Copper Resistance in *Pseudomonas syringae* Strains Isolated from Mango Is Encoded Mainly by Plasmids

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ABSTRACT

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Bacterial apical necrosis of mango, elicited by *Pseudomonas syringae* pv. *syringae*, limits fruit production in southern Spain and Portugal. Examination of a collection of *P. syringae* pv. *syringae* isolates for copper resistance showed that 59% were resistant to cupric sulfate. The survey of a mango orchard revealed an increase in frequencies of copper-resistant bacteria after repeated treatments with Bordeaux mixture. These data suggest that selection of copper-resistant strains could be a major

reason for control failures following management with copper bactericides. Most copper-resistant isolates harbored plasmids, although the majority of them contained a 62-kb plasmid that also was present in copper-sensitive strains. The 62-kb plasmids were differentiated by restriction enzyme analysis and hybridization to *copABCD* DNA. The most frequently found copper-resistant plasmid type (62.1) was transferable by conjugation. Southern blot hybridizations showed that genetic determinants partially homologous to *copABCD* were present in all the copper-resistant strains examined, and usually were associated with plasmids; these determinants were not detected in copper-sensitive strains. The selective pressure exerted by copper bactericide sprays on the diversity of copper resistance determinants in bacterial populations of mango is discussed.

Native to southern Asia, mango (*Mangifera indica* L.) is one of the most important fruit crops in the world. During the last 15 years, there has been a huge increase in mango production as markets have developed in Europe and North America (22). In Europe, most of the mango production is located in southern Spain and Portugal, where it has expanded steadily during the last 10 years. However, the commercial viability of this crop has been threatened by the frequent occurrence of bacterial apical necrosis, a newly reported disease of mango, which is caused by the phytopathogenic bacterium *Pseudomonas syringae* pv. *syringae* (6,8). Symptoms include necrosis of flower buds, leaves, stems, and flower panicles; it causes severe economic losses due to the decrease in fruit set.

Growers in Spain and Portugal make extensive use of registered copper compounds, mostly Bordeaux mixture, to control bacterial apical necrosis (8). In the last few years, however, growers have noticed a reduction in the efficacy of the treatments which, in turn, has led to an increase in the dosage and frequency of copper applications (6). This scenario is common for *P. syringae* or *Xanthomonas campestris* diseases that have been repeatedly managed with copper and the lack of control is usually due to the emergence of copper-resistant (Cu^r) bacteria (1,10,24,30,35,39). However, to the best of our knowledge, there are no published reports of the isolation of Cu^r *P. syringae* in southern Europe although, in Spain, copper compounds routinely are used for the management of bacterial diseases of vegetable and tree crops (23).

Copper-resistance determinants have been cloned and characterized from strains of *P. syringae* and *X. campestris*, and frequently

were found associated with native plasmids (5,9,19,21,34). Conjugative transfer of indigenous plasmids encoding copper resistance has been demonstrated for *P. syringae* and other phytopathogenic bacteria (3,36,39), contributing to the dissemination of copper resistance (37). The *copABCD* genes, isolated from a 35-kb plasmid of *P. syringae* pv. *tomato* (4), were the first such genes to be sequenced (25), and DNA cross-hybridizing to *copABCD* subsequently has been found in Cu^r strains of *P. syringae* from *impatiens* (11), *P. syringae* pv. *syringae* (31), and a variety of Cu^r and copper-sensitive (Cu^s) *Pseudomonas* spp. (12,43). In addition, *copA*, but not the rest of the *cop* operon, was shown to cross-hybridize to the cloned copper-resistance determinants from *X. campestris* pvs. *campestris* and *vesicatoria* (42). A second copper-resistance operon, located on the chromosome and designated *copJ* (28), has been found in a limited number of Cu^r *P. syringae* pv. *syringae* strains (31). Although *copJ* shares some structural similarities with *copABCD*, it seems that it mediates resistance by a different mechanism, and the two determinants do not to cross-hybridize (28,31). Also, purified DNA from certain Cu^r *P. syringae* pv. *syringae* strains did not hybridize to the cloned Cu^r genes previously described (35). In a separate study, colony hybridization experiments using *copABCD* and *copJ* as hybridization probes failed to detect a portion of the Cu^r strains of *P. syringae* pv. *syringae* (31). These studies suggest that *P. syringae* pv. *syringae* might harbor yet another type or types of Cu^r determinant.

In this study, we provide evidence that Cu^r strains of the pathogen and Cu^s bacterial population pockets that seem to be protected from the bactericides could be responsible for the ineffectiveness of the chemical management strategies currently used for bacterial apical necrosis. We found a rich diversity of Cu^r determinants among the *P. syringae* pv. *syringae* populations affecting mango trees, suggesting the acquisition of copper-resistance determinants from a variety of sources.

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MATERIALS AND METHODS

Isolation and phenotypic characterization of *P. syringae* pv. *syringae*. The 117 *P. syringae* pv. *syringae* strains studied in this work were isolated as previously described (8) from commercial mango orchards located in Faro (Portugal) and in different locations of Málaga and Huelva (Spain). Sampling was carried out from 1992 to 1999 in orchards repetitively treated with copper compounds, especially with Bordeaux mixture. Bacterial isolates were identified and characterized as described (7,8) by using biochemical, physiological, and pathogenicity tests, and by evaluating the production of virulence factors.

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. The 15 *P. syringae* pv. *syringae* strains isolated from mango trees described in Table 2 were selected from the 117 isolates obtained in this study as being representative of the different plasmid profiles and hybridization patterns observed. *Escherichia coli* was routinely grown in Luria-Bertani broth (LB) (29) at 37°C and *P. syringae* in King's medium B (KMB) or LB at 27°C. Resistance to copper was determined by using mannitol-glutamate-yeast extract medium (MGY) (3) amended with CuSO₄·5H₂O, using as inoculum bacteria that were previously grown for 48 h at 27°C on KMB. Isolates were considered resistant to copper when their growth on MGY + 0.8 mM CuSO₄ after 72 h at 27°C was equivalent to that on MGY in the same conditions. The minimal inhibitory concentrations (MICs) for copper were determined by inoculating isolates on MGY plates containing copper from 0 to 3.6 mM and measuring inhibition after 72 h at 27°C. When necessary, media were supplemented with antibiotics at the following

concentrations (µg/ml): ampicillin, 100; kanamycin, 30; and tetracycline, 15.

P. syringae pv. *syringae* FF5 is a copper-sensitive, plasmidless strain with a high efficiency of transformation by electroporation (35). A mating experiment with *E. coli* S17-λpir (mini-Tn5Km2) (14) as donor and strain FF5 as recipient was carried out to generate a kanamycin-resistant derivative by transposon mutagenesis. A stable kanamycin-resistant mutant was selected, designated FF5-km, and used as a copper-sensitive recipient strain in conjugation experiments.

P. syringae pv. *syringae* UMAF0049 is a Cu^r strain that contains a 62-kb pPT23A-like plasmid and an additional 88-kb plasmid (6). The introduction into UMAF0049 of pAKC, which contains the origin of replication of the native plasmid pPT23A from *P. syringae* pv. *tomato* PT23 (26), led to the loss of the 88-kb plasmid. After repeated subculturing without antibiotic selection, pAKC was lost and the resulting strain, containing only the native 62-kb plasmid, was retained and designated UMAF0049c4. The same procedure was followed with five other strains showing the same plasmid profile as UMAF0049 to obtain the corresponding derivatives cured of the 88-kb plasmid.

Copper-based bactericide treatment and estimation of bacterial populations on mango buds. From 1997 to 2000, two groups of 12 mango trees (cv. Tommy Atkins) each, affected by apical necrosis at La Redondela (Huelva, Spain), were sampled to determine the occurrence of *P. syringae* and the proportion of Cu^r bacteria in the bacterial population on buds. One group of trees was sprayed monthly with Bordeaux mixture (3 g/liter) from September to June during the 3 years of study, while the other group was used as a nontreated control. Bulk samples of five buds

TABLE 1. Bacterial strains and plasmids and their relevant characteristics

Strain, plasmid	Characteristics ^a	Reference, source
Bacterial strains		
<i>Escherichia coli</i> S17-1 λpir	Tp ^r Sm ^r recA, thi, pro, hsdR-M ⁺ , RP4:2-Tc:Mu:Km Tn7, λpir	14
<i>Pseudomonas syringae</i> pv. <i>tomato</i> PT23	Wild type, Cu ^r	3
<i>P. syringae</i> pv. <i>syringae</i> FF5	Plasmidless, Cu ^s , Km ^s	34
<i>P. syringae</i> pv. <i>syringae</i> FF5-km	FF5 derivative mutagenized with pUT/mini-Tn5km2, Cu ^s , Km ^r	This study
<i>P. syringae</i> pv. <i>syringae</i> UMAF0049c4	UMAF0049 derivative, cured of the 88-kb plasmid, harboring only 62-kb plasmid (62.1 type), Cu ^r (MIC: 1.8 mM)	This study
Plasmids, transposons		
pPT23D	Cu ^r plasmid from <i>P. syringae</i> pv. <i>tomato</i> PT23.2	4
pCOP2	copABCD cloned from pPT23D in pRK404, Cu ^r , Tc ^r	4
pCOPR1.1	copJ cloned from genomic DNA in pLAFR3, Cu ^r , Tc ^r	28
pUT/mini-Tn5Km2	Ap ^r , Km ^r , delivery plasmid for mini-Tn5Km2	14

^a Cu^s and Cu^r = copper sensitivity and resistance, respectively; Km^s and Km^r = kanamycin sensitivity and resistance, respectively; Tc^r = tetracycline resistance; MIC = minimal inhibitory concentration.

TABLE 2. Strains of *Pseudomonas syringae* pv. *syringae* isolated from mango trees representative of the different plasmid profiles observed and their minimal inhibitory concentration (MIC) values for cupric sulfate^a

Plasmid profile	Strain	MIC (mM)	Plasmid content (kb) ^y	Place of isolation
I	UMAF0049	1.8	88, 62 (62.1)	Algarrobo (Málaga, Spain)
	UMAF0081	1.8	62 (62.1)	Algarrobo (Málaga, Spain)
II	UMAF1012	2.0	62 (62.1)	Churriana (Málaga, Spain)
	UMAF2016	1.6	62 (62.1)	Faro (Portugal)
	UMAF0170	1.6	62 (62.2)	Estepona (Málaga, Spain)
	UMAF1110	1.6	62 (62.3a)	La Redondela (Huelva, Spain)
	UMAF1065	2.0	62 (62.3b)	Lepe (Huelva, Spain)
	UMAF0167	2.0	120	Torrox (Málaga, Spain)
IV	UMAF1128	2.0	62, 45	La Redondela (Huelva, Spain)
V	UMAF2026	1.6	45	Faro (Portugal)
VI ^z	UMAF1094	<0.8	62 (62.3c)	Lepe (Huelva, Spain)
	UMAF0158	<0.8	62 (62.4)	Algarrobo (Málaga, Spain)
	UMAF1029	<0.8	62 (62.5)	Lepe (Huelva, Spain)
Plasmidless	UMAF2025	2.0	...	Faro (Portugal)
	UMAF0174	<0.8	...	Benajarafe (Málaga, Spain)

^a Strains with MIC values of <0.8 mM are considered sensitive to copper.

^y Numbers and letters in parenthesis (62.1 to 62.5) indicate different restriction profiles with *EcoRI* of 62-kb plasmids.

^z Same plasmid profile as II, but considering only copper-sensitive strains.

were randomly collected from each of the 24 trees in February, April, and June, every year of the study. *P. syringae*-like and total bacterial populations were determined as previously described (8). For each sample, the proportion of Cu^r bacteria is expressed as the percentage of bacteria growing on MGY + 0.8 mM of CuSO₄ with respect to the total bacterial population growing in MGY. Data were subjected to analysis of variance (ANOVA) followed by the least significant difference test ($P = 0.01$), using SPSS software (SPSS Inc., Chicago).

Plasmid characterization. Plasmid DNA was isolated according to a modified alkaline lysis method (44) and separated by electrophoresis on 0.6% agarose gels (27). Plasmid size was estimated by comparison with plasmids from *P. syringae* pv. *tomato* PT23. Purified plasmid DNA digested with *Eco*RI was separated by electrophoresis on 1% agarose gels and examined for restriction fragment length polymorphisms.

Mating experiments. Biparental matings were carried out to determine whether copper resistance genes were located on conjugative plasmids. After overnight growth in LB, selected Cu^r *P. syringae* strains harboring 62-kb plasmids were mixed in a 1:1 ratio with the copper-sensitive strain *P. syringae* pv. *syringae* FF5-km, and the mixtures were incubated overnight on LB at 27°C. Transconjugants were selected on MGY amended with 1 mM copper sulfate and kanamycin, and their MIC values for copper determined as described above. All mating combinations were repeated at least three times.

Southern blot analysis. Approximately 5 µg of genomic or plasmid DNA was digested with *Eco*RI, separated by electrophoresis in 1% agarose gels, transferred to nylon membranes, and hybridized with radioactive probes following standard procedures (29). A 4.4-kb *Pst*I fragment from pCOP2, containing the *copABCD* genes from *P. syringae* pv. *tomato* PT23 (4,25), and a 2.8-kb *Bam*HI-*Pst*I fragment from pCOPR1.1, containing *copJ* from *P. syringae* pv. *syringae* AL513 (28), were labeled with [α -³²P]dCTP using the random primer method (29) and used as hybridization probes. Hybridizations and posthybridization washes at high-stringency (65°C) or low-stringency (42°C) conditions were carried out according to standard procedures (29). Hybridization signals were detected following a scan in a FUJI BAS 1500 equipment using a FUJIX BAS IP-CST 2040 cassette and analyzed with the MacBas software (FUJI Photo Film Co., Tokyo). All the different Southern blot experiments were performed at least twice.

RESULTS

Natural bacterial colonization of trees treated with Bordeaux mixture. Two groups of 12 mango trees each were sampled from 1997 to 2000 to determine the effect of treatment with Bordeaux mixture on the bacterial populations on buds. During these 3 years, both groups of trees were strongly affected by apical necrosis, with disease incidence ranging from 60 to 80% (data not shown). For all the sampling dates, more than 94% of the total isolates recovered from buds were fluorescent, oxidase-negative, arginine dihydrolase-negative pseudomonads and were consequently considered to be *P. syringae*-like. Both the total number of bacteria and the *P. syringae*-like counts did not differ significantly between buds from the trees treated with Bordeaux mixture and those from nontreated trees for any of the sampling dates; these population sizes were very similar throughout the experiment (Fig. 1A and B). However, after 1 year of treatment with Bordeaux mixture, the proportion of Cu^r bacteria on buds of treated trees increased sharply to around 50% and remained at that level during the second and third years (Fig. 1C). Although a slight increase in the Cu^r bacterial population on buds of nontreated trees also was observed during the experiment, these variations were not statistically significant.

Isolation of Cu^r *P. syringae* pv. *syringae* strains from mango orchards. Between 1992 and 1999, 117 *P. syringae* strains were isolated from mango trees, either healthy or showing typical

symptoms of bacterial apical necrosis, in eight different locations in southern Spain and Portugal. The isolates were phenotypically homogeneous, and all of them showed the following characteristics: they produced green fluorescent pigment; were oxidase and arginine dihydrolase negative; hydrolyzed esculin and gelatin; did not utilize sodium tartrate; were ice-nucleation active; elicited the hypersensitive response in tobacco; produced syringomycin; and were pathogenic on lilac, lemon, tomato, and pear, but not on bean pods (data not shown). Based on these characteristics, all these strains were identified as *P. syringae* pv. *syringae* (8).

MIC values for copper sulfate were determined for the 117 *P. syringae* pv. *syringae* strains (Table 3), and those growing on

Fig. 1. Populations of **A**, total, **B**, *Pseudomonas syringae*-like, and **C**, copper-resistant bacteria on buds of mango trees untreated or treated with Bordeaux mixture. Two groups of 12 trees each were sampled in February, April, and June, from 1998 to 2000: □ = Nontreated trees; ■ = trees subjected since September 1997 to a monthly spray of Bordeaux mixture (3 g/liter). Bulk samples of five buds were analyzed for each tree. The proportion of copper-resistant bacteria is the percentage of colonies obtained in mannitol-glutamate-yeast extract medium + 0.8 mM CuSO₄ with respect to the total bacterial population. Population or percentage values shown in the figure represent the arithmetical mean of the values obtained on each sampling date plus the standard deviation. Means followed by the same letter (in each bar) are not significantly different according to least significant difference test ($P = 0.01$).

MGY medium with 0.8 mM CuSO₄ were considered to be resistant to copper (Cu^r). Cu^r strains were isolated from all of the mango orchards studied, from healthy as well as from diseased trees, and on average represented 59% of the total number of strains. Their MIC values varied between 1.0 and 3.2 mM CuSO₄ (Table 3), although most of the strains had an MIC of 1.8 mM.

Plasmid analysis and characterization. Copper-resistance determinants in phytopathogenic bacteria are commonly associated with plasmids. Plasmid occurrence, therefore, was examined by agarose gel electrophoresis of DNA from 70 (40 Cu^r and 30 Cu^s) randomly selected *P. syringae* pv. *syringae* mango isolates. Results are summarized in Table 4, and the plasmid profiles of strains representing the different patterns observed are shown in Figure 2A. Plasmids were detected in 52 of the strains by electrophoresis in agarose gels; 5 additional strains, all Cu^r, harbored a 120-kb plasmid that was barely detectable on agarose gels and could be made readily apparent only in Southern blot hybridization experiments with the *copABCD* probe (Fig. 2, lane 11). Overall, there was no obvious correlation between plasmid sizes and resistance phenotype or the place of isolation. Only 3 of the Cu^r

strains were plasmidless, whereas 37 harbored plasmids, and 31 of them contained a 62-kb plasmid, either alone or together with other plasmids. A 62-kb plasmid was widely distributed among the Cu^s isolates, being present in 20 of them, although it did not show hybridization with the copper-resistance genes (*copABCD*) from *P. syringae* pv. *tomato* PT23, unlike the 62-kb plasmid present in Cu^r isolates (Fig. 2B). No plasmids were apparent in the 10 other Cu^s strains. According to plasmid content and the pattern of hybridization to the *copABCD* probe, the 70 strains examined could be assigned to six different plasmid profile groups (Table 4).

Different 62-kb plasmids were subjected to restriction analysis with *EcoRI* to compare the plasmids harbored by Cu^r strains with those present in Cu^s strains. Plasmids were purified from the 24 Cu^r isolates assigned to plasmid profile II, the 20 Cu^s isolates assigned to profile VI, and from derivatives of the 6 Cu^r isolates from profile I, which were obtained by curing their 88-kb plasmid as described above. Among the 50 strains studied, the 62-kb plasmids could be classified in five different *EcoRI* restriction patterns (Fig. 3, Table 4), which were designated 62.1 to 62.5. Consequently, plasmid profile groups II and VI included strains which harbored different plasmids, although they were indistinguishable by electrophoresis on agarose gels. Two of the restriction patterns (62.1 and 62.2) were exclusively present in Cu^r strains, and two other patterns were detected only in Cu^s strains (62.4 and 62.5). Only one of the patterns (62.3) was observed in Cu^r as well as in Cu^s strains, although the 62.3-type plasmids could be differentiated by their pattern of hybridization to a probe containing copper-resistance genes; thus, the hybridizing patterns designated as 62.3a and 62.3b were associated with Cu^r strains that hybridize with *copABCD* probe, whereas the nonhybridizing pattern (62.3c) was exclusively present in Cu^s strains. The plasmid types 62.2 (Cu^r) and 62.5 (Cu^s) differed only by specific bands of 19.7 and 2.5 kb, respectively (Fig. 3, lanes 2 and 6). The final size, obtained by adding up all the restriction fragments in each plasmid pattern (Fig. 3), suggested the presence of repeated bands below 7 kb for all the 62.3-type plasmids (lanes 3 and 4). The most frequently found plasmid type was 62.1, which was carried by 23 strains isolated from two locations in Málaga, Spain, and from one location in Portugal (Table 4). On the other hand, plasmid 62.2 and those of plasmid profiles IV and V had a very limited distri-

TABLE 3. Number of *Pseudomonas syringae* pv. *syringae* strains isolated from mango trees, belonging to different categories of minimal inhibitory concentration (MIC) values for cupric sulfate (mM)

Place of isolation	MIC (mM) interval for cupric sulfate				Total
	Sensitive	Resistant			
	<0.8	1.0–1.6	1.8–2.4	2.6–3.2	
Spain					
Málaga					
Algarrobo	10	8	19	3	40
Benajarafe	7	1	1	...	9
Churriana	...	...	4	...	4
Estepona	1	2	...	...	3
Torrox	...	...	2	...	2
Huelva					
Lepe	16	...	3	2	21
La Redondela	7	3	4	5	19
Portugal					
Faro	7	5	7	...	19
Total	48	19	40	10	117

TABLE 4. Number of copper-resistant (Cu^r) and copper-sensitive (Cu^s) *Pseudomonas syringae* pv. *syringae* strains belonging to different plasmid profile groups[†]

Place of isolation ^a	Cu ^r strains (n = 40)									Cu ^s strains (n = 30)			
	I ^v		II (62 kb) ^w				III		IV		V		P-less ^x
	88, 62 kb	62.1	62.2	62.3a	62.3b	120 kb	62, 45 kb	45 kb	P-less ^x	62.3c	62.4	62.5	P-less ^x
Algarrobo (Ma/SP)	6	7	...	...	...	3	...	...	...	...	1	...	4
Benajarafe (Ma/SP)	...	...	...	...	...	...	...	...	...	...	3	...	1
Churriana (Ma/SP)	...	3	...	...	...	...	...	...	...	...	...	...	...
Estepona (Ma/SP)	...	...	1	...	...	...	...	...	...	...	...	...	...
Torrox (Ma/SP)	...	...	...	...	...	2	...	...	...	...	...	...	...
Lepe (Hu/SP)	...	...	...	...	1	...	...	...	...	7	1	4	...
Redondela (Hu/SP)	...	...	...	1	4	...	1	...	2	4	...	...	...
Faro (POR)	...	7	...	...	...	...	...	1	1	...	...	...	5
Total	6	17	1	1	5 ^y	5	1	1	3 ^y	11	5	4	10
<i>copABCD</i> probe hybridization on	62.1	62.1	62.2	62.3a	62.3b ^y	120/LPC	45	45	LPC	NH ^z	NH	NH	NH

[†] The size of the plasmids on each plasmid profile group is indicated in parenthesis. The 40 copper-resistant and 30 copper-sensitive strains were randomly chosen from a collection of 117 strains isolated from mango trees in different locations of Spain and Portugal. The hybridization of these strains with the *copABCD* operon probe also are summarized.

^a Ma/SP = Málaga, Spain; Hu/SP = Huelva, Spain; and POR = Portugal.

^v The 62-kb plasmid from profile I is 62.1 type.

^w Profiles II and VI are differentiated because only the 62-kb plasmids from strains belonging to profile II hybridize to a fragment containing the copper-resistance genes *copABCD*. For profiles II and VI, numbers 62.1 to 62.5 indicate 62-kb plasmids, which are differentiated by their restriction profile with *EcoRI* or, in the case of the 62.3 (a to c), by their hybridization to the *copABCD* probe.

^x P-less = plasmidless.

^y In these strains, 62-kb plasmid or linearized plasmid and chromosomal DNA (LPC) hybridized with *copABCD* only at low stringency conditions. The rest of the copper-resistant strains showed positive hybridization at high stringency conditions.

^z NH = these strains did not hybridize with *copABCD* probe either at high- or low-stringency conditions.

bution and were represented in each case by only a single strain (Table 4).

Detection of homology to *cop* probes. Southern blot analyses of intact plasmids and digested plasmid and genomic DNA were performed (Figs. 2, 4, and 5, Table 4) to determine whether the Cu^r *P. syringae* pv. *syringae* isolates from mango contained DNA homologous to the previously described copper-resistance genes *copABCD* from *P. syringae* pv. *tomato* PT23 (4) and *copJ* from *P. syringae* pv. *syringae* AL513 (28). The previously selected 70 isolates (40 Cu^r and 30 Cu^s) were examined. A total of 32 Cu^r strains contained plasmids that showed moderate to strong cross-hybridization to the *copABCD* probe under high-stringency hybridization conditions (Table 4). Hybridization signals were observed for all the 62.1 plasmids, the 62.2 plasmid, one of the 62.3 plasmids (62.3a), the 120-kb plasmids, and the 45-kb plasmids (Fig. 2B, lanes 2 to 11). The 62.2 plasmid from the strain UMAF0170 had a stronger hybridization signal than the other tested strains. Additionally, the band corresponding to linear plasmid and chromosomal DNA also showed hybridization to the probe for strains containing 120-kb plasmids (Fig. 2, lane 11).

Analysis of plasmid and genomic DNA digested with *Eco*RI (Fig. 4, Table 4) revealed that the DNA homologous to the probe was located in a 19.7-kb fragment for all the plasmids of patterns 62.1, 62.2, and 62.3a; a 15-kb fragment for the 45-kb plasmid from profile IV; and a 12.7-kb fragment for 120-kb plasmids and the 45-kb plasmid from profile V (lanes 2–11). Thus, the 19.7-kb fragment seems to be common to many 62-kb plasmid groups (Fig. 3). Genomic DNA from strains harboring the 120-kb plasmids showed an additional hybridizing band of 19.7 kb, suggesting the occurrence of an extra copy of *copABCD* homologues in chromosomal DNA or the detection of an additional plasmid fragment not detected previously. Chromosomal DNA of the three plasmidless Cu^r isolates and plasmids of 62.3-type from the remaining five Cu^r strains (designated as 62.3b) showed hybridization to the *copABCD* probe under low-stringency conditions (42°C) but not under high-stringency conditions (65°C) (Figs. 2 and 5, lanes 7 and 12). No hybridizing bands were observed under

high- or low-stringency conditions with plasmid or chromosomal DNA from the 30 Cu^s strains, including the strains with the 62-kb plasmids and 62.3c pattern (Figs. 2, 4 and 5, lanes 13–16; Table 4). These results revealed the presence of DNA homologous to the *copABCD* operon in all the examined Cu^r strains of *P. syringae* but in none of the Cu^s strains.

No hybridization bands were observed with any of the 40 Cu^r and 30 Cu^s isolates tested using the 2.8-kb *Bam*HI-*Pst*I fragment containing *copJ* as a probe, either at high- or low-stringency hybridization conditions (data not shown).

Conjugation assays. The curing and mobilization of different 62-kb plasmids was attempted in order to confirm if the observed homology with the *copABCD* probe was indeed identifying the genes responsible for the Cu^r phenotype of their harboring strains. Curing of the plasmids was unsuccessful using conventional treatments, such as growth with ethidium bromide or acridine orange, or by incompatibility with the related pPT23A origin of replication included in pAKC (6). When strains UMAF0081 and UMAF0049c4, both containing 62.1 plasmids, were mated with FF5-km, copper resistance was transferred to FF5-km at frequencies ranging from 1.4×10^{-6} to 1.0×10^{-7} . No spontaneous Cu^r mutants were recovered in control platings of recipient cells alone. The MIC for copper sulfate for purified transconjugants from independent matings was 1.8 mM, the same determined for the donor strains, and all of them contained a plasmid that was indistinguishable from the 62.1 plasmid type on agarose gels (data not shown). Despite numerous attempts, we were unable to obtain Cu^r transconjugants of FF5-km using UMAF0170, which harbors a 62.2 plasmid, as donor strain.

DISCUSSION

Bacterial apical necrosis of mango (*Mangifera indica* L.), incited by *P. syringae* pv. *syringae* is one of the principal diseases

Fig. 2. Hybridization of intact native plasmids from 15 representative *Pseudomonas syringae* pv. *syringae* strains isolated from mango trees with a probe containing the copper-resistance genes *copABCD*. **A**, Agarose gel electrophoresis showing the different profiles detected. **B**, Southern blot analysis under high-stringency conditions of the gel shown in **A** using the 4.4-kb *Pst*I fragment containing the *copABCD* operon from *P. syringae* pv. *tomato* PT23 as a probe. Lane 1: *P. syringae* pv. *tomato* PT23 (used as molecular size marker and hybridization control). *P. syringae* pv. *syringae* isolates from mango trees are in lanes (plasmid profiles and the type of 62-kb plasmid harbored are indicated in parenthesis) 2, UMAF0049 (I, 62.1); 3, UMAF0081 (II, 62.1); 4, UMAF2016 (II, 62.1); 5, UMAF1012 (II, 62.1); 6, UMAF0170 (II, 62.2); 7, UMAF1065 (II, 62.3b); 8, UMAF1110 (II, 62.3a); 9, UMAF1128 (IV); 10, UMAF2026 (V); 11, UMAF0167 (III); 12, UMAF2025 (plasmidless); 13, UMAF1094 (VI, 62.3c); 14, UMAF0158 (VI, 62.4); 15, UMAF1029 (VI, 62.5); 16, UMAF0174 (plasmidless). Cu^r = copper-resistant strains and Cu^s = copper-sensitive strains. Plasmids size (kb) are listed at left and right. LPC indicates linear plasmid and chromosomal DNA.

Fig. 3. Graphic representation of the six different *Eco*RI restriction patterns observed among the 62-kb plasmids from copper-resistant (patterns 1 to 3) and copper-sensitive (patterns 4 to 6) *Pseudomonas syringae* pv. *syringae* strains. The patterns are designated as 62.1 (1); 62.2 (2); 62.3a and 62.3b (3); 62.3c (4); 62.4 (5), and 62.5 (6). Asterisks indicate fragments from 62.1, 62.2, and 62.3a patterns hybridizing at high stringency to a probe containing the copper-resistance genes *copABCD*. Molecular weight (kb) of linear DNA size standards is indicated at left.

which limits mango fruit production in orchards along the Southern coast of the Iberian peninsula (8). After a few years of treatment, growers have noticed a reduction in efficacy of copper compounds for the control of apical necrosis (8); our results suggest that the build-up of a Cu^r *P. syringae* pv. *syringae* population is responsible, at least in part, for the increased disease control failures. We isolated Cu^r *P. syringae* pv. *syringae* from all the sampled mango orchards in southern Spain and Portugal, where copper-based bactericides have been used for several years. Additionally, we have shown that repeated treatments with Bordeaux mixture on a commercial orchard could be correlated with a significant increase in the Cu^r bacterial population (Fig. 1). These data conclusively confirm that the extensive use of copper-based bactericides favors the occurrence of bacterial copper resistance (3,37), which may explain control failures following treatments with copper compounds (1,13,30,35,39). However, the overall proportion of Cu^r bacteria never was higher than approximately 60%, so that Cu^s populations might be due to bacteria colonizing areas of the trees that are not reached by the bactericides. In fact, it was previously shown (8) that buds of infested mango trees harbor a high population of *P. syringae* pv. *syringae* year round, and it is possible that bud architecture allows population pockets that are effectively shielded from harsh environmental conditions. This suggests that, in those cases where the bacteria already have colonized the buds internally, chemical control of bacterial apical necrosis using nonsystemic products would be only partially successful, even if the bacterial population is susceptible to the bactericide used.

All the Cu^r strains examined hybridized, either at high- (32 strains) or low-stringency conditions (8 strains), to a DNA fragment containing the structural genes for copper resistance (*copABCD*) from *P. syringae* pv. *tomato* PT23 (Figs. 2, 4, and 5), but not to a fragment containing the copper-resistance locus *copJ* from *P. syringae* AL513; therefore, *copABCD* homologous sequences could be involved in the observed copper resistance of *P. syringae* strains of mango. Copper-resistance determinants related to the *copABCD* operon have been described in recent years from different genera of bacteria in agricultural environments where

copper compounds are applied (12,19,28,31,42,43). As expected, the Cu^s strains did not hybridize to any of the probes, either at high- or low-stringency conditions. The 40 Cu^r *P. syringae* pv. *syringae* isolates examined were genetically very diverse, as judged by the multiplicity of plasmid profiles, the location of the putative copper-resistance genes, and the intensity of their hybridization to the *copABCD* probe, and could be classified into nine different groups (Table 4). This diversity appears to be reflected in the population structure of infested orchards: in those places where eight or more Cu^r isolates were obtained, they could be classified into three or more different genotypic groups (Table 4). However, it appears that certain Cu^r and Cu^s genotypes could be associated with specific geographical areas; for instance, the 62.3-type plasmids were found only in areas of Huelva. Nonetheless, and given the limited number of isolates tested, it would be necessary to examine more isolates to confirm this observation. Our results, therefore, might suggest that the emergence of copper resistance in the mango orchards studied could be due to the selection of local resistant genotypes rather than to the distribution of one or a few Cu^r genotypes in infested plant material. However, the isolation of Cu^r strains harboring 62.1 plasmids from orchards of Spain and Portugal, separated by up to 400 km, indicates that some of these bacteria might originate from nurseries propagating contaminated plants (39). In fact, *P. syringae* pv. *syringae* has, in general, a wide host range (23), and isolates from mango were indeed shown to be pathogenic to several other trees and herbaceous plants (8). Although this is, to our knowledge, the first report of Cu^r bacteria in Spain and Portugal, it is possible that the use of copper bactericides in other crops has allowed the selection and maintenance of Cu^r *P. syringae* pv. *syringae* populations (31) that could have subsequently colonized mangoes.

A much more intense hybridization of the *cop* probe to the 62-kb plasmid of strain UMAF0170 was observed; this suggests that the copper-resistance determinant of this strain is more closely related to the *cop* genes of *P. syringae* pv. *tomato* PT23. The Cu^r genetic determinants described here were also diverse, as judged by the intensity of their hybridization signals with the *copABCD* probe, which were moderately or very intense under

Fig. 4. Southern hybridization of **A**, *Eco*RI-digested plasmid DNA and **B**, total genomic DNA from 15 representative *Pseudomonas syringae* pv. *syringae* strains isolated from mango trees using the 4.4-kb *Pst*I fragment containing the copper-resistance genes *copABCD* from *P. syringae* pv. *tomato* PT23. Cu^r = copper-resistant strains and Cu^s = copper-sensitive strains. Lane 1: *P. syringae* pv. *tomato* PT23 (used as molecular size marker and hybridization control). *P. syringae* pv. *syringae* isolates from mango trees are in lanes (plasmid profiles and the type of 62-kb plasmid harbored are indicated in parenthesis) 2, UMAF0049 (I, 62.1); 3, UMAF0081 (II, 62.1); 4, UMAF2016 (II, 62.1); 5, UMAF1012 (II, 62.1); 6, UMAF 0170 (II, 62.2); 7, UMAF1065 (II, 62.3b); 8, UMAF1110 (II, 62.3a); 9, UMAF1128 (IV); 10, UMAF2026 (V); 11, UMAF0167 (III); 12, UMAF2025 (plasmidless); 13, UMAF1094 (VI, 62.3c); 14, UMAF0158 (VI, 62.4); 15, UMAF1029 (VI, 62.5); 16, UMAF0174 (plasmidless). Molecular weight (kb) of linear DNA size standards and hybridizing fragments are indicated at left and right, respectively.

Fig. 5. Southern hybridization of plasmid profile gels of selected *Pseudomonas syringae* pv. *syringae* isolates with a probe containing the copper-resistance genes *copABCD*. Hybridizations were carried out under **A**, high-stringency and **B**, low-stringency conditions. *P. syringae* pv. *syringae* isolates from mango trees are in lanes (plasmid profiles and the type of 62-kb plasmid harbored are indicated in parenthesis) 3, UMAF0081 (II, 62.1); 7, UMAF1065 (II, 62.3b); 12, UMAF2025 (plasmidless); and 15, UMAF1029 (VI, 62.5). The size of the hybridizing plasmids (62 kb) is marked at left. LPC indicates linear plasmid and chromosomal DNA.

high-stringency hybridization conditions or only apparent under low-stringency conditions (Figs. 2 and 5). These differences in hybridization are not likely to be due to the presence of mobile elements, because the probe contains only approximately 100 nt on either side of the open reading frames of the *copABCD* operon; the differences probably reflect different levels of homology or the conservation of only part of the Cu^r determinant. This last possibility is the most likely, because copper-resistance determinants that are only partially homologous to the *copABCD* genes from strain PT23 already have been described in other bacteria, such as *P. syringae* pv. *actinidae* (M. Nakajima, accession no. AB033419) and *Ralstonia metallidurans* (B. Borremans and D. van der Lelie, accession no. AJ278983). The diversity of copper-resistance determinants among *P. syringae* pv. *syringae* colonizing mango is intriguing, and suggests that they might play another role in bacterial metabolism, being selected for under the strong selective pressure of a copper-enriched environment. This is not unlikely; for example, *Aeromonas veronii* biovar *sobria* 3767, a pathogen of humans, carries a homologue of *copABCD* that is required for tolerance to copper as well as for adhesion to cultured epithelial human cells (16). The oxidase activities recently described for CopA-related proteins is another example (33). Similarly, an unrelated set of copper-resistance genes from *P. fluorescens* is involved in cytochrome *c* biogenesis and competitive fitness (43). The previously reported presence of *cop* homologues in copper-sensitive *Pseudomonas* strains (10,12) further supports the involvement of the *cop* homologues from *P. syringae* pv. *syringae* in other cellular functions.

Several plasmid profiles were observed in the *P. syringae* isolates, but a 62-kb plasmid was the most frequently detected, both in Cu^r and Cu^s strains (72.9%). In Cu^r isolates harboring this plasmid, *copABCD* homologues generally were associated with it. Plasmidic copper resistance has been reported in many phytopathogenic bacteria (3,5,10,11,35,39,42). Furthermore, all of the 62-kb plasmids cross-hybridized to the origin of replication from pPT23A (6), therefore belonging to the recently described pPT23A-like family (17), and to the *ruAB* genes (6), which confer tolerance to ultraviolet radiation (38,40,41). Thus, copper-resistance determinants may have been incorporated into the bacterial population along with other plasmid-encoded traits that could enhance the ecological fitness and persistence of their harboring strains (15,20). In fact, plasmids of the pPT23A-like family tend to encode determinants of importance for the ecology of diverse *P. syringae* pathovars, such as avirulence, extracellular virulence factors, and resistance to antibiotics (18,32,36). Additionally, it is important to note that mango plant surfaces are exposed to heavy solar radiation in southern Europe; it is conceivable that plasmid-encoded traits such as UV radiation tolerance could contribute to the maintenance of the carrying plasmid and, consequently, of any other genes that might be on the plasmid.

In contrast to our results, other authors have reported a strong conservation of Cu^r determinants among Cu^r bacteria isolated from the same field and from areas as distant from one another as California and Florida; albeit, in certain instances, the determinants were located on dissimilar plasmids (5,9,10,12,36). These bacteria, unlike *P. syringae* pv. *syringae*, were primarily narrow-host-range pathogens. The variety of habitats (mainly plants) that *P. syringae* pv. *syringae* may encounter could increase the diversity of its copper-resistance determinants. Cu^r determinants often are associated with conjugative or mobilizable plasmids (10,26,34,42). Also, Cu^r genes located on the *X. axonopodis* pv. *vesicatoria* (formerly *X. campestris*) (2) chromosome were shown to be transferred by conjugation, which supports the idea that their evolutionary success could be related to their capacity to reach a large number of bacteria through horizontal exchange (35,39). Plasmid-borne determinants are in general more easily established in bacterial populations (15) and the apparent prevalence of 62.1 plasmids might be due to its conjugative ability. It is possible, that

the current structure of the Cu^r population in mango orchards represents an early evolutionary phase that could change toward the predominance of a single, more fit, copper-resistance determinant.

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