

Regulation of Resistance to Copper in *Xanthomonas axonopodis* pv. vesicatoria

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Copper-resistant strains of *Xanthomonas axonopodis* pv. vesicatoria were previously shown to carry plasmid-borne copper resistance genes related to the *cop* and *pco* operons of *Pseudomonas syringae* and *Escherichia coli*, respectively. However, instead of the two-component (*copRS* and *pcoRS*) systems determining copper-inducible expression of the operons in *P. syringae* and *E. coli*, a novel open reading frame, *copL*, was found to be required for copper-inducible expression of the downstream multicopper oxidase *copA* in *X. axonopodis*. *copL* encodes a predicted protein product of 122 amino acids that is rich in histidine and cysteine residues, suggesting a possible direct interaction with copper. Deletions or frameshift mutations within *copL*, as well as an amino acid substitution generated at the putative start codon of *copL*, caused a loss of copper-inducible transcriptional activation of *copA*. A nonpolar insertion of a kanamycin resistance gene in *copL* resulted in copper sensitivity in the wild-type strain. However, repeated attempts to complement *copL* mutations in *trans* failed. Analysis of the genomic sequence databases shows that there are *copL* homologs upstream of *copAB* genes in *X. axonopodis* pv. citri, *X. campestris* pv. campestris, and *Xylella fastidiosa*. The cloned promoter area upstream of *copA* in *X. axonopodis* pv. vesicatoria did not function in *Pseudomonas syringae* or in *E. coli*, nor did the *P. syringae* *cop* promoter function in *Xanthomonas*. However, a transcriptional fusion of the *Xanthomonas* *cop* promoter with the *Pseudomonas copABCDRS* was able to confer resistance to copper in *Xanthomonas*, showing divergence in the mechanisms of regulation of the resistance to copper in phytopathogenic bacteria.

Both eukaryotic and prokaryotic cells require copper for normal growth. Copper is an essential cofactor of a number of enzymes involved in respiration, such as oxygenases and electron transport proteins (17). However, above a certain concentration, copper is toxic to the cell. Therefore, its intracellular levels must be tightly controlled (16). Copper has the ability to generate free radicals able to damage DNA and lipid membranes (22, 36). As a consequence, bacteria developed detoxification systems to protect themselves from toxic concentration of copper and still ensure they met their nutritional requirements. These systems have been found to be plasmid-, or chromosomally borne (for reviews, see references 38, 39, and 42).

For years, copper-containing compounds have been sprayed on vegetable and fruit crops to limit the spread of plant pathogenic bacteria and fungi. This continuous and popular use of copper-based antimicrobial compounds favored the spread of copper resistance genes among saprophytic and plant pathogenic bacteria (2, 10, 41). *Xanthomonas campestris* pv. vesicatoria, renamed *X. axonopodis* pv. vesicatoria (53), is a common plant pathogen of tomato and peppers. There have been several reports of copper-resistant *X. axonopodis* strains (5, 8, 10, 18, 19, 26, 30). Copper-resistant strains of other plant pathogenic bacteria have also been identified, including *Pseudomonas syringae* (2, 4, 12, 20, 41, 44, 45). Cloning and characterization of both plasmid and chromosomal copper resistance (*cop*) genes from these pathogens has shown that most are

related to each other (26, 27, 41, 55), and they are also related to the *pco* genes from enteric bacteria (6, 9, 46, 51; for a review, see reference 40). However, in spite of the considerable sequence similarities, there appear to be functional and regulatory differences between the *Pseudomonas cop* and enteric *pco* systems (7, for a review, see reference 40).

The *copABCD* operon of *P. syringae* (31) is specifically induced by copper (32). Copper-inducible expression requires a two-component regulatory system (*copRS*), which immediately follows the *copABCD* operon (34). CopR was purified and shown to bind to a conserved motif (*cop* box) at the –35 region of *cop* promoters from *P. syringae* (35). No sequences upstream from the *cop* box are required for copper-inducible expression (35). Chromosomal genes in some pseudomonads may substitute for the plasmid-borne *copRS* system (34), and expression of the *cop* promoter from *P. syringae* has only been observed in pseudomonads. In *Escherichia coli*, mutations in *pcoR* can also be complemented by a chromosomal gene (43). Expression of resistance to toxic levels of copper therefore appears to involve complex interactions between both plasmid and chromosomal genes. Cloned copper resistance genes from two species of *Xanthomonas* were also shown to be related to the *cop* operon from *P. syringae*, namely, homology to *copA* (26, 55).

We are interested in determining what natural barriers might exist to the dissemination of plasmid-borne copper resistance genes among different bacterial pathogens, possibly due to different features of the host background that may be required for expression of plasmid-borne resistance genes. In addition to the lack of expression of the cloned *cop* promoter from *P. syringae* in *Xanthomonas* (35), cloned copper resistance genes from *X. axonopodis* did not confer copper resistance to *P. syringae* or *E. coli* (10, 55). It was not known, however,

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TABLE 1. Bacterial strains and plasmids developed in this study

<i>Xanthomonas</i> strain or plasmid	Description ^a
<i>X. axonopodis</i> pv. <i>vesicatoria</i>	
7882.3	Cu ^s , Rif ^r , Kan ^r ; kanamycin cassette was inserted into the SalI site of <i>copL</i> in 7882 (10)
78518.2	Cu ^r Rif ^r ; the Cu ^r plasmid of 7882 was electroporated into strain 78518 (10)
Plasmids	
pCOP151	Sm ^r Cm ^r ; 1.9-kb BamHI fragment of pCOP120 (55) cloned in the BglII site of pMP190 (49) in the correct orientation to express <i>lacZ</i>
pCOP153B	Ap ^r ; 1.9-kb BamHI fragment of pCOP116 (55) cloned in pUC128 (25) in the anti- <i>lac</i> orientation
pCOP156	Ap ^r ; pCOP153B was digested with <i>KpnI</i> to result in a deletion of the 500-bp <i>KpnI</i> -BamHI fragment and then religated
pCOP157	Ap ^r ; pCOP153B was digested with <i>ClaI</i> to result in a deletion of the 1.4-kb <i>ClaI</i> -BamHI fragment and then religated
pCOP161	Sm ^r Cm ^r ; 1.5-kb <i>XbaI</i> - <i>KpnI</i> -fragment of pCOP156 cloned in pMP190
pCOP162	Sm ^r Cm ^r ; 510-bp <i>XbaI</i> - <i>KpnI</i> fragment of pCOP157 cloned in pMP190
pCOP166	Ap ^r ; pCOP153B was digested with <i>SphI</i> to result in a deletion of 0.6-kb <i>SphI</i> -BamHI fragment and then religated
pCOP168	Ap ^r ; pCOP156 was digested with <i>SacII</i> to result in a deletion of the 440-bp BamHI- <i>SacII</i> fragment and then religated
pCOP169	Sm ^r Cm ^r ; 1.0-kb (<i>SacII</i>) ^b - <i>XbaI</i> fragment of pCOP168 cloned in (<i>SalI</i>)- <i>XbaI</i> sites of pMP190
pCOP170	Sm ^r Cm ^r ; 1.0-kb (<i>XbaI</i>)- <i>SphI</i> fragment of pCOP166 cloned in (<i>XbaI</i>)- <i>KpnI</i> sites of pMP190
pCOP187	Tc ^r ; 510-bp BamHI- <i>HindIII</i> fragment of pCOP157 cloned in pRK415 (25)
pCOP189	Tc ^r ; 8.8-kb <i>HindIII</i> fragment of pDAC102 (34) cloned in the <i>HindIII</i> site of pCOP187, resulting in transcriptional fusion of the constitutive promoter with <i>copABCDRS</i> .
pCOP191	Ap ^r ; pCOP153B was digested with <i>ApaI</i> to result in a deletion of 900-bp <i>ApaI</i> -BamHI fragment and then religated
pCOP192	Ap ^r ; pCOP191 digested with BamHI and <i>StuI</i> , blunt ended, and religated
pCOP193	Ap ^r ; pCOP191 digested with <i>SalI</i> and <i>ClaI</i> , blunt ended, and religated
pCOP196	Ap ^r ; pCOP191 digested with <i>StuI</i> and <i>ApaI</i> , blunt ended and religated
pCOP197	Sm ^r Cm ^r ; the 985-bp <i>XbaI</i> - <i>KpnI</i> fragment of pCOP191 cloned in pMP190
pCOP198	Sm ^r Cm ^r ; the 735-bp <i>XbaI</i> - <i>KpnI</i> fragment of pCOP192 cloned in pMP190
pCOP199	Sm ^r Cm ^r ; the 935-bp <i>XbaI</i> - <i>KpnI</i> fragment of pCOP193 cloned in pMP190
pCOP202	Sm ^r ; Cm ^r ; the 250-bp <i>XbaI</i> - <i>KpnI</i> fragment of pCOP196 cloned in pMP190
pCOP203	Ap ^r ; the 470-bp <i>ClaI</i> - <i>ApaI</i> fragment of pCOP191 cloned in pUC128
pCOP204	Ap ^r ; the 200 bp <i>StuI</i> - <i>SalI</i> fragment of pCOP191 cloned into the <i>SmaI</i> - <i>SalI</i> sites of pUC128
pCOP205	Sm ^r Cm ^r ; the 470-bp <i>XbaI</i> - <i>KpnI</i> fragment of pCOP203 cloned in pMP190
pCOP206	Sm ^r Cm ^r ; the 200-bp <i>XbaI</i> - <i>KpnI</i> fragment of pCOP204 cloned in pMP190
pCOP210	Ap ^r ; pCOP192 was digested with <i>ClaI</i> blunt ended with <i>Klenow</i> and religated
pCOP214	Sm ^r ; Cm ^r ; the 725-bp <i>XbaI</i> - <i>KpnI</i> fragment of pCOP210 was cloned in pMP190
pCOPM1	Sm ^r ; Cm ^r ; substitution of first Met codon in <i>copL</i> (in pCOP192) to Val and subcloned into pMP190
pCOPM2	Sm ^r ; Cm ^r ; substitution of second Met codon in <i>copL</i> (in pCOP192) to Leu and subcloned into pMP190
pCOPM3	Sm ^r ; Cm ^r ; substitution of third Met codon in <i>copL</i> (in pCOP192) to Leu and subcloned into pMP190

^a Cu^r, copper resistance; Cu^s, copper sensitivity; Rif^r, rifampin resistance; Km^r, kanamycin resistance; Sm^r, streptomycin-resistance; Ap^r, ampicillin resistance; Tc^r, tetracycline resistance; Cm^r, chloramphenicol resistance.

^b Restriction sites in parentheses describe sites that have been blunt ended before ligation.

whether the genes from *Xanthomonas* were not expressed or whether the mechanism of resistance was not functional in the other genera. The objective of this study was to investigate the regulation of copper resistance in *X. axonopodis* in comparison to the related *cop* system in *P. syringae*.

The copper-resistant strain of *X. axonopodis* used for this study was isolated from a tomato field in California and found to harbor copper resistance genes on a nonconjugative 100-kb plasmid (10). The smallest fragment able to confer substantial resistance to copper to a copper-sensitive *X. axonopodis* strain was a 6.8-kb clone (55). Transposon mutagenesis with a promoterless *lacZ* gene was carried out by using *Tn1737* (52). A particular *Tn1737* insertion in the cloned copper resistance genes inactivated resistance to copper but did not show elevated β -galactosidase activity, suggesting the insertion occurred in the promoter area. The characterization of this promoter area and its ability to function in plant pathogens related to *X. axonopodis* pv. *vesicatoria* are the object of this report. Since copper is an important bactericide in agriculture, the

dissemination of plasmid-borne copper resistance among strains of a pathovar or between different pathovars, species, or genera of plant pathogenic bacteria has important implications for disease control efforts.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and plasmids. The bacterial strains and plasmids developed in the present study are listed in Table 1. Nutrient Agar (Difco) supplemented with yeast extract (1g/liter; NY) was used to maintain xanthomonads, whereas pseudomonads were grown on mannitol-glutamate agar (24) supplemented with yeast extract at 0.25 g/liter (MGY) at 28°C. Cultures of *E. coli* were grown in Luria-Bertani (LB) (33) broth at 37°C. Antibiotics were used at the following concentrations: rifampin, 50 μ g/ml; chloramphenicol, 20 μ g/ml; ampicillin, 50 μ g/ml; tetracycline, 10 μ g/ml; kanamycin, 25 μ g/ml; and streptomycin 50 μ g/ml. Nutrient broth (NY), MGY, and LB were used as liquid media to grow xanthomonads, pseudomonads, and *E. coli*, respectively. The antibiotic levels were decreased to half when bacteria were grown in liquid media. Constructed plasmids were introduced into *Pseudomonas* or *Xanthomonas* from *E. coli* strain S17-1 (47) by triparental matings on yeast-glucose-calcium carbonate agar with pRK2013 as the helper plasmid (15) or by electroporation. Mating mixtures or transformed cells were plated on media containing the

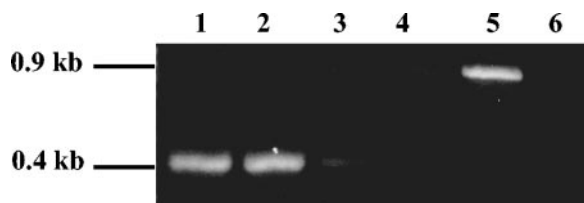


FIG. 1. RT-PCR analysis of *copL* and *copA* genes of *X. axonopodis* pv. *vesicatoria* 7882. Lanes 1 to 3, RT-PCR analysis for *copL*; lanes 4 to 6, RT-PCR analysis for *copA*. RT-PCR was performed on RNA isolated from cells grown in the absence (lanes 1 and 4) or presence (lanes 2 and 5) of 1 mM CuSO_4 . Lanes 3 and 6 show the results of the negative controls.

appropriate antibiotics. Strain 78518 (Cu^s [10]) was transformed with a CsCl-purified plasmid preparation from 7882 (10) by electroporation. Electroporated cells were plated onto NY agar containing CuSO_4 at 1.2 mM. A copper-resistant derivative of 78518 that had acquired the desired plasmid was designated 78518.2. The presence of the 100-kb copper-resistance plasmid was verified by plasmid isolation and comparing the plasmid size to the plasmid from the parent strain 7882.

General DNA manipulations, DNA sequencing, and sequence analysis. Molecular biology techniques were performed by using standard protocols described by Maniatis et al. (29). Transposon insertion mutagenesis (55) narrowed down the promoter area for the copper resistance determinants to a 1.9-kb BamHI fragment (pCOP153B) that was subcloned further into a BamHI-ApaI fragment (Table 1), which was sequenced. The resulting DNA and deduced protein sequences were analyzed with the University of Wisconsin Genetics Computer Group (14) and the BLAST programs provided by the National Center for Biotechnology Information. The alignment of the deduced amino acid sequences of CopL homologs were made with the Multalin software version 5.4.1 (11).

Isolation of RNA and primer extension. Bacterial cells of *X. axonopodis* pv. *vesicatoria* strain 7882 were grown overnight in LB broth without or with 7 mM CuSO_4 at 28°C. A total of 25 ml of the culture was pelleted, and the rapid RNA isolation procedure was followed (3). The integrity of the RNA was determined by visualization of the rRNA bands on a denaturing 1.2% agarose gel in the presence of formaldehyde. The primer extension procedure was done according to the method of Ausubel et al. (3). Briefly, 20 μg of RNA was annealed in $1 \times$ hybridization solution with 5×10^5 cpm of ^{32}P -end-labeled 18-mer oligonucleotides. To determine the 5' end of the RNA for *copL*, the oligonucleotide 5'-GCCTGCTCTTCATCACC-3' (hybridizing to nucleotides 409 to 426, Fig. 1) was used, and the products were analyzed by fractionation on a 6% polyacrylamide gel. The primer extension reactions were run side-by-side with dideoxy-sequencing reactions, primed with the same primer, as size markers.

RT-PCR analysis of *copL* and *copA*. *X. axonopodis* pv. *vesicatoria* strain 7882 cells were grown in NY until they reached an optical density at 600 nm of 0.5. The copper resistance genes were induced for 20 min by the addition of CuSO_4 to a final concentration of 1 mM. Control cultures were left untreated. Total RNA was isolated by using TRIzol (Invitrogen) and RNA samples were treated with RNase-free DNase I (Promega) for 1 h at 37°C. Reverse transcription-PCR (RT-PCR) analysis was performed by using the Access RT-PCR kit (Promega) according to the manufacturer's instructions. RT-PCRs were carried out in 25- μl reactions using either the L1-L2 primer pair or the L3-AUP primer pair for *copL* and *copA* gene, respectively. The sequences of the primers used were as follows: L1, 5'-CGGAATTCATGCTCGTCTTAACGGGG-3' (hybridizes to nucleotides 355 to 374, Fig. 1); L2, 5'-TAACTGCAGCTCGAGACGCTTAGCCGATCGGTG-3' (hybridizes to nucleotides 729 to 711); L3, 5'-CGCCTGCCTGCC TCATCTG-3' (hybridizes to nucleotides 684 to 703); and AUP, 5'-GGCCCGG CATCTTCTTCAAC-3' (hybridizes to nucleotides 622 to 642) of the *copA* gene. The full nucleotide sequence of *copA* and deduced amino acid sequences can be found in the GenBank entry AY536748. The RT reaction was performed at 48°C for 45 min, followed by a 2-min denaturation step at 94°C. For the amplification reactions, the conditions used were as follows: 40 cycles of 94°C for 30 s, 60°C for 1 min, and 68°C for 1 min, with a final extension step at 68°C for 5 min. The negative control comprised of samples subjected to the same reaction conditions, with the reverse transcriptase step being omitted. The amplified DNA fragments were separated on a 1.3% agarose gel.

Deletions and point mutations in *copL*. To test the effect of mutations and deletions in *copL* on the induction of *copA* transcription in response to copper, several subclones of the 1.9-kb BamHI fragment from pCOP120 (55) were

introduced into the promoterless β -galactosidase vector pMP190 (49). Similarly, amino acid substitutions at the first three methionines of CopL were carried out by PCR mutagenesis by using the "mega primer" method modified for improved amplification of the final product (1). The primers utilized for the mutagenesis were as follows (with introduced restriction endonuclease sites underlined and mutations in the methionine codon and changes to introduce endonuclease sites indicated in boldface): 5'-GATCGCCG**GTGCTCGTGCTTAAC**-3' for a valine substitution at the first methionine of CopL, 5'-TGC**GTCCGTAAGCTTGAA** TCCGGT-3' for a leucine substitution at the second methionine, and 5'-TATG AATCC**GAAGCTTGAAGAGCA**-3' for a leucine substitution at the third methionine of CopL. Three separate PCR amplification reactions were carried out by using pCOP192 as a template. The first PCR used one of the above primers and the pUC19 universal forward primer. The second reaction used the pUC19 universal reverse primer and a primer complementary to downstream *copL* sequences. For the third PCR, the products from the first two steps were mixed in a 5:1 molar ratio, and the final product was amplified with the pUC19 universal reverse and forward primers. The amplified fragments were gel purified, digested with XbaI and KpnI, and cloned into pUC128 (25). The clones bearing mutations were confirmed by restriction endonuclease digestion and sequencing. The resulting mutated versions of *copL* were cloned directionally upstream of the promoterless *lacZ* gene in pMP190 for assessment of the copper-inducible expression of *copA*.

β -Galactosidase assays. Strains of *Xanthomonas* containing the pMP190-based constructs obtained as described above were grown in NY to log phase and subcultured into NY or NY with 0.1 mM CuSO_4 for 16 to 20 h at 28°C. Strains of *Pseudomonas* and *E. coli* containing recombinant plasmids were grown in MGY amended with streptomycin at 5 $\mu\text{g}/\text{ml}$ and LB amended with chloramphenicol at 20 $\mu\text{g}/\text{ml}$. The inducing concentrations of CuSO_4 were 0.1 and 0.7 mM for *Pseudomonas* and *E. coli*, respectively. The β -galactosidase activities were assayed as described by Miller (33) with *o*-nitrophenyl- β -D-galactopyranoside as the substrate.

Marker-exchange mutagenesis of *copL*. A kanamycin resistance gene cassette from pMKm (37) was inserted into the SalI site of *copL* in pCOP156. The kanamycin resistance gene is transcribed in the opposite direction relative to that of *copL*. The mutated *copL* was subcloned into the broad-host-range plasmid pRK415 (25) and electroporated into the wild-type strain 7882. Marker exchange was carried out as described previously (28), with selection for kanamycin resistance and screening for loss of the plasmid-determined tetracycline resistance. The resulting mutant strain was designated 7882.3. The presence of the kanamycin cassette in *copL* on the indigenous copper resistance plasmid in 7882.3 was confirmed by Southern blot hybridization.

Test for induction of *copA* transcription by other metals. The metal salts used in the present study were: CuSO_4 , NiSO_4 , CrCl_2 , CoCl_2 , K_2CrO_4 , MnCl_2 , HgCl_2 , $\text{Y}(\text{NO}_3)_3$, NaCl , CsCl , LiCl_2 , AgNO_3 , $\text{Th}(\text{NO}_3)_3$, ZnSO_4 , CaCl_2 , $\text{Al}(\text{NO}_3)_3$, $\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2$, FeCl_3 , LaCl_3 , NdCl_3 , ErNO_3 , and CeCl_3 . The MIC (10) was calculated for each metal salt. One-tenth of the MIC concentration of each metal was used for induction, and in cases where the MIC was not accurately determined, the maximum concentration of the metals used in the test experiment was used for induction purposes (16 to 20 h). The test was performed in liquid cultures of NY at 28°C amended with different concentrations of the metals. Strain 7882(pCOP198) containing the intact *copL* and the 5' end of *copA* fused to the β -galactosidase reporter gene was tested for the ability of these metals to induce transcription of *copA*. Uninduced cultures of 7882(pCOP198) were used as a negative control, and the same strain grown in the presence of CuSO_4 was used as the positive control. β -Galactosidase activity was measured as described above.

Expression of the *Pseudomonas cop* operon in *Xanthomonas* under the control of the *Xanthomonas cop* promoter. To determine whether the lack of expression of the *Pseudomonas cop* operon in *X. axonopodis* is due to a transcriptional difference, a transcriptional fusion of the copper-inducible promoter from *X. axonopodis* with the *cop* operon of *P. syringae* was constructed (pDAC102 [34]). The resulting construct was introduced in a copper-sensitive (Cu^s) *X. axonopodis* pv. *vesicatoria* strain 78518, and its resistance to CuSO_4 was determined.

Nucleotide sequence accession number. The 987-bp sequence containing *copL* and the 5' end of *copA* was deposited in GenBank (accession number AY380578).

RESULTS

Sequence analysis of the promoter region of the copper resistance determinants from *X. axonopodis*. Plasmid pCOP151 (Table 1), containing a 1.9-kb fragment cloned in the *lacZ*

reporter vector pMP190, was further subcloned. A region of about 1 kb in the 5' portion of this fragment that was shown to be sufficient for the copper-dependent induction of the β -galactosidase reporter gene. This region was sequenced, revealing an open reading frame (ORF) of 369 bp, designated *copL*, located at the 5' end of the fragment. The 3' end of this ORF is also present in the published sequence of the copper resistance operon from *X. arboricola* pv. *juglandis* (26), but no sequences further upstream were included in that study.

Putative -10 and -35 regions were identified for *copL*, but no easily identifiable ribosome-binding site (RBS) was found. The lack of a strong RBS could suggest that CopL, a protein of 122 amino acids, is translated at very low levels. Although the putative -10 region (TAAAGT) is close to the conventional *E. coli* promoters, the putative -35 region (TTGTTC) matches the consensus motif for *Xanthomonas* (TTGTNN [23]).

Downstream of *copL*, there is a homolog to the multicopper oxidase *copA* starting at nucleotide 838. A putative RBS (TG-GAG) can be found -12 to -8 from the ATG of CopA. It is worth noting that the spacing of 113 bases between the stop codon of *copL* and the start codon of *copA* is larger than what is commonly found in bacteria for two genes part of an operon. The intergenic region could allow the binding of regulatory factors (A.E. Voloudakis and D. A. Cooksey, unpublished data). There is also an inverted repeat GGCGCC-N₄-GGCGCC starting nine bases downstream of the stop codon for *copL*.

The deduced amino acid sequence for CopL revealed the presence of potential metal-binding sites, such as C₃₇-X₂-H and C₇₃-X-C-X-C-H, as well as pairs of cysteines (C₆₇-C) and histidines (H₄₀-H and H₄₅-H). However, based on DNA or amino acid sequence data, *copL* was not related to any copper resistance systems known thus far. Careful examination of the genomic sequences of *X. axonopodis* pv. *citri*, *X. campestris* pv. *campestris* (13), and *Xylella fastidiosa* (48) revealed the presence of *copL*-like ORFs. A point of similarity between all four bacteria is the location of CopL upstream of *copAB*, which are homologous to the first two structural copper resistance genes of *X. campestris* pv. *juglandis* and *P. syringae* pv. *tomato*.

X. axonopodis pv. *vesicatoria* CopL was aligned with CopL_{XAC} (accession number NP_643936; 26% identity, 37% similarity) from *X. axonopodis* pv. *citri*, CopL_{XCC} (accession number AAM39894; 26% identity, 32% similarity) from *X. campestris* pv. *campestris*, and CopL_{XF} (accession number AAO27998; 27% identity, 39% similarity) from *Xylella fastidiosa*. The alignment revealed the conservation of several cysteine and histidine residues, which suggests they may have a functional role in the proteins.

There were no identifiable similarities in the region 5' to *copA* with the promoter region of the *cop* operon from *P. syringae* or already-characterized *Xanthomonas* promoters. Most notably, no similarity was observed to the *cop* box sequence, where the CopR regulatory protein was shown to bind in *P. syringae* (35). Sequence data indicated the presence of a strong RBS (TGGAG) located -11 to -8 bp upstream from the translation start codon of *copA*. Over this N-terminal region of CopA, the amino acid identity and similarity were 66 and 70%, respectively, with the CopA homolog of copper-resistant *X. arboricola* pv. *juglandis* (26).

Primer extension analysis of *copL*. An extension product was observed, corresponding to a 5' end of the RNA for *copL*. The extension products were observed with RNA isolated from induced and uninduced cells. However, uninduced cells gave a signal that was approximately three times weaker than the extension product from induced cells. There were no additional extension products observed when another primer, complementary to nucleotides 258 to 275, was used to detect the 5' end of mRNAs further upstream (data not shown).

RT-PCR analysis of *copL* and *copA*. To determine the presence of transcripts for *copL* and *copA* in the presence or absence of Cu²⁺ ions, an RT-PCR analysis was used. The results indicated the presence of a transcript for *copL*, regardless of the presence of copper. The expected size of the amplified product is 396 bp (Fig. 1, lanes 1 and 2). In contrast, a transcript for *copA* was only detected in the presence of Cu²⁺ (Fig. 1, lanes 4 and 5), as shown by the presence of an amplified product of 797 bp. This suggests that *copL* is transcribed from a constitutive promoter, but the expression of *copA* is copper dependent.

Deletion, insertion, and site-specific mutations in *copL*. A 740-bp StuI-ApaI fragment, which contained all of *copL* and the 5' end of *copA*, was the smallest subcloned fragment that gave full copper-inducible activity of *copA* in the *lacZ* reporter vector pMP190. Subclones containing only the 5' part of this fragment (the 5' end of *copL* and about 100 bp upstream from *copL*) had constitutive promoter activity (pCOP162, pCOP206, and pCOP170) (Fig. 2), probably corresponding to the transcriptional start site that was detected with or without copper exposure in this upstream region. However, a subclone of the 3' region (pCOP205), which included the last half of *copL* through the beginning of *copA*, did not have any promoter activity.

Specific mutations in *copL* were also made, including a frameshift mutation at the ClaI site (pCOP214; Fig. 2), a deletion between the SalI and ClaI sites (pCOP199; Fig. 2), and amino acid substitutions at the first three methionine codons of the *copL* ORF (pCOPM1, pCOPM2, and pCOPM3; Fig. 2). The frameshift mutation at the ClaI site and the SalI-ClaI deletion resulted in a complete loss copper-dependent induction of *copA*, again measured by expression of *lacZ* transcriptionally fused to these constructs (Fig. 2). Substitution of the first methionine codon of CopL with that of valine also abolished copper-dependent β -galactosidase expression, but substitution of the second and third methionines of *copL* with leucine did not. These results suggested that the translation of an intact message for *copL* is required for the copper-dependent transcription of *copA*.

A nonpolar *copL* insertional mutation was also constructed in the wild-type strain 7882 to determine its effect on the expression of the copper resistance phenotype. The wild-type *copL* gene in the indigenous copper resistance plasmid of 7882 was replaced by marker exchange mutagenesis with a derivative of *copL* containing a kanamycin resistance gene insertion at the SalI site with the direction of transcription of the kanamycin resistance gene in the opposite direction relative to *copL* and the downstream *cop* genes. The kanamycin cassette also did not have transcriptional terminators. The MIC of CuSO₄ was reduced from 2.0 mM in 7882 to 1.4 mM in the marker exchange mutant 7882.3, an MIC level that was higher than the

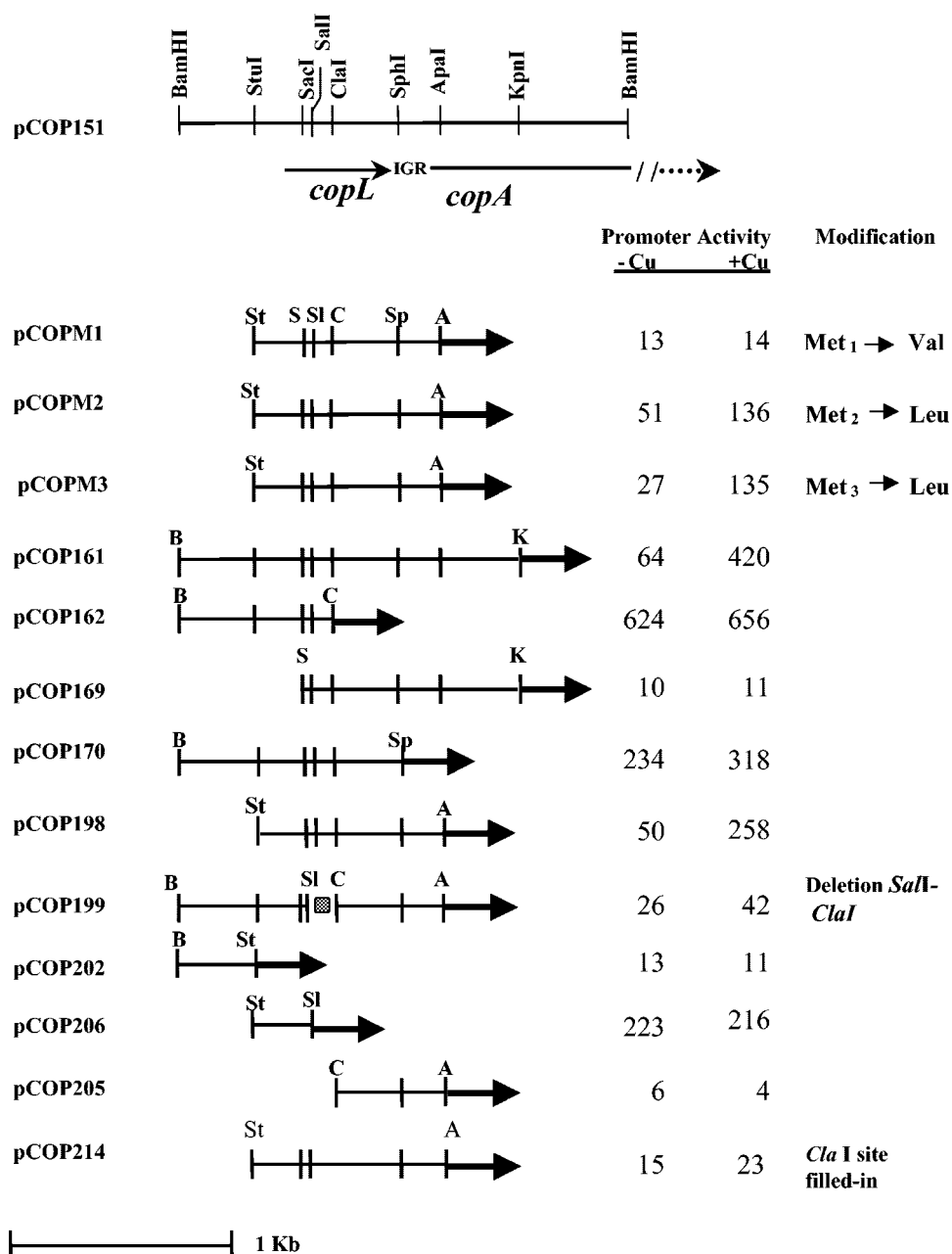


FIG. 2. Localization and deletion analysis of the 1.9-kb BamHI fragment containing *copL* and the 5' end of *copA* from *X. axonopodis* pv. *vesicatoria* strain 7882. All constructs were fused to *lacZ* in pMP190 and introduced into strain 7882. Vertical lines indicate important restriction sites. Promoter activity was measured as β -galactosidase activity (Miller units). β -Galactosidase assays for the three methionine replacement mutants—pCOPM1, pCOPM2, and pCOPM3—were carried out at a different time than the assays for the other mutants. The copper-inducible control for the methionine replacement mutants was pCOP197, which, in this assay, gave 42 Miller units for uninduced cells and 152 Miller units for induced cells. Alterations to restriction sites, deletions, and point mutations are indicated in the right-hand column. Abbreviations: St, StuI; S, SacII; Sl, Sall; C, ClaI; Sp, SphI; A, ApaI; K, KpnI. IGR, intergenic region.

MIC level of 0.6 mM obtained for the Cu^s strains 78518 and 7882.1.

Specific induction of the copper resistance determinants by copper. Of 22 different metal salts tested for induction of the copper resistance promoter-*lacZ* reporter construct (pCOP198) in *X. axonopodis*, only the copper salt (CuSO₄) resulted in a large increase in β -galactosidase production (data not shown). This

indicated that *copA* transcription is induced specifically by copper ions.

Complementation analysis of mutations in *copL*. Plasmids containing an intact, copper-inducible *copL-copA* region transcriptionally fused to *lacZ* (pCOP198; Fig. 2) or several derivatives with deletions (pCOP199 and pCOP205; Fig. 2) or a frame-shift mutation (pCOP214; Fig. 2) in *copL* were introduced

individually into strain 7882, 78518.2, or 78518(pCOP138). All three recipient strains contained intact *copL* and structural copper resistance genes, and thus the *copL* mutations were expected to be complemented in *trans*. However, none of the *copL* mutants was complemented in any of these backgrounds. The presence of the expected plasmids of these constructs was confirmed by plasmid isolation, electrophoresis, and Southern blotting. The integrity of the deletion and other mutations was confirmed by sequencing. In addition, introduction of *copL* on pCOP161 into the mutant strain 7882.3 containing a kanamycin resistance gene cassette inserted in *copL* failed to restore copper resistance to this strain (data not shown).

Specificity of promoter expression in *Xanthomonas*. The StuI-ApaI fragment of pCOP198 (Fig. 2), containing *copL* and the 5' end of *copA* transcriptionally fused to *lacZ*, showed copper-dependent expression of *copA* in both copper-resistant and copper-sensitive strains of *X. axonopodis* pv. *vesicatoria* (data not shown). A similar result was obtained when the construct was introduced in *X. axonopodis* pv. *vignicola*, *X. arboricola* pv. *pruni*, *X. axonopodis* pv. *phaseoli*, and a pectolytic xanthomonad (data not shown). However, no *copA* expression could be detected when this fragment or the subclone pCOP162 (Fig. 2) was introduced into *E. coli* strain DH5 α or into *P. syringae* pv. *syringae*, although pCOP162 showed constitutive *copA* expression in *Xanthomonas*. Similarly, when pCOP198 was introduced in strains PT23.2 (4) and PT12.2 (4) of *P. syringae* pv. *tomato*, which are known to contain *trans*-acting factors for the expression of the *Pseudomonas cop* promoter, there was no copper-dependent expression of *copA*.

In addition, we have shown previously (34) that the copper-inducible *cop* promoter from *P. syringae* was not expressed in *Xanthomonas*. In the present study, when we introduced plasmids containing *copL* (pCOP138) or *copRS* from *P. syringae* (pDAC102) into *X. axonopodis* strain 78518 that also carried pCOP38, a transcriptional fusion of the *P. syringae cop* promoter with *lacZ*, no β -galactosidase activity was detected, showing that the *P. syringae cop* promoter was still not active.

Expression of the *Pseudomonas cop* operon in *Xanthomonas* under the control of the *Xanthomonas cop* promoter. We constructed a transcriptional fusion of the constitutive *copL* promoter (included in the StuI-SaII fragment of pCOP206, Fig. 2) from *X. axonopodis* with the *P. syringae cop* operon. As a result, the recombinant DNA conferred copper resistance in *Xanthomonas* due to the expression of the *Pseudomonas cop* operon. The MIC of 78518(pCOP189) was 1.8 mM compared to 0.6 mM for the Cu^s 78518 (55).

DISCUSSION

Transposon mutagenesis of cloned copper resistance determinants in *X. axonopodis* pv. *vesicatoria* identified an area responsible for their transcriptional control (55). Sequencing of this region revealed an ORF of 369 bases coding for a protein of 122 amino acids, which was named CopL. Downstream of *copL* was found the 5' end of another ORF coding for a gene homolog to the multicopper oxidase *copA*. ORFs similar to *copL* could be found upstream of *copAB* in *X. campestris* pv. *campestris*, *X. axonopodis* pv. *citri*, and *Xylella fastidiosa*. The deduced amino acid sequence of CopL and other CopL-like sequences showed they have numerous cysteine and histidine residues, suggesting they may have a role in binding copper ions. However, no other recognizable motif identified thus far in proteins were present. CopL does not appear to be a negative regulator since its inactivation by marker exchange mutagenesis and point mutations did not result in a constitutive expression of *copA*. The size of CopL (122 amino acids) is similar to that of response regulators of many other bacterial regulatory systems (for a review, see reference 21). Other similarities to these regulators include a pair of aspartate residues near the N terminus (positions 34 and 36) and an aspartate residue at position 100 that is within a motif (LALGLDVMPLG) with some similarity to conserved motifs surrounding the aspartate phosphorylation site normally found near position 55. However, CopL is lacking the lysine (K) that is commonly present near the C terminus of response regulators (21).

Transcriptional analysis revealed that *copL* was transcribed constitutively, whereas *copA* was expressed only in the presence of copper. No *cop* box similar to the sequence found in the promoter area of the copper resistance genes in *P. syringae* was present, and no sequence previously determined to function as a promoter in *Xanthomonas* could be found in the intergenic region between *copL* and *copA*. This intergenic region does not reveal the presence of either an intrinsic terminator or a Rho-dependent terminator. However, the implementation of full copper resistance is dependent on the presence of an intact *copL* gene, coupled with the ability to translate CopL. In addition, the transcriptional control of *copA* in the presence of copper is also dependent on the sequence downstream of the SphI site located between *copL* and *copA*. The precise role of the intergenic region in the copper-dependent response of the downstream genes and its relationship to CopL activity remain to be determined.

The newly sequenced genomes of *X. axonopodis* pv. *citri* and *X. campestris* pv. *campestris* show the presence of three ORFs similar to *copL* and *copAB*. It was proposed that these chromosomal copies may have other functions than copper resistance. Another remarkable feature uncovered by sequencing the genomes of *X. axonopodis* pv. *citri* and *X. campestris* pv. *campestris* is the abundance of insertion sequence elements. A total of 109 insertion sequence elements were identified in *X. campestris* pv. *campestris*, and 87 were identified in *X. axonopodis* pv. *citri*. Mobile genetic elements have shown to be involved in the dissemination of antibiotic and copper resistance genes in *P. syringae* pv. *syringae* (50). Likewise, these elements could facilitate the transfer of the chromosomal copies of *copL*- and *copAB*-like genes to a plasmid. The involvement of plasmids in disseminating certain genes in plant pathogens has been largely documented (54). Eventually, the plasmid-borne copies of the structural genes would evolve other modes of regulation of their expression. The transcriptional fusions of *X. axonopodis copL* and truncated *copA* to the reporter gene β -galactosidase showed copper-dependent transcription of *copA* in all of the xanthomonads tested but not in *P. syringae* or *E. coli*. In addition, we have shown previously (34) that the copper-inducible *cop* promoter from *P. syringae* was not expressed in *Xanthomonas*. In the present study, the *cop* promoter from *P. syringae* was still not expressed in *Xanthomonas* when the *P. syringae copRS* regulatory genes, or *copL*, were supplied in *trans*. The *Pseudomonas cop* operon was found to

be functional in *Xanthomonas*, since a transcriptional fusion of the *X. axonopodis cop* promoter with the *P. syringae cop* operon provided resistance to the Cu^s strain 78518. This supports a functional conservation of the structural *cop* genes, but the mechanisms of regulation of *cop* genes have clearly diverged in these related genera of bacteria. One system has a copper-inducible two-component signal transduction mechanism, and the other is dependent on the *copL* regulatory gene.

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