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Functional Characterization of *copA* Gene Encoding Multicopper Oxidase in *Xanthomonas campestris* pv. *campestris*

Yi-Min Hsiao*

Department of Medical Laboratory Science and Biotechnology, Central Taiwan University of Science and Technology, Taichung 406, Taiwan

Yu-Fan Liu

Department of Biomedical Sciences, Chung Shan Medical University, Taichung 402, Taiwan

Pei-Yu Lee

Institute of Medical Biotechnology, Central Taiwan University of Science and Technology, Taichung 406, Taiwan

Pei-Chi Hsu

Institute of Medical Biotechnology, Central Taiwan University of Science and Technology, Taichung 406, Taiwan

Szu-Yu Tseng

Institute of Medical Biotechnology, Central Taiwan University of Science and Technology, Taichung 406, Taiwan

Yu-Chien Pan

Department of Medical Laboratory Science and Biotechnology, Central Taiwan University of Science and Technology, Taichung 406, Taiwan

ABSTRACT: The Gram-negative plant pathogenic *Xanthomonas campestris* pv. *campestris* (Xcc) is the causative agent of black rot in crucifers, a disease causing tremendous loss in agriculture. Copper-containing bactericides have been widely used to control this disease for many years, possibly leading to the development of copper resistance in Xcc. Homologues of copper resistance genes *copLAB* are present in the Xcc genome, but none has been characterized. In this study, mutations in *copL*, *copA*, and *copB* decreased Xcc copper tolerance. Among them, the *copA* mutant displayed the most significant reduction. The *copA* mutant also resulted in a reduction in virulence on the host cabbage. Sequence and mutational analysis demonstrated that *copA* encodes a multicopper oxidase and that CopA is able to catalyze the oxidation of 2,6-dimethoxyphenol. Alanine substitutions in each of the putative copper binding residues (H538, H583, C584, and H585) of CopA caused a loss of function including copper tolerance and oxidase activity. Furthermore, reporter assays showed that *copA* transcription is inducible in the presence of copper, subject to catabolite repression, and repressed under conditions of high osmolarity, nitrogen starvation, or oxygen limitation. This is the first time that multicopper oxidase has been characterized in the crucifer pathogen Xcc.

KEYWORDS: Multicopper oxidase, site-directed mutagenesis, reporter assay, *Xanthomonas*

INTRODUCTION

Copper compounds have been used to control bacterial and fungal diseases of agricultural crops for more than 100 years. This has favored the spread of copper resistance genes among saprophytic and plant pathogenic bacteria.[1,2] Subsequently, copper resistance has been described in several bacterial species that have been exposed to high levels of copper in agricultural environments, such as *Pseudomonas*[3−5] and *Xanthomonas*.[6,7]

P. syringae pv. *tomato*, a pathogen of tomatoes, is the first phytopathogen for which the copper resistance mechanism has been characterized at a molecular level.[3] Copper resistance genes in this organism are located on a 35-kb mobilizable plasmid pPT23D.[8] DNA encoding copper resistance has been cloned and sequenced from pPT23D and shown to contain four genes, designated *copABCD*, controlled by a copper-inducible promoter.[8−10] They are widespread in other *Pseudomonas* species, such as *P. cichorii*, *P. putida*, and *P. fluorescens*.[2] Deletion and site-specific frameshift mutation analysis suggest that *copA* and *copB* are essential for resistance. Although *copC* and *copD* are required for

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Table 1. Bacterial Strains and Plasmids Used in This Study[a]

	description	reference or source
E. coli		
DH5α	F$^-$ *endA1 hsdR17* (r_K^-) *supE44 thi-1* λ^- *recA1 deoR gyrA96 relA1* Δ(*argF-lacZYA*)*U169* ϕ80d*lacZ*DM15	58
BL21(DE3)	F$^-$ *ompT hsd*S_B ($r^-_B m^-_B$) *gal dcm* (DE3)	novagen
X. campestris pv. *campestris*		
Xc17	Virulent wild type strain isolated in Taiwan, Ap^r	37
CP17A	Xc17-derived mutant with a Gm^r cartridge inserted in *copA* gene, AP^r, Gm^r	this study
CP17B	Xc17-derived mutant with a Gm^r cartridge inserted in *copB* gene, AP^r, Gm^r	this study
CP17L	Xc17-derived mutant with a Gm^r cartridge inserted in *copL* gene, AP^r, Gm^r	this study
Plasmid		
yT&A	PCR cloning vector, Ap^r	yeastern
pTcopA	A 1853 bp RCR amplified fragment from *copA* (nucleotides −43 to +1810 relative to the translation start site) and cloned into yT&A	this study
pTcopAH538A	pTcopAderivative carrying an H → A mutation at position 538 of CopA	this study
pTcopAH583A	pTcopAderivative carrying an H → A mutation at position 583 of CopA	this study
pTcopAC584A	pTcopAderivative carrying a C → A mutation at position 584 of CopA	this study
pTcopAH585A	pTcopAderivative carrying an H → A mutation at position 585 of CopA	this study
pET30b	expression vector, Km^r	novagen
pETcopA	pET30b derivative carrying the 1806 bp *Nde*I-*Xho*I fragment of the Xc17 *copA* gene	this study
pOK12	*E. coli* general cloning vector, P15A *ori*, *lacZ*α fragment, Km^r	26
pOKcopA	pOK12 derivative carrying the 1806 bp *Nde*I-*Xho*I fragment of the Xc17 *copA* gene	this study
pOKcopB	pOK12 derivative carrying the 1482 bp *Nde*I-*Xho*I fragment of the Xc17 *copB* gene	this study
pOKcopL	pOK12 derivative carrying the 517 bp *Xho*I-*Xba*I fragment of the Xc17 *copL* gene	this study
pUCGM	Small broad-host-range Gm^r cartridge contained in pUC1918, a pUC19 derivative, Ap^r, Gm^r	27
pOKcopAG	the 889 bp *Hinc*II fragment of the pUCGM cloned into the *Hinc*II site of pOKcopA	this study
pOKcopBG	the 901 bp *Pst*I fragment of the pUCGM cloned into the *Pst*I site of pOKcopB	this study
pOKcopLG	the 901 bp *Pst*I fragment of the pUCGM cloned into the *Pst*I site of pOKcopL	this study
pRK415	Broad-host-range vector, RK2 *ori*, Tc^r	28
pRKcopA	the 1853 bp *Xba*I-*Eco*RI fragment of the pTcopA cloned into the *Xba*I and *Eco*RI sites of pRK415	this study
pRKcopAH538A	the 1853 bp *Xba*I-*Eco*RI fragment of the pTcopAH538A cloned into the *Xba*I and *Eco*RI sites of pRK415	this study
pRKcopAH583A	the 1853 bp *Xba*I-*Eco*RI fragment of the pTcopAH583A cloned into the *Xba*I and *Eco*RI sites of pRK415	this study
pRKcopAC584A	the 1853 bp *Xba*I-*Eco*RI fragment of the pTcopAC584A cloned into the *Xba*I and *Eco*RI sites of pRK415	this study
pRKcopAH585A	the 1853 bp *Xba*I-*Eco*RI fragment of the pTcopAH585A cloned into the *Xba*I and *Eco*RI sites of pRK415	this study
pFY13-9	promoter-probing vector derived from pRK415, using *lacZ* as the reporter, Tc^r	34
pFYcop692	the 692-bp fragment, −545/+147 relative to *copA* translation start site, cloned into the *Xho*I/*Xba*I sites of pFY13-9	this study
pFYcop517	the 517-bp fragment, −545/−29 relative to *copA* translation start site, cloned into the *Xho*I/*Xba*I sites of pFY13-9	this study
pFYcop491	the 491-bp fragment, −344/+147 relative to *copA* translation start site, cloned into the *Xho*I/*Xba*I sites of pFY13-9	this study
pFYcop316	the 316-bp fragment, −344/−29 relative to *copA* translation start site, cloned into the *Xho*I/*Xba*I sites of pFY13-9	this study

[a] Ap^r, ampicillin-resistant; Gm^r, gentamycin-resistant; Km^r, kanamycin-resistant; Tc^r, tetracycline-resistant.

full resistance, low-level resistance is conferred with the presence of only *copA* and *copB*. Immediately downstream from *copABCD* are two genes, *copS* and *copR*, which encode a two-component regulatory system involved in copper inducible expression of *copABCD*.[11]

In *Xanthomonas* species, copper resistance determinants have been described in *X. axonopodis* (formerly *campestris*) pv. *vesicatoria* strain 7882,[2,6] *X. arboricola* (formerly *campestris*) pv. *juglandis* strain C5,[7] and *X. axonopodis* pv. *citri* strain 306.[12] Copper resistance genes in *X. axonopodis* pv. *vesicatoria* strain 7882 are plasmid encoded, while those in *X. arboricola* pv. *juglandis* strain C5 and *X. axonopodis* pv. *citri* strain 306 are located on the chromosome. In *X. axonopodis* pv. *vesicatoria* strain 7882, the copper resistance genes *copLABMGF* have been cloned and sequenced but not studied further. It is only known that *copL* is required for copper-inducible expression of the downstream *copA*.[1] In *X. arboricola* pv. *juglandis* strain C5, four copper resistance genes encoding open reading frames ORF1 to ORF4 have been cloned and sequenced with the same general *copABCD* structure as the genes from *P. syringae*.[7] Similar to *P. syringae*, the four ORFs are required for the full expression of copper resistance, and ORF1 and ORF2 are essential for copper resistance.[7] The 3′ end of *copL* of *X. axonopodis* pv. *vesicatoria* strain 7882 is also present in the published sequence of the copper resistance operon from *X. arboricola* pv. *juglandis* strain C5,[7] but no sequences further upstream were included in that study. In *X. axonopodis* pv. *citri* strain 306, the *copLAB* homologues have been identified in the completed genome sequence,[13] and inactivation of the *copAB* operon results in copper sensitivity.[12] In a more recent report, genes encoding copper resistance were cloned from

Table 2. PCR Primers Used in This Study

primer	sequence[a]	direction and use[b]
1200XhoI	5′-CTCGAGATGGTATGCTCGGTGTACCT-3′	F, mutant construction, confirmation and promoter analysis
1401XhoI	5′-CTCGAGGTGTCATGAAGACATGGCCT-3′	F, promoter analysis
1702XbaI	5′-TCTAGATGCGCGTTGCCTGCACCGT-3′	F, complementation
1716XbaI	5′-TCTAGACAGGCAACGCGCATCGCAAT-3′	R, mutant construction, confirmation and promoter analysis
1745NdeI	5′-CATATGTCATTCGATCCCTTGTCC-3′	F, mutant construction, confirmation and overexpression
1891XbaI	5′-TCTAGAACGCAGGACGGCGGGCGT-3′	R, promoter analysis
3550NdeI	5′-CATATGAACGCCATGCAGACCGCTC-3′	F, mutant construction and confirmation
3550XhoI	5′-CTCGAGTGCCTCCACCCGCACTT-3′	R, mutant construction, confirmation and overexpression
3554*Eco*RI	5′-GAATTCATGCCTCCACCCGCACTTCG-3′	R, complementation
5031XhoI	5′-CTCGAGGAACCACACCCGCACGCC-3′	R, mutant construction, confirmation
H538AF	5′-GCAGCACCCCATC**GCC**CTGCACGGCATG-3′	F, point mutation
H583AF	5′-CCGCTGGGCCTAC**GCC**TGCCATCTGCTC-3′	F, point mutation
C584AF	5′-GCTGGGCCTACCAC**GCC**CATCTGCTCTACC-3′	F, point mutation
H585AF	5′-CTGGGCCTACCACTGC**GCT**CTGCTCTACCACATG-3′	F, point mutation
H538AR	5′- CATGCCGTGCAG**GGC**GATGGGGTGCTGC-3′	R, point mutation
H583AR	5′-GAGCAGATGGCA**GGC**GTAGGCCCAGCGG-3′	R, point mutation
C584AR	5′-GGTAGAGCAGATG**GGC**GTGGTAGGCCCAGC-3′	R, point mutation
H585AR	5′-CATGTGGTAGAGCAG**AGC**GCAGTGGTAGGCCCAG-3′	R, point mutation

[a] Added restriction enzyme sites are underlined. The mutated bases are in boldface and underlined. [b] F, forward direction; R, reverse direction.

X. citri subsp. *citri* strain A44 and *X. alfalfae* subsp. *citrumelonis* strain 1381.[14] ORFs related to the genes *copL*, *copA*, *copB*, *copM*, *copG*, *copC*, *copD*, and *copF* were identified in *X. citri* subsp. *citri* strain A44. The same ORFs, except for *copC* and *copD*, were also present in *X. alfalfae* subsp. *citrumelonis* strain 1381. *X. citri* subsp. *citri* strain A44 *copL*, *copA*, and *copB* appear to play a role in copper resistance. Cloning and characterization of both plasmid and chromosomal copper resistance genes from these pathogens have shown that most are related to each other. They are also related to the *cop* operon from *P. syringae*, namely, homologous to *copA* and *copB*.[7,12,14,15]

X. campestris pv. *campestris* (Xcc) is the causative agent of black rot in crucifers, a disease causing tremendous loss in agriculture.[16] Copper-containing bactericides have been used for many decades to reduce the severity of this disease. However, no copper resistance has been reported in this bacterium. In the fully sequenced Xcc genome, there is a chromosomal locus, *copLAB*, which shares nucleotide sequence similarity with copper resistance genes from *X. axonopodis* pv. *citri*, *X. axonopodis* pv. *vesicatoria*, and *X. arboricola* pv. *juglandis*. The biological functions of these genes have not been studied. The aims of the present work are to characterize these putative copper resistance genes in Xcc.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Media, and Culture Conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Luria−Bertani (LB) medium[17] was the general-purpose medium for cultivating *E. coli* and Xcc at 37 and 28 °C, respectively. For measurements of cell growth and promoter activity, XOLN and XVM2 media were used. The compositions of XOLN and XVM2 media are given elsewhere.[18,19] The stress conditions have been previously described.[20,21] Briefly, for high osmolarity, 0.3 M NaCl was added. For nitrogen starvation, tryptone, and yeast extract were omitted. For oxygen limitation, the cultures were supplemented with fumarate (0.25%) as an electron acceptor, overlaid with paraffin oil, and left to stand. For oxidative stress, 0.2 mM H_2O_2 was added. The following antibiotics were added when necessary: ampicillin (50 μg/mL), kanamycin (50 μg/mL), gentamycin (15 μg/mL), and tetracycline (15 μg/mL). Liquid cultures were shaken at 220 rpm. Solid media contained 1.5% agar.

DNA Techniques. Enzymes were purchased from Promega and Roche. Standard protocols have been described elsewhere.[22] PCR was carried out as previously described[23] using the primers listed in Table 2. DNA sequences on both strands were determined by the dideoxy chain termination method.[24] Transformation of *E. coli* was performed by the standard method[22] and that of Xcc by electroporation.[25]

Construction of *copA*, *copB*, and *copL* Mutants. Procedures for the construction of nonpolar *copA* mutant CP17A were as follows: the 1,806-bp *Nde*I-*Xho*I fragment containing the Xc17 *copA* gene was PCR amplified using the primer pair 1745NdeI/3550XhoI and cloned in *E. coli* vector pOK12,[26] which contained P15A *ori* and could not be maintained in Xcc, giving pOKcopA. A Gm^r cartridge from pUCGM[27] was inserted into the *Hin*cII site within the pOKcopA insert. The Gm^r cartridge was transcribed in the opposite direction relative to that of *copA* and did not possess the transcriptional terminator. The resultant plasmid, pOKcopAG, was electroporated into Xc17 allowing for double crossover via homologous recombination through the identical regions in the chromosome and the plasmid to replace the wild-type sequence. Similar methods were applied to generate the *copB* mutant CP17B and the *copL* mutant CP17L. Briefly, 1,482-bp and 517-bp DNA fragments containing *copB* and *copL* were amplified by PCR with primer pairs 3550NdeI/5031XhoI and 1200XhoI/1716XbaI, and cloned into pOK12 to give pOKcopB and pOKcopL, respectively. A Gm^r cartridge from pUCGM was inserted into the unique *Pst*I site within the pOKcopB and pOKcopL inserts, giving rise to pOKcopBG and pOKcopLB. Insertion of the Gm^r cartridge into the target gene was confirmed by PCR.

Complementation of *copA* Mutant. The 1,853-bp *Xba*I-*Eco*RI fragment encompassing the upstream 43 bp fragment plus the entire coding region of the Xc17 *copA* was PCR amplified using the primer pair 1702XbaI/3554*Eco*RI and ligated into the cloning vector yT&A (Yeastern), giving pTcopA. After sequence confirmation, the fragment

was excised from pTcopA with *Xba*I and *Eco*RI and cloned into the *Xba*I-*Eco*RI sites of the broad-host-range vector pRK415,[28] generating pRKcopA. This construct contained the *copA* gene downstream, oriented in the same direction as the *lac* promoter. For complementation of the *copA* mutant, plasmid pRKcopA was electroporated into the mutant CP17A.

Site-Directed Mutagenesis. Alanine substitution mutations were produced in CopA using the QuikChange site-directed mutagenesis kit from Stratagene according to the manufacturer's instructions. The mutations were constructed in the highly conserved copper binding motif of CopA (H538A, H583A, C584A, or H585A) using pTcopA as a template and the primers listed in Table 2. After DNA sequencing verification, the mutated *copA* was cloned into pRK415 to give pRKcopAH538A, pRKcopAH583A, pRKcopAC584A, and pRKcopAH585A. The constructs were then separately transferred to strain CP17A by electroporation.

Pathogenicity Test. The virulence of Xcc in cabbage was estimated after bacteria were introduced into the leaves by the leaf clipping method.[20] Lesion lengths were measured 14 days post-inoculation. Three independent experiments with six replicates were carried out.

Virulence Factor Determination. Exopolysaccharide production was measured as previously described.[29] Assays of the activities of extracellular protease and cellulase were performed on agar plates containing the appropriate substrate according to published methods.[30]

Cell Extract Preparation. Bacteria were grown overnight in LB medium and diluted with fresh LB (with or without 0.1 mM $CuSO_4$) to OD_{550} = 0.35, after which growth was allowed to continue. After 16 h, the cultures were centrifuged at 12000*g* at 4 °C for 2 min. The cells were then rinsed and responded in 0.1 M sodium phosphate buffer (pH 5.0). Cell extracts were obtained by sonication (cycles of 10 s pulse and 10 s rest on ice for 2 min). Following sonication, the cell debris and intact cells were removed by centrifugation, and the supernatant fraction was collected and used for the multicopper oxidase activity assay.

Multicopper Oxidase Activity Analysis. Qualitative evaluation of multicopper oxidase activity was performed on agar plates containing 2,6-dimethoxyphenol (DMP, Sigma) as substrate as previously described[31] with some modifications. Plates were prepared using a concentration of 1% agarose, 2 mM DMP in 0.1 M sodium phosphate buffer (pH 5.0), and 0.1 mM $CuSO_4$. Holes were made on the plates using the end of a pipet, and 10 μL of the sample was pipetted into each hole. Plates were incubated in the dark at 37 °C for 2 h, and the formation of an orange ring around each hole was assayed by visual inspection. Each experiment was performed at least three times with similar results. Quantitative assay of the multicopper oxidase activity was performed using DMP as the substrate according to a published method.[32] Briefly, an assay mixture (0.9 mL) consisting of 2 mM DMP in 0.1 M sodium phosphate buffer, pH 5.0, and 0.1 mM $CuSO_4$ was added to a 1.5 mL microcentrifuge tube. The reaction was started by adding 0.1 mL of an enzyme sample to the tube placed in a 37 °C water bath. After 10 min of incubation, the increase in absorbance of the mixture was measured at 468 nm and compared with that of a blank run under the same conditions. The optical density coefficient of the quinonic oxidized product, 3,3′,5,5′-tetramethoxydiphenylquinone, is 14800 M^{-1} cm^{-1}. One unit of multicopper oxidase activity was defined as the amount of enzyme needed to produce 1 μmol of 3,3′,5,5′-tetramethoxydiphenylquinone per min at 37 °C under the assay conditions. Protein contents were determined by the method of Bradford[33] using a protein assay reagent purchased from Bio-Rad Laboratories (Hercules) with BSA as the standard.

Copper Tolerance Assay. Strains to be assayed were grown in LB or XVM2 medium supplemented with $CuSO_4$ to final concentrations of 0, 100, 200, or 500 μM in LB and 0, 5, 10, 20, or 25 μM in XVM2 and incubated at 28 °C with shaking (220 rpm) for 24 h. Growth was evaluated by measuring OD_{550}.

Production of Recombinant CopA Protein. Plasmid pETcopA was constructed by inserting the 1,806-bp Xc17 *copA* coding region into the *Nde*I-*Xho*I sites of expression vector pET30b (Novagen) digested with the same enzymes. *E. coli* BL21(DE3) carrying pETmanA was grown in LB broth until OD_{595} of 0.5−0.6 was reached. At this point, protein production was induced by the addition of isopropyl thio-β-D-galactoside (IPTG) to a final concentration of 1 mM. Alternatively, pETcopA was used as a template to express the cloned *copA* with the S30 T7 High-Yield Protein Expression System from Promega following the manufacturer's instructions. For a negative control, pET30b was used to clarify the protein expression background on oxidase activity analysis. The reaction was incubated at 28 °C with shaking (220 rpm) for 1 h. The oxidase activity of synthesized protein was evaluated on a DMP-supplemented agar plate.

Promoter Activity Assay. Four reporter constructs were generated by cloning PCR fragments into the broad-host-range-promoter-probing vector pFY13-9, which used *lacZ* as the reporter.[34] Constructs pFYcop692, pFYcop517, pFYcop491, and pFYcop316 carried nt −545/+147, −545/−29, −344/+147, and −344/−29 regions relative to the *copA* translation start site, respectively. Xc17 harboring these constructs were grown overnight and inoculated into fresh media to obtain an initial OD_{550} of 0.35, after which growth was allowed to continue. Samples were taken in triplicate at designated intervals, and β-galactosidase activity was assayed as previously described, with the enzyme activity expressed in Miller units.[17]

Bioinformatic Analysis. Multiple sequence alignment of selected multicopper oxidases was generated with the ClustalX package. The three-dimensional structure of Xcc CopA was built by homology modeling using ModBase standard process. The template protein was *Cucurbita pepo* ascorbate oxidase (PDB code 1AOZ) with 27% shared identity with Xcc CopA. The dataset is TIMbarrel_templates, and the Modpipe version is 1.0. The figures were drawn with the program AccylyrsTM ViewerLite 4.2.

Statistical Analysis. Values are the means of three technical replicates per experiment, and each experiment was performed at least three times. Student's *t*-test was used to determine the statistical significance of differences between means.

RESULTS

***copLAB* Homologues Are Involved in Xcc Copper Tolerance.** To date, complete genomic sequences of three Xcc strains (ATCC33913, 8004, and B100) have been deposited in a public database.[13,35,36] A survey of the genome sequence data of Xcc reveals three genes (*copL*, *copA*, and *copB*) with homology to copper resistance-related genes in the fully sequenced genomes of these Xcc strains. The *copLAB* genes sit side by side on the chromosome and have the same transcriptional orientation. The ORF numbers of these genes in Xcc strains ATCC33913, 8004, and B100, respectively, are XCC0579, XC_3654, and xccb100_3773 for *copL*, XCC0577, XC_3656, and xccb100_3774 for *copA*, and XCC0576, XC_3657, and xccb100_3775for *copB*.[13,35,36] There are also homologues, XC5811, XC1624, and XC5812, in the genome sequence of strain Xc17. The genome sequence of this strain is almost complete (http://xcc.life.nthu.edu.tw).

To determine the function of *copLAB* in Xcc, a nonpolar mutant of each of the genes was constructed by mutagenizing the wild type strain Xc17 using homologous recombination (see the Materials and Methods section for details). The obtained mutants were designated CP17L, CP17A, and CP17B, respectively (Table 1). All strains grew at comparable rates and reached similar final OD_{550} values in the rich medium LB (6.29−6.93) or the defined minimal medium XVM2 (0.98−1.06) in the absence of copper. Differences in growth were observed in the presence of

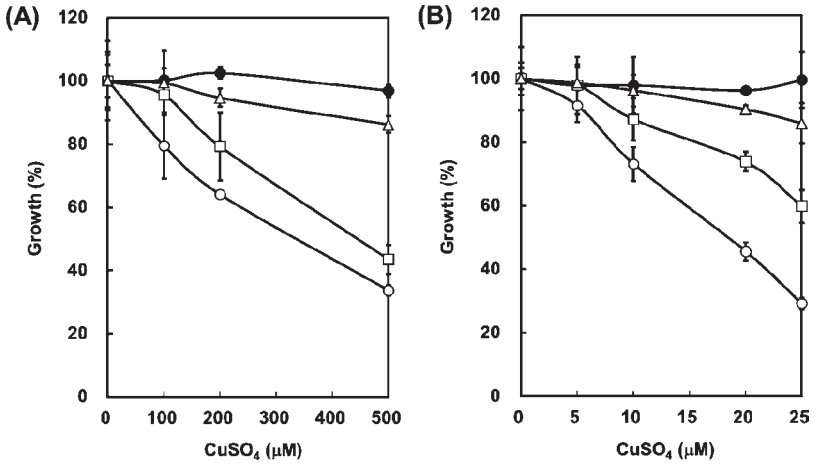

Figure 1. Effect of copper on Xcc growth. Overnight culture (50 μL) of each Xcc strain was inoculated into 2.5 mL of LB (A) or XVM2 (B) liquid medium containing the indicated copper sulfate concentrations. The cell density was measured at OD_{550} after 24 h. Results are the means of at least three independent experiments. The Xc17 (wild-type) is represented by shaded circles, the CP17L (*copL* mutant) by open triangles, the CP17B (*copB* mutant) by open squares, and the CP17A (*copA* mutant) by open circles. Error bars indicate standard deviations.

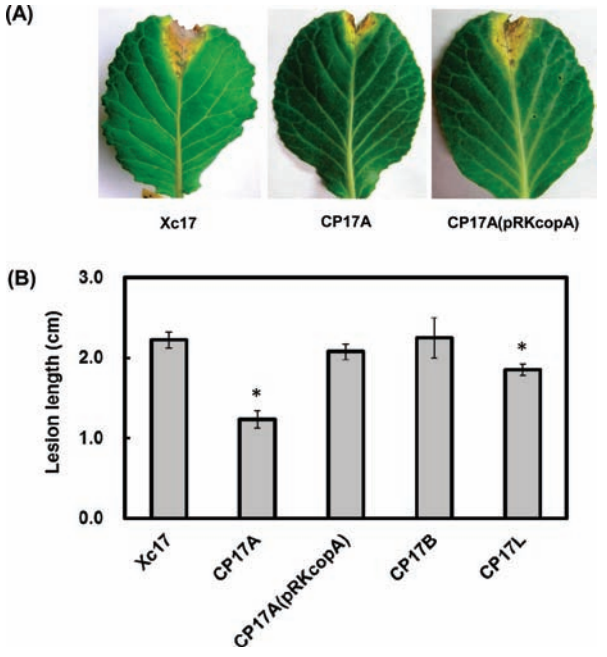

Figure 2. Effects of mutation of *copLAB* on the virulence of Xcc to cabbage. (A) Black rot symptoms caused by Xcc strains on inoculated leaves of the host plant cabbage. Photographs were taken on day 14 post-inoculation. (B) Average lesion lengths caused by Xcc strains. Values presented are the means ± standard deviations from three repeats, each with 6 leaves. Significance was tested by Student's *t* test (* indicates significance at $P < 0.05$).

copper. As observed in Figure 1, wild type Xc17 (shaded circles) grew well in LB supplemented with concentrations of up to 500 μM $CuSO_4$ (OD_{550} = 6.20) and in XVM2 supplemented with concentrations of up to 25 μM $CuSO_4$ (OD_{550} = 1.06). Under the same conditions, the *copL* mutant (CP17L, open triangles) revealed slight growth reduction with 86 and 75% of growth retained in LB supplemented with 500 μM copper (Figure 1A) and XVM2 supplemented with 25 μM copper (Figure 1B), respectively. In parallel experiments, both *copA* mutant (CP17A, open circles) and *copB* mutant (CP17B, open squares) exhibited significant growth reduction with retentions of 34 and 29% of growth for CP17A and 44 and 60% of growth for CP17B in LB and XVM2 in the presence of copper, respectively.

CopA Is Essential for the Full Virulence of Xcc. To investigate the association between *copLAB* and pathogenicity, the virulence of mutants was tested on host plant cabbage by the leaf-clipping method.[37] The rate of development of symptoms around the cuttings of the leaf edges inoculated with CP17L was slightly lower than that of those inoculated with Xc17, and no obvious difference was observed between the *copB* mutant and the wild type (data not shown). The strain with the mutation in *copA* had significantly reduced virulence compared with that of the wild type (Figure 2A,B). Introduction of the cloned gene into the *copA* mutant restored virulence to the wild type level (Figure 2). It has been suggested that insertion into *copA* does not cause a polar effect and does not affect the downstream *copB*. The lesion lengths were about 2.2, 1.2, 2.1, 2.3, and 1.9 cm, respectively, for Xc17 (wild-type), CP17A (*copA* mutant), CP17A(pRKcopA) (complemented), CP17B (*copB* mutant), and CP17L (*copL* mutant) at 14 days after inoculation (Figure 2B).

The virulence of Xcc depends upon a number of factors, including the ability to produce exopolysaccharides and extracellular enzymes (such as protease and cellulase).[38−40] To investigate whether a mutation in *copA* affects these pathogenicity-related factors, the exopolysaccharide yield and the activities of protease and cellulase produced by the *copA* mutant CP17A were determined. The exopolysaccharide productivity was about 1.22 mg/mL for Xc17 and 1.20 mg/mL for CP17A grown in LB media. The effect of *copA* mutation on the production of extracellular enzymes was evaluated on a substrate-supplementary plate assay. The diameters of the colonies formed by different cells on the same plate were similar. The diameters of the clearing zones including the colonies formed by Xc17 and CP17A were 1.76 and 1.72 cm and 2.01 and 1.98 cm, respectively, on skim milk and carboxymethyl cellulose supplementary plates. No significant differences in exopolysaccharide or extracellular enzyme levels were observed between CP17A and Xc17.

These results together with the results of the above-mentioned copper tolerance assay show that CopA plays a much more

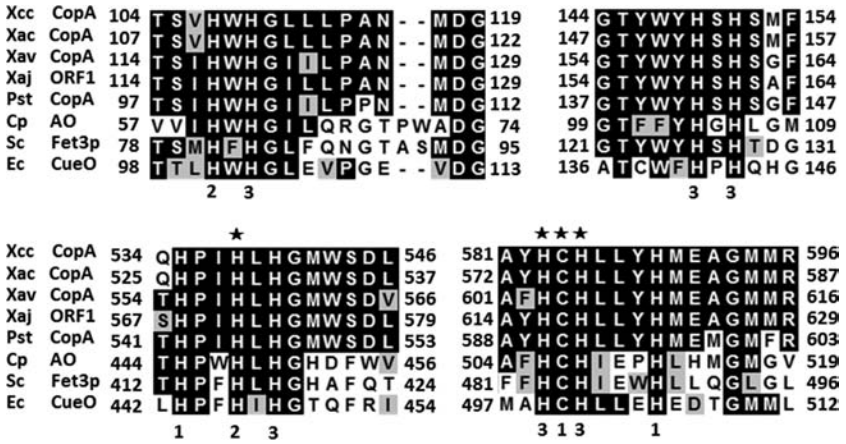

Figure 3. Amino acid sequences conserved among multicopper oxidases. The sequences are as follows: Xcc CopA (*X. campestris* pv. *campestris* CopA, Q8PCX4); Xac CopA (*X. axonopodis* pv. *citri* CopA, Q8PGI6); Xav CopA (*X. axonopodis* pv. *vesicatoria* CopA, Q5F0H7); Xaj ORF1 (*X. arboricola* pv. *juglandis* ORF1, Q56795); Pst CopA (*P. syringae* pv. *tomato* CopA, P12374); Cp AO (*Cucurbita pepo* ascorbate oxidase, 1AOZ); Sc Fet3p (*Saccharomyces cerevisiae* Fet3p, 1ZPU); and Ec CueO (*E. coli* CueO, 1KV7). Identical amino acids are indicated by black shading and similar amino acids by gray shading. Numbers indicate the positions of the amino acid residues of each protein. Potential ligands to the three different types of copper are indicated below the Ec CueO sequence by 1, 2, and 3, respectively. Asterisks above the Xcc CopA sequence denote the mutated residues.

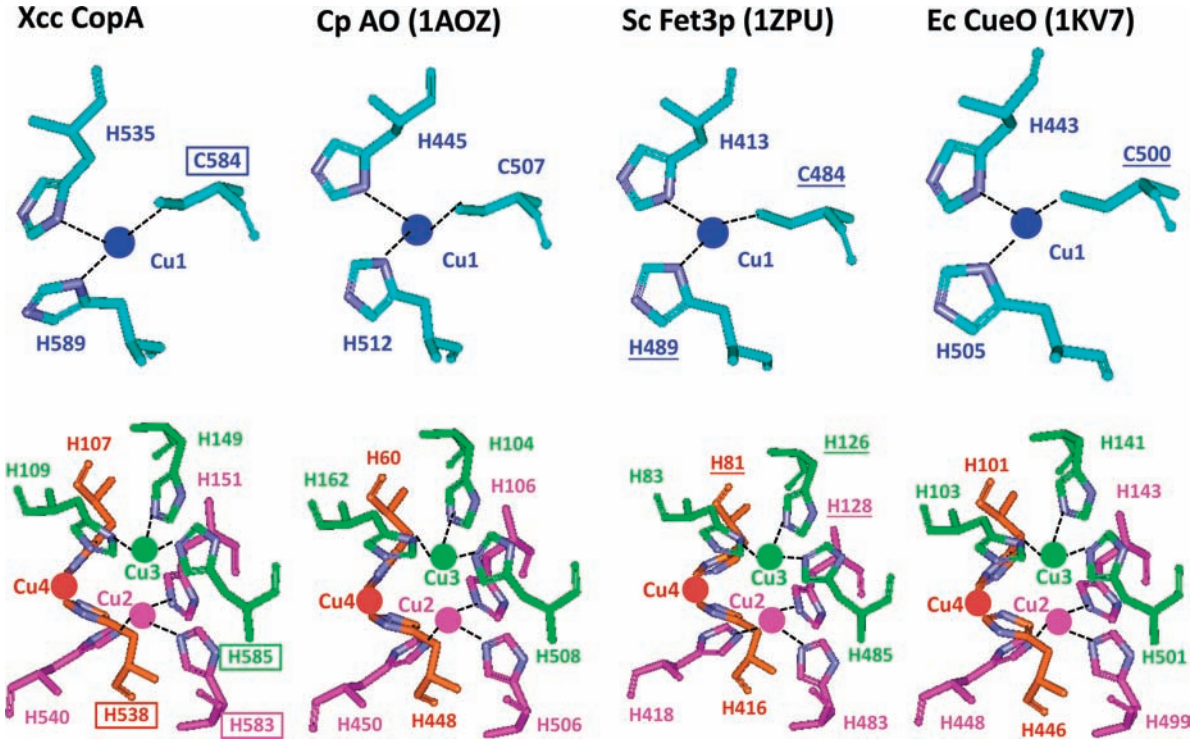

Figure 4. Stereo views showing the geometry of the type 1 (upper) and trinuclear (bottom) copper sites. The proposed Xcc CopA copper centers shown on the left use 1AOZ as the template. Type 1 copper for Cu1 and type 2 copper for Cu4 are represented as blue and brown spheres, respectively. The two type 3 coppers for Cu2 and Cu3 are indicated as pink and green spheres, respectively. Mutated amino acid residues in the present study (C584, H538, H583, and H585) are boxed. The amino acid residues characterized in other multicopper oxidases are underlined.

important physiological role than CopL or CopB in Xcc. Thus, we concentrated our attention on CopA in the following study.

Xcc CopA Is Similar to Other Multicopper Oxidases. Xcc *copA* encodes a protein of 602 amino acids with a calculated molecular mass of 66371 Da and a p*I* of 6.54. In the genome of the Xcc strain ATCC33913, CopA (UniProt accession number Q8PCX4) is annotated as copper resistance protein A and shows 86, 66, 65, and 62% shared identities with the homologous members of *X. axonopodis* pv. *citri* (CopA, Q8PGI6),[12] *X. axonopodis* pv. *vesicatoria* (CopA, Q5F0H7),[1] *X. arboricola* pv. *juglandis* (ORF1, Q56795),[7] and *P. syringae* pv. *tomato* (CopA, P12374),[10] respectively. It also shares ~30% identity with various characterized and crystallized multicopper oxidases from other organisms such as the ascorbate oxidase from *Cucurbita*

pepo (P37064, PDB code 1AOZ),[41,42] the multicopper oxidase Fet3p from *Saccharomyces cerevisiae* (P38993, PDB code 1ZPU),[43] and the blue copper oxidase CueO from *E. coli* (P36649, PDB code 1KV7).[44]

Multicopper oxidases are a family of enzymes typically composed of three plastocyanin-like domains (Cu-oxidase superfamily domains, PSSMID: 187413).[43] Sequence alignments indicate that multicopper oxidases feature four highly homologous regions, consisting of histidine-rich motifs that constitute the copper-binding sites.[45−47] The four highly conserved regions include "HPxHLHG" and "HCHxxxH" motifs at domain 3 as well as "HxH" and "WYHxH" motifs at domain 1 (x: any amino acids). In Xcc CopA, three Cu-oxidase superfamily domains were determined by NCBI Conserved Domains Database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=cdd) and are situated in 56−171 (domain 1, pfam07732), 216−362 (domain 2, pfam00394), and 482−602 (domain 3, pfam07731), respectively. Figure 3 shows amino acid sequence alignments of the four highly homologous regions from Xcc CopA, previously reported copper resistance proteins from other phytopathogens, and some crystallized multicopper oxidases. Also in Figure 3, a total of 10 histidines and 1 cysteine are conserved in multicopper oxidases and are thought to serve as the copper ligands.

The multicopper oxidases contain four copper centers: a type I copper (Cu1) and a trinuclear copper center comprising a type II copper (Cu4) and a type III copper pair (Cu2 and Cu3).[47,48] The type I copper center is located in domain 3, while the trinuclear copper center is embedded between domains 1 and 3 with both domains providing residues for copper coordination.[49] According to the crystal structures and results of mutant analysis, the amino acids responsible for binding the copper atoms in each of the type I, II, and III copper centers have been defined[41−44] and are shown in Figures 3 and 4. The three-dimensional structure of Xcc CopA built by homology modeling revealed a type I copper site (Figure 4, upper) and a trinuclear copper site (Figure 4, bottom), much like the arrangement found in *Cucurbita pepo* ascorbate oxidase, *Saccharomyces cerevisiae* Fet3p, and *E. coli* CueO. In Xcc CopA, it is suggested that (i) H535, C584, and H589 are related to type I copper (Cu1); (ii) H107 and H538 to type II copper (Cu4); and (iii) H109, H149, H151, H540, H583, and H585 to type III copper. Among them, H151, H540, and H583 form a Cu2 binding site, and H109, H149, and H585 form a Cu3 binding site (Figure 4).

Sequence analyses indicated that *copA* likely encodes a functional multicopper oxidase in Xcc. The relationships of conserved domains suggest that CopA, like other multicopper oxidases such as Fet3p and CueO, plays a role in cuprous oxidase activity and/or is essential in ion binding/trafficking to reduce the toxicity from elevated copper levels in the environment. In the present study, some residues involved in copper binding were mutated (C584 for Cu1, H583 for Cu2, H585 for Cu3, and H538 for Cu4), and their effects on copper tolerance and oxidase activity were evaluated.

Xcc CopA Possesses Dimethoxyphenol Oxidase Activity. A variety of multicopper oxidases have been shown to oxidize the substrate DMP producing a colored product.[32] To validate the putative multicopper oxidase activity of CopA, cellular extracts of different Xcc strains were assayed for DMP oxidase activity. Figure 5 reveals that (i) the wild type cells exhibit oxidase activity (29 mU/mg); (ii) the oxidase activity in cell extract from the *copA* mutant is clearly reduced (12 mU/mg); and that (iii) complementation of the *copA* mutant restores DMP oxidase activity (23 mU/mg).

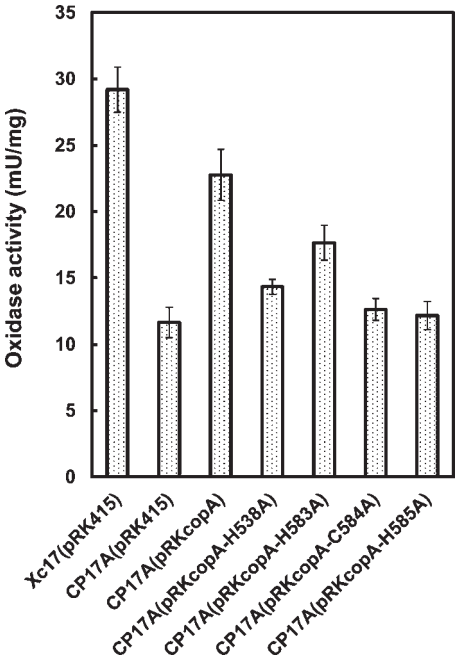

Figure 5. Detection of DMP oxidase activity in different Xcc strains. The *copA* mutant was complemented by in trans expression of the intact *copA* or mutated *copA* in which the conserved residues involved in copper binding were substituted with alanine. Measurement of the DMP oxidase activity is described in Materials and Methods. The experiments were repeated three times for each strain. Error bars indicate standard deviations.

To test the impact of the putative copper ligands described in Figures 3 and 4 on the oxidase activity of CopA, we generated four *copA* derivatives (H538A, H583A, C584A, and H585A) by site-directed mutagenesis. The mutated *copA* was cloned separately into the expression vector pRK415 for in trans expression in the mutant CP17A. While expression of the mutated *copA* (H538A and H583A) in CP17A partially restored DMP oxidase activity, expression of the other *copA* derivatives (C584A and H585A) failed to rescue the oxidase activity (Figure 5). These data suggest that the conserved residues in the copper binding motif are associated with the full activity of CopA.

Residues of CopA Implicated in Copper Binding Are Essential for Copper Tolerance. To determine the effect of the above variations in the copper binding motif on copper tolerance, we also examined the growth ability of CP17A complemented with wild type *copA* or different mutated *copA* in the presence or absence of copper. In trans expression of wild type *copA* in the *copA* mutant resulted in increased copper tolerance compared to that in the mutant strain, whereas expression of the *copA* variant H583A in the same *copA* mutant only partially restored copper tolerance (Figure 6). In addition, the ability to tolerate high copper levels was eliminated when CP17A was complemented with other mutated derivatives including pRKcopAH538A, pRKcopAC584A, and pRKcopA-H585A (Figure 6). These findings established that the copper tolerance function of CopA depends on its conserved amino acid residues in the putative copper binding motif.

Expression of *copA* Is Induced by Copper, Subject to Catabolite Repression, and Affected by Different Stress Conditions. In Xcc strain ATCC33913, the *copLAB* genes are flanked by the upstream *XCC0578* (*cysM*, 1,104 bp encoding a

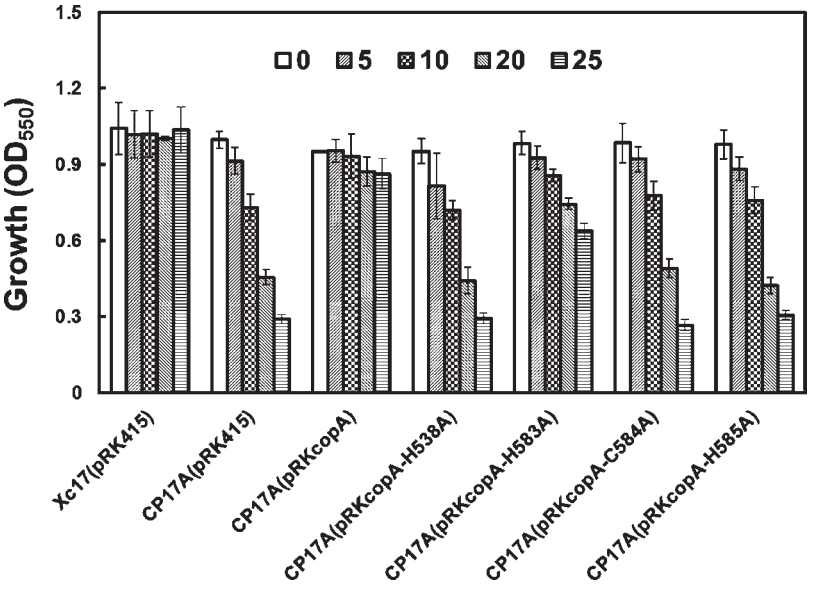

Figure 6. Role of the putative copper binding residues in CopA on Xcc copper tolerance. Overnight culture (50 μL) of each Xcc strain was inoculated into 2.5 mL of XVM2 medium supplemented with different concentrations of copper sulfate. The cell density was measured at OD_{550} after 24 h. Results are the means of at least three independent experiments. Error bars indicate standard deviations.

cysteine synthase) and the downstream *XCC0575* (*gloA*, 525 bp encoding the lactoylglutathionelyase) (Figure 7A).[13] *XCC0578* (*cysM*) is located upstream of *copL* in the opposite direction with a 130 bp intergenic space, whereas *XCC0575* (*gloA*) is located downstream of *copB* in the same orientation with a separation of 523 bp. Analysis of the region upstream of *copA* showed a putative ribosome-binding site (GGAG) 6 nt upstream of the CopA start codon. However, no identifiable promoter region matching the consensus sequence of *Xanthomonas*[50] was found. To characterize the upstream region of *copA*, four P*copA-lacZ* reporter constructs (Figure 7A) were used to assay promoter activity in Xc17 under different culture conditions. In preliminary experiments, β-galactosidase activities increased the following cell growth until about 24−48 h. Therefore, the β-galactosidase levels were measured at 24 h.

As shown in Figure 7B, when $CuSO_4$ was omitted, promoter activities were low in Xc17 carrying pFYcop692. In the presence of copper, significant incremental expression was found (7.2−13.4-fold) in Xc17(pFYcop692). In the case of Xc17(pFYcop517), there was a slight induction in expression (1.2−1.5-fold). On the contrary, the expression levels of Xc17(pFYcop491) and Xc17-(pFYcop316) were similar to those of Xc17(pFY13-9) which contained the empty plasmid (data not shown).

Xc17(pFYcop692) and Xc17(pFYcop517) expressed 611 and 2965 U of β-galactosidase, respectively, when cells were grown in XOLN containing glycerol without copper (Figure 7B). These were 1.7- and 1.9-fold increases, respectively, when compared with that of the glucose supplemented condition (354 and 1560 U). In glucose plus copper, Xc17(pFYcop692) and Xc17-(pFYcop517) expressed 4762 and 2279 U of β-galactosidase, with 89.4 and 62.6% of those levels expressed in glycerol plus copper, respectively. These observations of repression by glucose suggested that the expression of *copA* in Xcc is subject to catabolite repression.

Clp (cAMP receptor protein-like protein) and RpfF (an enoyl-CoA hydratase homologue required for the synthesis of diffusible signal factor, DSF) regulate a range of biological functions, including pathogenicity, multidrug resistance, oxidative stress, and aerobic respiration in Xcc.[51,52] To test for the involvement of Clp and RpfF in the transcription of *copA*, the fusion construct pFYcop692 was introduced into the *clp* mutant AU56E[53] and *rpf*F mutant RM17F,[20] and the resultant strains were subjected to β-galactosidase assays in LB medium with or without copper using Xc17(pFYcop692) for comparison. In the absence of copper, the β-galactosidase levels expressed in AU56E(pFYcop692) and RM17F(pFYcop692) were 329 and 358 U, respectively, similar to the level detected in Xc17-(pFYcop692) in the same medium (361 U, Figure 7B). When copper was present in LB medium, Xc17(pFYcop692), AU56E-(pFYcop692), and RM17F(pFYcop692) expressed 4307, 4064, and 4122 U of β-galactosidase, representing increases of 11.9-, 12.4- and 11.5-fold over the same strains grown without copper, respectively.

To gain more insight into the expression of *copA*, the β-galactosidase activities in Xc17(pFYcop692) and Xc17-(pFYcop517) were determined when cells were grown in XOLN containing glycerol as the carbon source and under a single stress condition (high osmolarity, nitrogen starvation, oxygen limitation, or oxidative stress) for 24 h. The β-galactosidase levels of Xc17(pFYcop692) under high osmolarity, nitrogen starvation, and oxygen limitation were 263 U, 466 U, and 412 U, respectively, or 43%, 76%, and 67% of normal levels (Figure 7C). Also shown in Figure 7C, the β-galactosidase activity under oxidative stress condition was the same as that under normal conditions (611 U vs 604 U). The β-galactosidase levels expressed by Xc17(pFYcop517) were 49%, 74%, 54%, and 105% of those under normal conditions for each of the above stress conditions, respectively (data not shown).

Xcc *copA* Gene Product Displays DMP Oxidase Activity. In order to investigate the enzymatic activities of the product encoded by *copA*, overexpression was induced in *E. coli* BL21-(DE3) carrying pETcopA. Compared with protein samples before IPTG addition, induction with IPTG for different times produced a 66-kDa-protein band (data not shown).

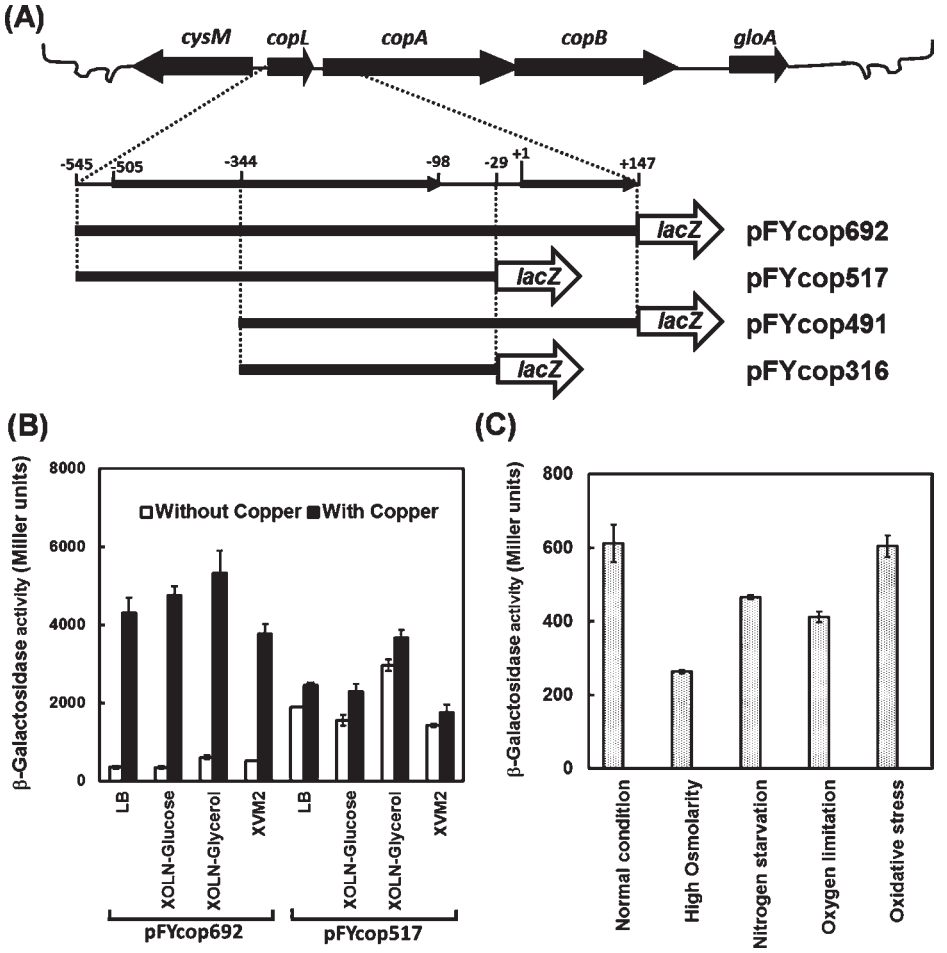

Figure 7. Promoter activities expressed from different fusion constructs in Xc17. (A) Genome organization of the Xcc *copLAB* genes and the upstream regions cloned into the promoter-probing vector pFY13-9 to form fusion constructs pFYcop692, pFYcop517, pFYcop491, and pFYcop316. (B) The promoter activity in Xc17 harboring pFYcop692 (left half) and pFYcop517 (right half). Cells were grown in different media in the presence (shaded bar) or absence (open bar) of copper sulfate. The concentrations of copper sulfate supplemented were 200 μM for LB and 20 μM for XOLN and XVM2. (C) The promoter activity in Xc17 harboring pFYcop692 under different stress conditions. Cells were grown in XOLN medium supplemented with glycerol. Promoter activity was measured as β-galactosidase activity (Miller units). Results are the means of at least three independent experiments. Error bars indicate standard deviations.

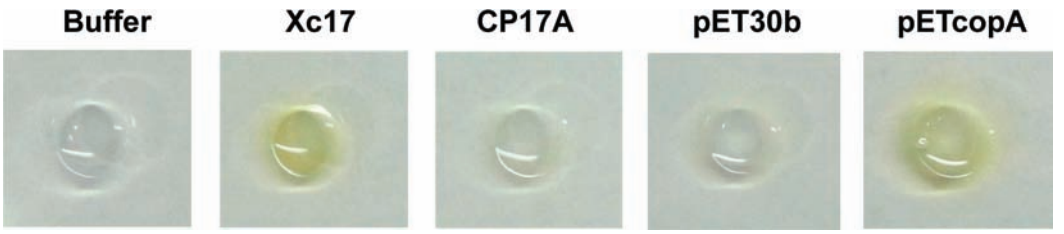

Figure 8. Plate assay for DMP oxidase activity. The DMP oxidase activity was evaluated using an agarose (1%) plate supplemented with 2 mM DMP and 0.1 mM $CuSO_4$. DMP oxidase activity appeared as an orange ring around the applied samples. Consistent results were obtained from three experiments with triplicate samples. Samples shown here are the buffer (0.1 M sodium phosphate buffer, negative control), Xc17 (cell extract in phosphate buffer from Xc17, the wild type), CP17A (cell extract in phosphate buffer from CP17A, the *copA* mutant), pET30b (recombinant proteins produced in the cell-free expression system using an expression vector pET30b as the template), and pETcopA (recombinant proteins produced in a cell-free expression system using pETcopA, *copA* cloned in pET30b, as the template).

Unfortunately, such a system caused CopA to accumulate as inclusion bodies. As a result, we failed to solubilize the induced cell free extract under this experimental condition. Instead, we used the *E. coli* extract-based cell-free protein synthesis system to express the recombinant CopA. The DMP oxidase activity of the recombinant CopA was detected in the DMP-supplemented agar plate as an orange ring surrounding the applied sample (Figure 8). Owing to the low level of protein produced by this system, sufficient purified protein could not be obtained to characterize the detailed enzymatic properties.

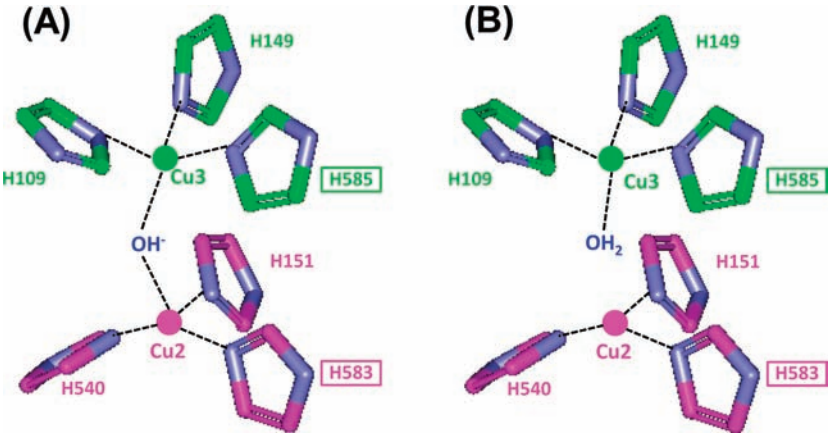

Figure 9. Proposed configuration change in Xcc CopA type III copper centers in oxidized (A) and reduced (B) forms. The color coding for the copper atoms and amino acids is as in Figure 6.

DISCUSSION

Previously, copper resistance genes have been studied in three *Xanthomonas* species: *X. axonopodis* pv. *vesicatoria* strain 7882 (plasmid-borne *copLABMGF*),[2,6] *X. arboricola* pv. *juglandis* strain C5 (chromosome encoded ORF1-4),[7] and *X. axonopodis* pv. *citri* strain 306 (chromosome encoded *copLAB*).[12] While this manuscript was being prepared, a paper was published on the genetics of copper resistance in another two *Xanthomonas* strains: *X. citri* subsp. *citri* strain A44 and *X. alfalfae* subsp. *citrumelonis* strain 1381.[14] The copper resistance determinants from these two citrus pathogens, *copLABMGCDF* and *copLABMGF*, are located on plasmids. Transposon mutagenesis of cloned copper resistance determinants in *X. citri* subsp. *citri* strain A44 revealed that *copL*, *copA*, and *copB* are the most important genes for copper resistance in this bacterium.

In the present work, we characterized *copLAB* from Xcc. In Xcc strain ATCC33913, *copLAB* is flanked by the upstream *cysM* and the downstream *gloA*.[13] Similar gene organization (*cysM-copL-copA-copB-gloA*) is also present in many other *Xanthomonas* for which genome sequences have been determined, including Xcc strains 8004 and B100,[35,36] *X. axonopodis* pv. *vesicatoria* strain 85−10,[54] *X. axonopodis* pv. *citri* strain 306,[13] and *X. oryzae* pv. *oryzae* strain KACC10331.[55] It is worth noting that in these *Xanthomonas* strains, homologues of several plasmid-borne copper resistance genes, such as *copCD* from *P. syringae* and *X. citri* subsp. *citri* strain A44 as well as *copMGF* from *X. axonopodis* pv. *vesicatoria* strain 7882 and *X. alfalfae* subsp. *citrumelonis* strain 1381, are absent from the genome. Because of the widespread distribution of copper resistance in bacteria, a comparative analysis of additional copper resistance systems might provide a better understanding of the functions of copper resistance genes, as well as information about their evolutionary relationships. Comparative analysis of copper resistance determinants from different species of *Xanthomonas* and *Pseudomonas* indicate that the *copAB* gene products play a major role in copper resistance.[12] Here, copper sensitivity analysis indicated that Xcc *copLAB* is required for copper tolerance and that CopA is the most important contributor.

Mutations in *copB* and *copL* do not cause significant defects in pathogenicity, whereas mutation in *copA* results in the attenuation of virulence. This is different from the situation in *X. axonopodis* pv. *citri* strain 306, where the *copA* mutant strain causes disease symptoms in a manner similar to that of the wild type strain.[12] It has been demonstrated that *copA*::Tn*5* *X. axonopodis* pv. *citri* strains cause delayed disease symptoms in the presence of copper.[12] Whether a similar situation exists in the Xcc *copA* mutant remains to be determined.

Virulence factor determination indicated that neither exopolysaccharide nor extracellular enzymes are affected by *copA* mutation. Xcc genome annotation revealed that different kinds of extracellular enzymes as well as numerous virulence determinants are associated with bacterial pathogenesis. As mutation in *copA* of Xcc does not affect the production of exopolysaccharide and extracellular enzymes (protease and cellulase), CopA most likely involves other unidentified virulence factor expressions. This topic merits further investigation.

Although several *Xanthomonas* CopA has been demonstrated to play a role in copper resistance, none has been characterized at the protein level. Sequence analysis indicated that Xcc *copA* encodes a multicopper oxidase. To date, no multicopper oxidase activity in Xcc has been described. Here, we demonstrated that Xcc possesses DMP oxidase activity and that CopA is implicated in this activity. Furthermore, the finding that a mutation in *copA* did not entirely reduce the level of DMP oxidase activity indicates that CopA is not the only enzyme that oxidizes DMP in Xcc.

From the site-directed mutagenesis data, the conserved residues in the putative copper binding motif are associated with the full oxidase activity as well as the copper tolerance function of CopA. Notably, expression of the mutated *copA* (pRKcopA-H583A) in CP17A partially restored CopA oxidase activity and its copper tolerance function. Among the four mutated amino acids, H583 was less important than the other three key residues (H538, C584, and H585). Typically, the type I copper center in multicopper oxidases mediates the electron transfer between the substrate and the trinuclear copper center formed by type II and a pair of type III copper centers, where a dioxygen molecule is converted into two water molecules.[47] When compared with other characterized and crystallized multicopper oxidases, (i) C584 is a type I copper binding site and functions in electron transfer; (ii) H538 is a type II copper binding site involved in redox reaction; (iii) H583 and H585 are type III copper binding sites and function in the binding and activation of dioxygen and in the intermediate status between oxidized and reduced forms, as shown in Figure 9. According to previous data regarding the type III copper site in *Saccharomyces cerevisiae* Fet3p and *Cucurbita pepo*

ascorbic oxidase,[42,56,57] Figure 9 reveals the proposed basic mechanism of dioxygen activation based on the two type III copper binding sites (Cu2 and Cu3) to explain why H583 is of less importance. First, in the oxidized form (Figure 9A), the oxygen atom is bound as OH^- and bridges the two type III copper (Cu2 and Cu3). Both H583 and H585 are essential for stabilizing the tetragonal intermediate configuration in the oxidized state. Second, in the reduced form (Figure 9B), the oxygen ligand bridging the copper ions Cu2 and Cu3 is released, and the coordination of Cu2 site is transformed from a tetragonal to a trigonal planar geometry. A water molecule, perhaps resulting from the protonation of the bridging OH^- upon reduction of the oxidized state, is bound to Cu3, and H585 (but not H583) contributes to the stabilization of this tetragonal configuration. H583 is only an intermediate for the "oxidized" form but not the "reduced" form. This is why the H583 is less important in oxidation activity and copper tolerance.

To date, only two reports have documented *copA* expression in *Xanthomonas*. One described *X. axonopodis* pv. *vesicatoria* strain 7882 plasmid-borne *copA*,[1] while the other described *X. axonopodis* pv. *citri* strain 306 chromosome encoded *copA*.[12] The former revealed that *copL* is required for copper-inducible expression of the downstream *copA* on reporter analysis.[1] The latter showed that *copA* expression and its gene product synthesis are induced by copper on Northern blot and Western blot.[12] In the present study, Xcc *copA* was induced by the addition of copper, and the region in response to the induction was located between −29 and +147. In addition, the −344/+147 region was not sufficient for significant expression. Intact *copL* and 5′ end of *copA* were necessary for copper-inducible activity of *copA* on the *lacZ* reporter assay. A similar phenomenon has also been found in *X. axonopodis* pv. *vesicatoria* strain 7882.[1] Reporter analysis indicated that *copA* expression is not affected by *clp* or *rpfF* mutation and that the copper inducible expression is independent of Clp and RpfF. Furthermore, of the four different stress conditions tested, only oxidative stress showed no significant change in β-galactosidase production, while high osmolarity, nitrogen starvation, and oxygen limitation reduced its expression, when compared with that in normal conditions.

In this study, we characterized *copLAB* in strain Xc17 of Xcc, which was originally isolated from cabbage in Taiwan. There were several important findings. First, *copL*, *copA*, and *copB* deletion mutants were constructed to evaluate their role in copper tolerance and pathogenicity of Xcc. Deletion of *copA* has more impact than *copL* and *copB* mutations. Second, CopA possesses multicopper oxidase activity, and the functionality of CopA is dependent on its conserved copper binding residues. In addition to presenting evidence to demonstrate that Xcc *copA* encodes a multicopper oxidase and is involved in pathogenicity and copper tolerance of Xcc, one significant outcome of our mutational analysis is confirmation of the sequence alignments used to suggest the identity of amino acid residues to the type I, type II, and type III copper in CopA. Third, the *copA* expression was evaluated under different culture conditions. Its transcription was induced by copper, independent of Clp and RpfF, subject to catabolite repression, and repressed under conditions of high osmolarity, nitrogen starvation, and oxygen limitation. This is the first time that copper resistance genes, as well as multicopper oxidase, have been characterized in Xcc. Knowledge obtained from this study is expected to facilitate further investigations of the expression and function of multicopper oxidase in Xcc, as well as the understanding of transcriptional regulation mechanisms of this phytopathogen.

AUTHOR INFORMATION

Corresponding Author

*Tel: 886-4-2239-1647 ext. 7052. Fax: 886-4-2239-6761. E-mail: ymhsiao@ctust.edu.tw.

Author Contributions

Y.-F.L. and P.-Y.L. contributed equally to this study.

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