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Recruitment and Rearrangement of Three Different Genetic Determinants into a Conjugative Plasmid Increase Copper Resistance in *Pseudomonas syringae*

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We describe the genetic organization of a copper-resistant plasmid containing *copG* and *cusCBA* genes in the plant pathogen *Pseudomonas syringae*. Chromosomal variants of *czcCBA* and a plasmid variant of *cusCBA* were present in different *P. syringae* pathovar strains. Transformation of the copper-sensitive *Pseudomonas syringae* pv. syringae FF5 strain with *copG* or *cusCBA* conferred copper resistance, and quantitative real-time PCR (qRT-PCR) experiments confirmed their induction by copper.

Pseudomonas syringae is a common foliar bacterium and causal agent of plant diseases in many different hosts worldwide, affecting both woody trees and herbaceous plants (1).

Copper bactericides have been widely used for decades to control bacterial infections in crop plants (2). However, the extensive use of copper bactericides can lead to many problems, among them the reduction of efficacy of this antimicrobial agent due to the selection of copper-resistant (Cu^r) strains (3, 4). Copper resistance determinants have been detected and described in several pathovars of *P. syringae* (3, 5–7) and are frequently encoded within native plasmids (3, 8, 9). These native Cu^r plasmids contribute to the dissemination of resistance genes among *P. syringae* strains from different pathovars (5, 10, 11). The *copABCD* genes, which were first described in a plasmid of *P. syringae* pv. tomato PT23 (8), is an important determinant of copper resistance in *P. syringae* and other phytopathogenic bacteria (6, 8, 9, 12–14). However, additional studies have shown that *P. syringae* may harbor other variants of Cu^r determinants (3, 7, 15).

The efflux system *czcCBA* and the analogous system *cusCBA* are probably the best-characterized members of the heavy metal efflux (HME)-RND (resistance-nodulation-cell division) family. The *czcCBA* system functions in the detoxification of cadmiun, zinc, and cobalt (16, 17), and the *cusCBA* system works in detoxifying monovalent cations, such as silver and copper (16, 18). These efflux systems have been widely studied in *Cupriavidus metallidurans* (formerly *Ralstonia metallidurans* or *Alcaligenes eutrophus*) (16, 19–22), *Escherichia coli* (18, 23), and *Pseudomonas putida* KT2440 (24–26), but no further studies have been carried out for the plant pathogen *P. syringae* pv. syringae.

In this work, we detected that the majority of the *cusCBA* genes from different *Pseudomonas* species could be wrongly annotated as *czcCBA* genes, based on the analysis of the conserved motifs of the RND transporter domains (27). This inaccurate annotation creates problems in the database regarding *czcCBA* or *cusCBA* genes in the *Pseudomonas* genus.

Our goal was to identify and characterize plasmid-encoded Cu^r genes in the *P. syringae* pv. syringae UMAF0081 strain. The bacterial strains used, and sizes of native plasmids harbored by the strains, are listed in Table 1. Each native plasmid examined is a member of the pPT23A plasmid family, a group of related plasmids broadly distributed within *P. syringae* and the related phytopathogen *Pseudomonas savastanoi* (32). These plasmids share the major replication gene *repA* (33), and phylogenetic analysis indicates that individual pPT23A family plasmids (PFPs) have been transferred between *P. syringae* pathovars, and individual plasmid-borne genes have been transferred among PFPs (34).

TABLE 1 Bacterial strains used in this study and their relevant characteristics

Bacterial strain	Origin	Host	Plasmid size (kb)[a]	Source or reference
Pseudomonas fluorescens Pf-5	United States	Soil	–	28
Pseudomonas syringae				
pv. garcae 2708	Africa	Coffee	72.6	NCPPB[c]
pv. syringae				
3910	Greece	Lemon	–	29
6–9	United States	Sweet cherry	61.6	30
7B44	United States	Ornamental pear	72.1	29
847	Italy	Cherry	–	29
B61	United States	Wheat	–	29
B728a	United States	Bean	–	31
B86-17	United States	Bean	54[b]	29
DAP11	Sweden	Willow	–	29
FF5	United States	Ornamental pear	–	7
PS270	Unknown	Apple	–	29
UMAF0081	Spain	Mango	61.6	3
UMAF0158	Spain	Mango	63.0	3
UMAF0170	Spain	Mango	64.5	3
UMAF1029	Spain	Mango	63.0	3
pv. tabaci 0893-29	Hungary	Tobacco	73.8	29

[a] –, no plasmid.
[b] The plasmid of this strain is currently being closed by sequencing; 54 kb is the provisional size determined.
[c] NCPPB, National Collection of Plant Pathogenic Bacteria (United Kingdom).

Based on a PFP sequencing project that included each plasmid listed in Table 1 (J. A. Gutiérrez-Barranquero, F. M. Cazorla, A. De Vicente, G. W. Sundin, unpublished data), the presence of *copG*, a

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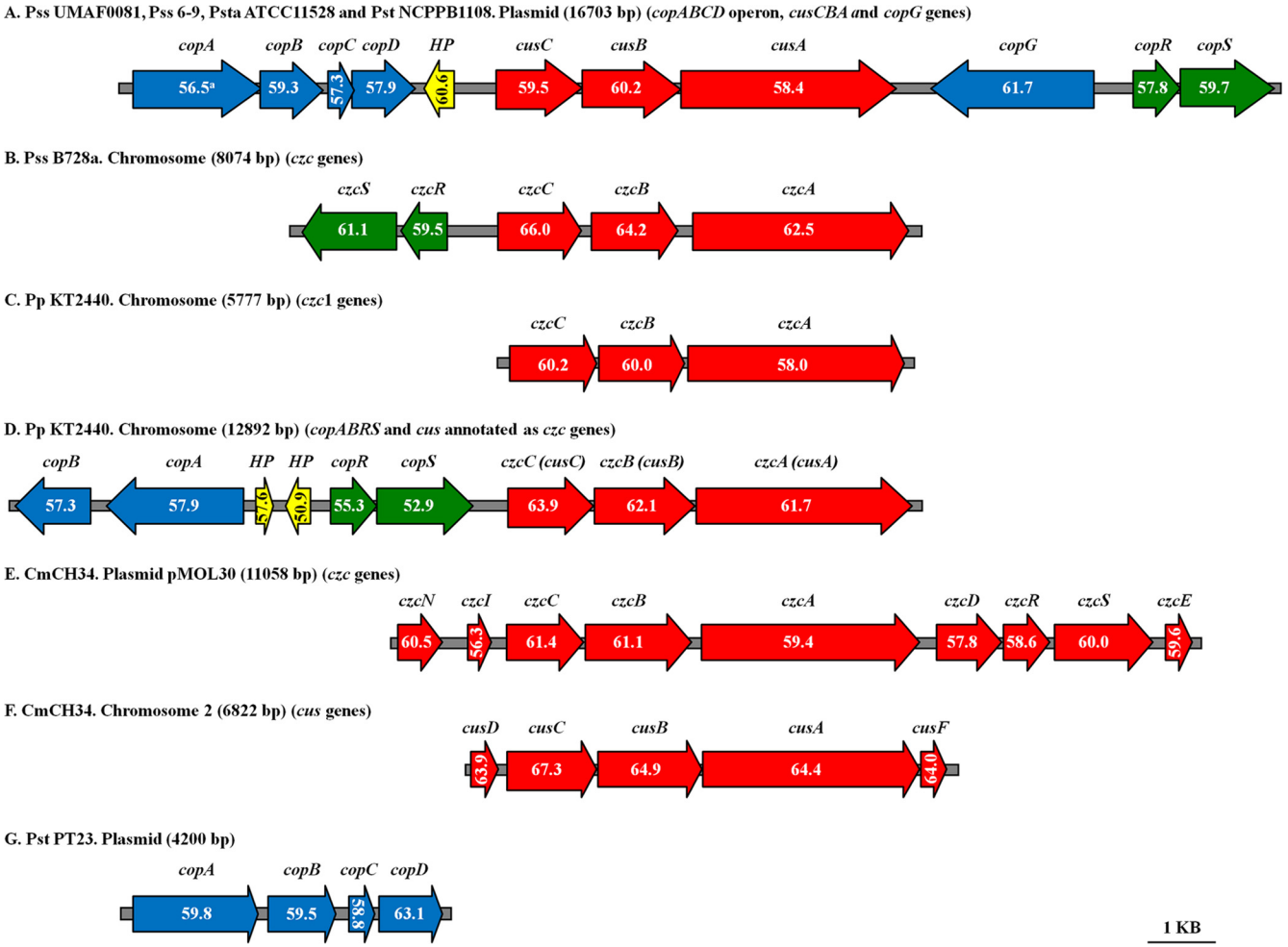

FIG 1 Genetic arrangement of the *czc*, *cus*, and *cop* genes located in different *Pseudomonas* and *Cupriavidus metallidurans* strains. (A) Arrangement of the *copG* and *cusCBA* genes within the *copABCDRS* operon in the 61.6-kb conjugative conserved native plasmid of *P. syringae* pv. syringae UMAF0081 and 6-9. Note that these sequences were also identified in draft genome sequences of *P. syringae* pv. tabaci ATCC 11528 and *P. syringae* pv. tomato NCPPB 1108. (B) Genetic organization of the *czc* operon in the chromosome of *P. syringae* pv. syringae B728a; (C) genetic organization of the *czc* operon in the chromosome of *Pseudomonas putida* KT2440; (D) genetic organization of the *cus* (incorrectly annotated as *czc*) and *copABRS* operons in the chromosome of *Pseudomonas putida* KT2440; (E) genetic organization of the *czc* operon in the plasmid pMOL30 of *Cupriavidus metallidurans* CH34; (F) genetic organization of the *cus* operon in chromosome 2 of *Cupriavidus metallidurans* CH34; (G) genetic organization of the *copABCD* operon from *P. syringae* pv. tomato PT23 present in the plasmid PT23D. The number inside each ORF denotes the percentage of GC content.

putative metal transporting P-type ATPase, and *cusCBA* (plasmid-encoded variant of *cus* genes) inserted within the *copABCD* operon (Fig. 1A) was observed and described for the first time associated with a conjugative-conserved native plasmid of 61.6 kb in two strains of *P. syringae* pv. syringae from different hosts and countries (*P. syringae* pv. syringae UMAF0081 and *P. syringae* pv. syringae 6-9). This novel arrangement has been also detected in the draft genomes of *P. syringae* pv. tabaci ATCC 11528 (35) and *P. syringae* pv. tomato NCPPB 1108 (36). The novel plasmid-encoded structure encompasses 16,703 bp and includes 11 open reading frames (ORFs) showing 99% nucleotide sequence identity and similarity among the four *P. syringae* strains. This genetic organization was then compared with the chromosomal and plasmid variants of *czcCBA* and *cusCBA* genes from different *Pseudomonas* species and *Cupriavidus metallidurans* CH34 (Fig. 1B to F) and also with the *copABCD* operon present in a native plasmid from *P. syringae* pv. tomato PT23 (Fig. 1G). The *cus* system of *Cupriavidus metallidurans* CH34 is located in chromosome 2 (2.5 Mbp), as is the *copABCDRS* operon (16, 37). However, sequence similarity and synteny with the *P. syringae* plasmid is low (i.e., query coverage of 32%). A different structure related to copper resistance can be found in the plasmid pMOL30 (38, 39), but with low similarity and synteny to the *P. syringae* plasmid (i.e., query coverage of 34%).

Thus, the presence of the novel structure led us to hypothesize that a combination of the plasmid-encoded *copABCD* and *cusCBA* genes along with the *copG* gene could be involved in the increase of copper resistance in the phytopathogenic bacterium *P. syringae* pv. syringae.

Fourteen strains of *P. syringae* pv. syringae from different plant hosts, including the Cu^r strains *P. syringae* pv. syringae UMAF0081 and 6-9, and two strains from different pathovars were studied. *Pseudomonas fluorescens* Pf-5 was used in this study as an external control. Strains and plasmids used in this study to

TABLE 2 Bacterial strains and plasmids used in this study to obtain the transformant strains

Strain or plasmid	Relevant characteristic(s)[d]	Source or reference
Bacteria		
Escherichia coli DH5α	*recA lac*ZDM15	GIBCO-BRL
P. syringae pv. syringae FF5	Sm^s Cu^s; plasmidless	7
Plasmids		
pCR2.1	Vector for TA cloning of amplicons and integration mutagenesis; Ap^r Km^r	Invitrogen Corporation
pCR2.1*cusCBA*	pCR2.1 with *cusCBA* genes from strain UMAF0081; Ap^r Km^r	This study
pCR2.1*copG*	pCR2.1 with *copG* gene from strain UMAF0081; Ap^r Km^r	This study
pBBR1MCS-5	lacPOZ′ mob, broad-host-range vector; Gm^r	40
pBBR1MCS-5*cusCBA*	pBBR1MCS-5 with *cusCBA* genes from strain UMAF0081; Gm^r	This study
pBBR1MCS-5*copG*	pBBR1MCS-5 with *copG* gene from strain UMAF0081; Gm^r	This study
Transformant strains		
FF5pBBR1MCS-5	FF5 transformed with the empty pBBR1MCS-5 vector; Cu^s	This study
FF5pBBR1*cusCBA*[a]	FF5 transformed with pBBR1MCS-5*cusCBA*; Cu^r	This study
FF5pBBR1*copG*[b]	FF5 transformed with pBBR1MCS-5*copG*; Cu^r	This study
Transconjugant strain		
UMAFCB[c]	Pss FF5-km (Km^r) × Pss UMAF0081 (Cu^r)	41

[a] Transformant strain of *Pseudomonas syringae* FF5 harboring the pBBR1MCS-5 plasmid with the *cusCBA* genes cloned.
[b] Transformant strain of *Pseudomonas syringae* FF5 harboring the pBBR1MCS-5 plasmid with the *copG* gene cloned.
[c] Transconjugant strain (Cu^r Km^r) selected from biparental matings of *P. syringae* pv. syringae FF5-km (Km^r) × *P. syringae* pv. syringae UMAF0081 (Cu^r). The plasmid of 61.6 kb was mobilized by conjugation.
[d] Ap^r, ampicillin resistant; Cu^r, copper resistant; Cu^s, copper sensitive; Gm^r, gentamicin resistant; Km^r, kanamycin resistant; Sm^s, streptomycin sentitive.

TABLE 3 MICs of cadmium, zinc, cobalt, and copper for the *Pseudomonas syringae* strains studied, including the transformant and the transconjugant strains

Bacterial strain	MIC[a] (mM)			
	$CdCl_2$	$ZnCl_2$	$CoCl_2$	$Cu_2SO_4 \cdot 5H_2O$[b]
Pseudomonas fluorescens Pf-5	>1	6	0.25	0.6
Pseudomonas syringae				
pv. garcae 2708	0.3	4	<0.01	0.4
pv. syringae				
3910	0.3	4	<0.01	0.4
6-9	0.3	4	<0.01	1.8
7B44	0.5	4	0.025	1.2
847	0.5	4	0.025	0.6
B61	0.5	4	<0.01	0.6
B728a	0.5	5	0.25	1.6
B86-17	1	4	0.25	0.6
DAP11	0.3	4	<0.01	0.4
FF5	0.3	4	<0.01	0.8
PS270	0.5	1	0.05	0.6
UMAF0081	0.3	4	<0.01	1.8
UMAF0158	0.3	4	<0.01	0.4
UMAF0170	0.3	4	0.05	1.2
UMAF1029	0.3	4	0.05	0.6
pv. tabaci 0893-29	0.3	4	0.025	0.6
Transformant strains				
FF5pBBR1MCS-5[c]	0.3	4	<0.01	0.8
FF5pBBR1*cusCBA*	0.3	4	<0.01	1.2
FF5pBBR1*copG*	0.3	4	<0.01	1.0
Transconjugant strain				
UMAFCB	0.3	4	<0.01	1.8

[a] The doses tested for the different heavy metals were as follows: $CdCl_2$, from 0 to 1; $ZnCl_2$, from 0.05 to 6; $CoCl_2$, from 0.01 to 1; and $Cu_2SO_4 \cdot 5H_2O$, from 0.2 to 1.8.
[b] Strains with MICs of ≤0.8 mM are considered sensitive to copper.
[c] Control strain: *P. syringae* pv. syringae FF5 transformed with the empty pBBR1MCS-5 vector.

obtain genetically derived strains are listed in Table 2. The bacterial strains were routinely grown in Luria-Bertani broth or agar (LB), at 28°C for all the *P. syringae* strains and at 37°C for the *E. coli* DH5α strain.

To determine functionality of *copG* and the *cusCBA* genes arranged with the *copABCD* operon, transformation experiments were performed. Specific primers were designed with restriction sites for double digestion and directional ligation into the pBBR1MCS-5 vector (40), based on *copG* and *cusCBA* sequences from the conjugative conserved native plasmid of the strains *P. syringae* pv. syringae UMAF0081 and *P. syringae* pv. syringae 6-9. Amplification was conducted using a high-fidelity *Taq* polymerase (Expand Long Range, dNTPack; Roche), and the PCR product was cloned into the pCR2.1 vector (Invitrogen Corporation) and transformed into competent *E. coli* DH5α cells. Subsequently, the PCR product was recovered from the pCR2.1 vector by double digestion, cloned into pBBR1MCS-5, and transformed into electrocompetent cells (42) of the copper-sensitive strain *P. syringae* pv. syringae FF5 (7). Determination of the MIC for copper, cadmium, zinc, and cobalt was then carried out for all *P. syringae* strains, the two transformant strains (FF5pBBR1*copG* and FF5pBBR1*cusCBA*), and the transconjugant strain (*P. syringae* pv. syringae UMAFCB) harboring the conjugative conserved native plasmid (Table 2). Agar plates of MG medium (5) supplemented with different concentrations of metals were used. The doses tested for the different heavy metals were $Cu_2SO_4 \cdot 5H_2O$, from 0.2 mM to 1.8 mM; $CdCl_2$, from 0.05 mM to 1 mM; $ZnCl_2$, from 0.05 mM to 6 mM; and $CoCl_2$, from 0.01 mM to 1 mM. The MIC values

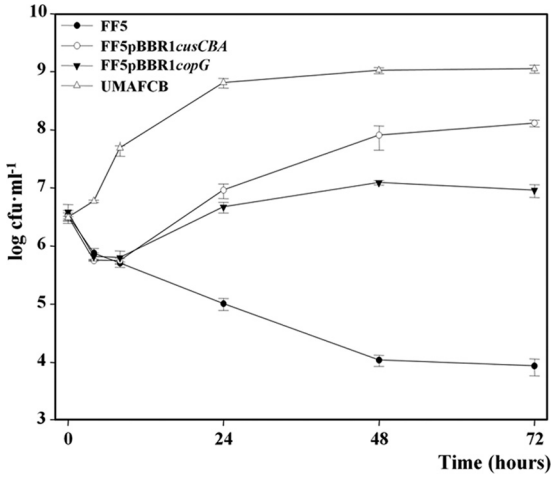

FIG 2 Growth curve of *Pseudomonas syringae* pv. syringae strains in MG liquid medium supplemented with 0.8 mM copper sulfate. *P. syringae* pv. syringae strains harboring different constructs were tested: wild-type copper-sensitive plasmidless strain (*P. syringae* pv. syringae FF5), transformant strains (*P. syringae* pv. syringae FF5pBBR1*cusCBA*, *P. syringae* pv. syringae FF5pBBR1*copG*), and the transconjugant strain (UMAFCB) harboring the 61.6-kb conserved native plasmid.

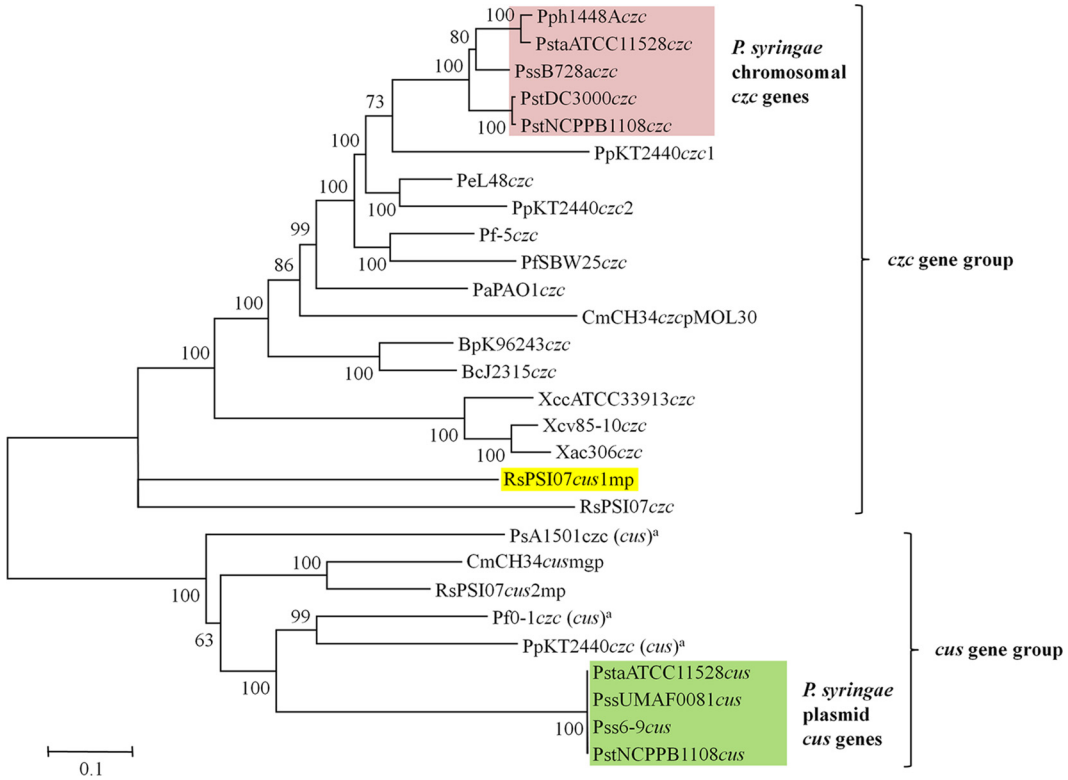

FIG 3 Phylogenetic distribution of *Pseudomonas syringae* strains and other related Gram-negative bacteria based on the sequences of the *czc* and *cus* genes from chromosomal and plasmid origin obtained from sequencing in this study or from the GenBank database. The neighbor-joining tree was constructed with combined sequences (*czcCBA* or *cusCBA* genes) using MEGA 5.05. Evolutionary distances are in units of nucleotide substitutions per site. Based on the sequences of the *cusCBA* plasmid-borne genes, strains of *P. syringae* pv. syringae of this study (UMAF0081 and 6-9) together with the strains ATCC 11528 (*P. syringae* pv. tabaci) and NCPPB 1108 (*P. syringae* pv. tomato) are grouped together and marked in green. Based on the sequences of the chromosomal *czc* genes, strains from different pathovars of *P. syringae*, including PstaATCC11528 and PstNCPPB1108, are grouped together and marked in pink. Bootstrap values (10,000 repetitions) are shown on branches. Sequences from the following strains were used in this analysis: *Burkholderia cenocepacia* (BcJ2315), *Burkholderia pseudomallei* (BpK96243), *Cupriavidus metallidurans* (CmCH34), *Pseudomonas aeruginosa* (PaPAO1), *Pseudomonas entomophila* (PeL48), *Pseudomonas fluorescens* (Pf-5, PfSBW25, and Pf0-1), *Pseudomonas putida* (PpKT2440), *Pseudomonas stutzeri* (PsA1501), *Pseudomonas syringae* pv. phaseolicola (Pph1448A), *Pseudomonas syringae* pv. syringae (PssB728a, PssUMAF0081, and Pss6-9), *Pseudomonas syringae* pv. tabaci (PstaATCC11528), *Pseudomonas syringae* pv. tomato (PstDC3000 and PstNCPPB1108), *Ralstonia solanacearum* (RsPSI07), *Xanthomonas anoxopodis* pv. citri (Xac306), *Xanthomonas campestris* pv. campestris (XccATCC33913), and *Xanthomonas campestris* pv. vesicatoria (Xcv85-10). [a], DNA sequences belong to the *cus* system based on the conserved motif domains but are annotated as *czc* genes.

(Table 3) displayed only a resistance pattern associated with the novel genes in the case of copper, among all the different strains tested. The *P. syringae* pv. syringae UMAF0081 and *P. syringae* pv. syringae 6-9 strains and the transconjugant strain UMAFCB showed the highest resistance level to copper. The transformant Cu^s strains confirmed that *copG* and *cusCBA* genes associated with the *copABCD* operon were involved in copper resistance (Table 3).

Next, the copper resistance of the transformant, the transconjugant (UMAFCB), and the wild-type copper-sensitive (*P. syringae* pv. syringae FF5) strains was also studied by examining growth in liquid MG medium supplemented with copper at 0.8 mM (Fig. 2). The transformant strains were able to grow at 0.8 mM copper. The bacterial counts of the FF5pBBR1*cusCBA* strain were higher than those of FF5pBBR1*copG*, in agreement with the previous results observed in the MIC analysis (Table 3). These experiments confirmed the role of both *copG* and *cusCBA* in the increase of copper resistance. As expected, *P. syringae* pv. syringae UMAFCB displayed the highest copper resistance level. All of these results seem to confirm that the novel plasmid arrangement of the *copG*, *cusCBA*, and *copABCD* operon is involved in increased levels of copper resistance.

Quantitative real-time PCR (qRT-PCR) analysis was conducted in order to determine if expression of the *copG* and *cusA* genes was induced by copper. RNA was extracted from four independent cultures of *P. syringae* pv. syringae UMAF0081 grown in MG liquid medium, two of them with copper at 0.8 mM. qRT-PCR analysis (triplicate technical replicates) on two independent RNA isolations under each condition (with or without copper) (43, 44) was carried out, as has been previously described (45) with minor modifications. The *copA* gene was used as a positive control, and the *rpoD* gene was used as a reference gene. The expression of the *cusA* and *copG* genes was clearly increased in the presence of copper (13-fold and 100-fold increases, respectively), and the baseline expression of the *cusA* gene was 3-fold higher than that of *copG*. These results correlate with the previous MIC (Table 3) and growth (Fig. 2) results.

A phylogenetic analysis was conducted using the complete sequences from chromosomal and plasmid *czcCBA* and *cusCBA* genes. Concatenated sequences were used for each strain for the multiple alignments using ClustalW2 software (46). Phylogenetic trees were constructed by using MEGA 5.05 (47) with neighbor-joining, minimum evolution and by eliminating all positions con-

taining gaps. Confidence levels of the branching points were determined using 10,000 bootstrap replicates. This tree was compared to that of a phylogenetic tree derived from a multilocus sequence analysis using partial sequences of *gyrB* and *rpoD* genes of *P. syringae* pv. syringae and other related strains (belonging to the genus *Pseudomonas*, *Burkholderia*, *Cupriavidus*, *Ralstonia*, and *Xanthomonas*). The phylogenetic distribution based on the housekeeping genes revealed a clear clustering of the *Pseudomonas* spp. as well as for *P. syringae* strains examined in this study (see Fig. S1 in the supplemental material). Likewise, when using *czc* genes for phylogenetic analysis, a similar clustering was observed. Interestingly, all the *cus* genes grouped together, forming a completely separate cluster, with only one exception (RsPSI07*cus*I) (Fig. 3). As far as we know, this is the first report of the presence of the *cus* system in a phytopathogenic bacterium such as *P. syringae* pv. syringae. In Fig. 3, some genes annotated as *czc* (PsA1501, Pf0-1, and PpKT2440) grouped with the plasmidic *cus* genes of *P. syringae*, but a deeper analysis of their sequences showed the typical domains of the *cus* genes (see Fig. S2 in the supplemental material). The incorrect annotations, as well as the scarce sequences of the *cus* system, led to difficulties in proper study of the phylogeny of this gene family. A correct annotation of the *cus* genes would be needed in order to elucidate the evolutionary history of this gene family.

In conclusion, we have described for the first time the presence of a *cus* system and its relationship with copper resistance in *Pseudomonas syringae*. Also in this study we report a novel arrangement of the *copABCD* operon, including insertions of *copG* and *cusCBA* encoded on a conjugative conserved native plasmid of 61.6 kb from two strains of *P. syringae* pv. syringae (6-9 and UMAF0081) that were isolated from different hosts and continents. Transformation experiments, MIC analysis, and qRT-PCR confirmed the role of the *copG* and *cusCBA* in the increase of copper resistance in *P. syringae*.

Nucleotide sequence accession numbers. Database searches were performed using the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). All the accession numbers from the different DNA sequences used in this study are summarized in Table S1 in the supplemental material. The newly determined *cus* sequences deposited in GenBank have the accession numbers JX645720 for *P. syringae* pv. syringae UMAF0081 and JX645721 for *P. syringae* pv. syringae 6-9. The sequences of the housekeeping genes *gyrB* and *rpoD* of *P. syringae* pv. syringae 6-9 have the accession numbers JX867861 and JX867862, respectively.

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