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Pathogenicity and copper tolerance in Australian *Xanthomonas* species associated with bacterial leaf spot

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ABSTRACT

Recent studies into the distribution of *Xanthomonas* species causing Bacterial Leaf Spot (BLS) in Australian solanaceous crops detail varied genomic profiles that may influence pathogenicity. These genomic studies are expanded upon here by reporting the pathogenicity, race and copper tolerance of the previously sequenced *Xanthomonas* strains. Capsicum (Yolo Wonder), tomato (Grosse Lisse) and differential lines of capsicum (Early Cal-Wonder) were used to determine pathogenicity and race. Copper tolerance of 44 *Xanthomonas* strains was measured by observing bacterial growth on copper sulphate amended media. Protein sequence associated with these traits was detected using genomic analysis and compared using protein alignments. Only strains of *X. euvesicatoria* (16 strains) were found to be pathogenic on both tomato and capsicum. These were determined to be race 4 and 9. High copper tolerance was detected in the majority of *Xanthomonas* strains tested. Multiple copper resistance and avirulence proteins were detected in genomic sequence. Relatively few of these were associated with plasmid sequences. The genomic basis for copper tolerance was determined to be complex, as the tolerance thresholds did not directly correlate with gene number or presence. Similarly, pathogenicity of the strains was also not always clearly linked with presence or absence of specific Avr genes. This study highlights the need for detailed and ongoing investigations into the function of these proteins and how they produce the phenotypes that affect crop production.

1. Introduction

Bacterial leaf spot (BLS) is caused by several *Xanthomonas* species in Australia as described by previous studies (Roach et al., 2017, 2019). Investigating virulence and biocide tolerance in these bacterial populations is key to effective management strategies, making both genetic and phenotypic studies of plant pathogens necessary for understanding these systems. The current knowledge of pathogenic race and copper tolerance in Australian BLS-associated *Xanthomonas* is limited, with copper tolerance most recently assessed more than a decade ago (Martin et al., 2004). Analysing the prevalence and the pathotypes of different races and copper tolerance will enable linking genetic data to field disease data and inform control recommendations.

BLS affects tomato, capsicum and chilli crops across Australia and outbreaks of varying severity are observed each season. Severe outbreaks routinely threaten Australian tomato, capsicum and chilli production, most recently valued at $645.2 m, $172.4 m and $9.4 m, respectively ("Australian Horticulture Statistics Handbook, 2016/17 - Vegetables," 2018). Strategies for BLS control in Australia are largely preventative in nature and include applications of copper formulations and planting resistant host cultivars, while antibiotics and various alternative products have also been used in other parts of the world (Strayer-Scherer et al., 2017). Copper tolerance in pathogens causing BLS has been recorded in Australia since the 1990s, with multiple copper sprays resulting in increased tolerance in populations of *Xanthomonas campestris* pv. *vesicatoria* (Martin et al., 2004). This observation has been made worldwide, with varying incidence of copper tolerance in *Xanthomonas* strains (Abbasi et al., 2015; Aguiar et al., 2000; Lugo et al., 2013). Determining copper tolerance in bacterial populations are necessary for effective disease control strategies, though detailed information on this phenomenon is complicated by the growing understanding that lab assays may not be as accurate or comparable as once

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thought (Griffin et al., 2018). Different methods for assessing growth on copper amended media and the varying ability of some media to bind copper makes comparisons difficult and highlights the complexity of copper ion interactions (Griffin et al., 2017; Hasman et al., 2009; Pernezny et al., 2008). As a response to the increasing awareness of copper and antibiotic tolerance, recent efforts have been focused on different metal formulations and biocontrol in the form of other bacterial species and phages (Balogh et al., 2003; Pontes et al., 2016; Strayer et al., 2015).

Bacterial genes involved in copper tolerance are generally located in plasmids of *Xanthomonas* strains (Behlau et al., 2011; Richard et al., 2017). The copper resistance operon carried on plasmids is reported for the causal agent of bacterial speck, *Pseudomonas syringae* pv. *tomato*, while the presence and arrangement of these genes in BLS-causing *Xanthomonas* species appears to be more variable (Griffin et al., 2017). The copLAB gene arrangement (copL, copA, copB) has been detected in multiple *Xanthomonas* pathogens including *X. euvesicatoria*, *X. vesicatoria* and *X. gardneri* (Richard et al., 2017; Voloudakis et al., 2005). Genes with homology to copM, copC, copG, copD and copF have been identified in *X. citri* (Behlau et al., 2011), while genes with homology to copR and copS of other genera appear to be rarely detected in *X. campestris* pv. *vesicatoria* (Basim et al., 2005). These genes have been observed in *Xanthomonas* plasmids since their characterisation, suggesting they are ubiquitous and readily transferred (Bender et al., 1990; Cooksey et al., 1990; Stall et al., 1986).

A challenge to effective disease control is quantifying the durability of disease resistance genes in host crops. Race determination describes bacterial populations that are able to evade certain host resistance genes to cause disease (Kousik and Ritchie, 1999). A significant challenge for capsicum and chilli (and tomato) breeding programs is the rapidly changing genetics of the pathogen impacting the durability of host resistance genes, as rapid shifts of bacterial pathogenic ability (races) have been observed in some growing regions (Pohronezny et al., 1992; Potnis et al., 2015). Bacterial populations with gene-for-gene relationships to specific host resistance genes in pepper and tomato are described as 'races'. These are determined by assessing differential reactions on specific plant lines that contain these resistance genes (Kousik and Ritchie, 1999). Currently there are four tomato races (T1-4) and eleven pepper races (P0-10) amongst *X. euvesicatoria*, *X. perforans*, *X. gardneri* and *X. vesicatoria* described (Potnis et al., 2015). Horizontal gene transfer within bacterial populations are responsible for shifts and the development of new races that evade plant resistance genes. Shifts in the race of *Xanthomonas* populations have been observed in multiple geographic locations since the description of these races (Dahlbeck and Stall, 1979; Kousik and Ritchie, 1996). Previous studies have also detected populations of multiple races within pepper and tomato fields (Araujo et al., 2017; Sahin and Miller, 1996).

This study aims to link *X. euvesicatoria* races and *Xanthomonas* pathogenicity with previously published effector data to investigate race distribution throughout the growing regions of Australia. We also aim to link genetic copper resistance elements with a standardised method for measuring copper sulphate tolerance in vitro. The data presented here will inform management strategies of BLS in Australia, as well as highlighting *Xanthomonas* species or races that have yet to be introduced to the Australian BLS pathosystem.

2. Methods

2.1. Isolate collection

Collection and characterisation data of Australian *Xanthomonas* strains from BLS-infected fields are detailed in Roach et al. (2017). A selection of these strains that represent the phylogenetic clades in the full data set (44 Australian strains, Table 1) with published draft genomes were subjected to race and copper tolerance testing as detailed below. This includes described strains of *X. euvesicatoria*, *X. vesicatoria, X. perforans*, *X. arboricola* as well as an uncharacterised *Xanthomonas* sp.

2.2. Pathogenicity and race determination

Pathogenicity on capsicum and tomato has been reported in (Roach et al., 2019). Pathogenicity on the alternative host was observed as small, dark lesions with yellow halo that displayed bacterial streaming. Differential lines of Early Cal-Wonder (ECW-10R, ECW-20R, ECW-30R), PI 235047, and a Jupiter susceptible check (Kousik and Ritchie, 1999) containing host resistance 'Bs' genes were inoculated with isolates of *X. euvesicatoria* according to the method provided by Syngenta® (personal communication). A line containing the Australian industry standard Xcv (*X. euvesicatoria*) hypersensitive resistance trait (*C. annuum* var. Daydream) was also included in this differential set. Overnight cultures of bacteria were suspended in dH_20 to concentrations of 1×10^8 cfu/ml and infiltrated to an approximate leaf surface area of 2 cm^2 with a 1 ml syringe (without the needle) into two leaves of a plant. Sterile distilled water was used as a negative control. The inoculated plants were aged between the 2nd and 10th true leaf stage and were kept in glasshouse conditions with mean monthly maximum temperatures of approximately 30 °C. Reactions were recorded daily for three dpi (days post inoculation) as resistant (+) or susceptible (−). Resistant (hypersensitive) responses were characterised as brown/black, necrotic or papery lesions developing from 24 to 48 h for Bs2 and 48–72 h for Bs3 and Bs4. Susceptible reactions were scored as no reaction or minor water-soaking within 3 days. Responses of ± were combined to determine race according to the established reaction profiles (Kearney and Staskawicz, 1990; Minsavage et al., 1990).

2.3. Copper tolerance

Copper sulphate ($CuSO_4.5H_2O$) amended CYEG (glucose) media was prepared according to published methods (Griffin et al., 2017; Pernezny et al., 2008). Media was adjusted to pH 7 and amended with 0, 0.1, 0.2, 0.5, 0.8, 1, 1.5, 2 and 5 mM $CuSO_4$ $5H_20$ and 1 M MES buffer (2-(N-Morpholino)ethanesulfonic acid, pH 6.9) to a final concentration of 20 mM and used within three days. Plates were spotted with 10 µl aliquots of 1×10^8 cfu/ml bacterial suspensions in dH_20 and growth assessed visually as positive or negative after two days of growth at *ca.* 25 °C (Griffin et al., 2017). Susceptible and resistant controls of *P. syringae* A1513R (Andersen et al., 1991) and DC3000 (Buell et al., 2003) were used in all three replicates of the experiment. The lowest concentration of copper sulphate that inhibited bacterial growth was recorded as the minimum inhibitory concentration (MIC), with these values then averaged across three replicates and rounded to the nearest tested concentration to give the reported MIC (Table 1). The threshold for determining copper tolerance was growth on media containing more than 0.2 mM copper sulphate as determined by the copper sensitive strain.

2.4. Computational identification of metal resistance and biocide resistance genes

Metal and biocide resistance proteins were detected computationally using the BacMet predicted database version 2.0 (Pal et al., 2014) and Blast+ version 2.6.0 (Madden, 2003). The dataset reported in Roach et al. (2019) was used in this analysis which included the 44 sequenced strains used in the above analyses with reconstructed plasmid sequence and additional GenBank sequence (Roach et al., 2019). Heat-maps of each resulting matrix of identified genes were generated in R version 1.0.136 (R core team, 2016) using the 'pheatmap' package (Kolde, 2015) and annotated in GIMP version 2.8.14 (The GIMP team, 2014). Additional CopL protein sequence was detected in sequenced genomes using the Blast+ protein algorithm with a custom database of all available GenBank *Xanthomonas* CopL aa (amino acid) sequence (e-value 0.00001).

Protein sequence of the identified copper tolerance genes was extracted and aligned with publicly available complete sequence of

Table 1
Metadata of *Xanthomonas* strains in this study, including region and date of isolation, species, in vitro copper sulphate tolerance and detected copper tolerance proteins (amino acid length and protein copy number). A = CopA, B = CopB, C = CopC, D = CopD, F = CopF, G = CopG, M = CopM, U = Cop unnamed.

Isolate #							Presence of 'Cop' protein																									
	Region	Date	Spp [a]	P [a]	T [a]	MIC (mM) [a]	A [b]			B [b]		C [b]		D [b]	F [b]			G [b]			M [b]			U [b]			L [c]					
BRIP 62432	Tenterfield	2015	xa	–	-[d]	0.8	596	622		423					796			180						116				94	90				
BRIP 62410	Stanthorpe	2015	xa	–	–	0.8	622	604		423					796			180						116				90					
BRIP 62412	Stanthorpe	2015	xa	–	–	0.8	604	622	623	423	425	127			309	796	792		180	174		250		116	117			125	90				
BRIP 62414	Stanthorpe	2015	xa	–	–	1	604	623	622	425	423	127			309	792	796		174	180		250		117	116			125	90				
BRIP 62416	Stanthorpe	2015	xa	–	–	0.8	622	596		423						796			180						116				90	125				
BRIP 62390	Gatton	2014	xe	+	+	1	594	622		374		127			309	808			121			256			116				91					
BRIP 62391	Gatton	2014	xe	+	+	1.5	622	594		374		127			309	808			121			256			116				91					
BRIP 62392	Gatton	2014	xe	+	+	1.5	594	622		374		127			309	808			121			256			116				91					
BRIP 62395	Gatton	2014	xe	+	+	1.5	622	594		374		127			309	808			121			256			116				91					
BRIP 62396	Gatton	2014	xe	+	+	1.5	594	622		113		127			309	808			121			256			116				91					
BRIP 62400	Gatton	2014	xe	+	+	1.5	622	594		374		127			309	808			121			256			116				91					
BRIP 62403	Gatton	2014	xe	+	+	1.5	594	622		374		127			309	808			121			256			116				91					
BRIP 62425	Stanthorpe	2015	xe	+	+	1	622	594		374		127			309	808			121			256			116				91					
BRIP 62438	Hawkesbury Heights	2015	xe	+	+	0.5	594																						91					
BRIP 62441	Bundaberg	2015	xe	+	+	1.5	622	594		374		127			309	808			121			256			116				91					
BRIP 62555	Bundaberg	2015	xe	+	+	1.5	594	622		374		127			309	808			121			256			116				91					
BRIP 62757	Bundaberg	2015	xe	+	+	1.5	594	622		374		127			309	808			121			256			116				91					
BRIP 62858	Bundaberg	2015	xe	+	+	1.5	622	594		374		127			309	808			121			256			116				91					
BRIP 62959	Bundaberg	2015	xe	+	+	1.5	594	622		374		127			309	808			121			256			116				91					
BRIP 63464	Bundaberg	2015	xe	+	+	1.5	594	622		374		127			309	808			121			256			116				91					
BRIP 39016	Christmas Creek	1973	xe	–	+	0.5	594																						92					
BRIP 38997	Bundaberg	1986	xe	+	+	1.5	622	594		374		127			309	808			121			256			116				91					
BRIP 62383	Bowen	2012	xp	–	+	2	594	444		330		127	127		309	608	608		121	174		256	250	116	108			89					
BRIP 62384	Bowen	2012	xp	–	+	1.5	622	594		423		127			309	808			121			256		116				89					
BRIP 62386	Bundaberg	2012	xp	–	+	2	444	594		332	330	76			309	608	608		174	121		250	256	111	116			89					
BRIP 62389	South Turramurra	2013	xp	–	+	1.5	622	594		332	330	127	127		309	608	608		174	121		250	256	117	116			89					
BRIP 62397	Brisbane	2014	xp	–	+	0.5	594																						89					
BRIP 62398	Bowen	2014	xp	–	+	1	622	594		422						808			180						116				90	89				
BRIP 62404	Bowen	2014	xp	–	+	1.5	594	622		423		127			309	808			121			256			116				89					
BRIP 62405	Bundaberg	2014	xp	–	+	2	594	623		425		127			309	608	608		174			250		108	116			89					
BRIP 63262	Bundaberg	2015	xp	–	+	2	444	594		332	330	127	127		309	608	608		174	121		250	256	117	116			89					
BRIP 63565	Bundaberg	2015	xp	–	+	1.5	594	622		423		127			309	808			121			256		116				89					
BRIP 63666	Bundaberg	2015	xp	–	+	1	594	622		332	330	127	127		309	608	608		174	121		250	256	116	111			89					
BRIP 62409	Stanthorpe	2015	x	–	–	0.8	622	604		423						796			180						116				86					
BRIP 62411	Stanthorpe	2015	x	–	–	0.8	622	596		423						796			180						116				86					
BRIP 62415	Stanthorpe	2015	x	–	–	0.8	622	596		423						796			180						116				86					
BRIP 62418	Stanthorpe	2015	x	–	–	2	444	596	622	423	330	332	127	127	309	608	608	796	180	121	174	256	250	117	116	116	90						
BRIP 62413	Stanthorpe	2015	xv	–	+	1.5	604	444		330	332	71			309	608	608		121	174		256	250	117	116								
BRIP 62423	Stanthorpe	2015	xv	–	+	2	604	444		330	332	127	127		309	608	608		121	174		256	250	117	116								
BRIP 62388	South Turramurra	2012	xv	–	+	2	604	622		423		127			309	808			121			256		116									
BRIP 62428	Tenterfield	2015	xv	–	+	2	604	444		330	332	127	127		309	608	608		121	174		256	250	117	116								
BRIP 62429	Tenterfield	2015	xv	–	+	2	444	604		330	332	127	127		309	608	608		121	174		256	250	116	117								
BRIP 38864	Bowen	1982	xv	–	+	2	622	606		423		127			309	808			121			256		116									
BRIP 38861	Victoria Point	1981	xv	–	+	1.5	622	606		423		127			309	808			121			256		116									

[a] Spp: Species identification and pathogenicity on host of isolation as determined by Roach et al. (2017, 2019); xa = *Xanthomonas arboricola*, xe = *X. euvesicatoria*, xp = *X. perforans*, x = uncharacterised *Xanthomonas* species, xv = *X. vesicatoria*, P: pathogenicity on capsicum, T: pathogenicity on tomato, MIC: minimum inhibitory concentration of copper sulphate tolerated by bacterial colonies.
[b] Names of copper tolerance proteins as listed in the BacMet database and Table S1.
[d] Some opportunistic activity detected in *X. arboricola* (Roach et al., 2017).
[c] Detected proteins with 30–40% homology to CopL in GenBank.

Table 2
Race determination by reactions on differential line and amino acid length of detected Avr proteins in sequenced *X. euvesicatoria* strains.

Strain ID	Resistance gene in differential line[a]				Race[b]	Length (aa) of detected protein			
	Bs1	Bs2	Bs3	Bs4		AvrBs1	AvrBs2	AvrBs2	AvrBs3/4
BRIP 62390	–	–	+	+	4	0	714	315	311
BRIP 62391	–	–	+	+	4	0	714	315	311
BRIP 62392	–	–	+	+	4	0	714	315	311
BRIP 62395	–	–	+	–	9	0	714	315	311
BRIP 62396	–	–	+	–	9	0	714	315	311
BRIP 62400	–	–	+	–	9	0	714	315	311
BRIP 62403	–	–	+	–	9	0	714	315	307
BRIP 62425	–	–	+	+	4	0	714[c]	315	311
BRIP 62438	–	–	+	–	9	0	714	315	323
BRIP 62441	–	–	+	+	4	0	714	315	311
BRIP 62555	–	–	+	–	9	0	714	315	311
BRIP 62757	–	–	+	–	9	0	714	315	311
BRIP 62858	–	–	+	–	9	0	714	315	307
BRIP 62959	–	–	+	–	9	0	714	315	311
BRIP 63464	–	–	+	–	9	0	714	315	307
BRIP 38997	–	–	+	–	9	0	714	315	311

[a] Based on necrotic, tan lesions developing from 24 to 48 h compared to the susceptible line. Some lesions were evident in the Bs2 line after 48 h.
[b] Pepper race as determined by differential line reactions.
[c] Residue change in protein sequence.

Xanthomonas copper resistance genes (Behlau et al., 2011). Annotations adjacent to detected Cop sequence with conserved aa lengths of 116, 69, 169 and 282 were also extracted and aligned with top Blast+ hits (protein database with an e-value of 0.00001) to determine identity and potential function in the copper tolerance operon. Amino acid sequence was aligned with the Clustal Omega version 1.2.2 (Sievers et al., 2011) plugin for Geneious version 9.1.7 (Kearse et al., 2012), and figures were annotated with GIMP version 2.8.14. Operon maps of identified copper tolerance proteins in draft genome and plasmid sequence were generated using the genoPlotR package (Guy et al., 2010) in R. The total number of detected copper resistance proteins was correlated with observed copper tolerance values using simple linear regression and visualised as a scatter plot in R to give the Pearson correlation coefficient for these two variables.

2.5. Computational identification of virulence genes

Whole genome protein sequence was screened for the presence of AvrBs3/4, AvrBs1.1 and AvrBs7 homologues (Bonas et al., 1993; Potnis et al., 2012) using the Blast+ protein algorithm with a custom database of these *Xanthomonas* protein sequences (e-value 0.00001). Amino acid sequence of previously identified AvrBs2 and AvrBs3 protein (Roach et al., 2019) were aligned with the Clustal Omega version 1.2.2 (Sievers et al., 2011) plugin for Geneious version 9.1.7 (Kearse et al., 2012) and figures were annotated with GIMP version 2.8.14.

3. Results

3.1. Race determination

Race reactions were generated for 16 *X. euvesicatoria* strains from capsicum and chilli in Gatton, Bundaberg and Hawkesbury heights (NSW). Reaction profiles determined pepper races 4 and 9 were present in *X. euvesicatoria* strains (Table 2). Hypersensitive reactions for Bs2 appeared at 24–48 h, and for Bs3 and Bs4 at 24–72 h. All strains showed no reaction on ECW-10R at 3 days. All strains were negative (pathogenic reaction) on the susceptible Jupiter line. Five strains were positive (hypersensitive response) on PI235047 within 3 days, while all others were negative. All strains tested on *C. annuum* var. Daydream resulted in resistant hypersensitive responses within 3 days.

3.2. Alignments of avirulence protein sequence

Avr proteins corresponding to the gene-for-gene differential line reactions were detected in *X. euvesicatoria* (Table 2). As reported previously, no copies of AvrBs1 were detected in these *X. euvesicatoria* strains, while all 16 strains appeared to contain the AvrBs2 and 12 contained homologues similar to AvrBs3/4 proteins (Roach et al., 2019). As the protein fragments were not long enough to determine if these were AvrBs3 or AvrBs4 they will be referred to as AvrBs3/4. Investigating the alignments of these proteins (Fig. 1) revealed each strain of *X. euvesicatoria* had two potential copies of AvrBs2; one of 714 aa that were nearly identical between the 16 strains, and one of 315 aa, that were also close to identical in amino acid sequence. The 714 aa protein was the same length as reported AvrBs2 protein (Swords et al., 1996), while the 315 aa protein represents a segment from the middle of the full AvrBs2 gene based on homology. An AvrBs3/4 protein of 311 aa was detected in 12 strains, three strains had protein sequence of 307 aa, and one strain had sequence of 323 aa. (Fig. 1, Table 2). The detected AvrBs3/4 protein were shorter than characterised *Xanthomonas* AvrBs3 (Bonas et al., 1989) and AvrBs4 (Bonas et al., 1993) proteins, and therefore could not be determined as either AvrBs3 or AvrBs4. AvrBs1.1 and AvrBs7 were not detected in any Australian *X. euvesicatoria* strains, though protein sequence homologous to both was detected in most *X. vesicatoria* strains (data not shown).

3.3. Copper tolerance determination

All 44 Australian *Xanthomonas* strains were unable to grow on media containing more than 1.5 mM $CuSO_4$ $5H_2O$ and all strains tolerated at least 0.5 mM $CuSO_4$ $5H_2O$ (Table 1). All strains grew on media containing no copper, and the tolerant and sensitive control strains had a MIC of 2 mM and 0.2 mM, respectively. Thirteen strains displayed the same copper tolerance as the resistant control (2 mM). On average, *X. vesicatoria*, *X. euvesicatoria* and *X. perforans* strains showed higher copper tolerance (2, 1.5 and 1.5, respectively) than the *X. arboricola* (0.8) and uncharacterised *Xanthomonas* strains (1).

3.4. Metal and biocide resistance genes

Homologues of twenty-six metal and biocide resistance proteins were identified in this dataset (Fig. 2, Table S1). None were unique to any species, and the profiles of detected proteins did not arrange strains by species. All strains contained proteins homologous to tolCsm, pcm, emrCsm, emrBsm and emrRsm. All but one strain (DAR 26933, Redland Bay 1977) contained at least one copy of CopA. Biocide resistance families (tolCsm, emrAsm, pcm, emrCsm, emrBsm and emrRsm) of salicylanilides, organo-sulphates, selenium, and hydrogen peroxide were

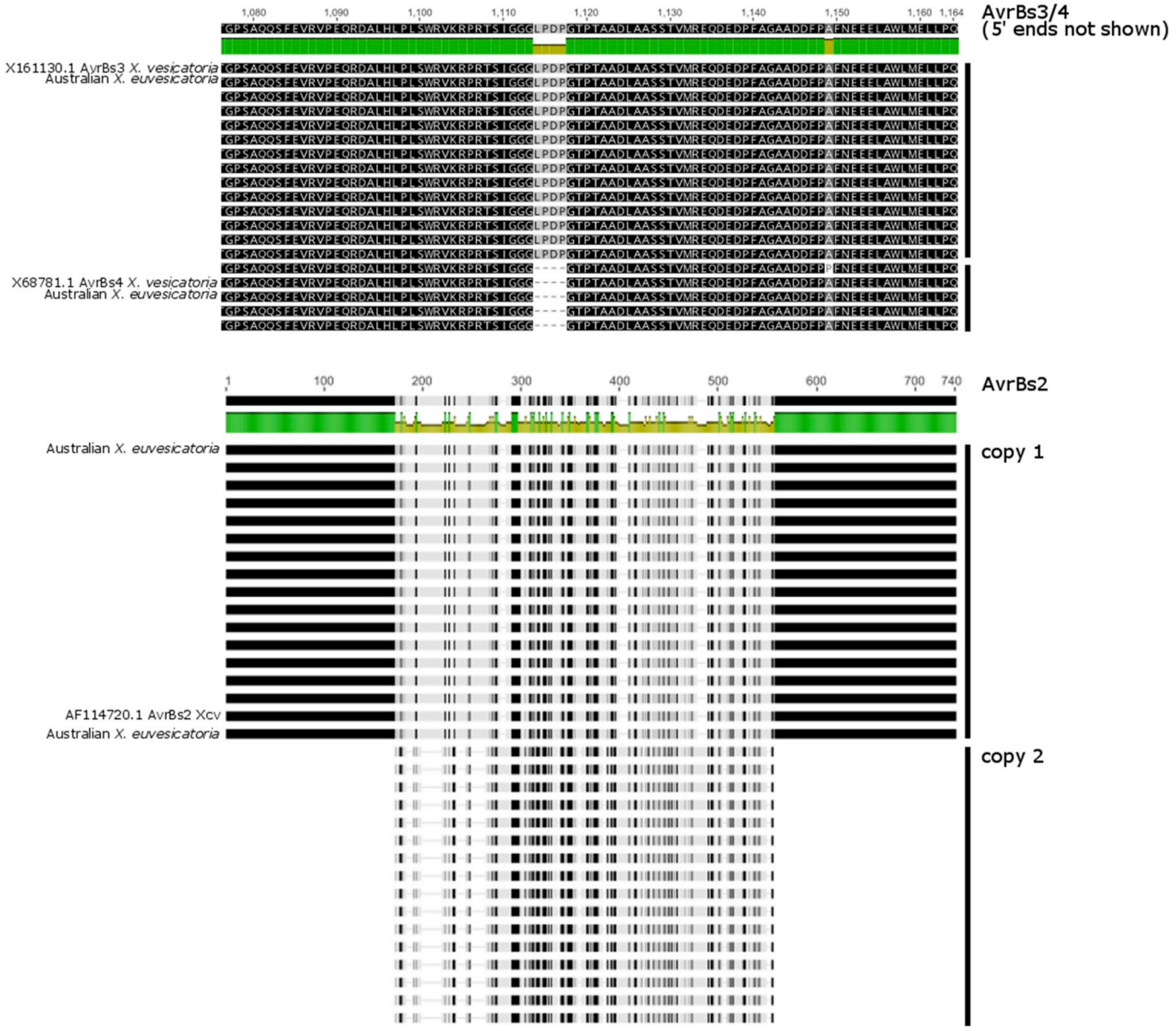

Fig. 1. Consensus sequence of amino acid alignments for each detected Avr protein in *X. euvesicatoria* strains. Amino acid similarity is indicated in grayscale, and in the consensus sequence above the alignment. The identical 5′ ends of AvrBs3/4 are not displayed.

also detected in the Australian strains (Table S1).

All 44 Australian strains contained at least one CopA protein of varying lengths. Copper tolerance proteins CopB, CopC, CopD, CopF, CopG, and CopM were also detected (Table 1). Proteins with low (30–40%) percent identity to CopL were detected using Blast searches as they were not detected in the genome sequence by the BacMet program. Cop proteins of the same length and number as the genomic proteins were identified in nine putative plasmids, with the addition of CopF and longer CopL sequences that were not detected in the corresponding whole genome sequence (Fig. 3). These putative plasmids were 88 kb in *X. vesicatoria* and 150 kb in *X. euvesicatoria* (strains BRIP 62390, 62403, 62425, 62757, 62858, 62959, 63464, 38864, and 38861) (Roach et al., 2019). Protein sequence homologous to a hypothetical transcriptional repressor was also detected in whole genome sequence ('unknown', Fig. 4). No other metal or biocide resistance proteins in the BacMet database were detected in any putative plasmid sequence. Individual effects of the detected genes on copper tolerance were not determined.

3.5. Alignments of Cop protein sequence

Alignments of the Cop proteins listed above from whole genome protein sequence of all strains listed in this study (Table 1) highlight differences in putative amino acid sequence and length (Fig. 4). CopC, CopD and a hypothetical protein identified in Behlau et al. (2017), "Cop-unnamed", are generally uniform apart from a few residue changes and some truncated sequences. Annotations adjacent to detected Cop sequence with conserved aa lengths of 116, 69, 169 and 282 were most similar to previously described protein associated with copper processing (Fig. 4). While the functions of these proteins have not been determined, they are included due to their consistent appearance in these draft copper tolerance operons. Alignments of these sequences are also included in Fig. 4.

4. Discussion

Xanthomonas strains isolated from Australian BLS-affected crops were previously characterised with genomic studies, describing genetic

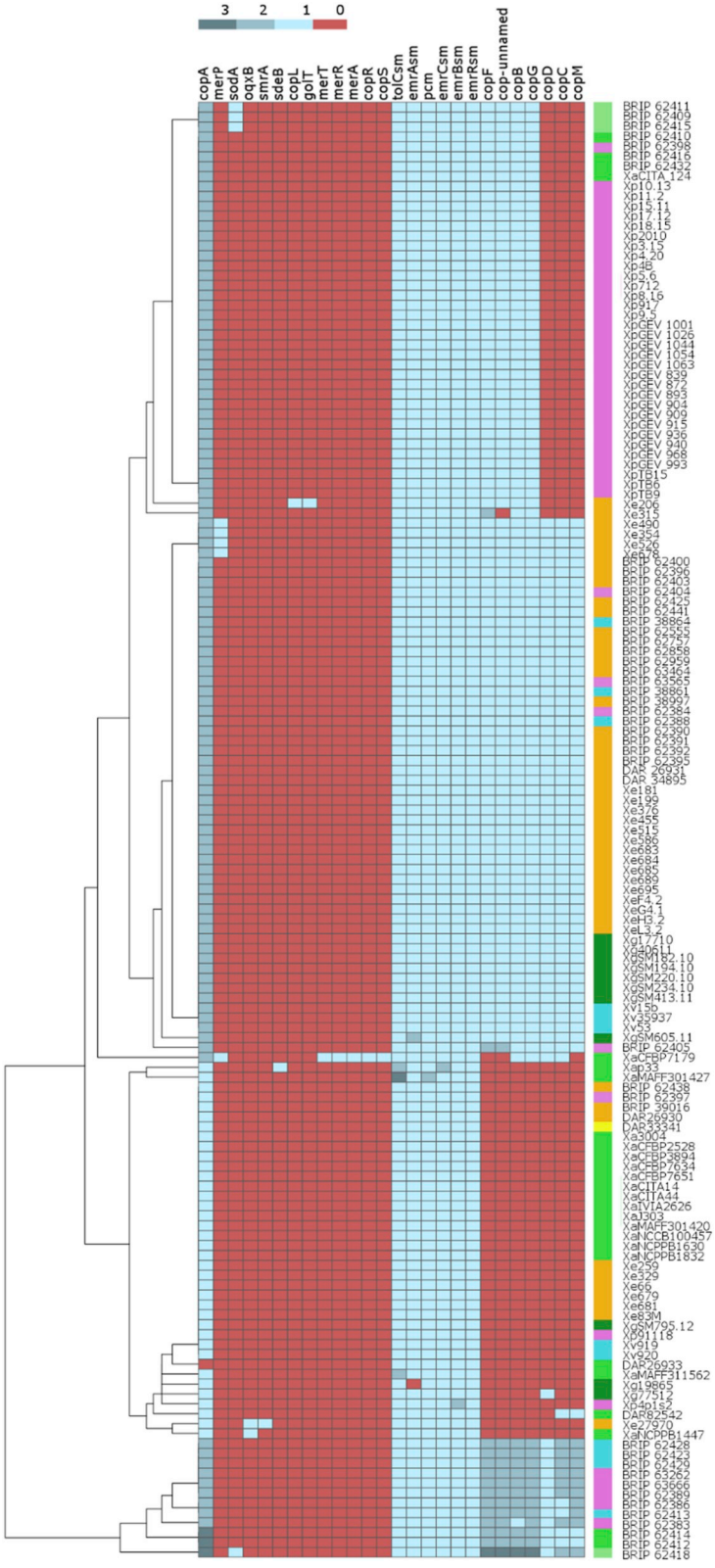

Fig. 2. Predicted metal and biocide resistance proteins from the BacMet database identified in draft genomes of 147 *Xanthomonas* strains. Protein names are listed on the X axis, and the strain name is listed on the Y axis. The scale describes copy number of identified proteins. The coloured bar above strain labels indicate species as follows: *X. perforans*; pink, *X. euvesicatoria*; orange, *X. arboricola*; green, *Xanthomonas* sp.; light green, *X. gardneri*; dark green, *X. vesicatoria*; blue, DAR 33341; yellow. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

diversity and specific effectors of the T3SS that impact pathogenicity, including Avr proteins (Roach et al., 2019). Using this genetic data, the phenotypes of pathogenicity and copper resistance examined in this study can be investigated in relation to the underlying pathogen genetics. The multiple races and copper tolerance identified in Australian *Xanthomonas* strains here emphasizes disease management issues that have been encountered worldwide, and are now detailed for Australian pathogen populations.

4.1. Host range of Australian Xanthomonas strains

While *X. euvesicatoria* is commonly reported as a pathogen of tomato

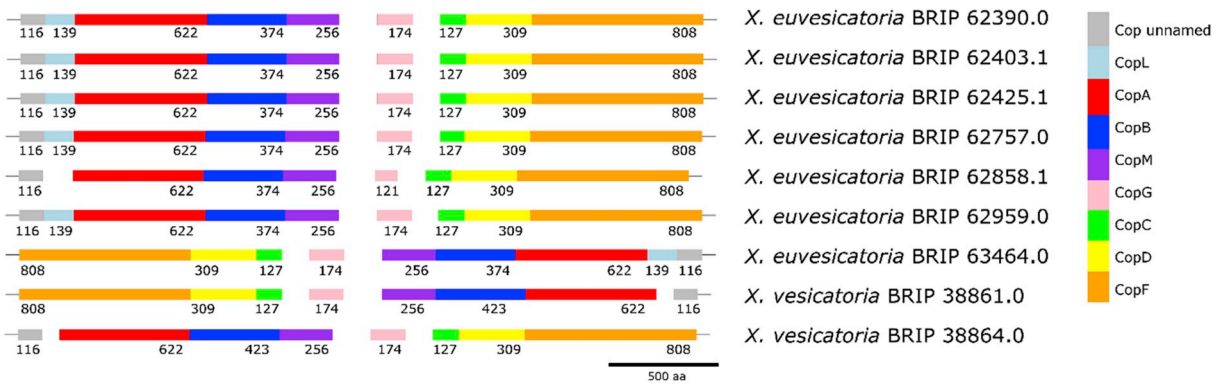

Fig. 3. Arrangement of identified Cop aa (amino acid) sequence in reconstructed plasmids of seven *X. euvesicatoria* and two *X. vesicatoria* strains. Colours relate to Cop names as described in the key and approximately correspond to aa length as shown by the scale. Coloured blocks indicate protein sequence, white gaps indicate uncharacterised sequence. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

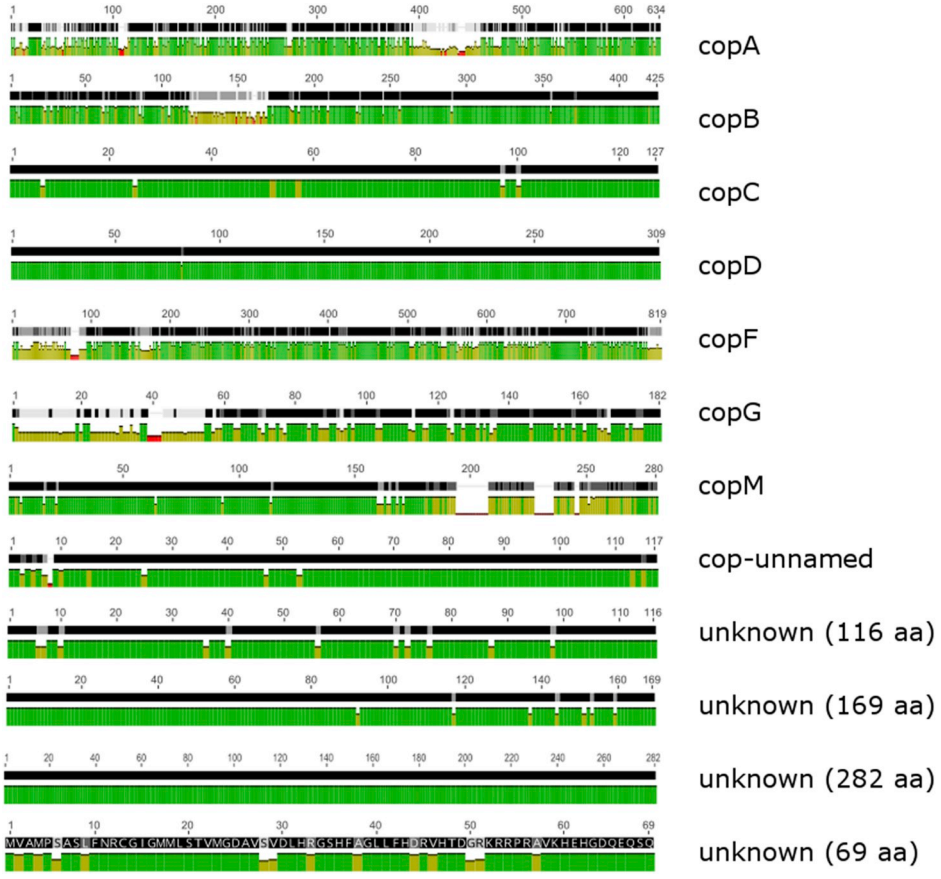

Fig. 4. Consensus sequence of amino acid alignments for each detected copper tolerance gene in all Australian *Xanthomonas* strains as listed in Table 1. Amino acid similarity is indicated in grayscale, and in the consensus sequence above the alignment. Alignments labelled 'unknown' are conserved protein sequences detected in Australian strains next to characterised Cop protein. The alignment labelled 'cop-unnamed' is an uncharacterised copper resistance protein from Behlau et al. (2017).

and pepper, all but two *X. euvesicatoria* strains (BRIP 39016 and DAR 26930) from Australian crops were found in capsicum and chilli (Roach et al., 2017, 2019). While *X. euvesicatoria* infections of tomato crops are not rare (Mbega et al., 2012; Potnis et al., 2015), recent reports indicate it is more common to observe *X. perforans* (and *X. gardneri*) in tomato and *X. euvesicatoria* in peppers (Ivey et al., 2016a; Ivey et al., 2016b; Myung et al., 2015; Schwartz et al., 2015). Prior to 1991, *X. euvesicatoria* was the main BLS pathogen on tomato in Florida (Potnis et al., 2015). More recent isolates from 1994 through 2006 revealed a large species and race shift of BLS pathogens in tomato crops of the southern USA. This indicates it was once more common to find *X. euvesicatoria* in tomato than it is today. As the only Australian *X. euvesicatoria* strains isolated from tomato were from 1973 to 1976, it is likely that Australian *X. euvesicatoria* populations reflect this host shift observed overseas.

It has been suggested the observed species shift is the result of bacteriocin production of *X. perforans* out-competing *X. euvesicatoria* in this niche (Lue et al., 2010). It has been previously determined (Roach et al., 2019) that *X. euvesicatoria* and *X. perforans* are genetically very similar, though we analyse these clades separately to describe the differences in phenotypes. The potential for these populations to compete and the observation that Australian *X. perforans* strains do not infect capsicum further support the utility of retaining the separate taxa.

It is generally accepted that the BLS-causing *Xanthomonas* species, while often associated with certain hosts, can be isolated from a variety of crops (Potnis et al., 2015). Recent studies of Australian and worldwide *Xanthomonas* effector profiles demonstrate the complexity of type III secretion system (T3SS) effectors, the plasmids that carry them and the role they play in pathogenicity (Roach et al., 2019; Potnis et al., 2015; Schwartz et al., 2015). While *X. perforans* is generally not considered pathogenic on pepper, one strain (Xp 2010) from Florida displayed dual infecting ability in pepper and tomato (Schwartz et al., 2015). An Australian strain, BRIP 62398, phylogenetically related to Xp2010 did not share this trait (Roach et al., 2019), as all tested Australian *X. perforans* strains were pathogenic only on tomato. This indicates pathogenicity traits are not necessarily reflected in phylogenies, and are a consequence of acquired genetic elements.

4.2. Detected pepper races highlight complexity of pathogenicity

A study of resistance to BLS pathogens in tomato and capsicum concluded a combination of on-farm hygiene, spray programs and resistance genes is necessary for effective disease control (Stall et al., 2009). Pyramiding resistance genes can provide disease resistance even if these genes have been defeated by bacterial pathogen populations. Conversely, under heavy disease pressure and adverse weather conditions, disease has still been observed in crops of resistant lines. Races of *Xanthomonas* describe populations with the ability to cause disease or hypersensitive reactions on host cultivars containing different resistance genes. These races have historically been determined using hypersensitive assays, with three races first described this way in 1969 (Cook and Stall, 1969; Kim and Hartmann, 1985). After the discovery of dominant host resistance genes, avirulence genes in the pathogen population were characterised as gene specific resistance (Swords et al., 1996), where host R genes recognise the avr genes in pathogens and induce a hypersensitive response. Mutations in these bacterial populations are responsible for the race shifts, which has been observed in multiple regions since the description of these races (Dahlbeck and Stall, 1979; Kousik and Ritchie, 1996). Variability in the timing of HR development and the phenotypes of HR with different host lines and pathogen strains have been characterised before, indicating the variability observed here in some strains is to be expected (Cook, 1973; Minsavage et al., 1990). Each host resistance gene results in a slightly different hypersensitive response for each plant line, indicating their mode of action for providing resistance is different for each gene (Hibberd et al., 1987).

Capsicum lines expressing Bs genes have been well characterised as providing resistance to BLS-causing *Xanthomonas* populations with corresponding Avr genes (Kim and Hartmann, 1985; Stall et al., 2009). The detected proteins require larger fragments to characterise. The characterisation of the *avrBs4* gene is not well defined as discussed below, though it is still unclear how this relates to the HR observed in Bs4 lines. Based on the differential line reactions, races 4 and 9 are present in Australian crops. The cultivar Daydream is a recent release that contains the industry standard XCV 1–5 and XCV 7–9 BLS resistance genes ("Daydream (RPP25150) - F1 Hybrid Capsicum Open Field," 2015). While these resistance genes are providing adequate protection currently, ongoing work is needed to maintain durable resistance. Characterising pathogen Avr genes that determine host specificity is an ongoing challenge necessary for enduring disease management in capsicum and tomato, likely involving more detailed investigations of type 3 effectors (Potnis et al., 2015).

4.3. Pepper races have a complex genetic basis

While the presence of AvrBs2 protein was detected previously in these *X. euvesicatoria* strains (Roach et al., 2019), further investigation reveals that two copies of this protein appear to be present (based on homology only); one of 714 aa and one of 315 aa. The 315 aa protein annotation represents the middle segment of the 714 aa protein. The possession of a truncated version in *X. euvesicatoria* suggests there may have been multiple acquisitions of this gene, possibly with one or more inactivation events. It has been noted that full AvrBs2 is needed for virulence (Kearney and Staskawicz, 1990), so the possibility of an additional truncated copy may not impact virulence at all.

The conservation of the AvrBs2 gene among many pathovars of *X. campestris* has been observed in many studies, and the detection of this protein in multiple *Xanthomonas* species (Roach et al., 2019) supports the suggestion that Bs2 recognises an essential pathogen gene (Kearney and Staskawicz, 1990; Swords et al., 1996). It has been demonstrated that the *avrBs2* is localised to the chromosome, as you would expect an essential, widely distributed gene to be (Minsavage et al., 1990). This same study found *avrBsT* and *avrBs3* to be carried on plasmids native to Xcv strains. To determine if the plasmid sequence of Australian *X. euvesicatoria* strains contain *avr* genes, physical pasmids will need to be sequenced for confirmation. One study of avirulence and copper tolerance genes on plasmids linked race shifts to copper usage, suggesting that the linkage of these genes on a transmissible plasmid may have supressed certain races as copper use increased (Pohronezny et al., 1992).

The detected homologues of AvrBs3 in *X. euvesicatoria* (Roach et al., 2019) were only a fragment (311 aa) of the full proteins (Bonas et al., 1993, 1989). AvrBs4, initially described as the *avrBs3-2* gene, is highly similar to AvrBs3 (Bonas et al., 1993), therefore the full nucleotide sequence of these genes with high read coverage will be necessary to determine presence of AvrBs3 or AvrBs4. As repeat regions present in these genes make accurate reads difficult, there is little AvrBs4 sequence available for comparison (Stall et al., 2009). Deletions and insertions have been observed in avirulence genes before, often contributing to shifts in pathogenic ability (Araujo et al., 2017; Kearney and Staskawicz, 1990). The specific functionality of these proteins and the observed deletions would require functional studies to fully characterise.

While the characterised Avr genes do provide effective crop protection in capsicum, additional host gene targets are being assessed to ensure durable disease resistance, particularly in tomato. It has been suggested that AvrBs1.1/AvrBs7 may interact with a new gene from C. *baccatum* var. *pendulum* (Potnis et al., 2012). No AvrBs1.1 or AvrBs7 was detected in Australian *X. euvesicatoria*, though proteins with homology to AvrBs1.1 (to a lesser extent AvrBs7) were detected in all *X. vesicatoria* strains (data not shown). While Australian grown commercial tomato cultivars don't have resistance genes, resistance against the XopJ4 effector in *X. perforans* has been identified in *Solanum pennellii* (Timilsina et al., 2016). Further investigations into these genes may present options for resistance breeding in tomato.

4.4. Copper tolerance in BLS-associated Xanthomonas species of Australia is widespread

The abundant copper tolerance observed in Australian *Xanthomonas* populations reflects the previous observations (Martin et al., 2004). Three of the tested Australian strains (BRIP 39016, BRIP 62438, BRIP 62397) that were most sensitive to copper at 0.5 mM MIC (though still more tolerant than the susceptible control at 0.2 mM MIC), were also separated by time and location from other collections. This reduced ability to tolerate copper may be due to the fact they were not isolated in growing regions exposed to current typical levels of copper sprays. A fitness cost to copper tolerance outside of selection pressure has been observed in *X. perforans*, suggesting the copper resistant phenotype is likely to be lost in populations not consistently exposed (Araujo et al., 2012). The high levels of copper tolerance in strains isolated from commercial crops in recent years supports this, as copper is widely used in these areas. Martin et al. (2004) demonstrated that the prevalence of tolerance increased in strains isolated pre-1987 to 2000. Based on their measurement of 40% tolerant strains in similar regions as sampled for this study our observation of 93% tolerant strains indicate the prevalence of copper tolerance has increased. This mirrors the situation recently reported for Australian *Pseudomonas syringae* pv. *tomato* strains

from tomato crops (Griffin et al., 2018, in press).

While direct comparison of copper resistance levels across studies is not possible due to differences in detection methods, the worldwide prevalence of generally high tolerance levels makes investigation into alternative control methods necessary. This also means bacterial resistance to alternative compounds and metals must be investigated. No protein sequence in Australian strains were homologous to publicly available silver resistance sequence, which would have implications for silver-based formulations that show promising control of disease (Strayer et al., 2015).

4.5. 'Cop' proteins of Australian Xanthomonas show specific arrangements

Multiple copies of amino acid sequence homologous to previously characterised copper resistance proteins were identified in this dataset, consistent with the high levels of tolerance observed. While the draft genome sequence may not detect all copper resistance genes or reliably predict the clusters, many detected homologues were still evident. The presence of the CopLAB and CopMGF clusters have been described in other *Xanthomonas* species (Behlau et al., 2017, 2011; Voloudakis et al., 2005), also showing that *copLAB* and *copMGF* genes are common to large *Xanthomonas* plasmids. CopLAB and CopMGF aa arrangements were also shown to be present on multiple scaffolds of genome assemblies, which is supported by the arrangement of Cop proteins in this study across the draft genomes. CopA has been described as a multi-copper oxidase that is essential for processing environmental copper (Hsiao et al., 2011), and the abundance of detected CopA in this study supports this. It has been demonstrated that the *copLAB* gene arrangement is responsible for tolerance (Behlau et al., 2011), though many of our most tolerant isolates have no detected CopL. Considering the low homology of these proteins, it is still possible they were too dissimilar to detect. All CopA proteins of 594 aa have adjacent CopL sequence, indicating this arrangement is likely still typical of *Xanthomonas* species.

Alignments of Cop aa sequence in Australian *Xanthomonas* show that certain proteins are highly conserved, with only a few residue changes in some strains. Other proteins show much more variation, which has also been observed in a study of *copL* and *copB* genes (Behlau et al., 2017). In particular, low homology between *copL* nucleotide sequences was noted and may explain the difficulty in detecting this protein in our sequence by homology. Our CopB aa sequence showed reduced homology in the n-terminal region, which reflected alignments of other copB genes that also demonstrated this region is necessary for full resistance (Behlau et al., 2017, 2011). The protein referred to here as 'Cop-unnamed', is most similar to the 116 aa hypothetical transcriptional repressor of *Xanthomonas alfalfae* subsp. *citrumelonis* (Behlau et al., 2017). The action of these genes individually and combined will need to be determined.

4.6. Conserved hypothetical protein sequence suggests novel copper tolerance genes

An annotated protein adjacent to all CopC proteins (except in BRIP 62386 and BRIP 62413) was the same length to the *Xanthomonas alfalfae* subsp. *citrumelonis* hypothetical transcriptional repressor (116 aa). While this hypothetical 116 aa (Fig. 4) has high homology to the hypothetical transcriptional repressor, it was not identified by our detection methods. All detected CopM proteins (apart from one in BRIP 62428) have an unidentified protein of 169 aa between it and a CopG protein. This 169 aa unidentified protein has homology to *S. maltophilia* putative transposases, but no function relating to copper tolerance in these strains is yet known. Several proteins matching a cytochrome *c* family protein from *X. perforans* were identified between CopA and Cop-unnamed sequence (except in *X. vesicatoria* strains BRIP 62423 and BRIP 62405). The consistent length and arrangement of these unidentified proteins indicates they may play an important role in CopLAB and CopMGF systems.

4.7. Few copper tolerance protein homologues were associated with reconstructed plasmid sequence

There are many studies describing the presence of these genes on plasmids (Bender et al., 1990; Richard et al., 2017; Voloudakis et al., 1993), though proteins homologous to described Cop proteins were more often associated with chromosome sequence than reconstructed circular sequence in this study. While these putative plasmids were only computationally identified, chromosomal *cop* genes have been described in Xcv (Basim et al., 2005), indicating the unique arrangement of these proteins on chromosomes may be more common in *Xanthomonas* than once thought. Copper homeostasis (*coh* genes) have been described in resistant and susceptible strains as a part of the bacterial copper metabolism as opposed to the plasmid borne copper resistance (*cop* genes). Several studies have noted the plasmid borne genes provide the majority of the copper resistance, while chromosomal genes are present in copper tolerant and sensitive strains (Behlau et al., 2017). There is increasing evidence that plasmid borne copper resistance genes may be integrated into the chromosome (Argüello et al., 2013). Closed genomes generated with long read sequencing would give greater resolution of copper tolerance homologues and their operon arrangements, further refining the patterns seen in these draft genomes.

The potential for plasmids to be readily transferred between *Xanthomonas* populations has been demonstrated before in *Xanthomonas* species (Canteros et al., 1995; Stall et al., 1986). The similarity of cop genes in otherwise distantly related species is further evidence of this (Behlau et al., 2017). CopLABMGF proteins of some *X. campestris* strains appear closely related to their counterparts in *X. vesicatoria*, likely a consequence of HGT between bacterial populations of a given niche. The presence of similar Cop protein in our *Xanthomonas* sp. and *X. arboricola* strains also suggests the transfer of these elements between different species is common, and supports the suggestion of Behlau et al. (2017) that unique clusters may have come from an unidentified environmental bacterial population.

5. Conclusions

This work presents many opportunities to further investigate how control of bacterial foliar disease may be achieved. New technologies such as lamp are being developed for use as field deployable diagnostics, which may also be used to detect a variety of virulence and resistance factors in bacteria. Resistance in commercial pepper lines is durable, though ongoing efforts will be needed to ensure this and improve it in tomato. The presence of resistance genes in bacteria may also be used to track outbreaks and pinpoint sources of disease. The presence of antibiotic resistance genes disseminated through HGT may also provide data on the movements of pathogen populations. The ability to protect crops with targeted diagnostics and quickly assess virulence of bacterial populations will not only inform management strategies, but could also be applied for quarantine purposes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cropro.2019.104923.

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