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*.

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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

Abbreviations: *Xac*, *Xanthomonas axonopodis* pv. citri; *Xav*, *Xanthomonas axonopodis* pv. vesicatoria; *Xcc*, *Xanthomonas campestris* pv. campestris; *Xcj*, *Xanthomonas campestris* pv. juglandis; *Xoo*, *Xanthomonas oryzae* pv. oryzae.

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 (*pcoABCDRE*) (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, *copABCDRE* (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. vesicatoria (*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 $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 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'-CGGAATTCATGTCTTTCGATCCCCCGTT-3') and CopA-R (5'-CGGAATTCATGCTTCCACCCG-3') were used for *copA* amplification, and primers CopB-F (5'-CGGAATTCATGAGCCGCTTTCGATGCA-3') and CopB-R (5'-CGGAATTCCTCAAAACCAAACGCG-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 CuSO_4 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, CdSO_4 , CuSO_4 , ZnSO_4 or AgNO_3 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, $5 \times$ SSPE, $5 \times$ 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 $1 \times$ SSC, 0.1 % SDS for 30 min, once in $0.5 \times$ SSC, 0.1 % SDS for 30 min, and once in $0.1 \times$ 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 (Epicentre 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 μ F, 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'-ACCTACAACAAAGCTCTCATCAACC-3') and KAN-2R (5'-GCAATGTAACATCAGAGATTTTGAG-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 (11 000 g, 30 min) the proteins in the supernatant were concentrated by precipitation with 20 % TCA on ice for 30 min. After centrifugation (11 000 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 80 000 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 μ g 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 OD₅₅₀. 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 μ l) 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 *pcp* 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.uci.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://genoma4.iq.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

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.

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

<i>X. citri</i>	1	-----MSRTPPPFSGHSTASG-----SRRRFVQGLALGGV
<i>X. oryzae</i>	1	MAVIGPARALRGFSQLCDASVLTMTERTGDVMSFSPCSDASSNG-----SRRRFVQGLALGGV
<i>X. campestris</i>	1	-----MSFDPLSNGGLARRRFVQGLALGGV
<i>X. juglandis</i>	1	-----MSRDSDDSRNGGPRHUSRRFVQGLALGGV
<i>X. vesicatoria</i>	1	-----MSHDDFRGPHGGPLLSRRRFVQGLALGGV
<i>P. tomato</i>	1	-----MESRTSRRTTFVGLAAGV
<i>P. syringae</i>	1	-----MPTRTSRRTTFVGLAASGL
<i>X. citri</i>	31	AASG-LWENDARAQAANTFVIR-GEQSGLCIGRLPVNFTGRPRSAITVNCSLPAPILLRW
<i>X. oryzae</i>	61	AVAGGLWRRDARAATPANTFVIR-GEQSGLCIGRLPVNFTGRPRSAITVNCSLPAPILLRW
<i>X. campestris</i>	26	AAATGLWRSBARATPVTFAVIRGGAALCIGRLPVNLTGRARSAITVNCSLPAPILLRW
<i>X. juglandis</i>	31	VAALLGLWPQLSWALKESQSNVLSGTEFDLAIGETPVNFTGRARSAITVNCSLPAPILLRW
<i>X. vesicatoria</i>	31	VAALLGLWPQASWALKESQSNVLSGTEFDLAIGETPVNFTGRARSAITVNCSLPAPILLRW
<i>P. tomato</i>	20	LGLGLWRSPPWAASSPALSVLSGTEFDLSIGETPVNFTGRARSAITVNCSLPAPILLRW
<i>P. syringae</i>	20	LGLGLWRSPPWAASPPALSVLSGTEFDLSIGETPVNFTGRARSAITVNCSLPAPILLRW
<i>X. citri</i>	89	REGDTVSVRVR-----NALTLQPTSHHHGILLPANMDGVPGMSFDGIAPQBYHYRFAI
<i>X. oryzae</i>	120	REGDTVSVRVR-----NALTLQPTSHHHGILLPANMDGVPGMSFDGIAPQBYHYRFAI
<i>X. campestris</i>	86	REGDTVSVRVR-----NALTLQPTSHHHGILLPANMDGVPGMSFDGIAPQBYHYRFAI
<i>X. juglandis</i>	91	REGDTVNLRVSNVLPASLHGHETSIIHHGILLPANMDGVPGMSFDGIAPQBYHYRFAI
<i>X. vesicatoria</i>	91	REGDTVNLRVSNVLPASLHGHETSIIHHGILLPANMDGVPGMSFDGIAPQBYHYRFAI
<i>P. tomato</i>	80	REGDTVNLRVRN-----RLTEATSHHHGILLPANMDGVPGMSFDGIAPQBYHYRFAI
<i>P. syringae</i>	80	REGDTVNLRVRN-----RLTEATSHHHGILLPANMDGVPGMSFDGIAPQBYHYRFAI
<i>X. citri</i>	144	ROSGTYWYHSHSMFOEQAGLYGAIVIDPLVEPPYRHDREHVVLSDWTDLDPALFRRLK
<i>X. oryzae</i>	175	ROSGTYWYHSHSMFOEQAGLYGAIVIDPLVEPPYRHDREHVVLSDWTDLDPALFRRLK
<i>X. campestris</i>	141	ROSGTYWYHSHSMFOEQAGLYGAIVIDPLVEPPYRHDREHVVLSDWTDLDPALFRRLK
<i>X. juglandis</i>	151	QOSGTYWYHSHSMFOEQAGLYGAIVIDPLVEPPYRHDREHVVLSDWTDLDPALFRRLK
<i>X. vesicatoria</i>	151	ROSGTYWYHSHSMFOEQAGLYGAIVIDPLVEPPYRHDREHVVLSDWTDLDPALFRRLK
<i>P. tomato</i>	134	ROSGTYWYHSHSMFOEQAGLYGAIVIDPLVEPPYRHDREHVVLSDWTDLDPALFRRLK
<i>P. syringae</i>	134	ROSGTYWYHSHSMFOEQAGLYGAIVIDPLVEPPYRHDREHVVLSDWTDLDPALFRRLK
<i>X. citri</i>	204	QMFSDHNYKRTVGDFVRDARDGLSATLADRMWGMGRMRTPTDLSDVNANTYTYLLNGV
<i>X. oryzae</i>	235	QMFSDHNYKRTVGDFVRDARDGLSATLADRMWGMGRMRTPTDLSDVNANTYTYLLNGV
<i>X. campestris</i>	200	QMFSDHNYKRTVGDFVRDARDGLSATLADRMWGMGRMRTPTDLSDVNANTYTYLLNGV
<i>X. juglandis</i>	211	KMFSDHNYKRTVGDFVRDARDGLSATLADRMWGMGRMRTPTDLSDVNANTYTYLLNGV
<i>X. vesicatoria</i>	211	KMFSDHNYKRTVGDFVRDARDGLSATLADRMWGMGRMRTPTDLSDVNANTYTYLLNGV
<i>P. tomato</i>	194	QMSDYNHKKRTVGDFVRDARDGLSATLADRMWGMGRMRTPTDLSDVNANTYTYLLNGV
<i>P. syringae</i>	194	QMSDYNHKKRTVGDFVRDARDGLSATLADRMWGMGRMRTPTDLSDVNANTYTYLLNGV
<i>X. citri</i>	264	APAGNWTGLFKPGEKVLRLRFINGSSMTYFDIRIPGLKMTVVAADGGYVHPVSDDELRIAA
<i>X. oryzae</i>	295	APAGNWTGLFKPGEKVLRLRFINGSSMTYFDIRIPGLKMTVVAADGGYVHPVSDDELRIAA
<i>X. campestris</i>	260	APAGNWTGLFKPGEKVLRLRFINGSSMTYFDIRIPGLKMTVVAADGGYVHPVSDDELRIAA
<i>X. juglandis</i>	271	TSIGNWTGLFKPGEKVLRLRFINGSSMTYFDIRIPGLKMTVVAADGGYVHPVSDDELRIAA
<i>X. vesicatoria</i>	271	TSIGNWTGLFKPGEKVLRLRFINGSSMTYFDIRIPGLKMTVVAADGGYVHPVSDDELRIAA
<i>P. tomato</i>	254	APAGNWTGLFKPGEKVLRLRFINGSSMTYFDIRIPGLKMTVVAADGGYVHPVSDDELRIAA
<i>P. syringae</i>	254	APAGNWTGLFKPGEKVLRLRFINGSSMTYFDIRIPGLKMTVVAADGGYVHPVSDDELRIAA
<i>X. citri</i>	324	AETFDVIVEPQGDAFTLFAQDMGRTGACGTTLAVRHGLQAPTPALDPRAILTMQDMGMD
<i>X. oryzae</i>	355	AETFDVIVEPQGDAFTLFAQDMGRTGACGTTLAVRHGLQAPTPALDPRAILTMQDMGMD
<i>X. campestris</i>	320	AETFDVIVEPQGDAFTLFAQDMGRTGACGTTLAVRHGLQAPTPALDPRAILTMQDMGMD
<i>X. juglandis</i>	331	AETFDVIVEPQGDAFTLFAQDMGRTGACGTTLAVRHGLQAPTPALDPRAILTMQDMGMD
<i>X. vesicatoria</i>	331	AETFDVIVEPQGDAFTLFAQDMGRTGACGTTLAVRHGLQAPTPALDPRAILTMQDMGMD
<i>P. tomato</i>	314	AETFDVIVEPQDAFTLFAQDMGRTGACGTTLAVRHGLQAPTPALDPRAILTMQDMGMD
<i>P. syringae</i>	314	AETFDVIVEPQDAFTLFAQDMGRTGACGTTLAVRHGLQAPTPALDPRAILTMQDMGMD
<i>X. citri</i>	384	DGMHGHDPHMHG-----GSHLAAHGMHTM
<i>X. oryzae</i>	415	DGMHGHDPHMHGOGTQGGDTTQ-----GDAAHAAHAMMPMPM
<i>X. campestris</i>	380	DGMHGHDPHMHGPAHQH-----ASPAD-----THAGHTVCHDPSAHAL
<i>X. juglandis</i>	391	HGMSGSGMSASDSSDSSSNKPMAMNMAGMAHEPGQRSAPAGADAHAGHDMGMSQS
<i>X. vesicatoria</i>	391	HGMSGSGMSASDSSDSSSNKPMAMNMAGMAHEPGQRSAPAGADAHAGHDMGMSQS
<i>P. tomato</i>	373	GMDHGHDPHMHGSGDGGADGMQTMSSMG-----GDSMPADHCKMSTMQ
<i>P. syringae</i>	373	GMEHGSVN-HAAPATHDMGMDHSMKG-----HDAMPQMDHATQTPAQK
<i>X. citri</i>	409	HSDDHKAHDK-----APHHPASETGNNPLDMRSNAPRLDDPGVGLRDNNGRRVLC
<i>X. oryzae</i>	454	HGHGSGNTQTK-----TPHCFASEDGNNPLDMRSNAPRLDDPGVGLRDNNGRRVLC
<i>X. campestris</i>	417	QHHGHPASSR-----APHHPASEDNNPLDMRSNAPRLDDPGVGLRDNNGRRVLC
<i>X. juglandis</i>	451	GSMFMDHAG-----MASHHPASEDNNPLDMRSNAPRLDDPGVGLRDNNGRRVLC
<i>X. vesicatoria</i>	439	GAMAGNQHG-----MASHHPASEDNNPLDMRSNAPRLDDPGVGLRDNNGRRVLC
<i>P. tomato</i>	417	GMDHGHDPHMHGSGDGGADGMQTMSSMG-----GDSMPADHCKMSTMQ
<i>P. syringae</i>	416	TPTP-----ASHHPASEDNNPLDMRSNAPRLDDPGVGLRDNNGRRVLC
<i>X. citri</i>	461	YADLHSLVFPDPPDGPREGREIELHLTGHEKFAWSFDGIASFASAPLLOYGERLRIVLVN
<i>X. oryzae</i>	506	YADLHSLVFPDPPDGPREGREIELHLTGHEKFAWSFDGIASFASAPLLOYGERLRIVLVN
<i>X. campestris</i>	469	YADLHSLVFPDPPDGPREGREIELHLTGHEKFAWSFDGIASFASAPLLOYGERLRIVLVN
<i>X. juglandis</i>	503	YADLHSLVFPDPPDGPREGREIELHLTGHEKFAWSFDGIASFASAPLLOYGERLRIVLVN
<i>X. vesicatoria</i>	490	YADLHSLVFPDPPDGPREGREIELHLTGHEKFAWSFDGIASFASAPLLOYGERLRIVLVN
<i>P. tomato</i>	477	YADLHSLVFPDPPDGPREGREIELHLTGHEKFAWSFDGIASFASAPLLOYGERLRIVLVN
<i>P. syringae</i>	460	YADLHSLVFPDPPDGPREGREIELHLTGHEKFAWSFDGIASFASAPLLOYGERLRIVLVN
<i>X. citri</i>	521	DTMMQHPHILHGMWSDLEDAGNFOVRKHTIDMPPGRRRTYRVRADALGRWAYHCHLLYH
<i>X. oryzae</i>	566	DTMMQHPHILHGMWSDLEDAGNFOVRKHTIDMPPGRRRTYRVRADALGRWAYHCHLLYH
<i>X. campestris</i>	529	DTMMQHPHILHGMWSDLEDAGNFOVRKHTIDMPPGRRRTYRVRADALGRWAYHCHLLYH
<i>X. juglandis</i>	563	DTMMQHPHILHGMWSDLEDAGNFOVRKHTIDMPPGRRRTYRVRADALGRWAYHCHLLYH
<i>X. vesicatoria</i>	550	DTMMQHPHILHGMWSDLEDAGNFOVRKHTIDMPPGRRRTYRVRADALGRWAYHCHLLYH
<i>P. tomato</i>	537	DTMMQHPHILHGMWSDLEDAGNFOVRKHTIDMPPGRRRTYRVRADALGRWAYHCHLLYH
<i>P. syringae</i>	520	DTMMQHPHILHGMWSDLEDAGNFOVRKHTIDMPPGRRRTYRVRADALGRWAYHCHLLYH
<i>X. citri</i>	581	MEAGMMREVRVEA
<i>X. oryzae</i>	626	MEAGMMREVRVEA
<i>X. campestris</i>	589	MEAGMMREVRVEA
<i>X. juglandis</i>	623	MEAGMMREVRVEA
<i>X. vesicatoria</i>	610	MEAGMMREVRVEA
<i>P. tomato</i>	597	MEAGMMREVRVEA
<i>P. syringae</i>	580	MEAGMMREVRVEA

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; *Xcj* ORF1, AAA72013. Arrows indicate amino acid residues of the putative type-1 copper site. The sequence alignment was performed using the CLUSTAL W program.

Fig. 3. Gene expression during growth. Growth in the presence of 1 mM CuSO_4 (a, b), or in the presence of 1 mM CdSO_4 , CuSO_4 , ZnSO_4 or AgNO_3 (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 CuSO_4 . (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 CdSO_4 , CuSO_4 , ZnSO_4 or AgNO_3 , 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 ^{32}P were used as probes.

of 1 mM CuSO_4 . 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

Fig. 4. Protein expression during growth. Growth in the absence (lane 1) and presence of 1 mM CuSO_4 (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 CuSO_4 . 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 CuSO_4 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.

Fig. 7. Ability of mutant and wild-type strains to induce canker symptoms in lemon leaves. (a, d) Wild-type (1) and mutant (2–4) strains in the absence of copper; (b, e) wild-type (1) and mutant (2–4) strains in the presence of 0.7 mM CuSO₄; (c, f) wild-type (1) and mutant (2–4) strains in the presence of 1.0 mM CuSO₄. (a, b, c) Water-soaking symptoms on lemon leaves 5 days a.i.; (d, e, f) citrus canker symptoms on lemon leaves 20 days a.i.

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.

Fig. 8. Growth of mutant and wild-type strains *in planta* (lemon leaves). Growth of wild-type (WT) and mutant (*copA*::Tn5) cells was evaluated in the absence and presence of 1 mM CuSO₄ after 2, 4, 6, 8, 10 and 12 days a.i. The results are the means of nine independent experiments. ■, Wild-type, no copper; ○, wild-type, 1 mM CuSO₄; ▲, *copA*::Tn5, no copper; ▽, *copA*::Tn5, 1 mM CuSO₄.

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 *Xcc* 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* transcript 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 *Xyllela 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 *copL* 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 1 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 once 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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REFERENCES

- Basim, H., Minsavage, G. V., Stall, R. E., Wang, J. F., Shanker, S. & Jones, J. B. (2005). Characterization of a unique chromosomal copper resistance gene cluster from *Xanthomonas campestris* pv. vesicatoria. *Appl Environ Microbiol* **71**, 8284–8291.
- Berks, B. C., Palmer, T. & Sargent, F. (2003). The Tat protein translocation pathway and its role in microbial physiology. *Adv Microb Physiol* **47**, 187–254.
- Bronstein, P. A., Marrichi, M., Cartinhour, S., Schneider, D. J. & DeLisa, M. P. (2005). Identification of a twin-arginine translocation system in *Pseudomonas syringae* pv. tomato DC3000 and its contribution to pathogenicity and fitness. *J Bacteriol* **187**, 8450–8461.
- Brown, N. L., Barrett, S. R., Camakaris, J., Lee, B. T. O. & Rouch, D. A. (1995). Molecular genetics and transport analysis on the copper-resistance determinant (*pco*) from *Escherichia coli* plasmid pRJ1004. *Mol Microbiol* **17**, 1153–1166.
- Caldelari, I., Mann, S., Crooks, C. & Palmer, T. (2006). The Tat pathway of the plant pathogen *Pseudomonas syringae* is required for optimal virulence. *Mol Plant Microbe Interact* **19**, 200–212.
- Cha, J. S. & Cooksey, D. A. (1991). Copper resistance in *Pseudomonas syringae* mediated by periplasmic and outer membrane proteins. *Proc Natl Acad Sci U S A* **88**, 8915–8919.
- Cooksey, D. A. (1987). Characterization of a copper resistance plasmid conserved in copper-resistant strains of *Pseudomonas syringae* pv. tomato. *Appl Environ Microbiol* **53**, 454–456.
- Cooksey, D. A., Azad, H. R., Cha, J. S. & Lim, C. K. (1990). Copper resistance gene homologs in pathogenic and saprophytic bacterial species from tomato. *Appl Environ Microbiol* **56**, 431–435.
- da Silva, A. C. R., Ferro, J. A., Reinach, F. C., Farah, C. S., Furlan, L. R., Quaggio, R. B., Monteiro-Vitorello, C. B., Van Sluys, M. A., Almeida, N. F. & other authors (2002). Comparison of the genomes of two *Xanthomonas* pathogens with differing host specificities. *Nature* **417**, 459–463.
- Dann, C. E., III, Wakeman, C. A., Sieling, C. L., Baker, S. C., Irnov, I. & Winkler, W. C. (2007). Structure and mechanism of a metal-sensing regulatory RNA. *Cell* **130**, 878–892.
- Feil, H., Feil, W. S., Chain, P., Larimer, F., DiBartolo, G., Copeland, A., Lykidis, A., Trong, S., Nolan, M. & other authors (2005). Comparison of the complete genome sequences of *Pseudomonas syringae* pv. *syringae* B728a and pv. *tomato* DC3000. *Proc Natl Acad Sci U S A* **102**, 11064–11069.
- Franke, S., Grass, G., Rensing, C. & Nies, D. H. (2003). Molecular analysis of the copper-transporting efflux system CusCFBA of *Escherichia coli*. *J Bacteriol* **185**, 3804–3812.
- Grass, G. & Rensing, C. (2001). CueO is a multi-copper oxidase that confers copper tolerance in *Escherichia coli*. *Biochem Biophys Res Commun* **286**, 902–908.
- Katzen, F., Becker, A., Zorreguieta, A., Puhler, A. & Ielpi, L. (1996). Promoter analysis of the *Xanthomonas campestris* pv. *campestris* gum operon directing biosynthesis of the xanthan polysaccharide. *J Bacteriol* **178**, 4313–4318.
- Khodursky, A. B., Bernstein, J. A., Peter, B. J., Rhodius, V., Wendisch, V. F. & Zimmer, D. P. (2003). *Escherichia coli* spotted double-strand DNA microarrays: RNA extraction, labeling, hybridization, quality control, and data management. *Methods Mol Biol* **224**, 61–78.
- Lee, Y. A., Henderson, M., Panapoulos, N. J. & Schroth, M. N. (1994). Molecular cloning, chromosomal mapping, and sequence analysis of copper resistance genes from *Xanthomonas campestris* pv. *juglandis*: homology with small blue copper proteins and multicopper oxidase. *J Bacteriol* **176**, 173–188.
- Lee, H. M., Tyan, S. W., Leu, W. M., Chen, L. Y., Chen, D. C. & Hu, N. T. (2001). Involvement of the XpsN protein in formation of the XpsL-XpsM complex in *Xanthomonas campestris* pv. *campestris* type II secretion apparatus. *J Bacteriol* **183**, 528–535.
- Mellano, M. A. & Cooksey, D. A. (1988). Nucleotide sequence and organization of copper resistance genes from *Pseudomonas syringae* pv. *tomato*. *J Bacteriol* **170**, 2879–2883.
- Messerschmidt, A., Rossi, A., Ladenstein, R., Huber, R., Bolognesi, M., Gatti, G., Marchesini, A., Petruzzelli, R. & Finazzi-Agró, A. (1989). X-ray crystal structure of the blue oxidase ascorbate oxidase from zucchini. Analysis of the polypeptide fold and a model of the copper sites and ligands. *J Mol Biol* **206**, 513–529.
- Mills, S. D., Lim, C. K. & Cooksey, D. A. (1994). Purification and characterization of CopR, a transcriptional activator protein that binds to a conserved domain (*cop* box) in copper-inducible promoters of *Pseudomonas syringae*. *Mol Gen Genet* **244**, 341–351.
- Munson, G. P., Lam, D. L., Outten, F. W. & O'Halloran, T. V. (2000). Identification of a copper-responsive two-component system on the chromosome of *Escherichia coli* K-12. *J Bacteriol* **182**, 5864–5871.

Outten, F. W., Outten, C. E., Hale, J. & O'Halloran, T. V. (2000). Transcriptional activation of an *Escherichia coli* copper efflux regulon by the chromosomal MerR homologue, CueR. *J Biol Chem* **275**, 31024–31029.

Outten, F. W., Huffman, D. L., Hale, J. A. & O'Halloran, T. V. (2001). The independent *cue* and *cus* systems confer copper tolerance during aerobic and anaerobic growth in *Escherichia coli*. *J Biol Chem* **276**, 30670–30677.

Rensing, C. & Grass, G. (2003). *Escherichia coli* mechanisms of copper homeostasis in a changing environment. *FEMS Microbiol Rev* **27**, 197–213.

Rensing, C., Fan, B., Sharma, R., Mitra, B. & Rosen, B. P. (2000). CopA: an *Escherichia coli* Cu(I)-translocating P-type ATPase. *Proc Natl Acad Sci U S A* **97**, 652–656.

Rouch, D. A. & Brown, N. L. (1997). Copper-inducible transcriptional regulation at two promoters in the *Escherichia coli* copper resistance determinant *pco*. *Microbiology* **143**, 1191–1202.

Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbour, NY: Cold Spring Harbour Laboratory.

Simpson, A. J. G., Reinach, F. C., Arruda, P., Abreu, F. A., Acencio, M., Alvarenga, R., Alves, L. M. C., Araya, J. E., Baia, G. S. & other authors (2000). The genome sequence of the plant pathogen *Xylella fastidiosa*. The *Xylella fastidiosa* consortium of the organization for nucleotide sequencing and analysis. *Nature* **406**, 151–157.

Sun, Q., Wu, W., Qian, W., Hu, J., Fang, R. & He, C. (2003). High-quality mutant libraries of *Xanthomonas oryzae* pv. *oryzae* and *X. campestris* pv. *campestris* generated by an efficient transposon mutagenesis system. *FEMS Microbiol Lett* **226**, 145–150.

Voloudakis, A. E., Reignier, T. M. & Cooksey, D. A. (2005). Regulation of resistance to copper in *Xanthomonas axonopodis* pv. *vesicatoria*. *Appl Environ Microbiol* **71**, 782–789.

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