The copper resistance operon copAB from Xanthomonas axonopodis pathovar citri: gene inactivation results in copper sensitivity

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Xanthomonas axonopodis pv. citri (Xac) causes citrus canker and the completion of the Xac genome sequence has opened up the possibility of investigating basic cellular mechanisms at the genomic level. Copper compounds have been extensively used in agriculture to control plant diseases. The copA and copB genes, identified by annotation of the Xac genome, encode homologues of proteins involved in copper resistance. A gene expression assay by Northern blotting revealed that copA and copB are expressed as a unique transcript specifically induced by copper. Synthesis of the gene products was also induced by copper, reaching a maximum level at 4 h after addition of copper to the culture medium. CopA was a cytosolic protein and CopB was detected in the cytoplasmic membrane. The gene encoding CopA was disrupted by the insertion of a transposon, leading to mutant strains that were unable to grow in culture medium containing copper, even at the lowest CuSO4 concentration tested (0.25 mM), whereas the wild-type strain was able to grow in the presence of 1 mM copper. Cell suspensions of the wild-type and mutant strains in different copper concentrations were inoculated in lemon leaves to analyse their ability to induce citrus canker symptoms. Cells of mutant strains showed higher sensitivity than the wild-type strain in the presence of copper, i.e. they were not able to induce citrus canker symptoms at high copper concentrations and exhibited a more retarded growth in planta.

INTRODUCTION

Normal growth and development of organisms depends on the appropriate pattern of gene expression and biochemical reactions necessary for biological transformations. Many important cofactors are required for such processes, one being copper, a trace metal that is important for normal growth and development, as it is essential for many enzymes involved in diverse biological processes. Although copper plays a crucial role in some biochemical transformations occurring in cellular processes, it can be toxic when it accumulates at concentrations exceeding those required. Thus, cells have evolved several systems to control copper homeostasis at the level of acquisition, intracellular distribution and copper efflux. Resistance mechanisms must be induced when concentrations exceed those required to meet basic cellular physiological needs.

The copper resistance mechanisms in Escherichia coli are the best understood, and two chromosomal systems have been identified, both catalysing the removal of excess copper from the cell. One is the two-component signal transduction system designated cus (Cu-sensing), consisting of the cusCFBA operon, whose transcription is regulated by the cusRS genes (Munson et al., 2000). The gene products work on copper efflux (Franke et al., 2003; Rensing & Grass, 2003). The other system, termed cue (Cu efflux), consists of the copper-responsive protein, CueR, that up-regulates the expression of two genes, copA and cueO (Outten et al., 2000). CopA is a copper-translocating P-type ATPase (Rensing et al., 2000) and CueO is a multicopper oxidase located in the periplasmic space (Grass & Rensing, 2001). While the CueR-regulated system is important for copper tolerance under aerobic conditions, the cusRS system is required for copper tolerance...
under anaerobic conditions (Outten et al., 2001), indicating that both systems are necessary for full copper tolerance. In addition to the chromosomal systems for copper homeostasis, plasmid determinants have also been described in E. coli. One such example is the pco (plasmid-borne copper resistance) system, consisting of seven genes (pcoABCDERS) (Brown et al., 1995) and regulated by the products of the genes pcoR and pcoS, proteins belonging to the family of the two-component sensor/responder phosphokinase regulatory system.

Copper resistance has become an important object of study in phytopathogenic bacteria as copper compounds have been widely used in agriculture to control plant diseases. As a consequence, plant-pathogenic bacteria have developed copper-resistance mechanisms, and Pseudomonas syringae pv. tomato was the first phytopathogen in which the mechanism was characterized at a molecular level (Cooksey, 1987). In this organism the genes are arranged in a single operon, copABCDRS (Cooksey, 1987), similar to the pco determinant from E. coli. PcoA and CopA both belong to the multicopper oxidase protein family; however, they must have different functions in the resistance mechanism since the pco system results in reduced cellular accumulation of copper and the cop system in increased uptake and sequestration (Brown et al., 1995). Copper-resistance determinants have also been described in the bacteria Xanthomonas campestris pv. juglandis (Xcj) (Lee et al., 1994) and in Xanthomonas axonopodis (formerly campestris) pv. vagatoria (Xav) (Basim et al., 2005; Cooksey et al., 1990). Although most of them are in some way related to each other and to the E. coli pco genes, there appear to be some functional and regulatory differences among them (Rensing & Grass, 2003). One such difference is the presence of the copL gene upstream from copA gene in a plasmid system described in Xav strain 7882, which is necessary for full copper resistance (Voloudakis et al., 2005). However, the same gene is not found in the chromosomal region carrying the copper-resistance genes in a different strain (strain XvP26) of the same phytopathogen (Basim et al., 2005). Thus, copper-resistance genes are organized into a regulatory network, which senses and responds differently to copper concentration, depending on the organism.

The comparative investigation of copper resistance genes has been facilitated as genomes from numerous bacterial phytopathogens have been completed. The genome of the bacterium X. axonopodis pv. citri (Xac), a pathogen that causes citrus canker, is now available (da Silva et al., 2002) and investigation of copper resistance genes are of great interest, since copper compounds are widely sprayed in citrus crops to control plant bacterial diseases. In this work we have investigated the involvement of copAB in the mechanism of Xac copper resistance. Our results show that they are organized in an operon whose transcription is induced by and is specific to copper. Furthermore, the requirement of the gene products for copper resistance was demonstrated by in vitro and in planta studies. These results are important for investigation of the regulatory mechanisms involved in copper resistance in Xanthomonas as they seem to be different from the well-studied mechanisms in Pseudomonas.

METHODS

Bacterial strains and growth conditions. Xac strain 306 (da Silva et al., 2002) was used as the source for genomic DNA. The bacterium was maintained in TSA medium (1 % tryptone, 1 % sucrose, 0.1 % sodium glutamate) containing 16 % glycerol at −80 °C. Growth experiments were performed in either TSA medium or nutrient agar (0.2 % beef extract, 0.5 % peptone, 0.5 % NaCl) at 28 °C, with copper concentrations adjusted by adding sterile 200 mM CuSO₄·5H₂O solution. The pH of the medium was adjusted to 7.0 by adding potassium phosphate buffer. Escherichia coli strain DH10B was used for plasmid amplifications.

Cloning of copA and copB coding region. The copA and copB full coding sequences were PCR-amplified from genomic DNA. The primers CopA-F (5′-CGGAAATTCATGCTTCTTGCATGCCCCGGTT-3′) and CopA-R (5′-CGGAATTCATGTCTTTCGATCCCCGGTT-3′) and CopB-R (5′-GCGGTTTCGATGCCAAGCTCCTGAG-3′) were used for copA amplification, and primers CopB-F (5′-CGGAATTCATGAGGCGCTTTCGATGCCAAGCTCCTGAG-3′) and CopB-R (5′-CGGAATTCATGAGGCGCTTTCGATCCCAACCCGGG-3′) for copB amplification. Primers were designed based on the gene sequences available at the Xac genome database (http://genoma4.iq.usp.br). The underlined sequences indicate EcoRI restriction sites. The amplified DNA fragments were cloned into pMOS vector (Amersham Biosciences) and the nucleotide sequences were confirmed by DNA sequencing.

Gene and protein expression analysis. Cells of Xac were grown in 100 (for RNA experiments) or 600 ml (for protein experiments) nutrient agar medium for 12 h in the absence of copper. At this time one sample (10 or 100 ml) was removed, and CuSO₄ was added to a final concentration of 1 mM. Samples of equal volumes were subsequently withdrawn at 0.5, 1, 2 and 4 h after addition of copper. In a different experiment, cells were grown for 12 h in the absence of copper and one sample was withdrawn. At this time, CdSO₄, CuSO₄, ZnSO₄ or AgNO₃ were added to a final concentration of 1 mM and samples were collected 0.5 h after addition of the metals. Total RNA was extracted by the hot phenol procedure (Khodursky et al., 2003) and gene expression was analysed by Northern blotting. Approximately 10 μg RNA was fractionated in 1.5% agarose-formaldehyde gel (Sambrook & Russell, 2001). After electrophoresis, the RNA was transferred to Hybond-N membrane (Amersham Biosciences) and hybridized at 42 °C in hybridization buffer [50% formamide, 5 × SSPE, 5 × Denhardt’s solution, containing 1 mM EDTA, 0.5 % SDS and 80 μl salmon sperm DNA (10 mg ml⁻¹)]. The whole coding sequences of copA and copB, radiolabelled by random priming, were used as probes. After overnight hybridization, blots were washed at 42 °C once in 1 × SSC, 0.1 % SDS for 30 min, once in 0.5 × SSC, 0.1 % SDS for 30 min, and once in 0.1 × SSC, 0.1 % SDS for 30 min, and exposed to X-ray film.

Protein expression was analysed by Western blotting. Bacterial pellets from a 100 ml culture were resuspended in 50 mM Tris/HCl, pH 8.0, 50 mM NaCl, 2 M urea, 1 mM benzamidine and 1 mM PMSF, and cells were lysed by sonication (20 cycles of 9 s). Cell debris was removed by centrifugation at 2700 g for 3 min at 4 °C. Total protein (40 μg for CopA and 80 μg for CopB) was separated by electrophoresis on 9 % (for CopA) or 12 % (for CopB) SDS-polyacrylamide gels and electrophoretically transferred onto a nitrocellulose membrane. Immunoblotting was performed with anti-CopA and anti-CopB polyclonal antibodies raised in rabbits and horseradish peroxidase-conjugated goat anti-rabbit...
IgG (Sigma). The detection of proteins was carried out with ECL Western blotting detection reagents (Amersham Biosciences).

copA transposon mutagenesis. To construct a copA mutant strain, the entire coding sequence was PCR-amplified from genomic DNA and cloned into the EcoRI site of pUC18. The transposon was inserted randomly into the plasmid using the EZ::TN <KAN-2> insertion kit, according to the instructions of the manufacturer (Epitcience Technologies). One plasmid with the transposon inserted into the copA coding sequence at 408 bp from the start codon was selected for further use. This plasmid was used to transform Xac by electroporation (2400 V, 25 μF, 300 Ω) (Sun et al., 2003) and recombinant clones were selected on TSA medium containing kanamycin (transposon selection marker). The integration of the transposon into the chromosome by homologous recombination was confirmed by Southern blotting analysis, PCR amplification of the entire sequence of the copA gene with the oligonucleotide pair CopA-F and CopA-R, and sequencing of the amplified fragment using the specific primers KAN-2F (5′-ACCTACAACAAAGCTCTCATAC-3′) and KAN-2R (5′-GCAATGTAACACGAGATTGTTG-3′). The mutant strains were named copA::Tn5.

Cell fractionation. Wild-type cells were grown in 200 ml nutrient agar medium for 12 h in the absence of copper. At this time, CuSO4 was added at a final concentration of 1 mM and cells were collected 4 h after the addition of copper. Cells were rinsed, resuspended in 10 ml 10 mM Tris/HCl, pH 8.0, buffer containing 20% sucrose and 3 mg lysozyme ml⁻¹ (Lee et al., 2001), and incubated on ice for 1 h to produce spheroplasts. After centrifugation (11000 g, 30 min) the proteins in the supernatant were concentrated by precipitation with 20% TCA on ice for 30 min. After centrifugation (11000 g, 10 min) the proteins (mainly periplasmic proteins) were washed with 70% ethanol and resuspended in 10 mM Tris/HCl, pH 8.0, 10 mM EDTA buffer. The pellet was gently rinsed in 10 mM Tris/HCl, pH 8.0, buffer containing 20% sucrose, resuspended in 10 mM Tris/HCl, pH 8.0, 10 mM MgCl2 buffer and the spheroplasts were lysed by sonication (cycles of 9 s sonication and 9 s on ice for 4 min). Cell debris and nucleic acids were removed by centrifugation at 7000 g for 15 min and the supernatant was centrifuged at 80000 g for 3 h to separate the cytoplasmic proteins in the supernatant. The pellet consisting of membrane proteins was resuspended in 10 mM Tris/HCl, pH 8.0, 10 mM EDTA buffer containing 0.5% Igepal CA-630. Total proteins in the cell fractions (periplasm, cytosol and cytoplasmic membrane) were quantified and approximately 40 μg was separated by electrophoresis on 9% (for CopA) or 12% (for CopB) SDS-polyacrylamide gels. CopA and CopB were identified by Western blotting as described above.

In vitro and in vivo growth of strain copA::Tn5. Cells of three mutant strains were evaluated in vitro for their ability to grow in the absence and presence of different concentrations of CuSO4. Wild-type and mutant strains were grown in nutrient agar medium, pH 7.0, with phosphate buffer in the absence and in the presence of 0.25, 0.5, 0.75 and 1.0 mM CuSO4, at 28 °C and 250 r.p.m. for 24 h. Growth was evaluated by measuring OD600. For in vivo analysis, lemon leaves were used as host for hypersensitivity tests of the wild-type and mutant strains. Plants were grown in chambers at 28 °C with artificial light, and the inoculum consisted of a bacterial suspension in phosphate buffer adjusted to an OD600 of 0.3. For hypersensitivity tests, wild-type and mutant strains were used to inoculate plant leaves in the absence and presence of 0.25, 0.5, 0.7, 1 and 2 mM CuSO4. A sample (100 μl) of cell suspension was infiltrated into the leaves by using a needleless syringe. Initial symptoms (water-soaking) were visualized 5 days after infection (a.i.) and symptoms characteristic of citrus canker disease (rust lesions) were visualized around 20 days.

Bacterial growth in lemon leaves was quantified after inoculating cells of wild-type and mutant strains in the absence and presence of 1 mM CuSO4. The inoculum consisted of a ten-times dilution of cell suspensions at an OD600 of 0.01. Leaf discs (0.9 cm diam.) were taken at 0, 2, 4, 6, 8, 10 and 12 days a.i. The discs were macerated in 1 ml sterile water in an Eppendorf tube, then the solutions were serially diluted and plated on TSA medium with (for mutant strain) and without kanamycin. The mean number of colonies from a minimum of five discs of the respective dilution was calculated and strain growth was quantified.

RESULTS

Genomic organization of copA and copB genes in Xac

During annotation of the Xac genome, homologues of the copA and copB genes were identified (da Silva et al., 2002). copA (ORF XAC3630) and copB (ORF XAC3631) are sequentially located in the genome sequence (position 4 304 624 to 4 307 493). No other identifiable copper-resistance-related gene was found at this locus (Fig. 1). Homologues of the C and D genes from the cop and pco operons are absent in the Xac genome, as well as ORFs 3 and 4 described for Xcj (Lee et al., 1994). Analysis of the region upstream of copA in Xac revealed the presence of ORF XAC3629 which encodes a conserved hypothetical protein of 152 aa with 26% identity and 37% similarity to the recently described CopL protein from Xav (Voloudakis et al., 2005). The distance between the stop codon of the ORF XAC3629 and the start codon of the copA gene is 112 nt.

According to the genome annotation, the stop codon of copA overlaps the start codon of copB (Fig. 1b). However, putative ribosome-binding sites (core sequence AGGA; www.ics.ucd.edu/~kibler/pubs/TR03) were not found at the end of copA; instead, some of them were identified at the beginning of the copB coding region (Fig. 1b). The putative sites were correctly located upstream to alternative ATG start codons, suggesting that another putative start codon for copB must be considered. Based on a sequence alignment of CopB proteins from different phytopathogens, we suggest the start codon as represented in Fig. 1(b). Based on a consensus sequence described for X. campestris (Katzen et al., 1996), suitable –10 and –35 promoter elements were identified upstream the copA ATG codon; however, the precise location of these elements as well as the transcription initiation site need to be determined experimentally.

The coding regions of copA and copB were PCR-amplified from Xac genomic DNA using primers based on the Xac genome sequence (http://genoam4.ig.usp.br). The copA gene encodes a 593 aa protein (CopA), which displays identity varying from 60–84% with homologues from phytopathogens such as Pseudomonas, Xanthomonas and Xylella. The 364 aa CopB protein displays 40–60% identity with homologues from the same phytopathogens. Sequence alignment of CopA proteins from different Pseudomonas and Xanthomonas species revealed the existence of two
domains: one highly conserved domain extending from the N-terminal region to the middle of the protein, and another highly conserved domain at the C-terminal region (Fig. 2). The C terminus contains the highly conserved domain His-X48-Cys-X4-His-X4-Met, a putative type-1 copper-binding site, found in multicopper oxidase enzymes (Fig. 2). In Xac this site involves the amino acid residues His526, Cys575, His580 and Met585. In addition, pairs of histidines, described as potential metal-binding sites, are present in the amino acid sequence. A phylogenetic analysis of CopA homologues revealed that the Xac protein clusters within a clade of Xanthomonas proteins distinct from Pseudomonas proteins (result not shown).

copA and copB gene expression is induced by copper and is specific to copper

We have determined that Xac strain 306 is able to grow at copper concentrations up to 1 mM; above this concentration cell growth is significantly reduced (results not shown). To verify whether copA and copB are transcriptionally regulated during growth in response to changing copper levels, we analysed the expression of both genes by Northern blotting. The complete coding sequences of copA (1782 bp) and copB (1095 bp) were used as probes to determine mRNA levels during growth in the absence of copper and at different times after exposure to CuSO₄. The copA and copB transcripts could be visualized only after the addition of copper (Fig. 3a, b, lanes 2–5), indicating that the copper led to elevated levels of induction of these genes. Transcripts could be detected 0.5 h after exposure to copper, and persisted for up to 4 h. This result suggests that the product of both genes may play a role in copper resistance in Xac. Moreover, only one transcript of 2.9 kb was detected by using either copA or copB genes as probes, which strongly supports the fact that both genes are organized in an operon (Fig. 3a, b). To further examine whether gene transcription regulation was copper-specific, other metal ions (cadmium, zinc and silver) were tested and copAB transcript levels were analysed. We observed no copAB transcripts in cultures grown in the presence of these metal ions; the copAB transcript was visualized only in the presence of copper (Fig. 3c, lane 4).

Gene expression was also analysed at the protein level. Protein expression was analysed in exponentially growing cells in the absence of copper and at different times after addition of CuSO₄ using polyclonal antibodies anti-CopA or anti-CopB. Both proteins were only detected after addition of CuSO₄ to the medium, reaching maximum levels at 4 h of exposure to copper (Fig. 4a for CopA and Fig. 4c for CopB). Thus, we conclude that copper induces both transcription and translation of the copAB operon.

Cellular location of CopA and CopB

Different cell fractions (periplasm, cytosol and cytoplasmic membrane) were obtained from cells grown in the presence

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**Fig. 1.** (a) Comparative analysis of the organization of copper resistance genes in phytopathogenic bacteria. The operon in Xac (NC_003919) was compared to operons from X. campestris pv. campestris (Xcc; NC_003902), X. oryzae pv. oryzae (Xoo; NC_006834), X. axonopodis pv. vesicatoria (Xav; AY536748), X. campestris pv. juglandis (Xcj; L19222), P. syringae pv. syringae (Pss; NC_007005) and P. syringae pv. tomato (Pst; M19930). (b) Intergenic region between copA and copB. The copA stop codon is shown in bold and italics, and the copB start codon is shown in bold and double-underlined. Putative ATG start codons for copB are shown in bold, and the start codon suggested by us is underlined. Putative ribosome-binding sites are boxed in grey.
### Fig. 2. Amino acid sequence alignment among CopA proteins from phytopathogenic bacteria.

Accession numbers:
- Xac CopA, AM38473
- P. syringae pv. tomato CopA, P12374
- P. syringae pv. syringae CopA, AAY36619
- Xav CopA, AAT07754
- Xoo CopA, AAW74010
- Xcc CopA, AAM39893
- Xaj ORF1, AAA72013

Arrows indicate amino acid residues of the putative type-1 copper site. The sequence alignment was performed using the CLUSTAL W program.
of 1 mM CuSO₄. The cellular location of both proteins was analysed by Western blotting using anti-CopA and anti-CopB antibodies (Fig. 5). CopA was detected only in the cytosol (Fig. 5b, lane 2), indicating that it is a cytosolic protein. A protein with a molecular mass greater than CopA was detected in the cytoplasmic membrane (Fig. 5b, lane 1), which is probably a product of unspecific hybridization since this protein was detected in crude extracts of Xac cells whether exposed to copper or not (Fig. 4a). CopB was detected only in the cytoplasmic membrane (Fig. 5a, lane 1).

**CopA and CopB are required for in vitro growth in the presence of copper**

As CopA has a putative C-terminal binding site for copper, we investigated the role of this protein in the copper

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**Fig. 3.** Gene expression during growth. Growth in the presence of 1 mM CuSO₄ (a, b), or in the presence of 1 mM CdSO₄, CuSO₄, ZnSO₄ or AgNO₃ (c). Total RNA from different times of growth was analysed. The upper panels show the hybridization; the lower panels show the loading of 23S rRNA after ethidium bromide staining of the same gel. (a, b) Lanes: 1, total RNA extracted from cells after 12 h of growth in the absence of copper; 2–5, total RNA extracted from cells at 0.5, 1, 2 and 4 h, respectively, after addition of 1 mM CuSO₄. (c) Lanes: 1, 3, 5 and 7, total RNA extracted from cells after 12 h of growth in the absence of metals; 2, 4, 6 and 8, total RNA extracted from cells 0.5 h after addition of 1 mM CdSO₄, CuSO₄, ZnSO₄ or AgNO₃, respectively. Total RNA (10 μg) was fractionated in 1.5% agarose-formaldehyde gel and the RNAs were transferred to nylon membranes and hybridized under the conditions described in Methods. The entire coding sequences of *copA* (a, c) and *copB* (b) labelled with ³²P were used as probes.

**Fig. 4.** Protein expression during growth. Growth in the absence (lane 1) and presence of 1 mM CuSO₄ (lanes 2–5). Lanes: 1, total protein from cells after 12 h of growth in the absence of copper; 2–5, total protein from cells at 0.5, 1, 2 and 4 h, respectively, after addition of 1 mM CuSO₄. Approximately 40 μg total protein for CopA (a, b) and 80 μg for CopB (c, d) was fractionated on an SDS-PAGE gel. Proteins were transferred to nitrocellulose membranes and blotted with polyclonal anti-CopA (a, b) and anti-CopB (c, d) antibodies raised in rabbits according to the conditions described in Methods. The numbers on the left represent standard molecular masses (kDa).

**Fig. 5.** Cellular location of CopA and CopB proteins. Xac cells exposed to 1 mM CuSO₄ were used to prepare cytoplasmic membrane (lane 1), cytosol (lane 2) and periplasm (lane 3) fractions. Proteins from each fraction were separated by electrophoresis on 9% (for CopA) or 12% (for CopB) SDS-polyacrylamide gels. Approximately 40 μg of each fraction was used for CopA and CopB analysis. Proteins were transferred to nitrocellulose membranes and blotted with polyclonal anti-CopA (b) and anti-CopB (a) antibodies raised in rabbits according to the conditions described in Methods. The numbers on the left represent standard molecular masses (kDa). Values represent typical results of four independent experiments.
resistance mechanism in Xac. We used transposon mutagenesis to construct a strain in which the copA gene was inactivated by the insertion of a transposon. The transposon was randomly inserted into a vector carrying copA in vitro and plasmids containing the transposon interrupting the gene sequence were selected. After DNA sequencing, two plasmids were selected: one containing the transposon inserted at nucleotide 72, and another containing the transposon inserted at nucleotide 408. The latter plasmid construction was utilized to transform Xac cells and clones were selected in medium containing kanamycin. A homologous recombination event allowed the integration of the transposon into the copA genomic locus. The integration event in five recombinant clones was analysed by Southern blotting using either the transposon or the copA coding region sequences as probes (results not shown). DNA sequencing of the copA gene amplified by PCR confirmed the insertion of the transposon into the copA sequence. Only three mutant strains were isolated.

One copA::Tn5 strain was analysed for its ability to synthesize CopA and CopB during growth in the presence of copper. The strain was not able to produce both proteins, even after addition of copper to the culture medium, showing a complete loss of copper induction. This result confirms that the mutation in copA is polar (compare Fig. 4b with Fig. 4a for CopA, and Fig. 4d with Fig. 4c for CopB) and, therefore, the copAB operon is inactive in the mutant strains. The three mutant strains were also analysed for their ability to grow at copper concentrations varying from 0.25 to 1 mM CuSO4, concentrations at which the wild-type strain can grow. All mutant strains were extremely sensitive to copper compared to the wild-type strain, exhibiting drastic growth reduction at copper concentrations as low as 0.25 mM (Fig. 6).

copA::Tn5 Xac strains cause delayed disease symptoms and show slower growth in plants in the presence of copper

To ascertain whether Cop proteins play a role in copper resistance when Xac cells infect plant hosts, wild-type and mutant strains were artificially inoculated into lemon leaves in the absence and presence of different concentrations of copper, and their ability to induce citrus canker symptoms into the plant host were compared. Wild-type and three mutant Xac strains were prepared for inoculations in the absence of copper (Fig. 7a, d) and in the presence of 0.7 (Fig. 7b, e) or 1.0 mM CuSO4 solution (Fig. 7c, f). Differences between the strains were not observed at lower copper concentrations. In the absence of copper, both wild-type and mutants induced citrus canker lesions that could be detected 5 (Fig. 7a) and 20 days a.i. (Fig. 7d). After inoculation in the presence of 0.7 mM CuSO4, the wild-type strain exhibited no difference in induced symptoms (Fig. 7b, 1 and e, 1) compared to the inoculations in the absence of copper (Fig. 7a, 1 and d, 1). However, mutant strains did not induce canker symptoms 5 days a.i. when inoculated in the presence of 0.7 mM copper (Fig. 7b, 2–4). At 20 days a.i. the mutant strains induced the same symptoms (Fig. 7e, 2–4) observed for the wild-type when inoculated in the presence of 0.7 mM copper (Fig. 7e, 1). When the copper concentration in the inoculation solution was raised to 1.0 mM, no hyperplastic symptoms were observed for either strain at 5 days a.i. (Fig. 7c), but at 20 days a.i. wild-type Xac developed the same type of lesion (Fig. 7f, 1) as observed either in the absence of copper (Fig. 7d, 1) or at 0.7 mM copper (Fig. 7e, 1). Under these conditions, the mutant strains (Fig. 7f, 2–4) were not able to induce canker lesions with the same intensity as the wild-type cells (Fig. 7f, 1). Therefore, the mutant strains were more sensitive than the wild-type strain to copper.

![Fig. 6. In vitro growth of wild-type strain (1) and copA::Tn5 mutant strains (2–4) in the absence and presence of copper. Cells were inoculated in culture medium containing different concentrations of CuSO4. Growth was measured by optical density at 550 nm after 24 h. Results are the means of at least three independent experiments.](image-url)
To quantify the effect of copper on the pathogen–host interaction, bacterial growth curves in planta were performed for the wild-type and one mutant strain (Fig. 8). In the absence of copper, the strains showed the same growth profile over 12 days of infection. When the strains were inoculated on a 1 mM copper solution, both growth curves showed reduction in the bacterial population compared to no copper addition. However, the growth of the wild-type was reduced more than 102-fold at 2 days a.i. and 104-fold at 12 days a.i. in the presence of 1.0 mM copper, while growth of the mutant strain was 103-fold lower at 2 days a.i. and 106-fold at 12 days a.i. in the presence of 1.0 mM copper (Fig. 8). Thus, in the presence of 1 mM copper, the mutant strain exhibited a more retarded growth in planta than the wild-type strain.

**DISCUSSION**

While metal ions are vital for many biological processes, such as transcription, respiration and growth, overaccumulation is detrimental for all sorts of organisms. In phytopathogens, an excess of copper induces resistance mechanisms, which are of great interest since copper compounds have been widely used in agriculture for disease control. In the Xac genome, two chromosomal genes were annotated as encoding homologues of the well-characterized CopA and CopB proteins from *Pseudomonas syringae* pv. *tomato* (Mellano & Cooksey, 1988). CopA and CopB are also found in *Xcj* and *Xav*. Both genes are sequentially located on the *Xac* chromosome and are transcribed as a unique transcript. In *Pseudomonas* spp., two other genes, *copC* and *copD*, are present in the *cop* operon. However, neither of these genes were identified in the *Xac*, *Xanthomonas campestris* pv. campestris (*Xcc*) or *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) genomes. BLAST analysis (http://genoma4.iq.usp.br) with the regulatory proteins PcoR and PcoS from *E. coli* (Brown et al., 1995), homologues of CopR and CopS, respectively, from *Pseudomonas syringae* pv. syringae, did identify a family of low-identity proteins all belonging to the two-component regulatory system in the *Xac* genome. Whether these *Xac* proteins play a role in copper resistance gene regulation remains to be determined. Comparative analysis of *cop* operons from different species of *Xanthomonas* and *Pseudomonas* revealed that the *cop* operon from *Xanthomonas* is one of the smallest operons, indicating...
that the copAB gene products may play a major role in the copper resistance mechanism and that the presence of only CopA and CopB allows this phytopathogen to develop copper resistance. This is consistent with the results described by Mellano & Cooksey (1988) which showed that the copA and copB genes from P. syringae pv. tomato are essential for resistance and that copC and copD are only required for maximum copper resistance. The fact that inactivation of the copAB operon leads to a mutant Xac strain, which is sensitive to copper, even at low concentrations (this work), reinforces this assumption.

Gene expression analysis of the cop operon demonstrated the presence of the copAB transcript only when copper was added to the culture medium. Following copAB transcription accumulation, CopA and CopB were induced, confirming copper-induced transcription coupled to translation. We could not identify regulatory DNA elements at the region upstream from the cop operon similar to the ‘copper box’ element located at the promoter regions of the pco operon in E. coli and the cop operon from P. syringae (Mills et al., 1994; Rouch & Brown, 1997). These systems are both regulated by two proteins belonging to the two-component regulatory system. This element was described as being essential for copper-inducible activity of both promoters. However, we identified a short ORF (XAC3629), upstream from the Xac copAB operon, that encodes a protein with 26 % identity to CopL from Xav strain 7882 (Voloudakis et al., 2005). The same gene is located upstream from the cop operon in Xcc (da Silva et al., 2002) and in the citrus phytopathogen Xylella fastidiosa (Simpson et al., 2000). Interestingly, Xav strain 7882 lacks copR and copS which encode proteins belonging to a two-component regulatory system. CopL was found to be required for full copper-inducible expression of copA in Xav. However, a possible role for CopL in copAB copper-inducible transcriptional regulation remains to be determined. Thus, two distinct mechanisms regulating cop gene transcription regulation seem to exist in phytopathogens: one dependent on the copper-inducible two-component signal transduction mechanism, and the other depending on the copR regulatory gene.

copA transcription in E. coli was also induced by copper and analysis of the copA promoter region revealed the presence of DNA elements characteristic of promoters regulated by the metalloregulatory proteins of the MerR family, transcriptional regulators found in a large number of Gram-positive and Gram-negative bacteria (Outten et al., 2000). However, CopA from E. coli is an ATPase (Rensing et al., 2000) that transports free copper from the cytoplasm to the periplasm, whereas CopA from phytopathogens, in general, may work by sequestering copper ions in the periplasm, suggesting a mechanism that prevents the build up of toxic levels of free copper ions in the cytoplasm (Cha & Cooksey, 1991). Sequence alignment of CopA proteins from phytopathogens suggested that the protein is arranged in a two-domain structure, with a putative type I binding site for copper at the C-terminal domain (Fig. 2). This site shows strong identity to the copper site in multicopper oxidases and is also found in CopA proteins from Pseudomonas and from different species of Xanthomonas. However, copper binding to this site has not yet been determined directly. A common characteristic among CopA proteins is the high histidine residue content (32 in Xac CopA), and these residues have been described as copper ligands in ascorbate oxidase (Messerschmidt et al., 1989), and could probably account for the large number of copper atoms bound by CopA. Cha & Cooksey (1991) demonstrated that CopA from P. syringae pv. tomato was able to bind up to 11 atoms of copper per molecule.

The CopA protein location in the cytosol of Xac is surprising based on its polypeptide sequence. While a putative N-terminal signal peptide was not identified by bioinformatic tools (http://bp.nuap.nagoya-u.ac.jp/sosui), the protein does have an N-terminal sequence that harbours the consensus SRRXFLK ‘twin arginine’ motif (17-SRRRFVQ-23 in Xac) and targets proteins for export to the periplasm by the Tat (twin arginine translocation) pathway (Berks et al., 2003). Deletion mutants in the Tat pathway of different pathovars of Pseudomonas syringae display a range of pleiotropic phenotypic changes, including defects in siderophore production and a decrease in copper resistance (Bronstein et al., 2005; Caldelari et al., 2006). CopB was demonstrated to be located in the cytoplasmic membrane of Xac, in agreement with the prediction of the presence of a putative signal peptide spanning the first 20 N-terminal amino acids (http://bp.nuap.nagoya-u.ac.jp/sosui). The cellular location of Cop proteins in other phytopathogens has not been investigated. In Pseudomonas syringae pv. tomato, both CopA and CopB were described as periplasmic proteins (Cha & Cooksey, 1991). However, copper resistance mechanisms differ even among different Pseudomonas pathovars (Feil et al., 2005). If CopA in Xac binds copper, based on the presence of the copper-binding site domain at the C terminus, one may ask in what form is copper available in the cytosol, and more importantly how do Cop proteins work in copper resistance in Xac? Recently Dann et al. (2007) described, for the first time, the existence of a metalloregulatory mechanism involving metal-dependent, allosteric regulation of RNA structure, suggesting the possibility of RNA-based metal sensors.

We have examined the participation of the copAB gene products in the copper resistance mechanism in Xac in two different ways. First, we analysed the specificity of transcription induction using several metal ions and found that transcription was only induced by copper. Among the metal ions tested, Zn(II) is the most similar trace element to Cu(II) and transcription induction of the cop operon was not observed with this metal. In Xac there is no evidence for the mechanism determining metal specificity, since genes encoding regulatory proteins are not part of the cop operon. Second, we inactivated the copAB operon by introducing a polar mutation into copA and analysed the mutant strain for its ability to grow in culture medium containing copper or
in host-plants inoculated in the presence of copper. These studies allowed us to confirm the in vivo role of the proteins in Xac copper resistance. The mutant strain was not able to grow on medium containing copper even at low concentrations. Although the mutant strain caused disease symptoms in a similar way to that observed in the wild-type strain in the absence of copper, the in planta cell growth was significantly impaired in the presence of copper and the appearance of symptoms was delayed compared to the wild-type strain. Finally, it is important to point out that the presence of the symptoms in plants inoculated with the mutant strain in the presence of 0.7 mM CuSO₄ may represent a movement of the bacteria from an environment of high copper concentration to one of low copper concentration inside the leaves. The role of CopA and CopB in copper resistance was corroborated by in planta growth curves in the presence of copper. Growth reduction of 10- to 100-fold was observed in the mutant strain compared to the wild-type strain at 2 and 12 days a.i., respectively. As far as we know this is the first evidence of both proteins mediating copper resistance in a phytopathogen–plant host interaction. The findings of this study reinforce the central role of CopA and CopB for copper resistance in Xac, but also indicate that the mechanism must involve the concerted action of different proteins. Improved knowledge of how these other proteins act on the global copper resistance mechanism will provide insights into new therapeutic targets against bacterial pathogens.

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