Research Article

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Copper resistance and genetic determinants in Chilean strains of *Clavibacter michiganensis* the causal agent of bacterial canker of tomato

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Abstract

Background: The control of *Clavibacter michiganensis*, the causal agent of bacterial canker in tomato, remains a significant challenge for crop cultivation. While copper-based products are the most commonly used bactericides, their efficacy against this pathogen is often inefficient. Therefore, the objective of this study was to determine the copper susceptibility of five Chilean *Clavibacter michiganensis* strains and to characterize their associated copper resistance gene repertoire.

Results: Chilean strains VQ28, VQ143, and VL527 showed moderate copper resistance, being able to grow at a concentration ≤ 0.32 mM of copper in CYEG medium. In contrast, strains OP3 and MSF322 showed higher copper resistance, growing at a copper-concentration ≤ 0.4 mM. The search for genes associated with copper resistance revealed the presence of the *copA, copC, copD, copZ, ycnI* and *ycnJ* genes and the *csoR1* regulator gene in the chromosomes of all the strains analyzed. The presence and location of the *csoR2* and *csoR3* regulators genes varied among the strains. Strains MSF322 and OP3, shown to be more tolerant to copper, possess a *copB* gene located in a plasmid which was not found in other Chilean strains. Notably, strain OP3, isolated in 2015 – years after the other strains – harbors copper resistance genes on plasmids highly similar to those in other Chilean strains, suggesting recent horizontal gene transfer.

Conclusion: Chilean strains of *Clavibacter michiganensis* exhibit moderate tolerance to copper, and the acquisition of new genes through horizontal gene transfer could play a crucial role in *Clavibacter michiganensis* copper resistance.

Supporting information may be found in the online version of this article.

Keywords: *Clavibacter michiganensis*; bacterial canker of tomato; copper resistance; copper-related genes; plasmids

1 INTRODUCTION

Clavibacter michiganensis (Cm) is a Gram-positive bacterium belonging to the phylum Actinomycetota, that causes bacterial canker of tomato (*Solanum lycopersicum*), one of the most devastating diseases affecting this crop.[1–4] Recent reviews[4,5] highlight the continued appearance and resurgence of Cm driven by the seed trade and the insufficient effectiveness of bactericides. Cm is a seed-transmitted bacterium that colonizes xylem vessels and distributes systemically in the host,[6] causing wilting, chlorosis and desiccation of foliage, cankers on stems, and occasionally lesions on fruits.[1–3]

The control of tomato bacterial canker persists as a serious problem for tomato cultivation, due to new outbreaks and first reports of its causal agent in different regions.[1,7] Bactericides mainly include copper-based products and antibiotics.[8–11] In Chile there are various commercial products based on copper in the forms of sulfate, hydroxide, oxychloride, and oxide. However, these treatments have been shown to be inefficient, since they only manage to reduce the concentration of the bacteria[3,12,13] and resistant strains to some of these products have been reported.[14,15]

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In previous work, Valenzuela *et al.*[16] reported the streptomycin resistance of Cm Chilean strains. The resistant Cm strains presented a mutation in the *rpsL* gene conferring resistance to streptomycin, probably associated with the intense use of antibiotics in tomato and other crops.

Copper is an essential trace element for all living organisms, acting as a cofactor in redox enzymes involved in respiration and oxidative stress responses. However, excess copper catalyzes Fenton-like reactions, disrupts membrane integrity, damages DNA, and induces protein misfolding, ultimately leading to cellular toxicity.[17–19] Therefore, cells have evolved mechanisms for copper homeostasis, which regulate the influx into the cell, and efflux of excess copper from the cell.[19] In bacteria, three proteins form the core of copper homeostasis: a copper exporting ATPase that pumps copper across the cytoplasmic membrane, a copper chaperone, which sequesters and directs cytoplasmic copper, and a copper-sensitive regulator that regulates the expression of these proteins. Gram-negative organisms possess additional components, such as copper export systems to export through the outer membrane, periplasmic multicopper oxidases (MCOs), and periplasmic copper chaperones.[20]

The genetic basis of copper resistance by Cm has been scarcely studied. In a comparative genomics study Tambong[21] described genes associated with copper homeostasis in *Clavibacter* subspecies (currently species), which included copper-translocating ATPases, chaperones, copper resistance and copper influx proteins. These genes are present in all Cm subspecies. We have previously sequenced the genome of five Chilean strains of Cm.[22,23] A study on the genetic diversity of Cm strains isolated from central Chile using Multilocus methods[24] resulted in low strain diversity. However, when analyzing virulence genes,[23] strains belonging to the same group showed differences in the repertoires and functionality of virulence genes. Differences were also observed in the expression of symptoms in tomato plants inoculated with the different strains. These results suggest that there may also be variations in gene repertoires and susceptibility to copper among Chilean strains and that strains that have been more exposed to continuous copper applications present a greater repertoire of resistance genes. Considering previous data, we hypothesized that some strains are more resistant due to a broader repertoire of copper-associated genes. The objective of this study was to determine the sensitivity to copper of five Chilean Cm strains, and their repertoires of genes associated with copper resistance.

2 MATERIALS AND METHODS

2.1 Bacterial strains and cultivation

Cm strains VQ28, VQ143, MSF322, VL527, and OP3, isolated from different regions of Chile between 1996 and 2015, were analyzed (Table 1). Approximate global positioning system (GPS) coordinates of sampling locations (latitude/longitude) of these strains are provided to enhance traceability and facilitate future spatial and epidemiological analyses. These strains were previously identified and characterized by their genetic diversity.[24] The results of the multilocus analysis grouped strains VQ28, VQ143 and OP3 into one cluster, strain MSF322 into a second cluster and VL527 into a third cluster. The genomes of these five strains were previously sequenced using Illumina and Nanopore platforms, and the assembly allowed obtaining the closed genome of four strains and the remaining strain in five contigs.[22,23] Cm strains were stored at −80 °C in 20% glycerol. In each experiment, the cultures were reactivated from glycerol in yeast–peptone–glucose agar (YPGA; 5 g L^{-1} yeast extract, 5 g L^{-1}, bactopeptone, 10 g L^{-1} glucose, 15 g L^{-1} agar), and incubated at 28 °C for 72 h for subsequent analysis.

2.2 *In vitro* evaluation of copper resistance of *Clavibacter michiganensis* strains

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) for the five Chilean Cm strains were determined based on the methodology described by Altimira *et al.*,[25] using CYEG, a low complexing mineral salt medium (1.7 g L^{-1} casitone, 0.35 g L^{-1} yeast extract, 2 g L^{-1} glycerol, 15 g L^{-1} agar), described by Andersen *et al.*[26] Bacterial liquid cultures were prepared overnight in YPG broth (5 g L^{-1} yeast extract, 5 g L^{-1} bactopeptone, 10 g L^{-1} glucose), and incubated in an orbital shaker at 28 °C. To assess the resistance levels, the highly copper-tolerant strain, *Cupriavidus metallidurans* CH34,[27] was used as positive control. The turbidity of the bacterial suspensions was measured in a spectrophotometer at 600 nm and the cultures were diluted in YPG broth to adjust turbidity to an optical density (OD) value of 0.3. Then 10 μL of each bacterial suspension, including the Chilean Cm strains and *Cupriavidus metallidurans*, were placed on a grid plate with CYEG medium supplemented with copper sulfate pentahydrate at 0, 0.04, 0.08, 0.16, 0.32, 0.4, 0.8, and 1.2 mM. Three replicates were performed for each strain on different plates. The plates were incubated at 28 °C for 72 h. The test was performed twice. An additional trial including only five Chilean strains (whose complete genome is available at the National Center for Biotechnology Information (NCBI) GenBank) and the resistant strain was performed. The MIC was defined as the lowest concentration where growth inhibition was observed, and MBC, as the lowest concentration where no bacterial growth was observed, after 72 h of incubation.

2.3 Search for copper resistance genes in *Clavibacter michiganensis*

The search for copper resistance genes was carried out in different data sources. Initially, the genome annotations of Cm strains available in the NCBI database[28] were searched, supplemented with the information reported by Tambong.[21] An E-value cutoff < 10–10, identity > 30% and coverage > 50% were used to filter the outcome. Additionally, a search for copper-related genes was performed in the BacMet Database (Antibacterial Biocide & Metal Resistance Genes Database, http://bacmet.biomedicine.gu.se/).[29] All annotated genes in the available genomes associated with copper resistance were listed and the sequences of these genes were searched and extracted from the genome of the reference strain *Clavibacter michiganensis* subsp. *michiganensis* NCPPB382 (accession number: AM711867.1; GI: 147829108). The detection and location of these genes in 32 available Cm genomes (Supporting Information Table S1), including five Chilean strains, was carried out using the BLAST+ tool.[30] Gene products of copper resistant genes were extracted from NCBI database, and the percentages of amino acid identities of the Cm strains were determined using the NCBI BLASTP tool,[30] selecting UniprotKB/Swiss-Prot(swissprot) database.

2.4 Plasmid sequence analysis

For plasmid and contigs comparison, multiple sequence alignments were performed, using the progressiveMauve tool with iterative refinement and default seed weight.[31] In addition, Cm contigs and plasmid sequences were aligned using the nucleotide

Table 1. *Clavibacter michiganensis* Chilean strains used in this study

Strain	Origin (region/locality)	Global positioning system (GPS) coordinates	Year of isolation	Submitted GenBank assembly	Assembly level	Genes (RefSeq)	Protein-coding (RefSeq)	Chromosome size (bp)	Plasmid name/size (bp)
VQ28	Valparaíso/Quillota	−32.5400, −71.1600	1996	GCA_019263765.1	Complete genome	3199	3125	32,04732	pVQ28-1/131.593 pVQ28-2/27.788
VQ143	Valparaíso/Quillota	−32.5400, −71.1600	2000	GCA_019263785.1	Complete genome	3116	3042	31,89 303	pVQ143/73.140
MSF322	Maule/Sagrada Familia	−35.1000, −71.5000	2005	GCA_011995665.1	Complete genome	3241	3160	32,84 014	pMSF1/38.824 pMSF2/76.361
VL527	Valparaíso/Limache	−33.0200, −71.2700	2012	GCA_011995885.1	Complete genome	3226	3145	33,21 579	pVL1/75.053
OP3	O'Higgins/Pichidegua	−34.3700, −71.3300	2015	GCA_011799785.1	Contig	3297	3215	5 contigs; Contig 1, 3 189 274 Contig 2, 131 602 Contig 3, 73 139 Contig 4, 38 824 Contig 5, 33 265	

BLAST tool.[30] Complementarily, contigs were analyzed using the Mash Screen strategy (maximum *P*-value 0.1) from the Plasmid Database PLSDB v.2024_05_31_v2 tool.[32]

3 RESULTS

3.1 Two strains of *Clavibacter michiganensis* were shown to be more tolerant to copper

The MIC and Minimum Bactericidal Concentration (MBC) of copper sulfate were determined for the five Chilean Cm strains in CYEG medium (Fig. 1). MIC values ranged from 0.16 to 0.4 mM (10–25 ppm copper). The MBC varied between 0.4 and 0.8 mM (25–50 ppm copper). Strains VQ28, VQ143 and VL527 were able to grow at concentrations ≤ 0.32 mM, while strains OP3 and MSF322 were able to grow at concentrations ≤ 0.4 mM copper in CYEG medium showing to be more tolerant to copper sulfate.

3.2 Only the two strains most tolerant to copper presented a *copB* resistance gene located in a plasmid

The search for genes in Cm genomes available in the NCBI database resulted in a total of ten genes (*copA, copC, copD, copZ, ycnI1, ycnI2, ycnJ, csoR1, csoR2* and *csoR3*) with annotations related to copper resistance, most of them annotated in strains NCPPB382 and CA00002 (Table S2). Annotated genes included regulators, transporters and chaperones. A comparison of gene sequences revealed that some genes found in strain NCPPB382, were homologous to genes found in strain CA00002 (i.e., *copP* was homologous to *copZ*, and gene CMM_2200 was homologous to gene *pcoC*). In the case of *copA*, identified with the same name in both strains, showed some differences in the number of base pairs (2475 bp in strain NCPPB382 and 2073 bp in strain CA00002). The location of copper related genes in the NCPPB382 strain genome was, from the origin of replication (3′-to-5′ direction) was (Fig. 2): *csoR2* (regulator); *ycnI1* (transporter); *ycnI2* next to *ycnJ* (transporters); an operon consisting of *csoR1* (regulator), *copZ* (chaperone), and *copA* (transporter); *copC* (copper resistance protein), and *copD* (copper resistance protein). The search using the BacMet database confirmed the presence of the ten copper-associated genes mentioned earlier. An additional transporter – *copB* gene – was found. The genes *copB* and *csoR3* were absent in strain NCPPB382.

The location of the genes in the genomes of the five Chilean Cm strains is similar, with some exceptions (Fig. 2). The presence of an operon including *csoR1, copZ*, and *copA* genes was found in the chromosome of the five Chilean strains. Upstream was found the *ycnJ* gene, and immediately beside the *ycnI* gene. Another copy of the *ycnI* gene was found closest to the origin of replication of the chromosome. In MSF322 and VL527 genomes, upstream gene *ycnI* the *csoR2* regulator was found, which is not present in the strain VQ143 and is found on a plasmid in strains OP3 and VQ28. Downstream of the operon, *copC* and *copD* genes were found in all strains. The *csoR3* gene was found in a plasmid in all strains, except in VQ28. Interestingly, only strains MSF322 and OP3, which showed greater resistance to copper, possess a *copB* gene. In the closed genome of the strain MSF322, this gene is located on the plasmid pMSF1, and in strain OP3 is located in the contig4 which is the same size and has a high identity with plasmid pMSF1 (see later). The *copB* gene in OP3 and MSF322 is flanked by an hypothetical protein and a recombinase family protein. When reviewing strains from other countries, this *copB* gene

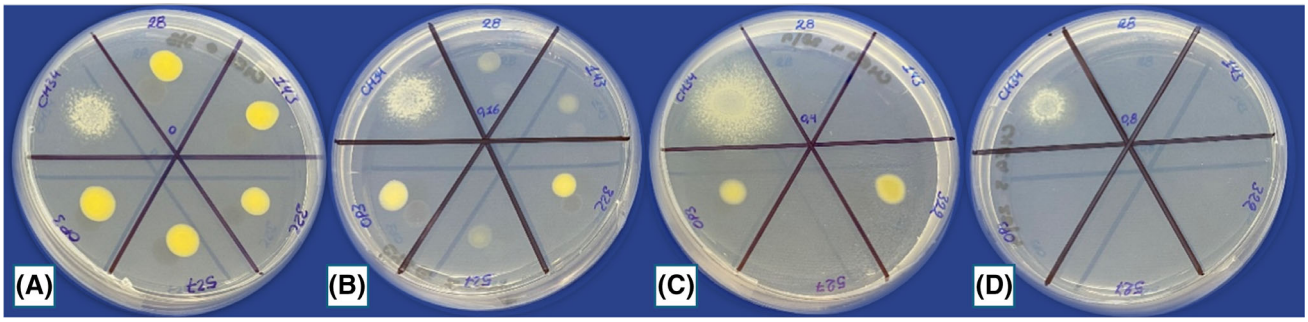

Figure 1. Determination of copper resistance in Chilean *Clavibacter michiganensis* strains. Plates showing different levels of copper resistance at 0 (A), 0.16 (B), 0.4 (C), and (D) 0.8 mM copper. Strains VQ28, VQ143 and VL527 were inhibited at 0.16 mM copper and showed no growth at 0.4 mM copper. Strains OP3 and MSF322 tolerate a concentration of 0.4 mM copper. The high copper resistant *Cupriavidus metallidurans* CH34 was included as a reference strain.

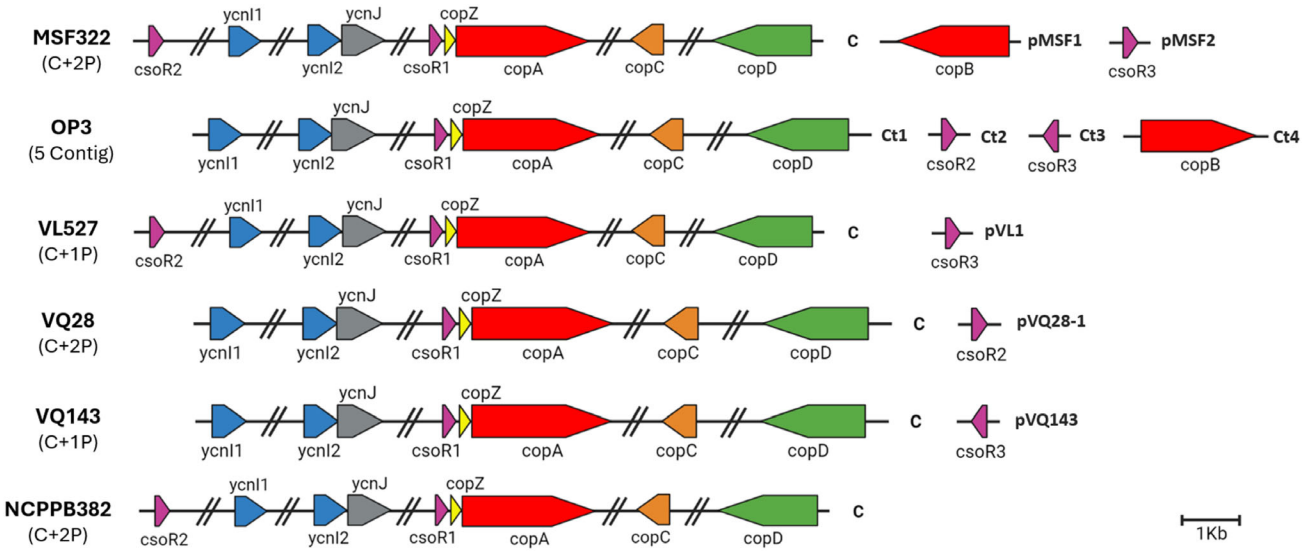

Figure 2. Copper resistance genes in Chilean *Clavibacter michiganensis* strains. Genetic distribution of gene associated to copper in the genomes from Chilean strains of *Clavibacter michiganensis* and the reference strain *Clavibacter michiganensis* NCPPB382. The *copB* gene is present in strains MSF322 and OP3. C, P, and Ct, correspond to the chromosome, plasmid, and contig, respectively.

was only found in the CFBP1465 strain from France and the CFBP7568 strain from the United States (Table S1).

When analyzing the gene products, it was observed that the size and amino acid sequence of protein CopA were variable between strains (824–832 amino acid). The gene products of CopB, CopC, CopD, CopZ, YcnI1, YcnI2, CsoR1, CsoR2 and CsoR3 were conserved with the same size and amino acid sequence, except YcnJ protein, where some differences in the amino acids were observed, but keeping the same quantity of amino acid between the strains (Table 2). Based on BLASTP analysis, gene products of *copA* of Chilean Cm strains showed 39% amino acid similarity with the copper-exporting P-type ATPase A, from *Staphylococcus haemolyticus* JCSC1435, except strain MSF322, which showed 47% amino acid similarity with the copper-exporting P-type ATPase A, of *Mycobacterium tuberculosis* CDC1551. The gene product of *copB*, present only in strains MSF322 and OP3, resulted in 42% similarity with the copper-exporting P-type ATPase B of *Enterococcus hirae* ATCC 9790. The genetic product of *copC* presented 42% amino acid similarity with the copper resistance protein C from *Pseudomonas syringae* pv. *tomato*; and the genetic product of *copD* gene resulted in 28% amino acid similarity with the copper homeostasis membrane protein CopD from *Escherichia coli*. The genetic product of *copZ* gene showed 37% amino acid similarity with the copper chaperone CopZ of *Bacillus subtilis* ssp. *subtilis* str. 168. The genetic products of *ycnI1, ycnI2* and *ycnJ* genes resulted in 33%, 35% and 27% amino acid similarity with the YcnI family protein, an uncharacterized protein YcnI and copper transport protein YcnJ from *B. subtilis* ssp. *subtilis* str. 168, respectively. The gene products of the *csoR1* and *csoR2* genes have 59% amino acid similarity with the copper-sensing transcriptional repressor CsoR of *B. subtilis* ssp. *subtilis* str. 168. The gene product of the *csoR3* gene has 57% amino acid similarity with the transcriptional regulator CsoR of *B. subtilis* ssp. *subtilis* str. 168 (Table 2).

3.3 The strain OP3 possesses three contigs with copper resistance genes that present homologies with plasmids of other Chilean Cm strains

When comparing the repertoires and location of the genes related to copper resistance, it was observed that some of them are located in plasmids. The *copB* gene is located in the plasmid pMSF1 (38 824 bp) of the strain MSF322 strain and in contig 4 (38 824 bp) of strain OP3 (Table 1). Comparing both sequences, a 99% identity was obtained. The *csoR2* gene was found in the

Table 2. Results of BLASTP copper resistance genes Chilean *Clavibacter michiganensis* strains (Uniprot/Swissprot and NCBI database)

Protein	Strain	Number of amino acids	NCBI annotation	Alignment description	Reference strain	Percent coverage (%)	Percent similarity (%)	Accession length	Accession
CopA	VQ28	831	Cation-translocating P-type ATPase	Copper-exporting P-type ATPase A	*Staphylococcus haemolyticus* JCSC1435	97	38.78	795	Q4L970.1
	VQ143	831	Cation-translocating P-type ATPase	Copper-exporting P-type ATPase A	*Staphylococcus haemolyticus* JCSC1435	97	38.78	795	Q4L970.1
	MSF322	824	Cation-translocating P-type ATPase	Copper-exporting P-type ATPase A	*Mycobacterium tuberculosis* CDC1551	97	46.95	761	P9WPU0.1
	VL527	827	Cation-translocating P-type ATPase	Copper-exporting P-type ATPase A	*Staphylococcus haemolyticus* JCSC1435	97	38.93	795	Q4L970.1
	OP3	832	Heavy metal translocating P-type ATPase	Copper-exporting P-type ATPase A	*Staphylococcus haemolyticus* JCSC1435	97	38.58	795	Q4L970.1
CopB	VQ28	NP	NP	NP	NP	NP	NP	NP	NP
	VQ143	NP	NP	NP	NP	NP	NP	NP	NP
	MSF322	702	Heavy metal translocating P-type ATPase	Copper-exporting P-type ATPase B	*Enterococcus hirae* ATCC 9790	98	41.56	745	P05425.2
	VL527	NP							
	OP3	702	Heavy metal translocating P-type ATPase	Copper-exporting P-type ATPase B	*Enterococcus hirae* ATCC 9790	98	41.56	745	P05425.2
CopC	VQ28	256	Copper resistance protein CopC	Copper resistance protein C	*Pseudomonas syringae* pv. *tomato*	49	41.67	126	P12376.1
	VQ143	256	Copper resistance protein CopC	Copper resistance protein C	*Pseudomonas syringae* pv. *tomato*	49	41.67	126	P12376.1
	MSF322	256	Copper resistance protein CopC	Copper resistance protein C	*Pseudomonas syringae* pv. *tomato*	49	41.67	126	P12376.1
	VL527	256	Copper resistance protein CopC	Copper resistance protein C	*Pseudomonas syringae* pv. *tomato*	49	41.67	126	P12376.1
	OP3	256	Copper resistance protein CopC	Copper resistance protein C	*Pseudomonas syringae* pv. *tomato*	49	41.67	126	P12376.1
CopD	VQ28	659	Cytochrome c oxidase assembly protein	Copper homeostasis membrane protein CopD	*Escherichia coli*	36	28.05	290	NPO78037.1
	VQ143	659	Cytochrome c oxidase assembly protein	Copper homeostasis membrane protein CopD	*Escherichia coli*	36	28.05	290	NPO78037.1
	MSF322	659	Cytochrome c oxidase assembly protein	Copper homeostasis membrane protein CopD	*Escherichia coli*	36	28.05	290	NPO78037.1
	VL527	659	Cytochrome c oxidase assembly protein	Copper homeostasis membrane protein CopD	*Escherichia coli*	36	28.05	290	NPO78037.1
	OP3	659	Cytochrome c oxidase assembly protein	Copper homeostasis membrane protein CopD	*Escherichia coli*	36	28.05	290	NPO78037.1
CopZ	VQ28	71	Heavy-metal-associated domain-containing protein	Copper chaperone CopZ	*Bacillus subtilis* subsp. *subtilis* str. 168	94	37.31	69	O32221.1

Table 2. Continued

Protein	Strain	Number of amino acids	NCBI annotation	Alignment description	Reference strain	Percent coverage (%)	Percent similarity (%)	Accession length	Accession
	VQ143	71	Heavy-metal-associated domain-containing protein	Copper chaperone CopZ	*Bacillus subtilis* subsp. *subtilis* str. 168	94	37.31	69	O32221.1
	MSF322	71	Heavy-metal-associated domain-containing protein	Copper chaperone CopZ	*Bacillus subtilis* subsp. *subtilis* str. 168	94	37.31	69	O32221.1
	VL527	71	Heavy-metal-associated domain-containing protein	Copper chaperone CopZ	*Bacillus subtilis* subsp. *subtilis* str. 168	94	37.31	69	O32221.1
	OP3	71	Heavy-metal-associated domain-containing protein	Copper chaperone CopZ	*Bacillus subtilis* subsp. *subtilis* str. 168	94	37.31	69	O32221.1
YcnI1	VQ28	234	YcnI family protein	YcnI family protein	*Bacillus subtilis*	37	33.33	204	WP_153255677.1
	VQ143	234	YcnI family protein	YcnI family protein	*Bacillus subtilis*	37	33.33	205	WP_153255677.2
	MSF322	234	YcnI family protein	YcnI family protein	*Bacillus subtilis*	37	33.33	206	WP_153255677.3
	VL527	234	YcnI family protein	YcnI family protein	*Bacillus subtilis*	37	33.33	207	WP_153255677.4
	OP3	234	YcnI family protein	YcnI family protein	*Bacillus subtilis*	37	33.33	208	WP_153255677.5
YcnI2	VQ28	254	YcnI family protein	Uncharacterized protein YcnI	*Bacillus subtilis* subsp. *subtilis* str. 168	94	35.27	204	P94431.1
	VQ143	254	YcnI family protein	Uncharacterized protein YcnI	*Bacillus subtilis* subsp. *subtilis* str. 168	94	35.27	204	P94431.1
	MSF322	254	YcnI family protein	Uncharacterized protein YcnI	*Bacillus subtilis* subsp. *subtilis* str. 168	94	35.27	204	P94431.1
	VL527	254	YcnI family protein	Uncharacterized protein YcnI	*Bacillus subtilis* subsp. *subtilis* str. 168	94	35.27	204	P94431.1
	OP3	254	YcnI family protein	Uncharacterized protein YcnI	*Bacillus subtilis* subsp. *subtilis* str. 168	94	35.27	204	P94431.1
YcnJ	VQ28	275	Copper resistance CopC family protein	Copper transport protein YcnJ	*Bacillus subtilis* subsp. *subtilis* str. 168	61	27.06	541	C0SP95.1
	VQ143	275	Copper resistance CopC family protein	Copper transport protein YcnJ	*Bacillus subtilis* subsp. *subtilis* str. 168	61	27.06	541	C0SP95.1
	MSF322	275	Copper resistance CopC family protein	Copper transport protein YcnJ	*Bacillus subtilis* subsp. *subtilis* str. 168	61	27.06	541	C0SP95.1
	VL527	275	Copper resistance CopC family protein	Copper transport protein YcnJ	*Bacillus subtilis* subsp. *subtilis* str. 168	61	27.06	541	C0SP95.1
	OP3	275	Copper resistance CopC family protein	Copper transport protein YcnJ	*Bacillus subtilis* subsp. *subtilis* str. 168	61	27.06	541	C0SP95.1
CsoR1	VQ28	93	Metal-sensitive transcriptional regulator	Copper-sensing transcriptional repressor CsoR	*Bacillus subtilis* subsp. *subtilis* str. 168	73	58.82	101	O32222.1
	VQ143	93	Metal-sensitive transcriptional regulator	Copper-sensing transcriptional repressor CsoR	*Bacillus subtilis* subsp. *subtilis* str. 168	73	58.82	101	O32222.1

Table 2. Continued

Protein	Strain	Number of amino acids	NCBI annotation	Alignment description	Reference strain	Percent coverage (%)	Percent similarity (%)	Accession length	Accession
	MSF322	93	Metal-sensitive transcriptional regulator	Copper-sensing transcriptional repressor CsoR	*Bacillus subtilis* subsp. *subtilis* str. 168	73	58.82	101	O32222.1
	VL527	93	Metal-sensitive transcriptional regulator	Copper-sensing transcriptional repressor CsoR	*Bacillus subtilis* subsp. *subtilis* str. 168	73	58.82	101	O32222.1
	OP3	93	Metal-sensitive transcriptional regulator	Copper-sensing transcriptional repressor CsoR	*Bacillus subtilis* subsp. *subtilis* str. 168	73	58.82	101	O32222.1
CsoR2	VQ28	105	Metal-sensitive transcriptional regulator	Uncharacterized protein YrkD, Transcriptional regulators CsoR (copper-sensitive operon repressor)	*Bacillus subtilis* subsp. *subtilis* str. 168	35	59.46	63	P54431.1
	VQ143	NP	NP	NP	NP	NP	NP	NP	NP
	MSF322	105	Metal-sensitive transcriptional regulator	Uncharacterized protein YrkD, Transcriptional regulators CsoR (copper-sensitive operon repressor)	*Bacillus subtilis* subsp. *subtilis* str. 168	35	59.46	63	P54431.1
	VL527	105	Metal-sensitive transcriptional regulator	Uncharacterized protein YrkD, Transcriptional regulators CsoR (copper-sensitive operon repressor)	*Bacillus subtilis* subsp. *subtilis* str. 168	35	59.46	64	P54431.2
	OP3	105	Metal-sensitive transcriptional regulator	Uncharacterized protein YrkD, Transcriptional regulators CsoR (copper-sensitive operon repressor)	*Bacillus subtilis* subsp. *subtilis* str. 168	35	59.46	63	P54431.1
CsoR3	VQ28	NP	NP	NP	NP	NP	NP	NP	NP
	VQ143	106	Metal-sensitive transcriptional regulator	Uncharacterized protein YrkD, Transcriptional regulators CsoR (copper-sensitive operon repressor)	*Bacillus subtilis* subsp. *subtilis* str. 168	35	56.76	63	P54431.1
	MSF322	106	Metal-sensitive transcriptional regulator	Uncharacterized protein YrkD, Transcriptional regulators CsoR (copper-	*Bacillus subtilis* subsp. *subtilis* str. 168	35	56.76	63	P54431.1

Table 2. Continued

Protein	Strain	Number of amino acids	NCBI annotation	Alignment description	Reference strain	Percent coverage (%)	Percent similarity (%)	Accession length	Accession
				sensitive operon repressor)					
	VL527	106	Metal-sensitive transcriptional regulator	Uncharacterized protein YkD, Transcriptional regulators CsoR (copper-sensitive operon repressor)	*Bacillus subtilis* subsp. *subtilis* str. 168	35	56.76	63	P54431.1
	OP3	106	Metal-sensitive transcriptional regulator	Uncharacterized protein YkD, Transcriptional regulators CsoR (copper-sensitive operon repressor)	*Bacillus subtilis* subsp. *subtilis* str. 168	35	56.76	63	P54431.1

Abbreviations: NCBI, National Center for Biotechnology Information; NP, not present.

plasmid pVQ28-1 (131 593 bp) of the strain VQ28 and in contig 2 (131 602 bp) of the strain OP3. Comparison of these sequences resulted in a homologous region of 87 Kb. Finally, the *csoR3* gene was found in the plasmid pMSF2 (76 361 bp) of strain MSF322, pVL1 (75 053 bp) of strain VL527, pVQ143 (73 140 bp) of strain VQ143 and in contig 3 (73 139 bp) of strain OP3. Interestingly, when comparing the sequences, homologous regions were observed between contig 3 and plasmid pVQ143. These homologies between the contigs of strain OP3, whose genome has not been closed, and the plasmids of the other Chilean strains would suggest that contigs 2, 3 and 4 of strain OP3 correspond to plasmids.

4 DISCUSSION

Copper remains the primary bactericide used for the control of phytopathogenic bacteria[33,34]; however, its frequent and intensive use is associated with several well-documented adverse effects. These include the accumulation of copper in agricultural soils, detrimental impacts on beneficial microbial communities, phytotoxic responses in crops, and the co-selection of resistance genes to both metals and antibiotics.[33,35,36] Furthermore, copper-driven selective pressure can enhance plasmid-mediated horizontal gene transfer, thereby facilitating the rapid spread of resistance determinants among pathogen bacterial populations.[37,38] Collectively, these concerns highlight the pressing need to develop alternative approaches and adopt integrated strategies for sustainable disease management. In order to study copper resistance mechanisms in Cm, five Chilean strains of Cm were analyzed. The CYEG medium with low mineral salt fixation was used, as it is commonly employed in copper resistance studies in phytopathogenic bacteria.[26] MIC values ranged from 0.16 to 0.4 mM and MBC values ranged from 0.4 to 0.8 mM. Strains OP3 and MSF322 were able to grow at a concentration ≤ 0.4 mM of copper in CYEG medium and were considered moderately resistant, whereas strains VQ28, VQ142 and VL542 were more sensitive to copper. De León *et al.*[39] reported MIC values of 150 μg mL^{-1} of copper sulfate (0.6 mM) and a MIC > 400 μg mL^{-1} of copper sulfate (> 1.6 mM) in YPGB/YPGA medium for Cm strains. Rajasekaran *et al.*[40] obtained MIC values for Cm of 125–250 μg mL^{-1} of copper sulfate (0.5–1.0 mM). Since the YPGA medium contains a higher concentration of components capable of chelating copper,[41,42] determinations using the CYEG medium are more accurate, which could explain the lower values obtained in this study. Reports of copper resistance carried out in other bacterial phytopathogenic species, report MIC values between 32 and 64 μg mL^{-1} (0.5 and 1 mM) of copper in CYE medium in copper-resistant strains of *Xanthomonas arboricola* pv. *juglandis* (Xaj).[43] Copper-resistant *Pseudomonas syringae* pv. *syringae* strains that grew at concentrations of 1 to 2 mM in CYE medium have been reported.[44,45] However, Loper *et al.*[46] and Jurgens and Babadoost[47] evaluated the copper resistance of *Erwinia amylovora* strains in CYE medium; none of the strains were able to grow at 0.16 mM, which was considered sensitive. These findings compared with the results obtained in this study, suggest that the Chilean strains are moderately resistant to copper. In Chile, Esterio *et al.*[48] reported Xaj strains capable of growing up to 64 μg mL^{-1} (1 mM) of copper. Moya-Elizondo *et al.*[49] reported Xaj strains capable of growing at concentrations ≥ 120 μg mL^{-1} (2 mM) of copper. The presence of these resistant strains in our country is related to excessive applications of copper products for the control of bacterial pathogens.Moreover, 12–14 applications per growing season in walnut trees fields in southern Chile have been reported.[49] The recommendation for using copper products in Chile to control phytopathogenic bacteria is to apply every 7 days and not exceed four to five

applications per season. These continuous applications of copper-based products lead to the appearance of more tolerant strains.

The search for genes associated with copper resistance in the genome of five Chilean strains of Cm, allowed us to determine the presence of ten genes. The *copA* and *copB* genes may be involved in copper efflux, while *ycnI* and *ycnJ* genes may be involved in the influx of copper to the cell. The system also includes a *copZ* gene that encodes a copper chaperone and two genes, *copC*, and *copD*, that encode copper resistance proteins. Additionally, three genes encode CsoR transcriptional repressors. Tambong[21] in a comparative genomics study between *Clavibacter* subspecies describes genes that encode the P-type copper translocating ATPase (*copA*), the CsoR repressor of the *copZA* operon, the copper(I) chaperone CopZ, two genes that encode the resistance protein CopC, and the conserved copper entry membrane protein YcnI. Additionally, a gene that encodes the copper resistance protein CopD. These genes were present in all subspecies. In our study, the genes *copB*, *ycnJ*, and two *csoR* genes were additionally found.

The location of the genes in the genomes of the five strains was very similar, with some exceptions. All strains showed an operon formed by the genes *csoR1*, *copZ*, and *copA*, and also presented the *ycnJ* gene and two copies of *ycnI* gene upstream of the operon and *copC* and *copD* genes downstream of the operon. The location and presence of *csoR2* and *csoR3* were variable among strains some of them were found in the chromosome and other located in the plasmids. The most interesting finding was the presence of *copB* gene only in MSF322 and OP3 strains, located in a plasmid, which were the strains that tolerated a higher concentration of copper. The presence of the *copB* gene does not correlate with either the MLSA genotypes or the virulence of the strains reported by Valenzuela *et al*.[23,24] A protein from the recombinase family was found next to *copB* gene in both strains, suggesting horizontal gene transfer events. Another interesting finding was that the plasmid with the *copB* gene in strain OP3 and MSF322 share 99% identity. Strain OP3 also presents two contigs carrying *csoR* regulatory genes, which are of similar size to the plasmids of strains VQ28 (*csoR2*) and strains MSF322, VL527 and VQ143 (*csoR3*), with homologous regions between them, suggesting horizontal gene transfer events. Although in strains of Cm the location of copper resistance determinants has been reported mostly in the chromosome, its location within plasmids has also been reported in the copper-resistant strains *P. syringae* pv. *syringae*[50] and *Xanthomonas citri* pv. *citri*.[51] These plasmids facilitate horizontal gene transfer. According to the location in the genome of Cm and comparing the copper homeostasis systems reported in other bacteria, this system could be divided into four subsystems. The first subsystem includes the CsoR2 repressor, YcnI, and YcnJ proteins, which have been described in *B. subtilis*.[52] The authors reported that copper acquisition is mediated by the YcnJ protein and is negatively regulated by CsoR. *Bacillus subtilis* cultures grown under copper-limiting conditions showed a significant up-regulation of the copper-responsive genes *ycnI, ycnJ, ycnK*, and *ycnL*. Therefore, this subsystem is composed of the YcnI, and YcnJ proteins may be responsible for the entry of copper into the bacterial cell in Cm and would be regulated by the CsoR repressor. The second subsystem is composed of the repressor CsoR1, the chaperone CopZ and the copper-exporting ATPase CopA. The third subsystem comprises the repressor CsoR3 and the copper-exporting ATPase CopB. A similar distribution has been described in *Lactococcus lactis*,[53] where the *copRZA* operon encodes the CopR repressor, a CopY-type repressor, the copper chaperone CopZ and the copper ATPase CopA. A second ATPase in *L. lactis* is encoded by the unlinked and monocistronic gene, which is also under the control of CopR. Transcriptional regulators in response to copper detect excess ions in the cell and modulate the transcription of genes and operons related to copper homeostasis, thus ensuring copper balance in the cell.[53] In *B. subtilis*, CsoR is a copper-sensitive transcriptional repressor that regulates the expression of the *copZA* operon that encodes a chaperone and a P-type copper efflux ATPase, respectively.[54,55] In turn, CopZ is responsible for sequestering copper or recycling the excess, transferring it to CopA to expel it from the bacterial cell.[56] The function of CopB in *L. lactis*, which has 55% similarity with the copper-exporting ATPase CopB of *Enterococcus hirae*, has not yet been demonstrated.[53] This subsystem may be responsible for the export of excess copper from the Cm bacterial cell, which may explain the higher resistance observed in the Chilean Cm strains. The fourth subsystem is made up of the CopC and CopD proteins. CopD/PcoD are membrane proteins located in the inner membrane and participate in copper transport. CopC/PcoC are copper-binding proteins located in the periplasm that have been described in Gram-negative bacteria.[57–59] Studies in *P. syringae* pv. *tomato* indicate that the *copA* and *copB* genes confer partial resistance to copper, while the *copC* and *copD* genes are required for greater resistance.[60] However, there is also evidence that *copC* and *copD* genes are required for copper entry across the membrane.[57] CopC from *P. syringae* can transfer copper from CopA to the CopD transporter located in the inner membrane, where copper is imported into the cytoplasm.[61] Transposon mutagenesis of cloned copper resistance determinants in *Xanthomonas citri* subsp. *citri* A44 revealed that *copL, copA*, and *copB* are the most important genes for copper resistance in this bacterium. Mutations in the genes *copM, copG*, *copC*, and *copD* resulted in little or no change in the strain's copper resistance.[62] There are still many questions about the functions of the different genes involved in copper homeostasis.[63] Additional studies are required to determine the role of these proteins in copper homeostasis in Cm.

The results of this study show that Chilean strains of Cm exhibit copper sensitivity and a variable repertoire of genes associated with copper homeostasis. Probably due to the multiple applications of copper to control the bacteria, the strains may have incorporated adaptation strategies, such as the acquisition of the *copB* gene, which would confer greater resistance. Given this situation, the search for new alternatives to control phytopathogenic bacteria is essential.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in NCBI at https://www.ncbi.nlm.nih.gov/.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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