

Evolution of copper resistance in the kiwifruit pathogen *Pseudomonas syringae* pv. *actinidiae* through acquisition of integrative conjugative elements and plasmids

Elena Colombi,^{1*} Christina Straub,¹ Sven Künzel,² Matthew D. Templeton,^{3,4} Honour C. McCann^{1,5†} and Paul B. Rainey^{1,2,6†}

¹New Zealand Institute for Advanced Study, Massey University, Auckland, New Zealand.

²Max Planck Institute for Evolutionary Biology, Plön, Germany.

³Plant and Food Research, Auckland, New Zealand.

⁴School of Biological Sciences, University of Auckland, Auckland, New Zealand.

⁵South China Botanical Institute, Chinese Academy of Sciences, Guangzhou, China.

⁶Ecole Supérieure de Physique et de Chimie Industrielles de la Ville de Paris (ESPCI Paris-Tech), PSL Research University, Paris, France.

Summary

Horizontal gene transfer can precipitate rapid evolutionary change. In 2010 the global pandemic of kiwifruit canker disease caused by *Pseudomonas syringae* pv. *actinidiae* (*Psa*) reached New Zealand. At the time of introduction, the single clone responsible for the outbreak was sensitive to copper, however, analysis of a sample of isolates taken in 2015 and 2016 showed that a quarter were copper resistant. Genome sequences of seven strains showed that copper resistance – comprising *czc/cusABC* and *copABCD* systems – along with resistance to arsenic and cadmium, was acquired via uptake of integrative conjugative elements (ICEs), but also plasmids. Comparative analysis showed ICEs to have a mosaic structure, with one being a tripartite arrangement of two different ICEs and a plasmid that were isolated in 1921 (USA), 1968 (NZ) and 1988 (Japan), from *P. syringae* pathogens of millet, wheat and kiwifruit respectively. Two of the *Psa* ICEs were nearly

identical to two ICEs isolated from kiwifruit leaf colonists prior to the introduction of *Psa* into NZ. Additionally, we show ICE transfer *in vitro* and *in planta*, analyze fitness consequences of ICE carriage, capture the *de novo* formation of novel recombinant ICEs, and explore ICE host-range.

Introduction

Horizontal gene transfer (HGT) is a potent evolutionary process that significantly shapes patterns of diversity in bacterial populations. Horizontally transmissible elements, including plasmids, phages and integrative conjugative elements (ICEs) move genes over broad phylogenetic distances and mediate abrupt changes in niche preferences that may even fuel speciation (Griffith, 1928; Médigue *et al.*, 1991; Lan and Reeves, 1996; Sullivan and Ronson, 1998; Ochman *et al.*, 2000; Ochman *et al.*, 2005; Retchless and Lawrence, 2007; Guglielmini *et al.*, 2011; Popa and Dagan, 2011; Polz *et al.*, 2013).

ICEs are plasmid-like entities with attributes of temperate phages that disseminate vertically as part of the bacterial chromosome and horizontally by virtue of endogenously encoded machinery for conjugative transfer (Wozniak and Waldor, 2010; Guglielmini *et al.*, 2011). Essential genetic modules include those mediating integration, excision, conjugation and regulation of conjugative activity (Mohd-Zain *et al.*, 2004; Juhas *et al.*, 2007; Roberts and Mullany, 2009). During the process of conjugation ICEs circularize and transfer to new hosts, leaving a copy in the original host genome (Wozniak and Waldor, 2010; Johnson and Grossman, 2015). Conjugation during pathogenesis is often regulated by environmental signals (Lovell *et al.*, 2009; Quiroz *et al.*, 2011; Vanga *et al.*, 2015).

In addition to a set of essential genes, ICEs often harbour ‘cargo’ genes of adaptive significance to their hosts. These include genes affecting biofilm formation, pathogenicity, antibiotic and heavy metal resistance, symbiosis and bacteriocin synthesis (Peters *et al.*, 1991; Rauch and De Vos, 1992; Ravatn *et al.*, 1998; Beaber *et al.*, 2002;

Received 18 August, 2016; accepted 2 January, 2017. *For correspondence. E-mail e.colombi@massey.ac.nz. Tel. (+64) 9 4140800 ext 43810. †Joint senior authors.

Drenkard and Ausubel, 2002; Burrus *et al.*, 2006; Ramsay *et al.*, 2006; Dimopoulou *et al.*, 2007; Kung *et al.*, 2010). The genetic information stored in cargo genes varies considerably resulting in ICEs that range in size from 20 kb to 500 kb (Johnson and Grossman, 2015).

In 2008, a distinct and particularly virulent form of the kiwifruit pathogen *Pseudomonas syringae* pv. *actinidiae* (*Psa*) was identified in Italy. It was subsequently disseminated throughout kiwifruit growing regions of the world causing a global pandemic that reached New Zealand (NZ) in 2010 (Balestra *et al.*, 2010; Abelleira *et al.*, 2011; Everett *et al.*, 2011; Vanneste *et al.*, 2011). Genomic analysis showed that although the pandemic was derived from a single clone it acquired a set of distinctive ICEs during the course of its global journey (Mazzaglia *et al.*, 2012; Butler *et al.*, 2013; McCann *et al.*, 2013). The NZ lineage carries *Psa*_{NZ13}|ICE_eno which harbours a 20 kb 'enolase' region' that is also found in otherwise divergent *Psa* ICEs (McCann *et al.*, 2013; McCann *et al.*, 2017).

Copper sprays have long been used in NZ to protect plants from a range of diseases. Since the arrival of *Psa* in NZ kiwifruit orchardists have employed copper-based products to protect vines. From 2011, an ongoing industry-based programme has been in place to monitor copper resistance. In 2014, evidence was first obtained of *Psa* isolates resistant to copper sulphate. Given that the clone of *Psa* originally introduced into NZ was sensitive to copper and lacked genes encoding copper resistance (McCann *et al.*, 2013), detection of copper resistance raised the possibility that this is an evolutionary response to the use of copper-based sprays.

Here, we report the phenotypic and genetic basis of copper resistance into NZ isolates of *Psa* and show that its emergence has been fuelled by HGT involving ICEs and plasmids. We additionally describe the mosaic structure of ICEs, show the dynamics of ICE transfer both *in vitro* and *in planta*, analyze fitness consequences of ICE carriage, capture the *de novo* formation of novel recombinant ICEs and explore ICE host-range.

Results

Occurrence of copper resistance in *Psa*

Psa NZ13, isolated in 2010 and representative of the clone introduced into NZ, lacks genes encoding copper resistance (McCann *et al.*, 2013) and is unable to grow at copper concentrations in excess of 0.8 mM CuSO₄ on MGY. Prior to 2014 no copper resistant or tolerant *Psa* strains had been reported in NZ. However, in 2014, two strains isolated from two different kiwifruit orchards, *Psa* NZ45 and *Psa* NZ47, displayed copper resistance, with a MIC of 1.2 mM CuSO₄. This finding prompted a small-scale sampling of both copper treated and untreated orchards in 2015/2016 encompassing the area where

resistance was first identified. From a sample of 213 strains isolated from seven orchards 59 were found to be copper resistant. Copper resistant isolates were recovered from both copper treated and untreated orchards. Additional copper resistant strains were procured from other kiwifruit-growing regions of NZ (Supporting Information Fig. S1).

ICE and plasmid-mediated acquisition of copper resistance in *Psa*

The genome of the focal copper resistant isolate, *Psa* NZ45, is a direct clonal descendant of the isolate originally introduced into NZ (*Psa* NZ13) with 2 SNPs in the 4,853,155 bp non-recombinant core genome alignment (McCann *et al.*, 2017), but differs in two significant regards. First, the 'native' ICE (*Psa*_{NZ13}|ICE_eno) at *att-1* (immediately upstream of *clpB*) is located at the second *att* site (*att-2*) immediately downstream of *queC* (Fig. 1). Second, the genome harbours a new 107 kb ICE (*Psa*_{NZ45}|ICE_Cu) integrated at the *att-1* site: *Psa*_{NZ45}|ICE_Cu carries genes encoding copper resistance (Figs 1 and 2A).

The genomes of six additional copper resistant *Psa* isolates were also sequenced (Table 1) and as with *Psa* NZ45, reads were aligned against the *Psa* NZ13 reference genome (McCann *et al.*, 2013; Templeton *et al.*, 2015). All six harbour mobile elements carrying genes encoding copper resistance. The diversity of these elements and genomic location is shown in Fig. 1 and their structure is represented in Fig. 2A. All isolates are direct clonal descendants of *Psa* NZ13 and thus share an almost identical genome with the exception of the determinants of copper resistance. In *Psa* NZ47, the genes encoding copper resistance are located on a 90 kb ICE (*Psa*_{NZ47}|ICE_Cu) integrated at the *att-1* site: the native *Psa*_{NZ13}|ICE_eno is located at the *att-2* site. *Psa* NZ62 carries an ICE identical to that found in *Psa*_{NZ47}|ICE_Cu (*Psa*_{NZ62}|ICE_Cu), but is integrated at the *att-2* site; the native ICE (*Psa*_{NZ13}|ICE_eno) is absent leaving the *att-1* site unoccupied. Isolate *Psa* NZ63 carries *Psa*_{NZ45}|ICE_Cu integrated at the *att-1* site, but, the native *Psa*_{NZ13}|ICE_eno has been lost. Copper resistance genes in isolate *Psa* NZ64 are also ICE-encoded, but the NZ64 ICE (*Psa*_{NZ64}|ICE_Cu) is genetically distinct from both *Psa*_{NZ47}|ICE_Cu and *Psa*_{NZ45}|ICE_Cu – at 130 kb, it is also the largest. In NZ64, *Psa*_{NZ64}|ICE_Cu is located at the *att-1* site and the *att-2* site contains the native (*Psa*_{NZ13}|ICE_eno) ICE. Isolates *Psa* NZ65 and NZ66 both harbour copper resistance genes on a near identical, 120 kb previously undescribed plasmid (p*Psa*_{NZ65} and p*Psa*_{NZ66} respectively). The only significant difference among the plasmids is the location of a streptomycin resistance-encoding transposon (see below): both isolates have the original *Psa*_{NZ13}|ICE_eno integrated at the *att-2* site (Fig. 1). *Psa* harbouring copper

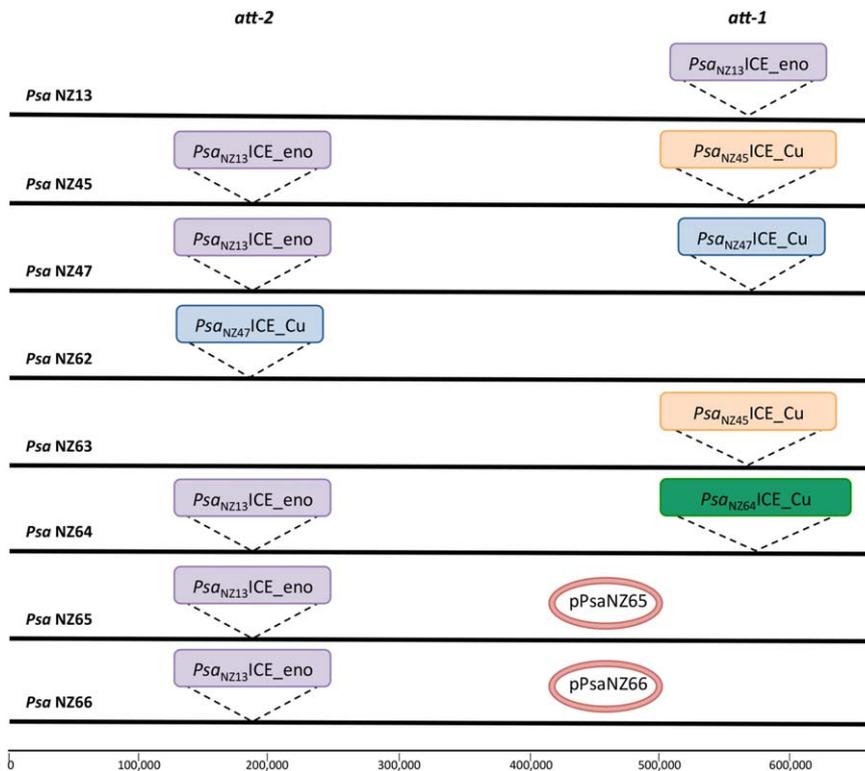


Fig. 1. Genomic location of *Psa* ICEs. In purple the *Psa*_{NZ13}|ICE_eno (100 kb), in orange *Psa*_{NZ45}|ICE_Cu (107 kb), in blue the *Psa*_{NZ47}|ICE_Cu (90 kb), in green the *Psa*_{NZ64}|ICE_Cu (130 kb), pPsaNZ65 and pPsaNZ66 plasmids are 111 kb. Each island is bounded by 52 bp *att* sequences overlapping tRNALys. In *Psa* NZ13 the *att-1* site is located at 5,534,632 bp, *att-2* at 1,733,972 bp. The figure is not to scale (the entire genome of 6.7 Mbp is indicated as a single black line). Both *Psa*_{NZ13}|ICE_eno and *Psa*_{NZ47}|ICE_Cu ICEs were detected in *Psa* NZ47 by sequencing, but analysis of independent colonies from the freezer stock show that *Psa*_{NZ13}|ICE_eno is prone to loss.

resistance-encoding ICEs have a MIC CuSO₄ of 1.2 mM while the MIC of plasmid-carrying *Psa* is 1.5 mM (Table 1).

That such a small sample of isolates is each unique with regard to the copper resistance-encoding element points to highly dynamic processes shaping their evolution. Such dynamism has been previously noted among enolase-encoding ICEs obtained from a global collection of epidemic *Psa* isolates (McCann *et al.*, 2013) and has been observed elsewhere (Burrus *et al.*, 2006; Wozniak and Waldor, 2010). In this study, our samples came from a relatively small geographical location. Two ICEs, *Psa*_{NZ64}|ICE_Cu and *Psa*_{NZ47}|ICE_Cu, were found in different isolates sampled from the same orchard (one year apart), although the two isolates containing near identical plasmids were isolated from orchards located 100 km apart. Two isolates sampled one year apart from the same location (neighboring orchards in Te Puke) carry the same ICE (*Psa*_{NZ45}|ICE_Cu = *Psa*_{NZ63}|ICE_Cu; *Psa*_{NZ47}|ICE_Cu = *Psa*_{NZ62}|ICE_Cu) (Table 1).

The dynamics of ICE evolution becomes especially evident when placed in the broader context possible by comparisons to ICEs recorded in DNA databases. The core genes of the copper resistance-encoding ICEs from NZ *Psa* isolates are syntenuous with the core genes of the family of ICEs that includes PPHGI-1 (isolated in 1984 from bean in Ethiopia (Teverson, 1991; Pitman *et al.*, 2005)) and the three ICEs previously described from *Psa* found in New Zealand (2010), Italy (2008) and China (2010) (McCann *et al.*, 2013; Butler *et al.*, 2013;

E. Colombi, unpublished). *Psa*_{NZ45}|ICE_Cu is a mosaic of DNA from two known ICEs and a plasmid. It shares regions of near perfect identity (over 66 kb) with ICEs present in the otherwise divergent host genomes of *P. syringae* pv. *panici* (*Ppa*, LGM2367) isolated from proso millet in Madison (USA) in the 1920s (over the first 38 kb it differs by just 12 SNPs, and one 144 bp indel), *P. syringae* pv. *atrofasciens* (*Paf*, ICMP4394) isolated in NZ in 1968 from wheat and a 70.5 kb plasmid present in a non-pandemic *Psa* strain (J2), isolated in Japan in 1988 (Fig. 2B).

Two of the ICEs described here have also been found in non-*Psa Pseudomonas* isolated from kiwifruit leaves. *Psa*_{NZ47}|ICE_Cu shows 99.7% pairwise nucleotide identity with an ICE found in *P. marginalis* ICMP 11289 isolated in 1991 from *A. deliciosa* in Katikati (NZ). *Psa*_{NZ64}|ICE_Cu is almost identical (99.5% nucleotide pairwise identity) to an ICE from *P. syringae* pv. *actinidifoliorum* (*Pfm*) ICMP19497, isolated from kiwifruit in 2010 in Te Puke (NZ) (Visnovsky *et al.*, 2016) (Table 1). Additionally, a 48 kb segment of *Psa*_{NZ64}|ICE_Cu encoding copper resistance genes shares 99.3% nucleotide pairwise identity with a locus found in *P. azotoformans* strain S4 (Fang *et al.*, 2016), which was isolated from soil in 2014 in Lijiang (China). However, the locus from *P. azotoformans* is not associated with an ICE.

Genetic determinants of copper resistance

Copper resistance is typically conferred by operons encoding either copper efflux (*cusABC*) and/or sequestration

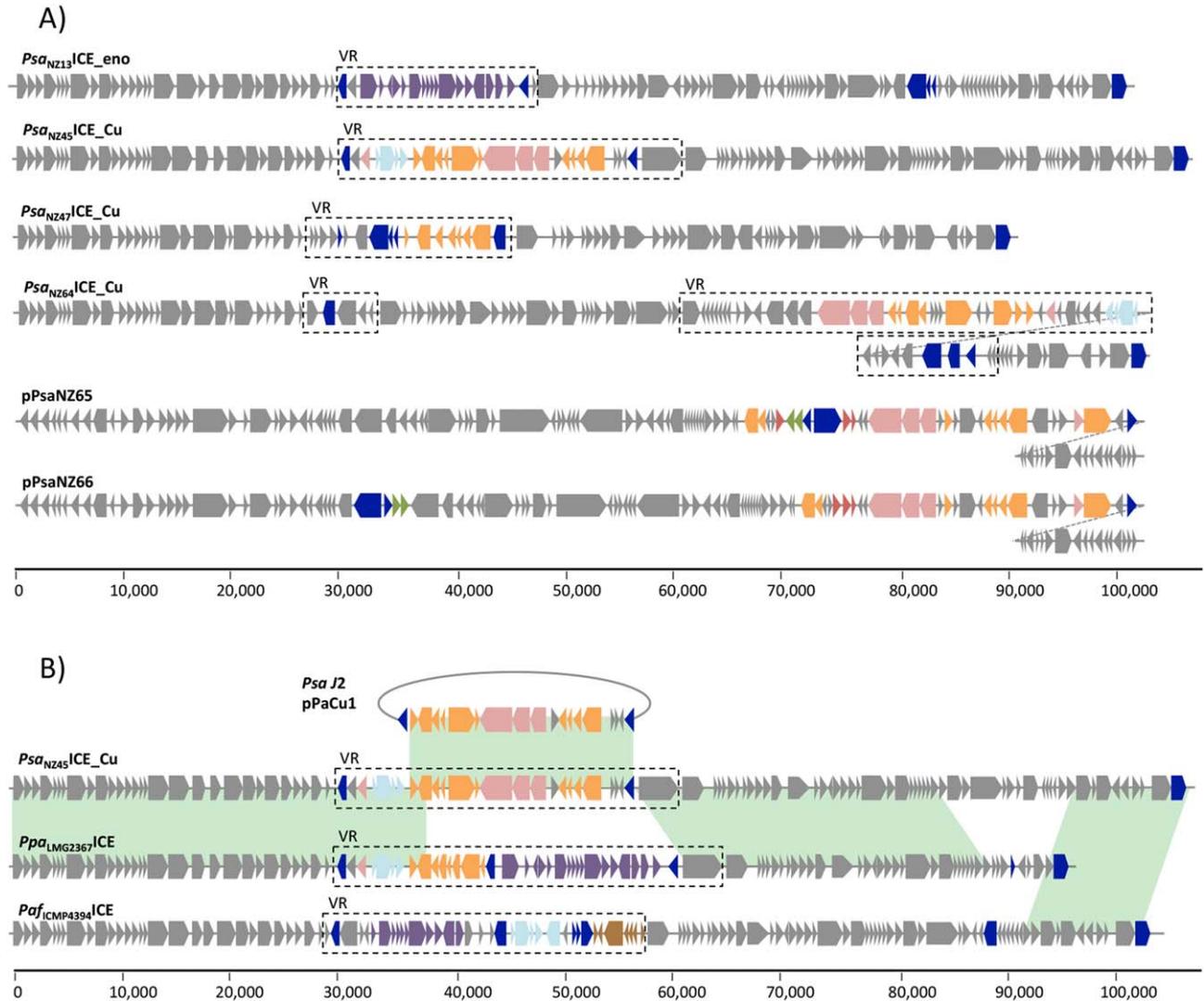


Fig. 2. Genetic organization of ICEs and plasmids acquired by *Psa* and mosaicism of *Psa*_{NZ45}|ICE_Cu.

A. Blue boxes are mobile genes (transposases or recombinases), purple boxes define the 'enolase region', orange boxes depict copper resistance genes, azure boxes are arsenic resistance genes, pink boxes are genes belonging to the *czc/cus* system, green boxes are streptomycin resistance genes, red boxes are cation transporter ATPases, brown boxes denote genes encoding mercury resistance. Core 'backbone' and other cargo genes are depicted as grey boxes. Dotted diagonal lines indicate continuation of the element.

B. Areas in green show more than 99% pairwise nucleotide identity. *Psa*_{NZ45}|ICE_Cu and *Ppa*_{LGM2367}|ICE share identity both in the first 38 kb and 20 kb downstream of VR. The remaining 20 kb of the *Psa*_{NZ45}|ICE_Cu VR is almost identical to pPaCu1 (it differs by just 2 SNPs). The last 12.5 kb of *Psa*_{NZ45}|ICE_Cu is identical to *Paf*_{ICMP4394}|ICE.

(*copABCD*) systems, both of which can be under the regulation of the *copRS/cusRS* two-component regulatory system (Bondarczuk and Piotrowska-Seget, 2013). ICEs identified in *Psa* isolates harbour operons encoding examples of both resistance mechanisms (and regulators), plus genetic determinants of resistance to other metal ions. In each instance, the resistance genes are located within 'variable regions' (VR) of ICEs into which cargo genes preferentially integrate (Fig. 2A). Delineation of these variable regions comes from detailed analysis of 28 unique ICEs from the *Pph* 1302A and *Psa* families that will be

published elsewhere (E. Colombi, unpublished). Overall there are notable similarities and differences in the organization of the variable regions.

As shown in Fig. 2B, the first 38 kb of *Psa*_{NZ45}|ICE_Cu is almost identical (99.7% identical at the nucleotide level) to *Ppa*_{LGM2367}|ICE. This region spans the core genes, but extends ~8.2 kb into the variable cargo genes with just two SNPs distinguishing the two ICEs (across the 8.2 kb variable region). Encoded within this region is an integrase, arsenic resistance genes (*arsRBCH*), a gene implicated in cadmium and cobalt resistance (*czcD*) and the *copRS*

Table 1. List of genomes used in this study.

Isolate ID	Place of Isolation	Year of Isolation	Host of Isolation	Orchard's copper Programme	MIC to CuSO ₄	GenBank Accession	ICMP number
<i>Psa</i> NZ13	Te Puke, NZ	2010	Kiwifruit	NA	0.8 mM	CP011972-3	18884
<i>Psa</i> NZ45	Te Puke, NZ	2014	Kiwifruit	Full spray	1.2 mM	CP017007-8	20586
<i>Psa</i> NZ47	Te Puke, NZ	2014	Kiwifruit	Spray free	1.2 mM	CP017009-11	22180
<i>Psa</i> NZ62	Te Puke, NZ	2015	Kiwifruit	Organic	1.2 mM	MOMK00000000	22181
<i>Psa</i> NZ63	Te Puke, NZ	2015	Kiwifruit	To minimum	1.2 mM	MOML00000000	22182
<i>Psa</i> NZ64	Te Puke, NZ	2016	Kiwifruit	Spray free	1.2 mM	MOMM00000000	22183
<i>Psa</i> NZ65	Te Puke, NZ	2016	Kiwifruit	Full spray	1.5 mM	MOMN00000000	22184
<i>Psa</i> NZ66	Coromandel, NZ	2016	Kiwifruit	Full spray	1.5 mM	MOMJ00000000	22185
<i>Ppa</i> LGM2367	Madison, USA	1921–1922	Proso millet	NA	NA	ALAC00000000	
<i>Paf</i> ICMP4394	Auckland, NZ	1968	Wheat	NA	NA	LJPO00000000	4394
<i>P. marginalis</i> ICMP4394	Katikati, NZ	1991	Kiwifruit	NA	NA	LKGX00000000	4394
<i>Psa</i> J2	Japan	1988	Kiwifruit	NA	NA	AGNQ00000000	
<i>Pfm</i> ICMP19497	Te Puke, NZ	2010	Kiwifruit	NA	NA	LKBQ00000000	19497

regulatory system. Partway through *copS* the two ICEs diverge at a recombination breakpoint with the downstream variable region from *Psa*_{NZ45}ICE_Cu being homologous to a set of copper resistance genes found on plasmid pPaCu1 from the divergent (non-pandemic) Japanese isolate of *Psa* (J2) (Nakajima *et al.*, 2002). This region comprises a putative copper transporting ATPase encoded by *copG* (Gutiérrez-Barranquero *et al.*, 2013), *cusABC* genes involved in the detoxification of monovalent cations, including copper and silver (Mergeay *et al.*, 2003; Rensing and Grass, 2003) and *copABCD* (Fig. 2B). The

last 4 kb of the variable region of *Psa*_{NZ45}ICE_Cu shares almost complete identity with *Ppa*_{LGM2367}ICE (Fig. 2B).

Detail of the diversity of copper resistance (and related metal resistance) genes is shown in Fig. 3. All elements (ICEs and plasmids) harbour the *copRS* regulatory system and, with the exception of *Psa*_{NZ47}ICE_Cu, all carry both *cusABC* and *copABCD*, although their organization varies. For example, while *copABCD* is typical, in *Psa*_{NZ64}ICE_Cu *copAB* and *copCD* are organized as two separate operons (Fig. 3). The putative copper ABC transport system encoded by *copG* is a common feature, and determinants

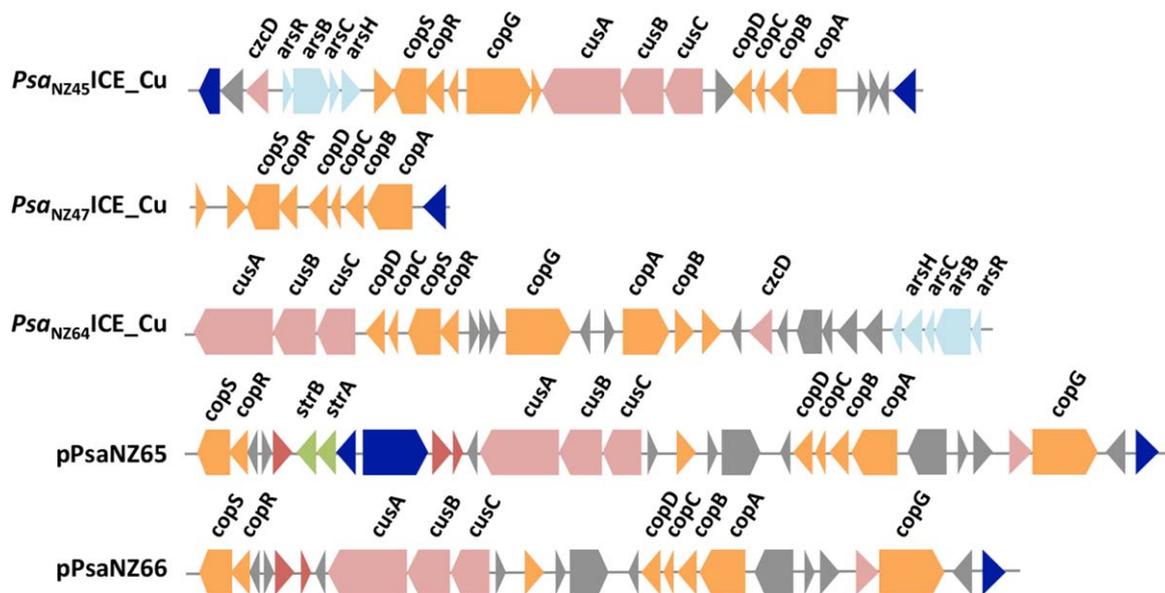


Fig. 3. Genetic organization of metal resistance loci. Blue boxes are mobile genes (transposases or recombinases), orange boxes depict copper resistance genes, azure boxes are arsenic resistance genes, pink boxes are genes belonging to the *czc/cus* system, green boxes are streptomycin resistance genes and other genes are depicted as grey boxes.

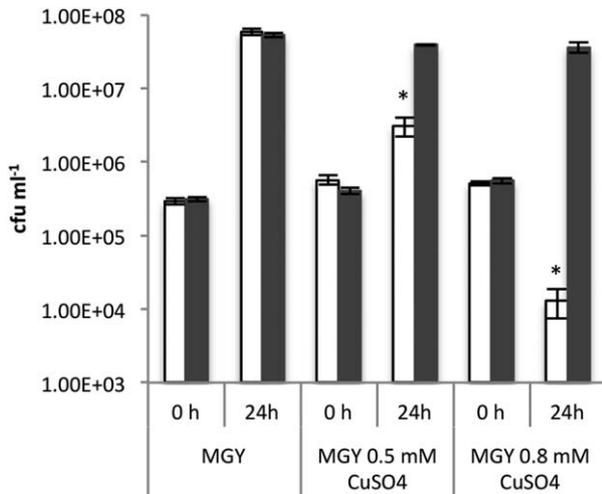


Fig. 4. Effect of copper ions on growth of *Psa* NZ13 and *Psa* NZ45. *Psa* NZ13 (white bars) and *Psa* NZ45 (grey bars) were grown for 24 h in shaken MGY culture and MGY supplemented with 0.5 mM and 0.8 mM CuSO₄. Data are means and standard deviation of three independent cultures. The asterisk indicates significant difference $P < .05$ (one tailed *t*-test).

of arsenic resistance are present in both *Psa*_{NZ45}ICE_Cu and *Psa*_{NZ64}ICE_Cu. The putative cadmium and related metal resistance gene, *czcD*, is also present on these two ICEs. As noted above, a transposon carrying determinants of streptomycin resistance (*strAB*) is present on plasmids pPsaNZ65 and pPsaNZ66. The transposon is of the Tn3 family and the cassette bears identity to streptomycin resistance carrying transposons found in *P. syringae* pv. *syringae* B728a (Feil *et al.*, 2005), but also on plasmid pMRVIM0713 from *Pseudomonas aeruginosa* strain MRSN17623 (GenBank: KP975076.1), plasmid pMK1-C from *Klebsiella pneumoniae* strain PMK1 (Stoesser *et al.*, 2014) and plasmid pTi carried by *Agrobacterium tumefaciens* LBA4213 (Ach5) (GenBank: CP007228.1).

At the level of the operons determining copper resistance there is marked genetic diversity, however, with the exception of CopR, there is little evidence of within operon recombination. The CusABC system is carried on pPsaNZ65 and pPsaNZ66 (but these are identical) and the ICEs *Psa*_{NZ45}ICE_Cu and *Psa*_{NZ64}ICE_Cu: CusA, CusB and CusC show 75.8%, 50.0% and 44.8% pairwise amino acid identity respectively; phylogenetic trees based on protein sequences show congruence (Supporting Information Fig. S2). The CopABCD system is present on *Psa*_{NZ45}ICE_Cu, *Psa*_{NZ47}ICE_Cu, *Psa*_{NZ64}ICE_Cu (but CopAB and CopCD are in different locations (Fig. 3)) and plasmid pPsaNZ65 (and pPsaNZ66): CopA, CopB, CopC and CopD show 76.4%, 63.1%, 79.1% and 60.8% pairwise amino acid identity respectively. With the exception of CopC (where bootstrap support is low) phylogenetic trees

for each protein show the same overall arrangement (Supporting Information Fig. S3). The two component regulatory system *copRS* is also present on each of the elements with the amino acid sequences of CopR showing 84.3% and those of CopS 63.0% pairwise amino acid identity. Phylogenetic trees show CopS from *Psa*_{NZ64}ICE_Cu to be the most divergent, and those from *Psa*_{NZ45}ICE_Cu and *Psa*_{NZ47}ICE_Cu being most similar: CopR shows the same phylogenetic arrangement, however, CopR sequences from *Psa*_{NZ45}ICE_Cu and *Psa*_{NZ47}ICE_Cu are identical at the protein level suggesting a recent recombination event (Supporting Information Fig. S4).

*Psa*_{NZ45}ICE_Cu imposes no detectable fitness cost and confers a selective advantage in vitro in the presence of copper

To determine whether ICE carriage confers a fitness cost, we took advantage of the fact that *Psa* NZ13 and *Psa* NZ45 are essentially isogenic, with the exception of the additional ICE in *Psa* NZ45 (*Psa*_{NZ45}ICE_Cu). Each strain was grown alone and density of cells monitored over a 72 h period with samples taken every 24 h. In the absence of copper sulphate, no difference in cell density was detected; however, in the presence of 0.5 and 0.8 mM CuSO₄ the density of *Psa* NZ13 was reduced (Fig. 4). There is thus no apparent fitness cost associated with carriage of *Psa*_{NZ45}ICE_Cu in the absence of copper sulphate, but there is a fitness advantage in copper-containing environments.

Although carriage of *Psa*_{NZ45}ICE_Cu appeared not to affect the growth of *Psa* NZ45 in the absence of copper, a more precise measure of fitness was sought by performing competition experiments in which *Psa* NZ13 and *Psa* NZ45 were co-cultured. For this experiment *Psa* NZ13 was marked with a kanamycin resistance cassette so that it could be distinguished from kanamycin sensitive, copper resistant *Psa* NZ45. Over a 24 h period where the two strains (founded at equal density) competed for the same resources (in shaken MGY medium without copper sulphate), the fitness of *Psa* NZ45 was not significantly different to *Psa* NZ13 (0.29 ± 0.2 ; mean and SEM from three independent experiments, each comprised of three replicates), indicating no significant detectable cost of carriage of *Psa*_{NZ45}ICE_Cu.

Given that the mechanism of copper resistance in *Psa* NZ45 – based upon *copABCD* – likely involves sequestration of copper ions we considered the possibility that this isolate might confer cross protection to non-copper resistant isolates, such as *Psa* NZ13. To this end, we performed co-culture experiments at sub-inhibitory and inhibitory copper sulphate concentrations. Growth of *Psa* NZ13 at sub-inhibitory concentrations of copper sulphate was significantly impaired by the presence of *Psa* NZ45

and this was especially evident at 48 and 72 h (Supporting Information Fig. S5). At the inhibitory copper sulphate concentration, *Psa* NZ13 appeared to benefit from the presence of *Psa* NZ45. Again, this was most evident at 48 and 72 h (Supporting Information Fig. S5).

*Psa*_{NZ45}ICE_Cu imposes no detectable fitness cost but confers a minor selective advantage in planta

Cost and benefit of carrying *Psa*_{NZ45}ICE_Cu was also evaluated during endophytic colonization of kiwifruit leaves. No significant difference was observed in growth of singly-inoculated *Psa* NZ13 and *Psa* NZ45 in both the absence and presence of a commonly used commercial copper-based product (Nordox75 (0.375 g l⁻¹)) (Supporting Information Fig. S6). Competition assays in the presence or absence of Nordox75 confirmed carriage of *Psa*_{NZ45}ICE_Cu imposes no significant fitness cost or advantage during endophytic growth (Fig. 5 and Supporting Information Fig. S7). However, in the presence of Nordox75 there was a significant advantage to epiphytic cells carrying *Psa*_{NZ45}ICE_Cu observable at day 3, but not at day 7 (Fig. 5 and Supporting Information Fig. S7).

*Psa*_{NZ45}ICE_Cu transfer dynamics in vitro and in planta

To determine whether *Psa*_{NZ45}ICE_Cu is active and capable of self-transmission samples from the *Psa* NZ45 and *Psa* NZ13 mixtures from the co-cultivation experiments were also plated on MGY medium containing both kanamycin and copper sulphate. Copper resistant, kanamycin resistant transconjugants were detected both *in vitro* and *in planta*. This means that a fraction of *Psa* NZ13 strains acquired *Psa*_{NZ45}ICE_Cu. These transconjugants marginally inflate the counts of *Psa* NZ45, however, the number of transconjugants (see below) was several orders of magnitude less than *Psa* NZ13, thus having no appreciable effect on the measures of relative fitness.

At 24 h in shaken MGY broth transconjugants were present at a frequency of $5.04 \pm 2.25 \times 10^{-3}$ per recipient cell (mean and SEM from three independent experiments, each comprised of three replicates). Analysis of samples from *in planta* experiments showed that at 3 days, transconjugants were present at a frequency of $2.05 \pm 0.63 \times 10^{-2}$ per recipient cell (mean and SEM from three independent experiments, each comprised of five replicates). On plants in the presence of Nordox (0.375 g l⁻¹) the frequency of transconjugants was $9.37 \pm 1.56 \times 10^{-2}$ per recipient three days after inoculation (mean and SEM from three independent experiments, each comprised of five replicates). Transfer was also observed in M9 agar, on M9 agar supplemented with 0.5 mM CuSO₄, on M9 agar supplemented with a macerate of Hort16A fruit with transconjugants present (at 48 h) at a frequency of

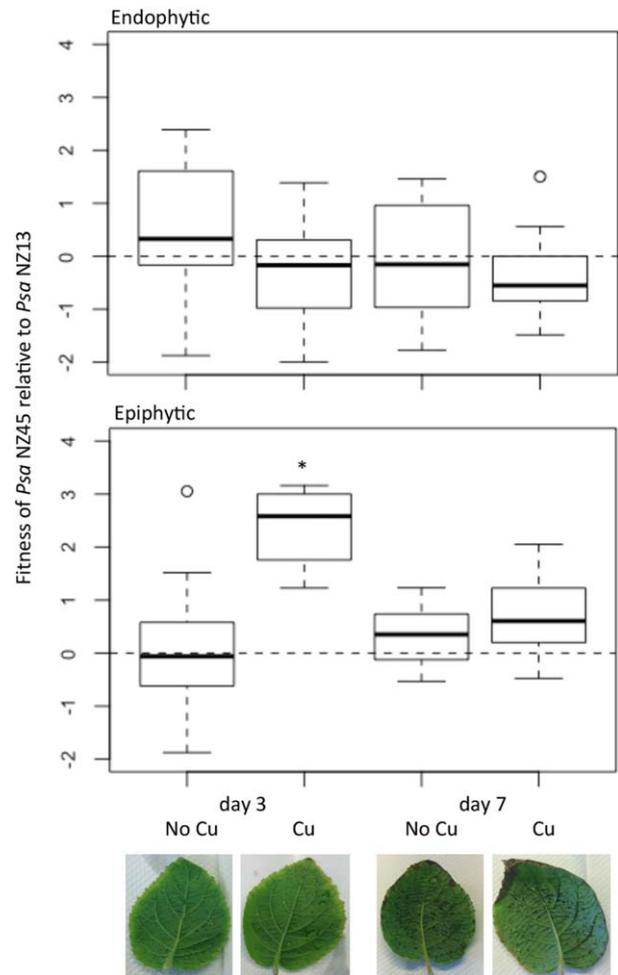


Fig. 5. Competition assay of *Psa* NZ45 and *Psa* NZ13 *in* and *super* *planta*. The difference in fitness between *Psa* NZ45 and *Psa* NZ13 was assessed endophytically and epiphytically on leaves of the kiwifruit cultivar Hort16A. Data represent the difference in the Malthusian parameters of three experiments of five replicates. The copper product Nordox75 (0.375 g l⁻¹) was sprayed adaxially and abaxially until run off (Cu). The asterisk indicates significant difference $P < .05$ from 0 (one tailed *t*-test).

$2.16 \pm 0.9 \times 10^{-5}$, $1.11 \pm 0.4 \times 10^{-5}$ and $1.98 \pm 0.8 \times 10^{-5}$ per recipient cell respectively.

To explore the dynamics of transfer *in vitro*, samples from shaken MGY cultures were taken hourly, for 6 h, and then at 24 h. The data, presented in Fig. 6, show acquisition of *Psa*_{NZ45}ICE_Cu by *Psa* NZ13 within 1 h of the mating mix being established (approximately 1 recipient per 10⁵ recipient cells). The frequency was relatively invariant over the subsequent 6 h period, but rose to approximately 1 recipient in 10³ cells at 24 h.

Detection of ICE transfer just 1 h after mixing donor and recipient cells promoted a further experiment in which transconjugants were assayed at 10 min intervals. From three independent experiments, each with five replicates,

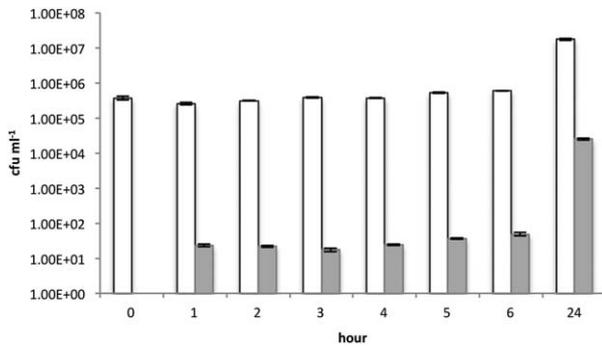


Fig. 6. *In vitro* transfer of *Psa*_{NZ45}|ICE_Cu from *Psa* NZ45 to *Psa* NZ13. Colony forming units of the recipient *Psa* NZ13 (white bars) and *Psa* NZ13 carrying *Psa*_{NZ45}|ICE_Cu (transconjugants, grey bars) was monitored during co-cultivation. Data are means and standard deviation of three independent cultures

transconjugants were detected at 30 min (approximately 4×10^{-7} transconjugants per recipient cell).

Analysis of co-cultivation experiments from kiwifruit leaves showed evidence that *Psa*_{NZ45}|ICE_Cu also transferred *in planta*. The frequency of transconjugants at day 3 and day 7 was approximately 1 per 50 recipient cells and the frequency of transconjugants was not affected by changes in the initial founding ratios of donor and recipient cells (Supporting Information Table S1). Overall, the frequency of transconjugants was approximately three orders of magnitude greater *in planta* than *in vitro*.

ICE displacement and recombination

To check the genetic composition of transconjugants and to investigate whether *Psa*_{NZ45}|ICE_Cu integration in recipient cells occurred at the *att-1* or *att-2* site, a set of primers were designed to identify the location of ICE integration in the *Psa* NZ13 genome (Supporting Information Table S2). Eleven independently generated transconjugants from shaken MGY culture were screened. As expected, successful amplification of primers annealing to *copABCD* in

*Psa*_{NZ45}|ICE_Cu was observed in all transconjugants, while amplification of the *enolase* gene primers (indicative of the presence of the native *Psa*_{NZ13}|ICE_eno) occurred only in *Psa* NZ13 and *Psa* NZ45 (Supporting Information Fig. S8). However, in two transconjugants only the *IntPsaNZ13-att-1* primer pair resulted in amplification, suggesting that recombination between *Psa*_{NZ45}|ICE_Cu and *Psa*_{NZ13}|ICE_eno had occurred (Supporting Information Fig. S9). Genome sequencing of one of these transconjugants revealed a recombination event inside the variable region of the ICE that produced a chimeric ICE identical to *Psa*_{NZ45}|ICE_Cu up to and including the CuR operon, with the remainder identical to the downstream segment of *Psa*_{NZ13}|ICE_eno (Fig. 7).

*Psa*_{NZ45}|ICE_Cu can be transferred to a range of *P. syringae* strains

The host range of the *Psa*_{NZ45}|ICE_Cu was characterized using a panel of nine different *Pseudomonas* strains as recipients, representing the diversity of *P. syringae* and the genus more broadly. Transfer of *Psa*_{NZ45}|ICE_Cu to *Psa* J31, *Pfm* NZ9 and *P. syringae* pv. *phaseolicola* (*Pph*) 1448a (on M9 agar plates) was observed with the frequency of transconjugants per recipient cell being $7.64 \pm 1.7 \times 10^{-6}$, $7.74 \pm 2.5 \times 10^{-7}$ and $1.23 \pm 0.2 \times 10^{-4}$ respectively. No transconjugants were detected for *P. aeruginosa* PAO1, *P. fluorescens* SBW25, *P. syringae* pv. *tomato* DC3000 or *Psa* K28, despite the fact that these three strains have both *att* sites.

Discussion

The importance and impact of HGT on the evolution of microbial populations has long been recognized (Sullivan *et al.*, 1995; Lilley and Bailey, 1997; Ochman *et al.*, 2005; 2000; Wozniak and Waldor, 2010; Polz *et al.*, 2013). Here we have captured the real time evolution of copper resistance in a plant pathogen, in an agricultural setting, and

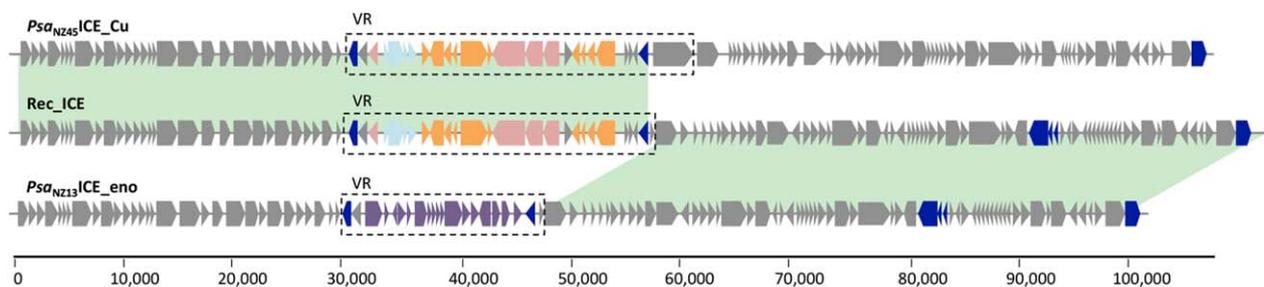


Fig. 7. Structure and mosaicism of the recombinant ICE (Rec_ICE) in transconjugant *Psa* NZ13. Areas highlighted in green show 100% pairwise identity. The recombination break point is inside the variable region (VR). Blue boxes are mobile genes (transposases or recombinases), purple boxes define the 'enolase region' (McCann *et al.* 2013), orange boxes depict copper resistance genes, azure boxes are arsenic resistance genes and pink boxes are genes belonging to the *czc/cus* system. Core 'backbone' and other cargo genes are depicted as grey boxes.

shown that movement of copper resistance genes occurs primarily via ICEs. Of the seven copper resistant *Psa* isolates analyzed, five contain copper resistance-encoding ICEs – three unique ICEs in total – with variable placement within the *Psa* genome, including movement and instability of the native ICE (*Psa*_{NZ13}ICE_eno). Further evidence of dynamism comes from *in vitro* and *in planta* studies, which show not only transfer to isogenic *Psa* and unrelated *P. syringae* strains, but also the ready formation of chimeras between *Psa*_{NZ45}ICE_Cu and *Psa*_{NZ13}ICE_eno. Mosaicism of ICEs has been reported elsewhere and is often promoted by the presence of tandem copies (Garriss *et al.*, 2009; Wozniak and Waldor, 2010). The ease with which ICEs move between strains and capacity for intra-ICE recombination emphasizes the futility of drawing conclusions on strain phylogeny based on ICE phylogeny (McCann *et al.*, 2013), but also the impossibility of understanding ICE evolution based on the phylogeny of ICEs themselves.

Evidence of the formation of chimeric ICEs extends beyond the ICEs studied here. *Psa*_{NZ45}ICE_Cu is a recombinant of two previously reported ICEs and a plasmid: most surprising is the fact that the recombinant components are derived from elements isolated from three geographic regions (USA, Japan and New Zealand) from three different plants (millet, kiwifruit and wheat) and spanning almost 100 years. Additionally, two of the copper resistance-encoding ICEs found in *Psa* (*Psa*_{NZ47}ICE_Cu and *Psa*_{NZ64}ICE_Cu) have been reported in other kiwifruit leaf colonizing organisms emphasizing the ease by which self-transmissible elements can move between members of a single community. Clearly, the potency of evolution fuelled by ICEs with the *P. syringae* complex is remarkable, with impacts likely extending well beyond that inferred from the analysis of genome sequences (Fondi *et al.*, 2016).

Evidence of the spectrum and dynamic of transfer inferred from the genomic analysis of natural isolates is bolstered by demonstration of the *in vitro* and *in planta* transfer of *Psa*_{NZ45}ICE_Cu. The fact that *Psa*_{NZ45}ICE_Cu can be detected in a recipient strain just 30 min after mixing with a donor strain (in shaken broth culture) points to an as yet undetermined proficiency for transfer. At the same time, the frequency of transconjugants *in planta* are several orders of magnitude greater than *in vitro* suggesting even greater potential for transfer in the natural environment.

The selective causes underpinning the evolution of copper resistance in *Psa* is uncertain and to date copper resistance in *Psa* is not known to have evolved outside of NZ. While it is tempting to blame use of copper sprays by orchardists, it is possible that the evolution of copper resistant *Psa* is a more general response to copper levels in New Zealand soils, copper emission to the atmosphere from anthropomorphic activities (Hong *et al.*, 1996; Staehlin *et al.*, 2016), combined with the use of copper-based

sprays in NZ agriculture (Morgan and Taylor, 2004; Dean, 2016). Support for this stems from the fact that *Psa*_{NZ47}ICE_Cu shows almost perfect identity with an ICE found in *P. marginalis* (ICMP 11289) from kiwifruit isolated in 1991 (in NZ). In addition, copper resistance-encoding ICEs were found in both copper treated and untreated orchards.

In previous studies of copper resistance in *P. syringae*, beneficial effects due to carriage of resistance genes have been detected during assays of epiphytic growth (Sundin *et al.*, 1989; Menkissoglu and Lindow, 1991). In our work, the impact of the copper resistance-encoding ICEs on fitness *in planta* – in the presence of copper sprays – appears to be minimal. The selective advantage conferred by *Psa*_{NZ45}ICE_Cu was statistically significant only for epiphytic growth at day 3 in the presence of Nordox75. The lack of observable benefit at day 7 likely reflects tissue breakdown and escape of endophytic bacteria through stomata, causing inflation of the number of epiphytic bacteria (Fig. 5).

From the perspective of pathogen control using copper-based sprays there are at least three reasons to treat our detection of a minimal advantage to carriage of genetic determinants of copper resistance with caution. First, for reasons mentioned above and encountered elsewhere (Beattie and Lindow, 1999; Hirano and Upper, 2000) it is difficult to accurately assess fitness of microbes on plants and it is possible that our measures underestimate the contribution of copper resistance to growth in the presence of copper: even a 1% increase in fitness over 24 h, which is beyond experimental capacity to detect, can have significant long-term consequences. Second, the presence of copper resistance genes means opportunity for levels of resistance to increase through, for example, promoter mutations that increase levels of transcription of resistance determinants, or through acquisition of additional copper resistance-encoding genes. Third, and perhaps most significantly, is the fact that the copper resistance-encoding ICEs confer no measurable fitness cost even in the absence of copper. This suggests that these elements will not be readily lost from *Psa* populations even if copper-based sprays were eliminated (Andersson and Hughes, 2010; Neale *et al.*, 2016). That some strains of the global pandemic now contain two ICEs gives reason to suspect elevated evolutionary potential among these isolates.

While the focus of our investigation has been copper resistance, the ICEs reported here carry a cargo of additional genes, some of which are implicated in resistance to other metals. In some instances, the cargo genes have no similarity to genes of known function (some of the grey boxes in Fig. 2A). ICEs and similar laterally transferred elements provide opportunity for genes unrelated to copper resistance, for example gene connected to virulence, to hitchhike and rapidly spread. In this regard, the two plasmids characterized here are of interest: both carry

determinants of streptomycin resistance – an antibiotic that is also sprayed on NZ kiwifruit orchards in order to control *Psa*. The potential for hitchhiking has been previously noted in the context of antibiotic resistance-encoding plasmids (Gullberg *et al.*, 2014).

Recognition of ICEs along with their potential to change the course of microbial evolution extends less than twenty years (Wozniak and Waldor, 2010). While it might be argued that this potential is no different from that long realized via conjugative plasmids, or phage (Ochman *et al.*, 2000), ICEs, being a composite of both, seem to have an edge. Unlike conjugative plasmids that rarely integrate into the host genome, ICEs integrate as a matter of course and are largely immune to segregational loss; additionally, fitness consequences as a result of carriage are likely to be minimal. Unlike temperate phages, ICEs do not kill the host upon transfer, but they can nonetheless mediate transfer upon encountering transfer proficient conditions. Having control over both vertical and horizontal modes of transmission, while minimizing costs for host cells, marks these elements as especially potent vehicles of microbial evolution.

Experimental procedures

Strains and culture condition

All *Pseudomonas* strains were cultured in King's B medium at 28°C, *E. coli* was cultured in Luria Bertani medium at 37°C. All liquid overnight cultures were shaken at 250 rpm. Both kanamycin and nitrofurantoin were used at 50 µg ml⁻¹.

DNA extraction and genome sequencing

For genome sequencing, DNA samples were extracted using the Promega Wizard® Genomic DNA Purification Kit following the recommended protocol. *Psa* NZ45 was sequenced using the PacBio platform, the remainder were sequenced using the Illumina HiSeq platform.

Genomic reconstruction of ICEs

ICEs identified in genome sequences were used as query sequences for BLAST searches of the NCBI WGS database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Contigs were subsequently downloaded and where ICEs were represented by two contigs they were concatenated in Geneious (<http://www.geneious.com>, Kearse *et al.*, 2012). Concatenation was only required in two instances. ICEs were annotated using the RAST server (<http://rast.nmpdr.org>, Aziz *et al.*, 2008) and manually curated. Alignments were performed using Geneious.

Psa isolation from kiwifruit orchards

1 cm² kiwifruit leaf disks were macerated in 200 µl 10 mM MgCl₂. The macerate was plated on *Pseudomonas* selective

media amended with cetrimide, fucidin and cephalosporin (Oxoid) and incubated at 28°C for 3 days. *Psa* was identified using either diagnostic PCR or LAMP assays (Rees-George *et al.*, 2010; Ruinelli *et al.*, 2017).

Copper resistance assays

Copper resistance was evaluated by determining the minimal concentration of copper that inhibited growth (minimal inhibitory concentration, MIC) on mannitol-glutamate yeast extract medium (MGY) plates supplemented with CuSO₄·5H₂O (Bender and Cooksey, 1986; Cha and Cooksey 1993). *Psa* strains were considered resistant when their MIC exceeded 0.8 mM CuSO₄.

Mutant development

A Tn5 transposon was used to generate kanamycin resistant (*kanR*) strains. *E. coli* S17-1 Tn5*Shah Sgid1* (Zhang *et al.*, 2015) was used as donor and *E. coli* pK2013 (Ditta *et al.*, 1980) as helper. Helper, donor and recipients were grown overnight. 200 µl of helper and donor and 2 ml of recipient were separately washed with 10 mM MgCl₂ and then mixed together and washed again. The mix was then re-suspended in 30 µl of 10 mM MgCl₂, plated on pre-warmed LB agar plates and incubated for 24–48 h at 28°C. Cells were then harvested, resuspended in 1 ml of sterile 10 mM MgCl₂ and plated on KB kanamycin nitrofurantoin plates. Selected mutants were screened for normal growth in KB, LB and M9.

In vitro growth

Overnight cultures of *Psa* NZ13^{kanR} and *Psa* NZ45 were used to determine the *in vitro* growth of each strain in MGY alone or supplemented with 0.5 and 0.8 mM CuSO₄. 10 ml liquid MGY cultures were established with a starting density of 10⁵ cfu ml⁻¹ and shaken for up to 3 days. Bacterial growth was monitored by plating on KB kanamycin (*Psa* NZ13^{kanR}), MGY 0.8 mM CuSO₄ (*Psa* NZ45). Three replicates per strain and media combination was used, and the experiment was repeated three times.

In planta growth

Clonally propagated *Actinidia chinensis* var. 'Hort16A' plantlets were maintained at 20°C with a light/dark period of 14/10 h, 70% constant humidity. *Psa* NZ13^{kanR} and *Psa* NZ45 were grown on KB agar plates for 48 h at 28°C. Inoculum with a final optical density (OD₆₀₀) of 0.2 of either strain was prepared in 50 ml 10 mM MgCl₂ with 0.002% of Silwet. Three to four week old plantlets were inoculated by dipping the aerial parts in the inoculum solution for 5 sec. Five separate plantlets were dip inoculated for each treatment. For experiments assessing *in planta* growth in copper-sprayed plantlets, Nordox75 was used at the recommended dosage of 0.375 g l⁻¹ (www.kvh.org.nz/spray_products). Dip-inoculated plantlets were allowed to dry, then sprayed adaxially and abaxially with Nordox75 until runoff to ensure complete coverage. Bacterial growth was monitored 0, 3 and 7 days post inoculation. 1 cm² disk leaves were cut using a sterile cork borer, surface

sterilized in 70% ethanol and ground in 200 μ l 10 mM MgCl₂. Serial dilutions of the homogenate were plated on KB kanamycin to count *Psa* NZ13^{kanR} and MGY 0.8 mM CuSO₄ to count *Psa* NZ45. Each experiment was repeated three times.

In vitro and in planta competition assays

In vitro and *in planta* competition assays were conducted as described earlier for single strains, except that *Psa* NZ45 and *Psa* NZ13^{kanR} were coinoculated in a 1:1 mix. Endophytic bacteria were harvested as above. To measure the epiphytic population, leaves were detached and the petiole sealed with wax. Each leaf was placed in a sterile bag containing 35 ml of 10 mM MgCl₂. Bags were gently shaken for 3 min, leaf washes were transferred into 50 ml centrifuge tubes and the solution centrifuged for 10 min at 4000 rpm. The supernatant was discarded and the pellet resuspended in 200 μ l 10 mM MgCl₂. Bacterial growth was monitored by plating serial dilutions on KB kanamycin (*Psa* NZ13^{kanR}), MGY 0.8 mM CuSO₄ (*Psa* NZ45) and on MGY kanamycin 0.8 mM CuSO₄ (*Psa* NZ13^{kanR} that acquired copper resistance). *In vitro* assays had three replicates per strain, *in planta* assays were conducted using five replicates, each experiment was repeated three times. Fitness was calculated as the difference between their Malthusian Parameters: $\ln[N_{PsaNZ45}(t_1)/N_{PsaNZ45}(t_0)] - \ln[N_{PsaNZ13}(t_1)/N_{PsaNZ13}(t_0)]$ (Lenski *et al.*, 1991).

ICE integration screening

Primers used in this study are listed in Supporting Information Table S2. Four primers were designed to detect the genomic location of ICE integration: two specific for the integrases at the end of each ICEs (*IntPsaNZ45*, *IntPsaNZ13*) and two for the ICE insertion site on the chromosome, annealing to the *clpB* (*att-1* site) and *queC* (*att-2* site) genes. The primer combination (*IntPsaNZ45-att-2*, *IntPsaNZ45-att-1*, *IntPsaNZ13-att-1* and *IntPsaNZ13-att-2*) indicates the location of the ICEs. Another two sets of primers were designed to amplify either *CuR* (*copA*) or *enolase* genes present in the VR of *Psa*_{NZ45}-CE_{Cu} and *Psa*_{NZ13}-ICE_{eno} respectively. PCRs were performed using Thermo Scientific *Taq* DNA Polymerase following the manufacturer's instructions.

ICE mobilization assay

Psa NZ45 was used as the ICE donor. Strains tested in the mobilization assays are listed in order of divergence relative to the donor: *Psa* K28 (biovar 2) (McCann *et al.*, 2013), *Psa* J31 (biovar 1) (McCann *et al.*, 2013), *P. syringae* pv. *actinidifoliorum* NZ9 (McCann *et al.*, 2013), *P. syringae* pv. *tomato* DC3000 (Buell *et al.*, 2003), *P. syringae* pv. *phaseolicola* 1448a (Teverson, 1991), *P. syringae* H24 and H33 (isolated from kiwifruit; C. Straub, unpublished data), *P. fluorescens* SBW25 (Zhang *et al.*, 2006) and *P. aeruginosa* PAO1 (Holloway, 1955). The copper sulphate MIC was determined for all tested recipient strains, which were all tagged with kanamycin Tn5. A biparental mating was performed using 2 ml and 200 μ l of washed recipient and *Psa* NZ45 cells respectively. The cells were mixed, centrifuged briefly and resuspended in 30 μ l of 10 mM MgCl₂ alone, 10 mM MgCl₂ with 0.5 mM CuSO₄ or 30

μ l of 1 cm² kiwifruit plantlet macerate in 200 μ l of 10 mM MgCl₂ as required. The cell mixture was plated onto solid media (M9 plates) and incubated at 28°C for 48 h. Cells were then harvested and resuspended in 1 ml of sterile 10 mM MgCl₂. Serial dilutions were plated on KB kanamycin to count the total number of recipients and on MGY amended with kanamycin and copper sulphate to count transconjugants.

Biosecurity and approval

All works were performed in approved facilities and in accord with APP201675, APP201730, APP202231.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site

Fig. S1. New Zealand kiwifruit growing regions with isolation sites of copper resistant *Psa*. Map was modified from regional classification map of June 2016 (Kiwi Vine Health (KVH)).

Fig. S2. UPMGA tree of the Cus system proteins in *Psa* NZ. Bootstrap values are shown at each node.

Fig. S3. UPMGA trees of the Cop proteins genes in *Psa* NZ. Bootstrap values are shown at each node.

Fig. S4. UPMGA trees of CopR and CopS proteins in *Psa* NZ. Bootstrap values are shown at each node.

Fig. S5. Density of single and co-cultured *Psa* in liquid MGY supplemented with 0.5 m and 0.8 mM CuSO₄. *Psa* NZ13 was cultured alone (white bars) or co-cultured with *Psa* NZ45 (grey bars). Data are means and standard deviation of three independent cultures. Asterisk indicates significance at 5% level by one-tailed *t*-test.

Fig. S6. *In planta* growth of *Psa* NZ13 and *Psa* NZ45. The single growth of *Psa* NZ13 (white bars) and *Psa* NZ45 (grey bars) was assessed endophytically on leaves of the kiwifruit cultivar Hort16A. The copper product Nordox75 (0.375 g l⁻¹) was sprayed adaxially and abaxially until run off. Data are means and standard deviation of five replicates. One tailed *t*-test showed no statistical difference in growth between of *Psa* NZ13 and NZ45 in absence or presence of copper.

Fig. S7. Growth of co-inoculated *Psa* NZ13 and *Psa* NZ45 on kiwifruit leaves. Growth of *Psa* NZ13 (white bars) and *Psa* NZ45 (grey bars) was assessed endophytically (A) and epiphytically (B) on leaves of the kiwifruit cultivar Hort16A. The copper product Nordox75 (0.375 g l⁻¹) was sprayed adaxially and abaxially until run off. Data are means and standard deviation of five replicates. One tailed *t*-test showed no statistical difference in growth between of *Psa* NZ13 and NZ45 in absence or presence of copper.

Fig. S8. Analysis of the presence of the variable region (VR) of *Psa*_{NZ45}ICE_Cu and *Psa*_{NZ13}ICE_eno in 11 *Psa* NZ13 transconjugants. PCRs were carried out to detect *copA* (VR of *Psa*_{NZ45}ICE_Cu) or *enolase* genes (VR of *Psa*_{NZ13}ICE_eno). Controls of *Psa* NZ13 and *Psa* NZ45 show one and two bands, indicative of *Psa*_{NZ13}ICE_eno in *Psa* NZ13 and both *Psa*_{NZ13}ICE_eno *Psa*_{NZ45}ICE_Cu and in *Psa* NZ45, respectively. All transconjugants, lanes 1–11 have acquired *Psa*_{NZ45}ICE_Cu.

Fig. S9. Analysis of the insertion site of the *Psa*_{NZ13}ICE_eno and *Psa*_{NZ45}ICE_Cu in 11 *Psa* NZ13 transconjugants. PCRs were to detect the integration of *Psa*_{NZ13}ICE_eno in the *att-1* or *att-2* sites (*intPsaNZ13-att-1* and *intPsaNZ13-att-2*) and the integration of *Psa*_{NZ45}ICE_Cu in the *att-1* or *att-2* sites (*intPsaNZ45-att-1* and *intPsaNZ45-att-2*). Controls of *Psa* NZ13 and *Psa* NZ45 show that in *Psa* NZ13 the *Psa*_{NZ13}ICE_eno is integrated in the *att-1* site and in *Psa* NZ45 the *Psa*_{NZ13}ICE_eno is integrated in the *att-1* and the *Psa*_{NZ45}ICE_Cu in the *att-2* site.

Table S1. *In planta* transfer of *Psa*_{NZ45}ICE_cu from *Psa* NZ45 to *Psa* NZ13 at different founding ratios of donor and recipient. Donor and recipient strains were dip-inoculated onto Hort16A leaves at different founding ratios and frequency of recipients determined at days 3 and 7.

Table S2. Primers used for *Psa*_{NZ13}ICE_eno and *Psa*_{NZ45}ICE_Cu detection and integration loci.