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Claudine Boyer, Pierre Lefeuvre, Damien Richard, Kanta Kumar Lobin, Olivier Pruvost. Complete Genome Sequence of a Copper-Resistant *Xanthomonas campestris* pv . *campestris* Strain Isolated from Broccoli in Mauritius Suggests Adaptive Gene Gain Through Horizontal Gene Transfer. *Phytopathology*, 2024, 114 (2), pp.328-333. 10.1094/PHYTO-05-23-0177-SC . hal-04548066

HAL Id: hal-04548066

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Submitted on 16 Apr 2024

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Complete Genome Sequence of a Copper-Resistant *Xanthomonas campestris* pv. *campestris* Strain Isolated from Broccoli in Mauritius Suggests Adaptive Gene Gain Through Horizontal Gene Transfer

Claudine Boyer,¹ Pierre Lefeuvre,¹ Damien Richard,¹ Kanta Kumar Lobin,² and Olivier Pruvost^{1,†} 

¹ CIRAD, UMR PVBMT, F-97410 Saint Pierre, La Réunion, France

² FAREI, Le Reduit, Mauritius

Accepted for publication 7 August 2023.

Abstract

Bacterial adaptation is facilitated by the presence of mobile genetic elements and horizontal gene transfer of genes, such as those coding for virulence factors or resistance to antimicrobial compounds. A hybrid assembly of Nanopore MinIon long-read and Illumina short-read data was produced from a copper-resistant *Xanthomonas campestris* pv. *campestris* strain isolated from symptomatic broccoli leaves in Mauritius. We obtained a 5.2-Mb high-quality chromosome and no plasmid. We found four genomic

islands, three of which were characterized as integrative conjugative elements or integrative mobilizable elements. These genomic islands carried type III effectors and the copper resistance *copLABMGF* system involved in pathogenicity and environmental adaptation, respectively.

Keywords: adaptation, antimicrobial compounds, black rot of crucifers, type III effectors

Black rot is the most important disease of cruciferous vegetable crops, including broccoli (*Brassica oleracea* L.), and induces substantial economic losses worldwide. Typical disease symptoms caused by *Xanthomonas campestris* pv. *campestris* (*Xcc*) are chlorotic to necrotic spots, as a result of stomatal infection, and V-shaped lesions, which start at the leaf margins, as a result of hydathode infection. *Xcc* is a seedborne pathogen, which facilitates its spread over long distances. Black rot is controlled using various strategies, including seed testing, cropping practices, resistant cultivars, and chemical control often involving copper-based pesticides (Vicente and Holub 2013).

Copper is an essential element to aerobic organisms. However, excessive intracellular copper concentrations are toxic to bacteria (Giachino and Waldron 2020). Intracellular copper concentration in bacteria is regulated by copper homeostasis systems (for review, see Andrei et al. 2020; Argüello et al. 2013), a function that is to some extent performed in xanthomonads by the products of the chromosome-borne *cohL*, *cohA*, and *cohB* genes (Behlau et al. 2011; Marin et al. 2019; Potnis et al. 2011). *CohA* is a multicopper oxidase, which presents a Cu(I) binding site and protects cells against Cu(I) stress through its oxidation to the less toxic Cu(II) form (Andrei et al. 2020). *CohB* is an ATPase, which binds and transports copper ions in the extracellular environment when copper concentrations approach toxic levels (Argüello et al. 2007; Bissig et al. 2001). The *cohL* gene is involved in copper sensing and may act as a regulatory RNA. Moreover, the *cohL-cohA* intergenic region forms a complex stem-loop secondary structure that may control *cohA* expression (de Freitas et al. 2019). Copper sprays have been extensively used by

farmers for decades to prevent and control fungal and bacterial crop diseases, including those caused by xanthomonads (Lamichhane et al. 2018). As a result, copper resistance has developed in several *Xanthomonas* species (Behlau et al. 2013, 2017; Richard et al. 2017b). Several genetic systems for copper resistance have been reported in the genus *Xanthomonas* (Behlau et al. 2011; Lee et al. 1994; Voloudakis et al. 2005). In *Xcc*, the sole report of copper resistance to date was associated with a plasmid-borne *copLAB* (Behlau et al. 2017) frequently present in copper-resistant strains worldwide (Behlau et al. 2011; Richard et al. 2017b). However, two recent studies reported the integration of the copper resistance *copLAB* system into the chromosome of some strains of *X. euvesicatoria* pv. *perforans* and *X. citri* pv. *viticola*, respectively (Bibi et al. 2023; Rana et al. 2023).

In the context of (i) the high prevalence of black rot in Mauritius (Southwest Indian Ocean region) and (ii) the emergence of copper resistance worldwide in multiple *Xanthomonas* species (including in Mauritius) (Richard et al. 2017a), we collected samples of *Xanthomonas*-like isolates between 2019 and 2021. Samples were recovered from cruciferous host species in 123 fields and phenotypically screened for copper resistance. In 2021, copper resistance was detected in strains from the Phoenix locality. Here, using a hybrid MinIon/Illumina sequencing method, the high-quality genome sequence for the Mauritian copper-resistant *Xcc* strain CFBP 9145, isolated from typical black rot symptoms on broccoli, evidenced the presence of several genomic islands hosting type III effectors and copper resistance genes.

Bacterial culture was performed on YPGA medium (yeast extract 7 g liter⁻¹, peptone 7 g liter⁻¹, glucose 7 g liter⁻¹, and agar 18 g liter⁻¹, pH 7.2) at 28°C for 72 h. Copper resistance was detected by growth on YPGA supplemented with 200 ppm of CuSO₄, and the PCR amplification of *copL* and *copD* genes (including negative and positive controls) yielded amplicons of the expected size (Behlau et al. 2013; Richard et al. 2017b). Genomic DNA extracted from pure culture, using the Wizard genomic DNA purification kit (Promega, France), was used for library preparation. We used TruSeq (150 × 2, read length) and rapid sequencing protocols for Illumina NovaSeq 6000 and Oxford Nanopore MinIon R9.4.1, respectively.

[†]Corresponding author: O. Pruvost; olivier.pruvost@cirad.fr

Funding: Support was provided by the Interreg Epibio, the Conseil Régional de La Réunion, the European Observation Network for Territorial Development and Cohesion (GURDT I2016-1731-0006632), and CIRAD.

e-Xtra: Supplementary material is available online.

The author(s) declare no conflict of interest.

Adapters from raw reads were trimmed using Trimmomatic v0.36 (ILLUMINACLIP:NexteraPE-PE.fa:2:30:10 SLIDING-WINDOW:4:15 MINLEN:150) (Bolger et al. 2014) and Porechop v0.2.3 (<https://github.com/rrwick/Porechop/>) with default parameters. In total, we obtained 5,197,800 short paired-end reads (5.2 Mb, ~299× coverage) and 15,836 long reads (5.2 Mb, ~32× coverage). The raw error rate for long reads was 9.87% (497,481 bases). De novo co-assembly was performed using Unicycler v0.4.9b with default parameters (Wick et al. 2017). Completeness and contamination were estimated using CheckM v1.2.2 (Parks et al. 2015). The genome was annotated using Prokka v1.14.6 (Seemann 2014). Whole-genome average nucleotide identity (ANI) scores were calculated using fastANI v1.33 (Jain et al.

2018). Type III effectors were predicted using BLASTx searches compared with the EuroXanth database for type III effectors (<https://internet.myds.me/dokuwiki/doku.php?id=bacteria:t3e:t3e>). An effector was considered to be present if it displayed 80% or greater amino acid identity over 80% of the query length. Conjugative systems were found with the MacSyFinder CONJscan module (Abby et al. 2014; Cury et al. 2017). The transcription activator-like (TAL) effector repeat number was corrected using long reads covering this region and polished with Illumina reads, using bcftools v1.10.2. The repeat variable diresidues (RVD) sequence was obtained using AnnoTale v1-3 (Grau et al. 2016). Functional and phylogenetic analyses were performed using Quetel v1.1 (Pérez-Quintero et al. 2015). Mobile genetic elements were predicted using the online tools ICEfinder v1.0 (Liu et al. 2019) and PHASTER (Arndt et al. 2016), respectively.

The assembly yielded a single circularized 5,204,395-bp chromosome with a GC content of 64.9%, with 100% completeness and 0% contamination, carrying 4,453 coding sequences (CDSs), 59 tRNAs, and 2 rRNA operons (GenBank accession CP124745).

The highest whole-genome ANI scores (98.74%) against the genome of pathotype strains of *X. campestris* pathovars associated with Brassicaceae (Table 1) (Vicente and Holub 2013) placed

TABLE 1. Average nucleotide identity (ANI) scores between CFBP 9145 and four pathotype strains of *Xanthomonas campestris*

Strain	Pathovar	ANI score (%)	RefSeq assembly accession
ATCC 33913	<i>campestris</i>	98.74	GCF_000007145.1
NCPBP 347	<i>armoraciae</i>	98.17	GCF_020731405
NCPBP 1946	<i>raphani</i>	97.25	GCF_020813075.1
NCPBP 937	<i>incanae</i>	96.60	GCF_020813295

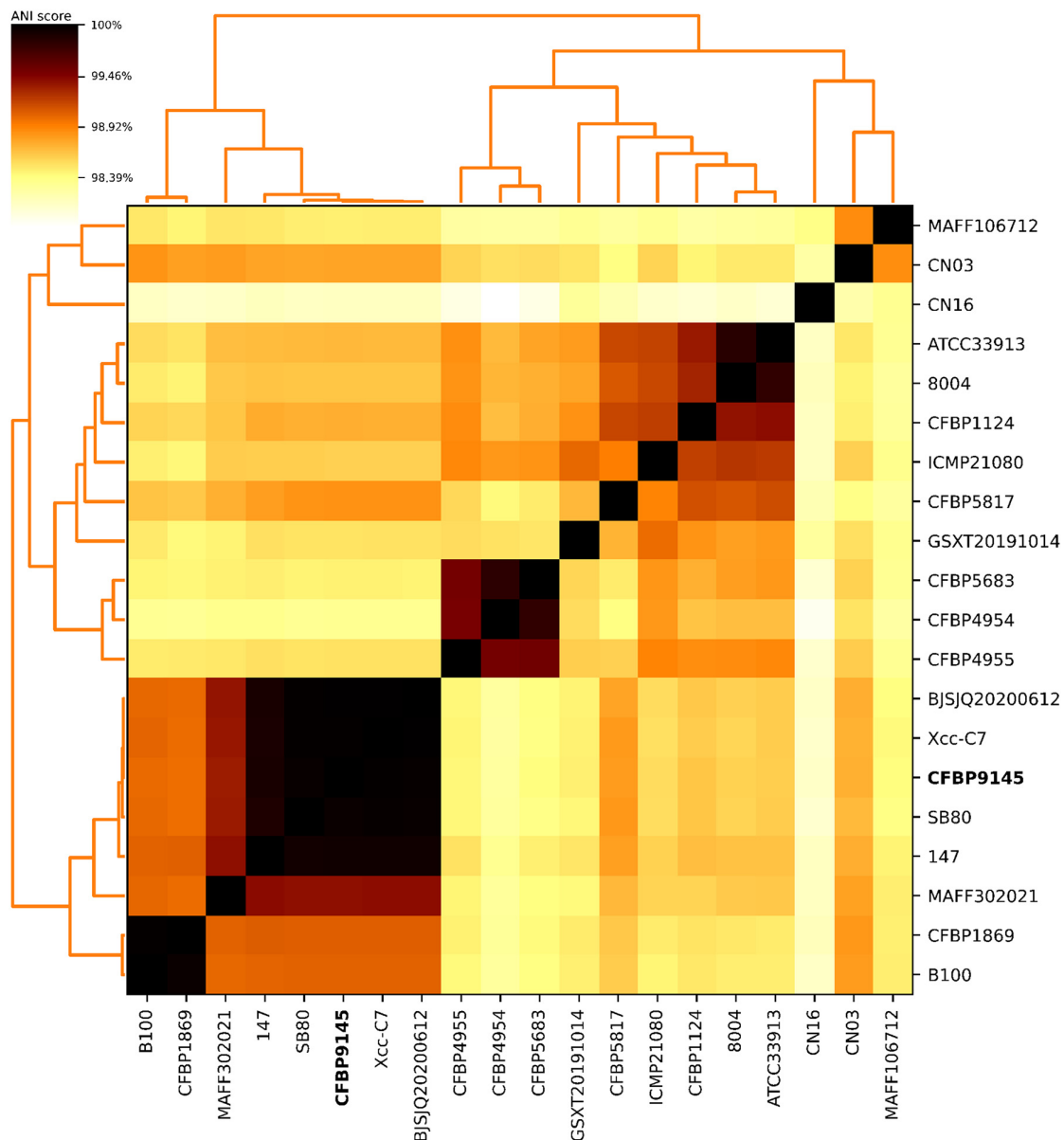


Fig. 1. Heatmap of pairwise average nucleotide identity (ANI) scores and clustering tree between *Xanthomonas campestris* pv. *campestris* (*Xcc*) selected genomes.

CFBP 9145 in the *campestris* pathovar, consistent with the symptomatology observed in the field. To identify the closest strains, pairwise ANIs were computed on 20 *Xcc* chromosomes, including CFBP 9145, its most closely related strains, and strains representative of *Xcc* genetic diversity (Guy et al. 2013) (Fig. 1; Supplementary Table S1). The strain CFBP 9145 was found to be most closely related to strains BJSJQ20200612 (Chen et al. 2022), SB80 (Erken Meral et al. 2022), *Xcc*-C7 (Kesharwani et al. 2023), and 147 (Guy et al. 2013) isolated from China, Turkey, India, and Brazil, with 99.986, 99.980, 99.978, and 99.866% ANI scores, respectively.

Here, we report a 93.3-kb chromosomal genomic island flanked by the *tRNA_{uri}* gene of the *Xcc* strain CFBP 9145, with a 62.2% GC content and 88 CDSs. The first ~50 kb were composed of a rearranged mosaic of regions present in the chromosome of the *Xcc* strain GSXT20191014 (NZ_CP069084.1; best hit score of 99.97% identity for 93% of query cover) isolated from cabbage in China (Chen et al. 2022). The remaining ~40 kb shared 100% identity with a region of the pLH3.1 plasmid (NZ_CP018472.1) from the *X. euvesicatoria* pv. *perforans* strain LH3, previously found to encode for copper resistance (Richard et al. 2017a) (Fig. 2). Similar to LH3 but unlike most other sequenced strains, CFBP 9145 hosted the copper resistance gene cluster *copLABMGF*, with *copC* and *copD* being absent. CFBP 9145 therefore presents with a genetic organization of copper resistance genes identical to that originally reported for *X. euvesicatoria* pv. *citrumelonis* strain 1381 (Behlau et al. 2011). These genes can be hosted on a plasmid, as in LH3 and 1381, or on the chromosome, as in *X. euvesicatoria* pv. *perforans* strain Xp2010 and *X. citri* pv. *viticola* (Behlau et al. 2012; Bibi et al. 2023; Rana et al. 2023; Richard et al. 2017a). All these strains shared 91% or more nucleotide identity on all genes in the *copLABMGF* cluster (Table 2). Similar to LH3 but unlike most other sequenced strains, the arsenic resistance gene cluster (*arsBHCR*) was also absent (Richard et al. 2017a). Nucleotide comparison revealed a low level of identity (62%) with the *copLABMGF* gene cluster identified in *Xcc*-BrA1. The latter is a distant relative of other reported *copLAB* genes from *Xanthomonas* and *Stenotrophomonas* (Behlau et al. 2017). The *copL*, *copA*, and *copB* genes in CFBP 9145 (position 2,558,734 to 2,562,256 bp) shared 51.7, 66.9, and 62.9% nucleotide identity with the copper homeostasis genes *cohL*, *cohA*, and *cohB*, respectively. The latter genes were also located on the chromosome (position 4,402,437 to 4,406,228 bp) and shared 92.9, 97.9, and 98.3% nucleotide identity with the *cohL*, *cohA*, and *cohB* genes in *Xcc* pathotype strain ATCC 33913.

Integrative elements represent one of the many known mobile genetic elements, including integrative conjugative elements (ICEs)

and integrative mobilizable elements (IMEs). Both encode their own excision and integration machinery. ICEs can be horizontally transferred to other bacterial cells using a type IV conjugative pilus (T4SS) (Johnson and Grossman 2015), whereas IMEs use the conjugation machinery of an unrelated co-resident conjugative element for their own transfer (Oliveira et al. 2017).

Two putative IMEs (3,124,879 to 3,164,552 bp, 39.6 kb in length, 61.2% GC content, 47 CDSs and 3,588,005 to 3,629,010 bp, 41.0 kb in length, 63% GC content, 39 CDSs, referred to as *IME Xcc1* and *IME Xcc2*, respectively) were predicted in the CFBP 9145 chromosome (Fig. 3). Flanked by a *tRNA_{gly}* and direct repeats referred to as *attL/attR* sites, a putative ICE of 87.6 kb in length, 60.2% GC content, and 91 CDSs was also predicted, adjacent to the genomic island for copper resistance (2,408,348 to 2,495,962 bp). Among the components of the ICE conjugative apparatus (Smillie et al. 2010), *traI* and *traD* genes from the MOB_H family, relaxase, and type IV coupling protein (T4CP) were predicted with a type IV secretion system (T4SS). This was composed of *tfc* genes related to a class G membrane-associated mating-pair formation complex (MPF_G) (Fig. 3) (Guglielmini et al. 2014). The structure and sequence of this conjugative system suggest that this ICE is related to the ICE_{Ecl} family found in Gammaproteobacteria (Delavat et al. 2017). A closely related ICE (*ICEXca2*) was recently reported in *Xcc* strain CN03 (99.8% of nucleotide identity on 100% query cover) (de Assis et al. 2022).

The pathogenicity of xanthomonads largely relies on effector proteins secreted in host cells by a type III secretion system (T3SS) (Büttner 2016; Jacques et al. 2016; Kay and Bonas 2009).

CFBP 9145 hosts 28 homologous non-TAL type III effectors within a repertoire of 33 genes. Interestingly, eight of these (*xopG*, *xopP*, *xopAL1*, *xopA*, *xopAH*, *xopE3*, *xopH*, and *avrBs1*) (Fig. 3; Fig. 4A) are located in the integrative elements described previously. CFBP 9145 also contains a truncated version of *xopF1*, consistent with previous data, but unlike other *Xcc* strains (Potnis et al. 2011).

Three of the four *Xcc* strains that were found to be very close to CFBP 9145 using the ANI score presented the same repertoire as CFBP 9145. The only exception was the Brazilian strain 147, which hosts a distinct and truncated allele of *xopD* (*xopD2*), instead of *xopD1* (Fig. 4A). At least two of the non-TAL type III effectors, which were detected on the ICE present in the CFBP 9145 genome, were previously reported as genes that modulate the host specificity of *Xcc* (He et al. 2007; Vicente and Holub 2013).

In the genus *Xanthomonas*, TAL effector proteins consist of 1.5 to 33.5 tandem repeats of 33 to 34 amino acids in length. The pair of

TABLE 2. BLASTn identity percentages between copper resistance genes in strain CFBP 9145 and other *Xanthomonas* strains hosting the *copLABMGF* system

CFBP 9145 vs.	Taxon	<i>copL</i>	<i>copA</i>	<i>copB</i>	<i>copM</i>	<i>copG</i>	<i>copF</i>
LH3	<i>X. euvesicatoria</i> pv. <i>perforans</i>	100.00	100.00	100.00	100.00	100.00	100.00
CCRMXcv80	<i>X. citri</i> pv. <i>viticola</i>	99.05	99.47	97.29	96.92	97.97	97.32
CCRMXcv17	<i>X. citri</i> pv. <i>viticola</i>	99.05	99.47	97.29	96.92	97.97	97.32
CFBP 7764	<i>X. citri</i> pv. <i>viticola</i>	99.05	99.47	97.29	96.92	97.97	97.32
Xac1381	<i>X. euvesicatoria</i> pv. <i>citrumelonis</i>	93.81	95.83	91.28	96.32	99.82	100.00
Xp2010	<i>X. euvesicatoria</i> pv. <i>perforans</i>	93.81	95.83	91.28	93.36	96.87	94.89

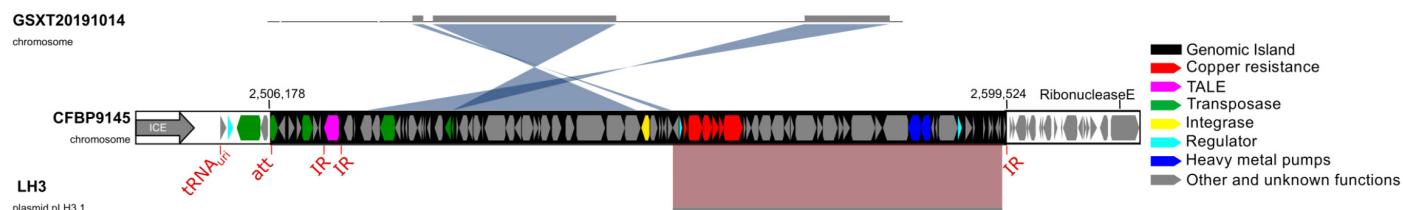


Fig. 2. Gene content of the genomic island hosting copper resistance genes, with a focus on the mosaic structure of CFBP 9145 compared with the closest genomes publicly available (figure produced using mauve_snapshot_2015-02-13). Genes are colored according to the function of the protein that they encode. IR: inverted repeat.

residues in positions 12 and 13 constitutes the RVD, which drives the specificity of the nucleotide-binding mechanism. Translocated into the host plant nucleus, they bind eucaryote promoter target sites, the effector-binding element sequences, and activate the expression of plant genes, which aid in bacterial infection or induce plant defense (Mak et al. 2013; Moscou and Bogdanov 2009).

Located in the specific *Xcc* mosaic part of the genomic island hosting copper resistance genes (Fig. 2), a single TAL effector of

11.5 repeats shared 100% RVD sequence homology with the TAL effector *hax3* (*tal12a*) from the EBE group I (Fig. 4B), a gene previously found to contribute to the development of disease symptoms on *B. oleracea* var. *botrytis* (Denancé et al. 2018). This TAL effector was integrated into a Tn3-like mobile insertion cassette in strain CFBP 9145 (Ferreira et al. 2015).

Herein, we highlight the cardinal importance of horizontal transfer of adaptive genes in the plant-pathogenic *Xanthomonas* genus.

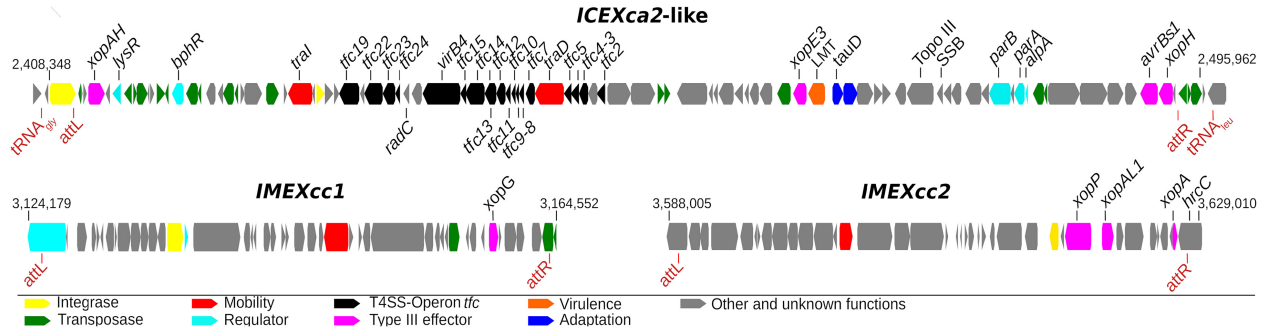


Fig. 3. Gene content of the integrative elements *Xca2*-like (integrative conjugative element), *Xcc1* (integrative mobilizable element), and *Xcc2* (integrative mobilizable element). Genes are colored according to the function of the protein that they encode. LMT: lytic murein transglycosylase; topo III: topoisomerase III; and SSB: single-strand DNA-binding protein.

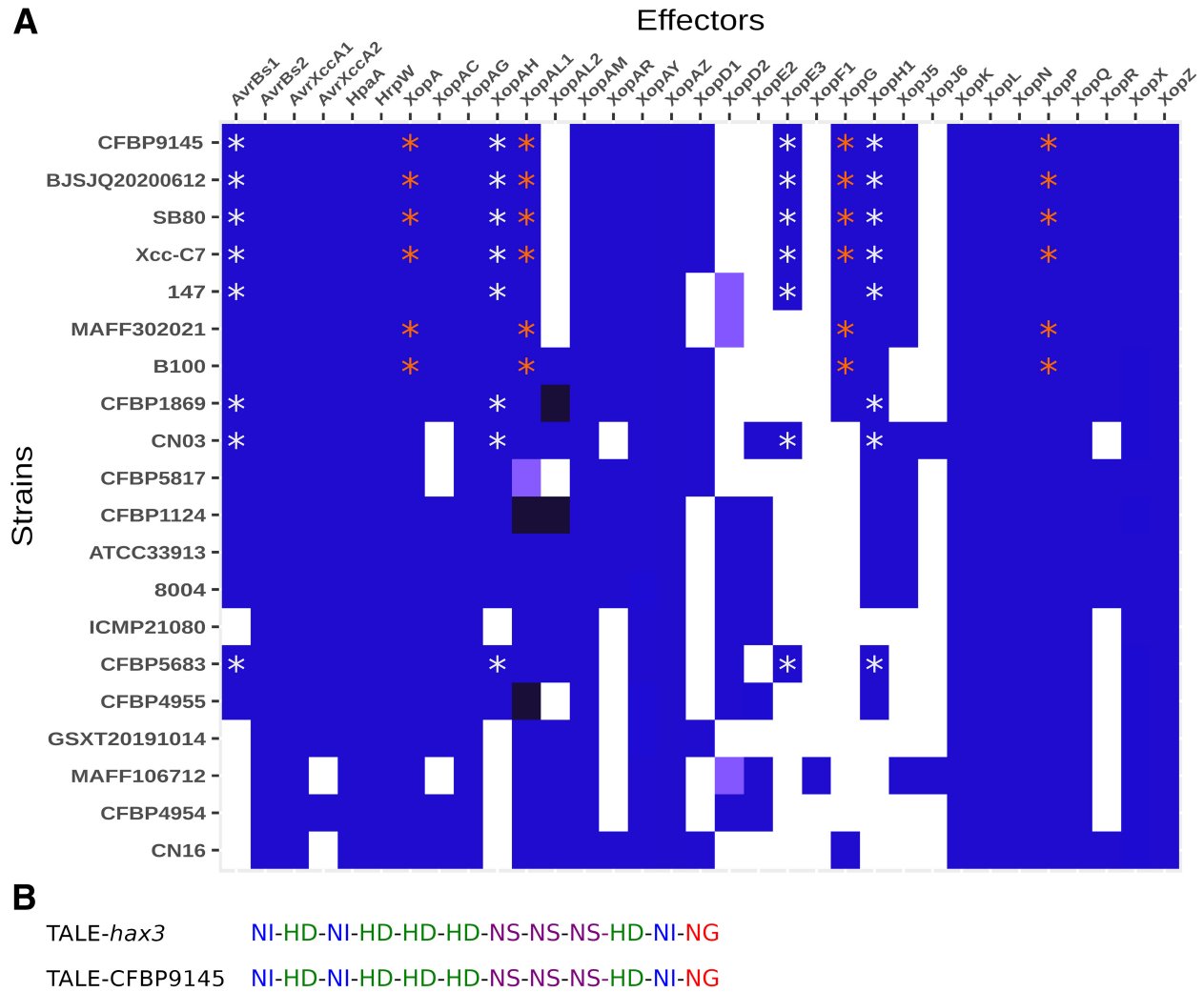


Fig. 4. A, Composition of non-TAL (transcription activator-like) type III effectors between genomes representative of genetic diversity in *Xanthomonas campestris* pv. *campestris* (*Xcc*), ranked in ascending order of average nucleotide identity scores, compared with CFBP 9145. **B,** TAL effector repeat variable diresidues sequence correspondence. The presence or absence of specific genes is indicated using a blue or white square, respectively. Truncated genes ($\geq 80\%$ amino acid identity over 60 to 80% of the query length) are shown with purple squares. Black squares correspond to PCR data from Guy et al. (2013) but not found in complete genomes from NCBI accessions. Effectors associated with integrative conjugative and integrative mobilizable elements are shown by white and orange stars, respectively.

Four genomic islands, three of which had the structure of integrative elements (ICEs and IMEs), were identified in the chromosome of the crucifer pathogen *Xcc*. These genes include several type III effectors showing a strain-specific presence in genomes. Some of these genes were previously reported to increase strain virulence or contribute to strain host range modulation. We also found a gene cluster conferring resistance to copper, a pesticide used on a massive scale to control many bacterial and fungal crop pathogens. The present study further illustrates the importance of horizontal gene transfer in the rapid adaptation of bacterial pathogens to anthropogenic selection pressure. A growing number of reports that document copper resistance in plant-pathogenic bacteria advocate the implementation of thorough surveillance and management programs.

Acknowledgments

We thank the plant protection platform 3P, IBISA.

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