL* from *X. citri* subsp. *citri* A44 and chromosome-borne *copL* from *X. citri* subsp. *citri* 306 and 85-10 is approximately 40%.

Transposon mutagenesis of copper resistance genes from *Xanthomonas citri* subsp. *citri*. Transposon mutagenesis of cloned copper resistance determinants in *X. citri* subsp. *citri* A44 revealed that *copL*, *copA*, and *copB* are the most important genes for copper resistance in *X. citri* subsp. *citri*. Transconjugant *X. perforans* 91-118 strains carrying mutated pXccCu2 were plated on MGY agar supplemented with different concentrations of copper. Mutation of *copL* and *copA* lowered copper resistance to levels tolerated by copper-sensitive strains. Irrespective of the mutation site in the genes *copL*, *copA*, and *copB*, mutants had resistance reduced to 50, 50, and 75 mg liter^{-1} of copper, respectively (Table 3; Fig. 3). As a reference, the transconjugant *X. perforans* 91-118 harboring pXccCu2 or pXacCu2 can resist up to 300 mg liter^{-1} of copper on MGY, and wild-type (WT) *X. citri* subsp. *citri* A44 and *X. alfalfae* subsp. *citrumelonis* 1381 were able to grow on MGY supplemented with 400 mg liter^{-1} of copper sulfate. Mutations in the N-terminal region of *copM*, which has high homology with the same region in other copper-resistant strains, such as *X. alfalfae* subsp. *citrumelonis* 1381, *X. axonopodis* pv. vesicatoria 7882, and *S. maltophilia* K279a, reduced copper resistance slightly, and mutants were able to grow with up to 200 mg liter^{-1} of copper (Table 3; Fig. 3). Irrespective of the insertion site in the gene, no change in copper resistance was observed when the transposon was inserted in *copG*, *copC*, and *copD* or in ORF1, which is homologous to a transcriptional repressor

TABLE 3. Sites of transposon insertion in selected copper genes in pXccCu2 from *X. citri* subsp. *citri* A44 and the effect of mutagenesis on the level of copper resistance

Mutant	Region mutated	Mutation site in the gene (bp)	Gene size (bp)	Portion deleted (%)	Tolerated concn of copper (mg liter^{-1})[a]
M60	Upstream of *cop* genes	1,029 upstream of *copL*			300
M114	Upstream of *cop* genes	717 upstream of *copL*			300
M257	Hypothetical repressor	16	327	95	300
M357	*copL*	26	420	94	50
M377	*copL*	401	420	5	50
M206	Between *copL* and *copA*	2 upstream of *copA*			50
M122	*copA*	659	1,872	65	50
M08	*copA*	788	1,872	58	50
M06	*copA*	1190	1,872	36	50
M167	*copA*	1389	1,872	26	50
M125	*copA*	1821	1,872	3	50
M169	*copB*	113	1,269	91	75
M160	*copB*	376	1,269	70	75
M46	*copB*	647	1,269	49	75
M10	*copB*	817	1,269	36	75
M120	*copM*	90	771	88	200
M48	*copM*	138	771	82	200
M149	*copG*	67	525	87	300
M155	*copG*	271	525	48	300
M89	*copG*	505	525	4	300
M159	*copC*	341	384	11	300
M101	*copD*	194	930	79	300
M98	*copD*	686	930	26	300

[a] Maximum tolerated concentration of copper, as copper sulfate pentahydrate, added to mannitol-glutamate-yeast extract (MGY) agar.

gene and is located upstream of *copL* (Table 3; Fig. 3). Insertional mutations in the region upstream of ORF1 did not affect resistance to copper (Table 3; Fig. 3).

DISCUSSION

This is the first time copper resistance has been characterized in the citrus pathogens *X. citri* subsp. *citri* and *X. alfalfae* subsp. *citrumelonis*. We localized the determinants for copper resistance to a 7.9-kb region in *X. citri* subsp. *citri* strain A44 from Argentina and an 8.1-kb fragment in *X. alfalfae* subsp. *citrumelonis* strain 1381 from Florida. Sequencing of these fragments revealed 10 and 7 ORFs associated with copper resistance in *X. citri* subsp. *citri* and *X. alfalfae* subsp. *citrumelonis*, respectively. In *X. citri* subsp. *citri*, ORF2, ORF3, and ORF4 were required for a high level of resistance in transconjugant screening. These three ORFs have high homology with *copL*, *copA*, and *copB*, respectively, from *S. maltophilia* K279a (17) and *X. axonopodis* pv. vesicatoria 7882 (44). Insertional mutation of ORF7, ORF8, ORF9, and ORF10, the first and last two of which exhibit homology to *copG*, *copC*, and *copD*, respectively, from *S. maltophilia* K279a (17), had no observable effects on copper resistance when tested in the *X. perforans* 91-118 transconjugant background. Likewise, mutation of ORF1, which is homologous to a hypothetical transcriptional repressor gene from several bacteria, did not affect copper resistance. Mutation of ORF5, which is homologous to *copM* (also referred to as cytochrome *c*) from *S. maltophilia* K279a (17) slightly reduced the copper resistance of transconjugants.

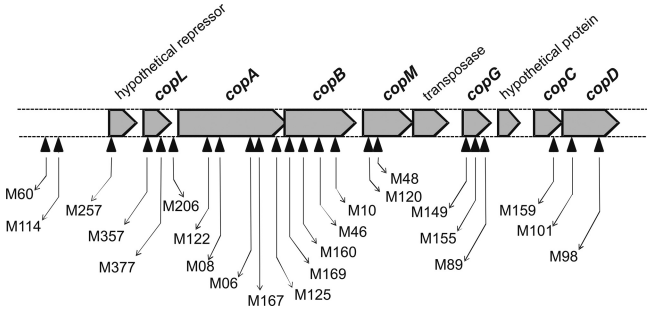

FIG. 3. Transposon insertion sites within the copper resistance determinants of pXccCu2 from *Xanthomonas citri* subsp. *citri* strain A44. Black triangles indicate sites of transposon insertion.

Copper resistance of transconjugant strains of different Cu^s *Xanthomonas* species carrying pXccCu2, which harbors the copper determinants from *X. citri* subsp. *citri*, showed a slight reduction of resistance on MGY agar (from 400 to 300 mg liter^{-1}) compared to the WT strain, *X. citri* subsp. *citri* A44. Such a decrease in resistance could be due to the fact that *copF* is absent and *copD* is incomplete in pXccCu2. However, the fact that the same behavior was observed for WT *X. alfalfae* subsp. *citrumelonis* 1381 and its clone pXacCu2, which harbors all the same genes identified in pXccCu2 and also includes *copF*, suggests either that *copF* is not important for resistance and the slight decrease of resistance was due to the fact that the cloned copper resistance determinants were expressed in a different strain or that other genes might be involved in full copper resistance. If the latter is correct, the presumptive additional gene(s) is likely to be located far from the cloned gene cluster. No other ORF related to copper resistance was found upstream of ORF1 in pXccCu2 and *S. maltophilia* K279a or downstream of *copF* in *S. maltophilia* K279a. As discussed earlier, the organizations, sizes, and nucleotide sequences of genes in *S. maltophilia* K279a, which belongs to the *Xanthomonadaceae* family, and pXccCu2 are highly similar,

thus making *S. maltophilia* K279a a reliable reference for comparison. *S. maltophilia* is ubiquitous in aqueous environments, soil, and plants, including water, urine, and respiratory secretions, and was grouped in the genus *Xanthomonas* before becoming the type species of the genus *Stenotrophomonas* (30).

Comparison of copper resistance determinants in *X. citri* subsp. *citri* A44, *X. alfalfae* subsp. *citrumelonis* 1381, *S. maltophilia* K279a (17), and *X. axonopodis* pv. vesicatoria 7882 (44) revealed that high homology (≥92%) of nucleotide sequences is maintained among these strains only for *copLAB*, 70% of *copM*, which is positioned immediately downstream of *copB*, and *copF*, located at the end of the gene cluster in all strains. Although we could not determine the importance of *copF* for copper resistance by insertional mutation because it was absent from our subclone, pXccCu2, we were able to demonstrate that the conserved regions in *copLAB* and *copM* have direct involvement in copper resistance. *copLAB* is essential for copper resistance, and the N terminus of the product of *copM* is necessary for full resistance.

The individual functions of the homologous genes identified in pXccCu2 and pXacCu2 for conferring copper resistance in *Xanthomonas* have not been completely revealed. Except with *copL*, which was demonstrated to be involved in the regulation of copper resistance (44), the roles of genes have been presumed based on the roles of homologous genes from other organisms. It seems that CopA and CopB are copper binding proteins, CopM is a cytochrome *c* oxidase involved in electron transport, CopG is a hypothetical export protein, CopC and copD are transmembrane transporter proteins, and CopF is a putative copper-transporting p-type ATPase (17, 44).

The results presented in this study with transposon mutagenesis of copper resistance genes from *X. citri* subsp. *citri* corroborate the supposition that *copL* is essential for the regulation of *copA* and *copB*. We presume that the disruption of *copL*, which completely abolished copper resistance, affects the transcription of *copA* and *copB*. Likewise, mutation of *copA*, which also eliminated copper resistance, may have led to the absence of copper binding proteins, both CopA and CopB. Moreover, the fact that disruption of *copB* did not fully reduce copper resistance indicates that partial resistance was being conferred by an intact *copA* gene. In this case, CopA proteins were synthesized and bound to copper in the absence of an intact, functional *copB* gene. Thus, mutation of *copA* may have had a polar effect, knocking out *copB*, whereas *copB* did not affect *copA*.

Homologs of the copper resistance genes *copLAB* cloned from *X. citri* subsp. *citri* A44 and *X. alfalfae* subsp. *citrumelonis* 1381 are present on the chromosomes of Cu^r strains, such as *X. vesicatoria* 1111 (31), and strains that have been determined to be Cu^s, such as *X. citri* subsp. *citri* 306 (18) and *X. vesicatoria* 85-10 (41). Homologs of these genes are also present in many other *Xanthomonas* strains, including *Xanthomonas oryzae* pv. oryzae, *X. campestris* pv. vesicatoria, and *X. campestris* pv. campestris, whose resistance or sensitivity to copper is unconfirmed. On the chromosome, the homologs display the same organizational pattern observed for the resistance genes from Cu^r strains; however, no other ORF related to copper resistance has been identified downstream of chromosomal *copLAB* in *X. citri* subsp. *citri* 306 and *X. vesicatoria* 85-10, as was demonstrated for the actual resistance genes from *X. citri* subsp. *citri* A44 and *X. alfalfae* subsp. *citrumelonis* 1381.

The presence of homologs of copper resistance genes on the chromosome has been previously reported for other bacteria. Chromosomal genes that hybridize with the *cop* operon were detected in Cu^r and Cu^s strains of *Pseudomonas* (16). In *P. syringae*, *cop* homologs have been detected in more than 20 Cu^s strains from eight pathovars (23). Furthermore, it has been demonstrated that in several strains of *P. syringe*, these chromosomal homologs can activate the plasmid-borne *cop* promoter (23, 27), reflecting the possible chromosomal origin of the plasmid-borne resistance genes.

Clearly, the annotated, chromosomal *copLAB* operon from xanthomonads is not responsible for copper resistance, but it is likely to be necessary for homeostasis and/or tolerance. Teixeira et al. (40) demonstrated that chromosomal *copAB* from *X. citri* subsp. *citri* 306 is responsive to copper amendments; however, this strain was mistakenly rated as copper resistant. This was probably due to pH adjustments made to the medium with potassium phosphate buffer (40), which has been shown to reduce the actual concentration of copper available in the medium (29). While strains harboring the copper resistance genes *copLAB*, which are highly similar (≥90%) to the ones cloned in this study, can grow on MGY agar amended with up to 400 mg $liter^{-1}$ of Cu, strains that have only the chromosomal *copLAB* genes, such as *X. citri* subsp. *citri* 306, grow with up to 75 mg $liter^{-1}$ of Cu and, hence, are Cu^s. Thus, to avoid further confusion or misinterpretation, we suggest that the nomenclature of chromosomal homologs of *copL*, *copA*, and *copB* in xanthomonads, which are probably copper homeostasis genes, should be changed to *cohL*, *cohA*, and *cohB*, respectively, as a reference to copper homeostasis genes.

It remains to be determined whether *cop* and *coh* genes interact and how important these genes are for the bacteria. Although the similarities of the amino acid sequences of the product of *copA* from *X. citri* subsp. *citri* A44 and of the products of chromosomal homologs from *X. citri* subsp. *citri* 306 and *X. vesicatoria* 85-10 are not high (51 and 57%, respectively), Teixeira et al. (40) have demonstrated that the disruption of *cohA* from *X. citri* subsp. *citri* 306 increases sensitivity to copper, indicating a role for this gene in copper metabolism, possibly with *cohB*, whose product has high similarity in its amino acid sequence with that of the product of the plasmid-borne *copB* gene (77%). Thus, both *copAB* and *cohAB* genes may encode copper binding proteins. However, the differences in the amino acid sequences of their products may account for the reduced capacity of the CohAB protein to bind copper compared to that of CopAB. Moreover, the homology of *copL*, which has been demonstrated to regulate the expression of *copAB* in *Xanthomonas vesicatoria* (44), to *cohL* from *X. citri* subsp. *citri* 306 and *X. vesicatoria* 85-10 is low (approximately 40%). This indicates that *copAB* and *cohAB* might be regulated differently by *copL* and *cohL*, respectively, which in turn is reflected in their differing levels of expression, copper binding affinities, and abilities to tolerate copper in the environment. Given that copper is an essential element for the metabolism of bacteria, *coh* genes may be responsible for binding low levels of the metal to cells (36)—amounts just enough for the housekeeping metabolism and not sufficient to account for high levels of copper resistance.

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