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In vitro exploration of the *Xanthomonas hortorum* pv. *vitians* genome using transposon insertion sequencing and comparative genomics to discriminate between core and contextual essential genes

Lucas Morinière^{1,*}, Solène Lecomte¹, Erwan Gueguen² and Franck Bertolla^{1,*}

Abstract

The essential genome of a bacterium encompasses core genes associated with basic cellular processes and conditionally essential genes dependent upon environmental conditions or the genetic context. Comprehensive knowledge of those gene sets allows for a better understanding of fundamental bacterial biology and offers new perspectives for antimicrobial drug research against detrimental bacteria such as pathogens. We investigated the essential genome of *Xanthomonas hortorum* pv. *vitians*, a gammaproteobacterial plant pathogen of lettuce (*Lactuca sativa* L.) which belongs to the plant-pathogen reservoir genus *Xanthomonas* and is affiliated to the family *Xanthomonadaceae*. No practical means of disease control or prevention against this pathogen is currently available, and its molecular biology is virtually unknown. To reach a comprehensive overview of the essential genome of *X. hortorum* pv. *vitians* LM16734, we developed a mixed approach combining high-quality full genome sequencing, saturated transposon insertion sequencing (Tn-Seq) in optimal growth conditions, and coupled computational analyses such as comparative genomics, synteny assessment and phylogenomics. Among the 370 essential loci identified by Tn-Seq, a majority was bound to critical cell processes conserved across bacteria. The remaining genes were either related to specific ecological features of *Xanthomonas* or *Xanthomonadaceae* species, or acquired through horizontal gene transfer of mobile genetic elements and associated with ancestral parasitic gene behaviour and bacterial defence systems. Our study sheds new light on our usual concepts about gene essentiality and is pioneering in the molecular and genomic study of *X. hortorum* pv. *vitians*.

DATA SUMMARY

All new genome and sequencing data were deposited in the corresponding databases of the National Center for Biotechnology Information (NCBI, <https://www.ncbi.nlm.nih.gov>), and are associated with BioProject PRJNA530964 and BioSample SAMN11340225. The Illumina and PacBio raw sequencing reads of the complete genome sequencing of strain LM16734 are

accessible at the Sequence Read Archive (SRA) database under accession numbers SRR12066994–SRR12066995. The manually revised annotated assembly carried out at the MicroScope annotation platform (<https://mage.genoscope.cns.fr>) was deposited in the GenBank database under accession number GCA_014338485.1. The transposon insertion sequencing reads of the two duplicates were also deposited in the SRA database under accession numbers

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Keywords: *Xanthomonas hortorum*; Tn-Seq; bacterial leaf spot of lettuce; mobile genetic elements; *Xanthomonadaceae*; essential genome.

Abbreviations: BLSL, bacterial leaf spot of lettuce; CDS, coding sequence; COG, Cluster of Orthologous Groups; DEG, database of essential genes; EPS, exopolysaccharide; HMM, hidden markov model; KEGG, kyoto encyclopediae of genes and genomes; LB, luria-bertani; MGE, mobile genetic element; MTase, methyltransferase; OLD, overcoming lysogenization defect; PCR, polymerase chain reaction; REase, restriction endonuclease; R-M, restriction-modification; Tn-Seq, transposon insertion sequencing; TSA / TSB, tryptic soy agar / tryptic soy broth; TTR, trimmed total reads.

The NCBI GenBank assembly and SRA accession numbers for the complete *X. hortorum* pv. *vitians* LM16734 genome and raw sequencing reads are GCA_014338485.1 and SRR12066994–SRR12066995, respectively. Transposon insertion sequencing reads are available at SRA under accessions SRR12067051–SRR12067052. All data are associated with BioProject PRJNA530964. Supplementary Material can be found at 10.6084/m9.figshare.13573523.

Data statement: All supporting data, code and protocols have been provided within the article or through supplementary data files. Seven supplementary tables and three supplementary figures are available with the online version of this article.

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SRR12067051–SRR12067052. The GenBank assembly accession numbers for the other published genome data used in this study are given in Table S1 (available in the online version of this article). Tables S1–S7 and Figs S1–S3 are available at figshare with the following doi: <https://figshare.com/s/aab51b0deba42f67e969>.

INTRODUCTION

Evolutionary forces shape bacterial genomes through persistence and loss of genes in relation to the fitness cost or benefit they provide to the organism or population. However, for most genes, the conferred fitness depends on occupied environments and ecological niches, or on the lifestyle adopted by the bacterium [1, 2]. A minority of coding sequences are considered indispensable regardless of the environmental context, as they regulate critical cellular processes such as DNA replication, cell wall integrity or energy metabolism [3]. These coding sequences and structural elements as a whole are referred to in the literature as the minimal gene set [4] or essential genome [5] of a given bacterium. However, gene essentiality is usually deduced from indirect observations of lethal mutant phenotypes in a given medium. Thus, observed essentiality could either result from direct alteration of critical functions required for cell survival (e.g. DNA replication, membrane synthesis), or context-dependent effects related to the genome content or environmental conditions (e.g. toxin–antitoxin systems, auxotrophy) [6, 7]. Consequently, the essentiality of some genes varies among strains within the same species [8–10]. Therefore, the essential genome of a bacterium can be seen as a combination of ‘core’ and ‘accessory’ essential genes, and the latter depend on environmental conditions or the genetic context.

Essential genomes have been historically assessed through systematic single-gene knock-out or deletion experiments [11, 12], or identification of conserved genes by comparative genomics [13, 14]. Yet, both methods have substantial limitations. For example, computational approaches based on protein sequence homology yielded artificially small essential genomes because similar functions can be encoded by non-orthologous genes, while experimental approaches were time-consuming and subject to variability due to methodological differences [3, 15]. However, the development and extensive application of genome-wide transposon insertion sequencing – abbreviated Tn-Seq [16] – in the last decade, and of its variants referred to as INSeq [17], TraDIS [18] or HITS [19] has allowed for highly efficient and systematic identification of the essential features of cell survival in a genome. Briefly, Tn-Seq experiments use high-throughput sequencing of a pooled population of mutants covering the entire genome to assess the impact of each mutation site on overall fitness and survival. Experiments can be carried out both in optimal growth conditions to identify genes commonly considered as absolutely essential [5, 20–26], and in diverse environmental conditions (e.g. *in vivo*, in synthetic communities) to reveal conditionally essential genes [27–32]. Essential genomes have been so far characterized *in vitro* for at least 63 bacteria from various phyla (DEG database, accessed September

Impact Statement

The genus *Xanthomonas* constitutes a huge reservoir of economically and ecologically important plant-pathogenic species that pressure agroecosystems and threaten global food sustainability. Amongst these, *Xanthomonas hortorum* pv. *vitiens* causes a bacterial leaf spot of lettuce. This disease attacks all varieties of lettuce around the world, and no sustainable and operational means of control has yet been found. Moreover, little is known about the genetic and molecular functioning of *X. hortorum* species. Previous studies conducted on other organisms have shown that transposon insertion sequencing (Tn-Seq) is a powerful tool for acquiring fundamental knowledge on cell functioning and critical cellular processes because it reliably identifies prospective targets for new antimicrobial compounds. Using the discriminative power of highly saturated genome-wide Tn-Seq and high-quality complete genome sequencing, the present study offers an extensive description and analysis of *X. hortorum* pv. *vitiens* LM16734 genes required for cell survival in optimal growth conditions. Portrayal of the essential genome is accompanied by a critical reflection on the concepts underlying gene essentiality based on gene conservation, synteny and phylogenomic analyses. In this way, we discriminated between core essential genes of xanthomonads and horizontally acquired contextual essential genes in this first *Xanthomonas* essential genome.

2020) [33]. However, research has been mostly focused on human-pathogenic bacteria (51 essential genomes out of 63) because systematic essentiality assessment is considered a promising way to identify new targets for antimicrobial drugs [34]. However, apart from the two major α -proteobacterial plant pathogens *Agrobacterium fabrum* [35] and *Ralstonia solanacearum* [36], no other essential genomes of plant pathogens, especially from the γ -proteobacterial class, have been investigated.

The genus *Xanthomonas* is a large reservoir of important plant-pathogenic γ -proteobacteria, as *Xanthomonas* species are the causative agents of diseases of almost 400 plant species, some of which are major crops worldwide (rice, cabbage, tomato, citrus, etc.) [37]. They belong to the same taxonomic family – *Xanthomonadaceae* – as the environmental and opportunistic pathogenic genus *Stenotrophomonas* and the devastating plant-pathogenic genus *Xylella*. While some *Xanthomonas* species have been extensively studied, e.g. major *X. campestris* or *X. oryzae* species [38], there still remains a lot to discover about most species. Among these, *Xanthomonas hortorum*, which comprises seven distinct pathovars and an increasing number of non-classified pathogenic strains attacking a wide variety of plants, has been scarcely investigated, perhaps partially because of its complex and until recently unresolved

taxonomy [39]. Indeed, apart from a few studies on some type III effectors of the tomato pathogen *X. hortorum* pv. *gardneri* (formerly known as '*X. gardneri*') [40, 41], and a noticeable genomic study of *X. hortorum* pv. *carotae* [42], extensive functional genomic studies and mutagenesis experiments have not yet been attempted on *X. hortorum*.

Our research work focuses primarily on *X. hortorum* pv. *vitians*, the causal agent of the bacterial leaf spot of lettuce disease (BLSL). This foliar disease is characterized by an outburst of small water-soaked lesions that later coalesce to form large necrotic patches [43]. Although sporadic, BLSL seems to have been re-emerging all around the world since the 1990s [39], sometimes dramatically reducing plant yield and quality, and increasing post-harvest losses [44]. Moreover, this pathogen can disseminate across the environment and persist mostly through seed infestation [45], but also in buried plant debris, irrigation water and epiphytically on a wide variety of weeds [46, 47]. It has become a concerning threat for lettuce production in the absence of efficient and practical means of disease control or prevention [48]. Understanding the architecture and evolution of the *X. hortorum* pv. *vitians* genome would certainly support the many attempts made to select resistant lettuce cultivars [49] and prevent predictable resistance by identifying virulence and resistance genes.

The present study aims at giving a comprehensive description of the essential genome of *X. hortorum* pv. *vitians* in optimal growth conditions, and provides an overview of the conservation and horizontal transfer of essential genes within the family *Xanthomonadaceae*. Using high-resolution complete genome sequencing and the design of a highly saturated transposon mutant library, we combined the discriminative power of genome-wide Tn-Seq experiments with critical insights into gene essentiality brought by multiple computational analyses. This innovative approach allowed us to identify core essential features conserved at various taxonomic levels, and genetic-context-dependent essential genes acquired through horizontal transfer events. This primary functional genomic study of *X. hortorum* pv. *vitians* will push forward investigations of this under-investigated species and lead to advances in both fundamental genomic understanding and disease control research.

METHODS

Bacterial strains, plasmids and growth conditions

Xanthomonas hortorum pv. *vitians* strain LM16734 (=CFBP 8638) was isolated from a diseased lettuce in 2016 in the Rhône-Alpes region, France [39], and routinely cultured at 28 °C on 1/10th tryptic soy agar (TSA) plates or in 1/10th tryptic soy broth (TSB). *Escherichia coli* MFDpir/pSamEc [27] was grown at 37 °C in LB medium supplemented with diaminopimelic acid (DAP), for which it is auxotrophic, at 57 µg.ml⁻¹. When required, the media were supplemented with 100 µg.ml⁻¹ ampicillin and 25 µg.ml⁻¹ kanamycin. The pSamEc plasmid is a mobilizable suicide vector carrying (i) the ampicillin resistance gene *bla*, (ii) a Himar1-C9 transposase gene under the control of the *lac* promoter and (iii) a kanamycin

resistance gene flanked by mariner inverted repeat sequences containing *MmeI* restriction sites. The strains were kept at -80 °C in 30 % (v/v) glycerol vials for long-term storage.

Complete genome sequencing, assembly and annotation

High-quality complete genome sequencing of *X. hortorum* pv. *vitians* LM16734 was achieved using a combination of long-read and short-read technologies. Genomic DNA was extracted and purified from overnight broth cultures using the standard phenol-chloroform method [50], then sent to GATC Biotech for PacBio long-read sequencing with a PacBio RS instrument (Pacific Biosciences) and to Eurofins Genomics (Eurofins Scientific) for Illumina HiSeq 2x150 bp short-read sequencing on a NovaSeq 6000 instrument (Illumina Inc.). Hybrid assembly of raw reads from the two sequencing strategies was performed using UNICYCLER v0.4.2 [51], and the resulting assembly was deposited and annotated on the MicroScope platform (<https://mage.genoscope.cns.fr>) [52]. Annotation of the genes of interest was cured manually based on homology and synteny analysis following the MicroScope recommendations. The replication origin (*oriC*) was identified based on its homology with the origin of the *X. hortorum* B07-007 genome recorded in the DoriC v10 database (DoriC ID: ORI97015374) [53]. The replication terminus sequence (*dif*) was detected by BLAST-searching the *dif* site of *X. campestris* pv. *campestris* 17 [54]. Both predicted *oriC* and *dif* sequences are presented in Table S7. Finally, leading and lagging strands of replication were determined by analysing the GC-skew bias computed with the GenSkew web tool (<http://genskew.csb.univie.ac.at/>).

Construction of the transposon library, DNA preparation and sequencing

A transposon mutant library was generated by performing a conjugation of *X. hortorum* pv. *vitians* LM16734 with a donor strain of the mariner transposon Himar1-C9. Briefly, six mixed solutions containing 15 ml of an overnight culture of strain LM16734 and *Escherichia coli* MFDpir/pSamEc were mixed in equal parts, centrifuged at 3344 x g for 20 min, and resuspended each in 200 µl of TSB supplemented with DAP at 57 µg ml⁻¹. The resulting suspensions were deposited on over-dried TSA plates and incubated at 28 °C for 2 h, then resuspended in 1 ml of TSB. Conjugation efficiencies were evaluated by plating serially diluted aliquots on TSA plates supplemented with kanamycin. The remaining suspensions of the conjugation experiments were diluted to 1/5th and plated on TSA kanamycin plates, and then incubated at 28 °C for 3 days. Finally, the plates were washed with 800 µl of TSB, and colonies were resuspended with a spreader. Bacteria were subsequently mixed with 30 % (v/v) glycerol for long-term storage at -80 °C. The library theoretically consisted of 250,000 individual colonies, i.e. individual transposition events, at a concentration of 10¹⁰ CFU.ml⁻¹.

Library DNA preparation and sequencing procedures were derived from Royet *et al.* [27]. The genomic DNA of the

mutant library was extracted in two replicates from 125 µl aliquots using a Promega Wizard Genomic DNA Purification kit (Promega). Then, 50 µg of DNA from each sample was digested with 50 U of *MmeI* in a final volume of 1.2 ml at 37°C for 1.5 h. Digestions were heat-inactivated for 20 min at 80°C and purified with a QIAquick PCR Purification kit (Qiagen). The samples were then concentrated with a vacuum concentrator to obtain final volumes of 20 µl, which were deposited on 1% (w/v) agarose gels. Each gel was cut out to extract the digested 1.3–1.8 kb fragments containing the transposon and adjacent genomic DNA using a QIAquick Gel Extraction kit (Qiagen). For each sample, 500 ng of digested and purified DNA was ligated to 0.44 µM of double-stranded barcoded adapters obtained as described previously [27] with 2000 U of T4 DNA ligase in a final volume of 50 µl at 16°C overnight. Five 18-cycle PCRs were performed per sample to amplify the genomic DNA adjacent to the transposon. Each reaction mix contained 100 ng of DNA, 1 U of Q5 DNA polymerase (New England Biolabs), 1× Q5 buffer, 0.2 mM of dNTPs, and 0.4 µM of the LIB_PCR_5 and LIB_PCR_3 primers [32]. The five reaction products per sample were pooled and concentrated with a vacuum concentrator to obtain 25 µl samples that were deposited on a 1.8% (w/v) agarose gel. The 125 bp bands were cut out and extracted with a QIAquick Gel Extraction kit (Qiagen). All gel electrophoresis and gel extraction steps were carried out on a blue-light transilluminator to avoid DNA damage, and DNA was stained with GelGreen (Biotium). Finally, DNA samples were dialysed for 4 h on 0.025 µm nitrocellulose membranes (Milipore) deposited on sterile ultra-purified water. Thirty nanograms of each DNA library were sent to MGX (CNRS Sequencing Service) for quality control assessment and high-throughput sequencing on Illumina HiSeq 2500 instruments (Illumina Inc.).

Assessment of genome essentiality

Raw reads were treated with CUTADAPT v1.15 [55] to retain only those containing the mariner inverted left repeat (ACAGGTTGGATGATAAGTCCCGGTCTT) and cut the adapter sequence. Then, sequencing yield differences between the two replicates were normalized to the lowest yield by random subsampling with SEQTK v1.2 (<https://github.com/lh3/seqtk>). Trimmed read datasets were mapped to unique TA sites on independent replicons without mismatches on the genome using a modified version of the TPP script [27] from the TRANSIT software program v2.0.2 [56]. Gene essentiality was determined by analysing the datasets with TRANSIT v2.3.4 using the Hidden Markov Model method (HMM) [57]. We performed Trimmed Total Reads (TTR normalization, summed the read counts of the two replicates, applied a LOESS correction for genome positional bias, and discarded the read counts in the 10% N-terminus and C-terminus of the ORF that may have led to erroneous interpretations. Eventually, reciprocal BLASTN of genes classified as essential, growth-defect and growth-advantage were performed with BLAST+ v2.6.0 [58]

against the genome to exclude the genes present in multiple copies on the same or different replicons, considered as false positives. Visualizations of the circular genome and local genomic regions were plotted with DNAPLOTTER [59].

Gene set enrichment analysis and cellular pathway mapping

Cluster of Orthologous Groups (COG)- [60] based annotation of genes was retrieved from the MicroScope platform. Gene set enrichment analyses were performed by computing the proportion of each COG category in the different gene states (i.e. non-essential, essential, growth-defect and growth-advantage) reported against their global proportion in the genome. The fold-change values were log-transformed to determine over- and under-represented COG categories depending on gene state. Kyoto Encyclopedia of Genes and Genomes (KEGG) (<https://www.kegg.jp/>) annotations were assigned to genes with BLASTKOALA [61] and mapped to *Xanthomonas hortorum* reference pathways with KEGG MAPPER [62].

Analysis of essential gene conservation at multiple taxonomic levels

Conservation of the essential genes identified within the genus *Xanthomonas* and within the family *Xanthomonadaceae* was investigated. First, GenBank assemblies of 72 genomes exhaustively representing the taxonomic diversity of *Xanthomonas*, three genomes of *Stenotrophomonas maltophilia* and three genomes of *Xylella fastidiosa* subspecies *fastidiosa*, *multiplex* and *pauca* were retrieved from the NCBI database. Genome accession numbers, taxonomic names and general assembly statistics are given in Table S1. Then, a whole-genome-based phylogeny was constructed using PHYLOPHLAN2 v0.40 (<https://bitbucket.org/nsegata/phylophlan/wiki/phylophlan2>) [63]. Briefly, a core of ~350 single-copy highly conserved proteins from the PhyloPhlan database were retrieved from the genomes using USEARCH v11.0.667 [64], aligned and concatenated with MUSCLE v3.8.31 [65], and a phylogeny was built with FASTTREE v2.1.11 SSE3 [66]. Robustness was assessed locally using 1000 resamples of the Shimodaira–Hasegawa (SH) test [67]. Previously identified homologues of the essential protein-coding genes were then searched for among the 78 genomes using BLASTP with thresholds of 50% coverage, 30% amino-acid identity and an e-value <10⁻⁵. To check for potential false negatives caused by erroneous automatic annotation, sequences were also searched for using TBLASTN and the same thresholds, and homologous nucleotide sequences were searched for using BLASTN with thresholds of 50% coverage, 70% nucleotide identity and an e-value <10⁻⁵. Finally, essential and growth-defect genes were searched for in the DEG10 v15.2 database of essential genes (accessed June 2020) [33] with BLASTP using thresholds of 50% coverage, 30% amino-acid identity and an e-value <10⁻⁵. The phylogenetic tree and BLAST results were plotted together with the *ggtree* R package [68].

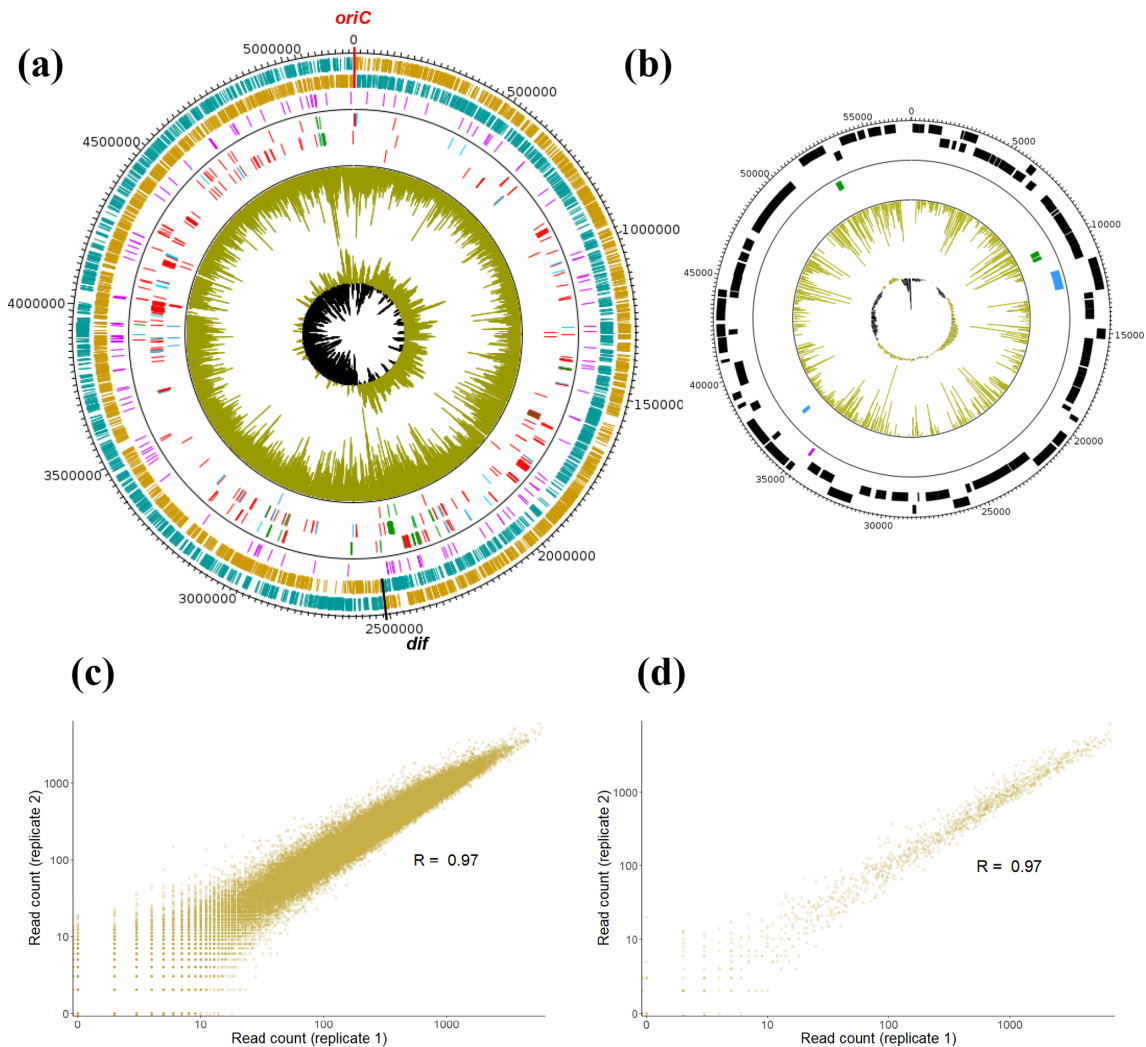


Fig. 1. Complete genome atlas of *X. hortorum* pv. *vitians* LM16734. The localizations of essential, growth-defect and growth-advantage loci identified by Tn-Seq are indicated on (a) the circular chromosome (5,213,310 bp) and (b) plasmid pLM16734 (57,250 bp). The outermost ring track is a clockwise-arranged base pair (bp) ruler, with ticks every 20,000 bp for (a) and every 200 bp for (b). The next two tracks represent predicted protein-coding gene sequences on the forward and reverse strands, respectively. The chromosome replication origin (*oriC*) and terminus (*dif*) are marked as red and black ticks in (a), respectively, and protein-coding genes on the leading and lagging strands of replication are shown in orange and blue, respectively. The third (purple) track shows predicted ncRNA sequences on both strands. After the separation line, the next two tracks represent essential (red), growth-defect (blue) or growth-advantage (green) protein-coding genes on the forward and reverse strands, while the sixth track indicates important RNAs with the same colour code. Finally, the seventh track (gold) shows the mean distribution of read counts between the two technical replicates throughout the entire genome, and the eighth track depicts the GC-skew (positive in gold and negative in black). (c, d) Correlations between the two technical replicates of the Tn-Seq experiment. The log-transformed numbers of reads per TA site are plotted and compared between replicates, and Pearson correlation coefficients are indicated for (a) the circular chromosome ($R=0.97$) and (b) plasmid pLM16734 ($R=0.97$).

RESULTS AND DISCUSSION

Complete genome sequencing of *X. hortorum* pv. *vitians* LM16734 and design of a highly saturated transposon mutant library

The complete genome of *Xanthomonas hortorum* pv. *vitians* LM16734 was sequenced using a combination of PacBio long-read and Illumina HiSeq short-read technologies. PacBio sequencing yielded 94,722 reads with a mean read length of 10,717 bp, while Illumina HiSeq

sequencing yielded more than 7.2 million paired-end reads with a mean read length of 151 bp. Hybrid assembly constructed with UNICYCLER resulted in two replicons: a circular chromosome of 5,213,310 bp (Fig. 1a) and a native plasmid of 57,250 bp, designated pLM16734 (Fig. 1b), with an overall sequencing depth of 587.5 \times . The presence of a pLM16734-like plasmid might be a common feature among *X. hortorum* pv. *vitians* strains. Plasmid detection experiments performed on 18 strains representative of the

Table 1. Transposon-insertion sequencing (Tn-Seq) statistics

Replicate	Sequencing yield	No. of reads after normalization [*]	No. of Tn-end containing reads	Replicon	Read count (unique TA sites)	TA hits [†]	Insertion density [†]	Mean read count over non-zero TA [‡]
1	23,662,661	23,179,825	20,267,199	Chromosome	20,168,343	60,186	70.5	335.1
				pLM16734	938,617	1,197	88.0	784.1
2	23,179,825	23,179,825	20,309,279	Chromosome	20,209,37	60,715	71.2	332.9
				pLM16734	92,4781	1,218	89.5	759.3

^{*}Numbers of reads were normalized based on the lowest sequencing yield (i.e. replicate 2).

[†]The complete *X. hortorum* pv. *vitians* LM16734 genome contains 85 314 TA sites in the chromosome and 1361 in plasmid pLM16734. Insertion density was calculated as the ratio of TA sites with at least one read mapped over the total number of TA sites in the replicon.

[‡]Mean value of mapped reads per TA site with at least one read.

known diversity of this lettuce pathogen indeed revealed that they all contained a single native plasmid ranging from around 40 to 60 kb [39]. The curated annotation carried out at the MicroScope platform detected 4,909 predicted protein-coding sequences (CDSs), 56 tRNAs, six rRNAs, one transfer-messenger RNA (tmRNA), seven regulatory riboswitches and 77 non-coding RNAs (ncRNAs) on the chromosome, and 89 CDSs and one ncRNA on plasmid pLM16734. The protein-coding density was therefore 87.6% on the chromosome and 79.1% on pLM16734, similar to values observed in the complete genome of *X. hortorum* pv. *carotae* M081 and other xanthomonads [42].

We designed a transposon mutant library for our Tn-Seq experiment by mating recipient strain LM16734 with an *E. coli* MFDpir donor strain containing a transferable *Himar9* mariner transposon on the suicide vector pSamEc. Approximately 250,000 individual transconjugants were recovered on 1/10th TSA rich medium supplemented with kanamycin and pooled. Then, two DNA libraries were prepared from two different aliquots of the mutant pool and massively sequenced by Illumina sequencing. The sequencing yielded more than 23 million reads in each replicate, which were normalized to the lowest value (Table 1). Of these 23 million reads, 87.4% (replicate 1) and 87.6% (replicate 2) were considered as ‘true’ TA-site-disrupted reads because they carried the *Himar9* end sequence. The fact that 99.5% of the *Himar9*-end-harboring reads mapped to unique TA sites on the chromosome in both replicates certified the quasi-certain complete absence of persistence of the vector within the mutant library. This phenomenon often occurs in mariner-based Tn-Seq studies that do not use a suicide vector, and can result in a drastic reduction in the number of informative reads mapping the genome because vector sequences can be amplified during the PCR steps [9, 22]. It is worth noting that the discrepancy between the total number of *Himar9*-end-harboring reads (~20.3 million) and the sum of reads mapped to the two

replicons (~21.1 million) suggested that some sequences of plasmid pLM16734 were identical or highly similar to some chromosomal regions. Mapping these filtered reads to the genome also revealed that out of the 85,314 and 1,361 TA sites potentially disrupted by the transposon on the chromosome and the plasmid, respectively, 60,186 (replicate 1) to 60,715 (replicate 2) of the chromosomal TA sites and 1,197 (replicate 1) to 1,218 (replicate 2) of the plasmid TA sites contained at least one insertion (Table 1). Hence, the insertion density achieved was 70.5–71.2% on the chromosome and 88–89.5% on the plasmid, i.e. one insertion every 85 bp on the chromosome and one every 47 bp on the plasmid on average (Fig. 1a, b). The TA site saturation of the library is a critical parameter for statistical inference of essentiality in Tn-Seq studies [57, 69]. Saturation achieved was higher in the present study than in most mariner-based Tn-Seq studies [10, 22, 24, 57]. Furthermore, correlations between the number of reads at each TA site (i.e. the read count) of the two replicates were optimal on both replicons as asserted by Pearson correlation coefficients of 0.97 in both cases (Fig. 1c, d). In brief, previous analyses of our complete genome sequence and mutant library representativeness showed that we achieved a high quality in all key-point statistics that strongly supported further statistical gene state interpretations.

***In vitro* identification of essential and fitness-related genes**

The computational assessment of gene essentiality by Tn-Seq methods generally relies on examination of the number of disrupted sites within a given gene relative to the total number of potential transposon insertion sites (i.e. the insertion rate). This rate theoretically reflects the tolerance of the organism to the loss of the encoded function [57, 70]. Yet, other approaches for Tn-Seq data analysis use the number of reads at insertion sites (i.e. the read count) as a quantitative measure by calculating the mean read count in a sliding window to

identify regions of the genome presenting lower insertion values [71]. Both methods have their drawbacks: the first tend to overestimate the number of essential genes [71] and are sensitive to insertions in the N- and C-terminal regions of genes, incorrectly concluding that they are non-essential [72], while the second have been criticized for their lack of statistical significance [56, 57]. We decided to use the HMM-based approach offered by TRANSIT because it uses both the qualitative insertion rate and quantitative read count parameters. Moreover, it assesses the probability of essentiality for each individual TA site, so that domains of essentiality can be identified in genes. It also performs a four-state classification, as genes and regions can be labelled as essential, non-essential, growth-defect or growth-advantage. The growth-defect state, also referred to as critical in other studies [10, 22], corresponds to observations that a deficit rather than complete lack of insertions within a region might be a sign that it is not directly vital for the cell, but that its disruption would impair the growth of the organism [16]. Likewise, genes or regions presenting a significantly higher read count than the rest of the genome are classified as growth-advantage, meaning that their disruption would lead to a higher growth rate, possibly because they represent an unnecessary metabolic cost in the studied conditions (e.g. secretion of an extracellular enzyme or toxin) [57].

Our HMM-based analysis conducted with TRANSIT primarily identified 571 essential chromosomal loci. Nine were subsequently considered as artefactual CDSs after manual examination on the MicroScope comparative genomics platform, and another 192 were discarded because they were duplicated in the genome, on the same or different replicons, and thus were impossible to classify according to our workflow. Nineteen loci computationally identified as growth-defect were also discarded manually because they were duplicated. Finally, 34 more loci were impossible to categorize because they did not contain TA sites. All these loci were therefore classified as ‘undetermined’ and are detailed in Table S2. Following these refinement steps, 370 chromosomal loci were positively identified as essential (Table S3), consisting of 359 protein-coding genes, eight tRNAs and three ncRNAs. Additionally, 44 protein-coding genes and six tRNAs were categorized as growth-defect (Table S4), and 77 protein-coding genes, one tRNA and one ncRNA were considered as growth-advantage after curation (Table S5). The initial analysis and subsequent manual curation identified three growth-defect and three growth-advantage genes on plasmid pLM16734 (Table S6).

Analysis of essential genes of *X. hortorum* pv. *vitians* associated with core bacterial features

A striking observation one can make about the number of essential genes in various bacteria is that most of them appear to rely on a restricted set of 300–500 essential genes despite important variability in terms of genome size, architecture or lifestyles [33]. To determine whether the essential genes we identified in *X. hortorum* pv. *vitians* were common across the bacterial kingdom, we searched

for homologues in the Database of Essential Genes (DEG10 v15.2, accessed June 2020), which at the time contained 48 essential genomes from 29 Gram-negative bacteria, 17 from Gram-positive bacteria and two from *Mollicutes*. An overwhelming majority (93.5%) of the essential genes we identified in *X. hortorum* pv. *vitians* had at least one putative homologue in the database (Table S3). Therefore, it seems that essential genes and functions are widely conserved across bacteria. Moreover, the comparison of our growth-defect gene set to the DEG database revealed that 86% of them also had putative homologues in the database, suggesting that they could have been annotated as essential under more stringent experimental conditions.

To further compare the key cellular functions of *X. hortorum* pv. *vitians* and those described in other bacteria, we classified genes into general functional categories or metabolic pathways based on the COG and KEGG databases. The COG classification of essential genes showed that almost half of them were clustered in only three categories: 24% were related to fundamental processes of translation and ribosome biogenesis (J), 11% were associated with energy production and conversion (C), and another 11% were related to cell wall and membrane biogenesis (M) (Fig. 2a). However, it appeared more accurate to consider how enriched the COG categories were in the essential gene set considering their representation in the complete genome. Cellular processes presenting an enrichment in the essential gene set were associated with translation and ribosome structure (J), coenzyme metabolism (H), intracellular trafficking and secretion (U), energy production (C), cell division (D), cell wall and membrane biogenesis (M), and nucleotide metabolism (F) (Fig. 2b). Similar analyses in other bacteria also showed that translation and ribosome structure were the key essential functions of bacterial cells, while the other COG categories enriched in our gene set were usually identified as essential but varied in terms of importance and gene numbers [8, 23, 24, 26, 73]. Other COG-based analyses of non-essential, growth-defect and growth-advantage genes are not discussed here but can be found in Fig. S1. In addition, KEGG pathway clustering reached better precision in the determination of crucial cellular pathways, and underlined that most of the genes required for aminoacyl-tRNA biosynthesis, ribosome structure, protein export, DNA replication and lipopolysaccharide biosynthesis were essential (Table 2). About half of the genes involved in peptidoglycan biosynthesis, the citrate cycle, oxidative phosphorylation and RNA degradation were also required for cell survival. Unlike what was described in other organisms [10, 22, 25, 73], our analysis revealed that genes of the glucose catabolism pathways were apparently not essential for *X. hortorum* pv. *vitians* survival in rich medium (Fig. S2), even though glucose is the main carbon source in TSA medium. The only essential gene in the pentose phosphate pathway encoded the ribose-phosphate pyrophosphokinase (*prsA*, XHV734_3940), which catalyses the conversion of ribose-5-phosphate into 5-phosphoribosyl-1-pyrophosphate, a key compound

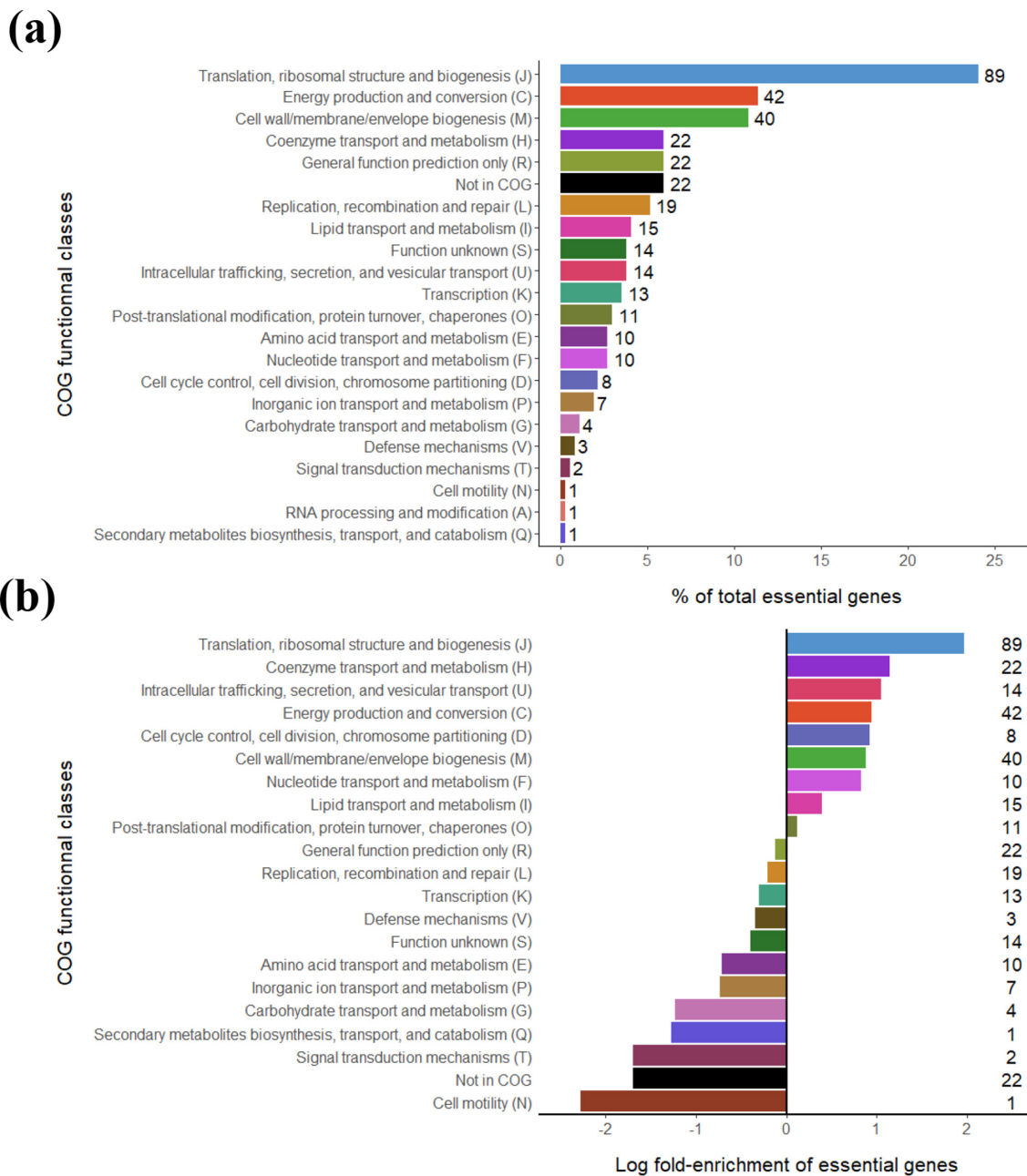


Fig. 2. Clustering of essential genes based on Cluster of Orthologous Groups (COG) functional classes. (a) Proportion of each class in the essential gene set, with the number of genes next to the bars; (b) log-fold enrichment of COG classes, calculated as the ratio of the proportion of a given class in the essential gene set to its overall proportion in the genome.

in the biosynthesis pathways of the essential purine and pyrimidine bases [74]. More interestingly, the only essential gene associated with the glycolysis pathway encoded the phosphopyruvate hydratase, also known as enolase (*eno*, XHV734_2903), which converts 2-phospho-D-glycerate into phosphoenolpyruvate. Considering that this enzyme is multifunctional – it is also involved in the RNA degradosome [75], a pathway containing seven essential genes (Table 2) – this secondary function of the enolase probably accounts more for its essentiality phenotype than does its

role in glycolysis. Overall, these two examples highlight the fact that some genes in our essential gene set act as metabolic hubs, and this makes it difficult to precisely determine which of their many roles is critical and responsible for the observed essentiality phenotype. The absence of essential genes in the usually key pathways of glucose catabolism is probably a reflection of the ability of xanthomonads to rely on the Entner-Doudoroff alternative glucose catabolism pathway (Fig. S2) [76]. A metabolic flux study of the utilization of ^{13}C -labelled glucose in *X. campestris* revealed

Table 2. Essential KEGG pathways in *X. hortorum* pv. *vitians* LM16734 (non-exhaustive)

KEGG pathways	Essential+Critical genes [*]	Total genes [†]
Aminoacyl-tRNA biosynthesis	22+1	24
Ribosome	48	54
Protein export	10+1	16
DNA replication	10	15
Lipopolysaccharide biosynthesis	11	17
Peptidoglycan biosynthesis	12	21
Citrate cycle	13	24
Oxidative phosphorylation	26	51
RNA degradation	7	15
Fatty acid biosynthesis	6+1	19
Pyrimidine metabolism	6+2	25
Amino sugar and nucleotide sugar metabolism	6	34
Purine metabolism	8	55
Biosynthesis of amino acids	9+3	105

^{*}Number of genes identified as essential and growth-defect.

[†]Total number of genes in the model pathway in the *X. campestris* pv. *campestris* ATCC 33913[†] organism-specific KEGG database.

that it was mostly processed through the Entner-Doudoroff pathway to be converted into pyruvate; yet, it could also be marginally handled by the glycolysis and pentose phosphate pathways [77]. Therefore, we can reasonably conclude that no matter which of the three glucose catabolism pathways was disrupted in *X. hortorum* pv. *vitians* in our experiment, the others would be able to take over and prevent the occurrence of deleterious phenotypes. In a nutshell, most of the *X. hortorum* pv. *vitians* essential genome overlapped with key cellular functions described in other organisms, while displaying a few peculiar features.

Conservation of essential genes among *Xanthomonadaceae* and investigations of specific essential features and mobile genetic elements

As we intended to go beyond the description of common essential bacterial functions, we further looked into the specific essential genes showing no homology in the DEG database and investigated the conservation of our essential gene set among the genus *Xanthomonas*, and also to some extent the family *Xanthomonadaceae*. To that end, we built a genome-based phylogeny of all the known species and pathogens of *Xanthomonas* species, *Stenotrophomonas maltophilia* and *Xylella fastidiosa* using relevant type and pathotype strain genomes (Table S1), and analysed the conservation of *X. hortorum* pv. *vitians* essential genes across the tree (Fig. 3).

We deliberately chose weakly stringent coverage and identity thresholds to look at the conservation of protein functions and deal with quality heterogeneity in genome assemblies and annotations, but we checked for inconsistencies and potential artefacts manually. Hence, 334 out of 359 protein-coding genes were shared among all investigated *Xanthomonadaceae* (Table S3). The remaining 25 genes could be distinguished into two groups according to their distribution pattern in the phylogeny: (i) those that were mostly conserved among *Xanthomonadaceae* or displayed a phylogeny-related distribution (Fig. 3, filled inverse triangles), and (ii) those that showed a heterogeneous distribution unrelated to phylogeny (Fig. 3, empty inverse triangles).

The genes included in the first category (phylogeny-related distribution) were mainly related to known genes and functions. For example, the six genes of the *pha* operon, which encodes the monovalent cation/proton antiporter system Pha also referred to as Mrp or Mnh, were essential according to our analysis as no in-frame transposon insertions were observed (Fig. 4a). These genes are conserved in all *Xanthomonas* species and *S. maltophilia*, but completely absent in *X. fastidiosa* (Fig. 3). Note that all six genes in the operon are required for the antiporter to be functional [78]. Antiporters of the Pha family are widely distributed across bacteria and usually involved in cytoplasmic pH homeostasis and efflux of toxic cations, but also in a broad range of other resistance and environmental adaptation phenotypes [79]. The *pha* operon is essential for a few bacteria to grow in rich medium [11, 35, 80]. Its absence in *X. fastidiosa* could be a consequence of the extreme genome reduction undergone by this pathogen, which made it fit for only a few restricted homeostatic environments [81], while *Xanthomonas* species and *S. maltophilia* are adapted to living and surviving in more variable and stressful environments. Thus, they might partly rely on Pha antiporters to buffer physicochemical toxicity in challenging environments (e.g. epiphytic life for *Xanthomonas* species).

Similarly, three components of the *gum* gene cluster were also essential (Fig. 4b). This set of 12 genes (*gumB* to *gumM*) encodes the synthesis and export of the well-known *in planta* virulence factor and biotechnologically important exopolysaccharide (EPS) xanthan. The three genes – *gumE*, *gumI* and *gumJ* – turned out to be conserved in most *Xanthomonas* species but absent in *X. sacchari* and *X. albilineans* (Fig. 3), as previously reported [82]. They had no homologues in the DEG database (Table S2), except *gumJ* that showed low but significant homology (85% coverage and 25% amino acid identity, e-value of $3.27e^{-20}$) with the succinoglycan biosynthesis transport protein ExoT of *Agrobacterium tumefaciens*, essential in rich medium [35]. They were also absent in *S. maltophilia*, in line with the inability of *Stenotrophomonas* to synthesize xanthan as one of the major features that led to their differentiation from the genus *Xanthomonas* [83]. Concerning *X. fastidiosa*, it was shown that the absence of *gumG*, *I* and *L* leads to the formation of another type of EPS [84], and independent acquisition of the *gum* cluster by *Xanthomonas* species and *Xylella* species may account

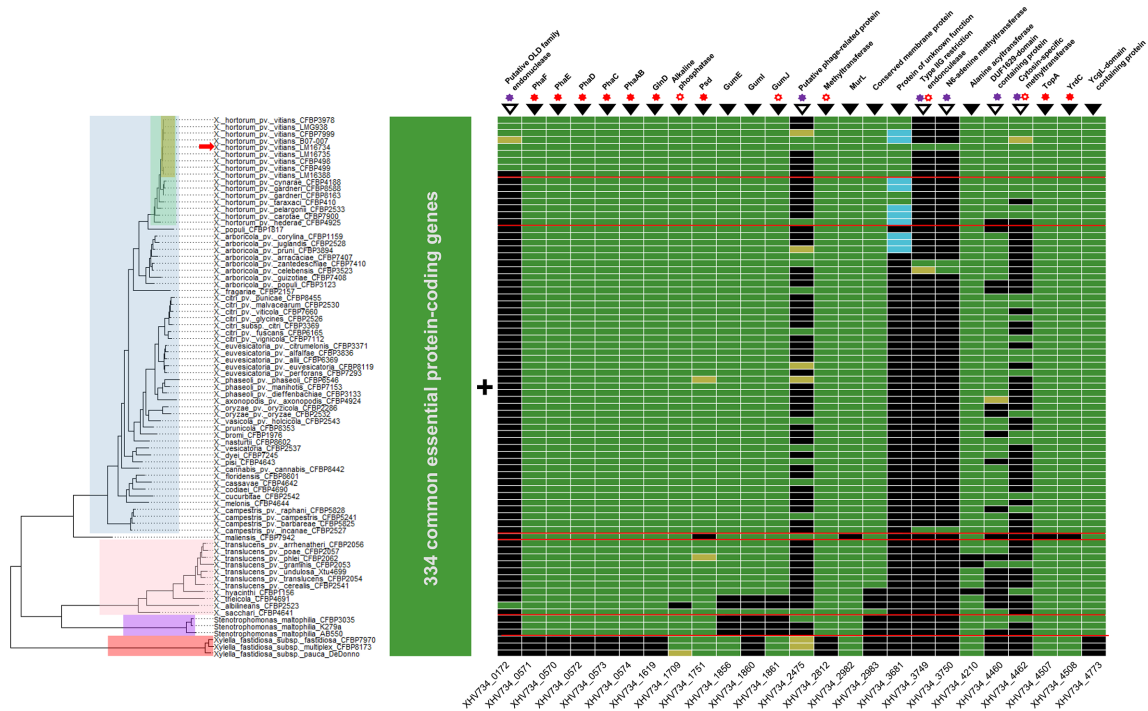


Fig. 3. Conservation of the essential genes of *X. hortorum* pv. *vitians* LM16734 in the family *Xanthomonadaceae*. Colours on the phylogenetic tree indicate taxonomic levels: yellow = *X. hortorum* pv. *vitians*, green = *X. hortorum*, blue = *Xanthomonas* clade I, pink = *Xanthomonas* clade II, purple = *Stenotrophomonas maltophilia*, and red = *Xylella fastidiosa*. Taxonomic separations are also indicated by red lines on the gene conservation matrix. The position of the *X. hortorum* pv. *vitians* LM16734 genome in the phylogeny is highlighted by a red arrow. In the matrix, green boxes indicate detection with BLASTp (>50% coverage, >30% identity, e-value >10⁻⁵), yellow boxes detection with tBLASTn (>50% coverage, >30% identity, e-value >10⁻⁵), and blue boxes detection with BLASTn (>50% coverage, >70% identity, e-value >10⁻⁵). The numbers of gene loci are shown below the matrix, and gene product annotations are indicated above it. Empty and filled black inverted triangles refer to the two distribution patterns discussed in the text. Filled red asterisks indicate that at least one homologue was detected in the DEG10 v15.2 essential gene database with BLASTp (>50% coverage, >30% identity, e-value >10⁻⁵), while empty red asterisks indicate lower homology thresholds (>40% coverage and/or >20% identity and e-value >10⁻⁵). Finally, purple asterisks highlight the presence of mobile genetic-element-associated genes (transposase, integrase, reverse transcriptase) in the neighbourhood of the gene (<10 kb distance).

for these differences [85]. Interestingly, a previous study by Katzen *et al.* [86] reported the impossibility to create insertion mutants of *gumB*, *C*, *E*, *J* and *M* in *X. campestris* pv. *campestris*. Yet, Kim *et al.* [87] later claimed that only the knock-out mutation of *gumJ* was lethal to *X. oryzae* pv. *oryzae*. Lethality of *gum* gene mutations might thus be species-specific, probably because of complex transcriptional patterns that are likely to differ among *Xanthomonas* species [88–90]. In our case, considering that *gumJ* and *gumE* are responsible for the translocation of the lipid-linked sugar repeat units from the cytoplasm to the periplasm and for the polymerization of these units in the periplasm, respectively [76, 91], their disruption would eventually cause EPS units to accumulate in the cytoplasm or the periplasm. Some lipid-linked intermediates of xanthan biosynthesis have been hypothesized to be toxic when they accumulate inside the cell [86], an assumption further supported by the demonstration that intermediate components of a similar extracellular EPS of *Streptococcus pneumoniae* were indeed toxic [92]. Therefore, the toxicity of the lipid-linked repeat units of xanthan probably accounts for the lethal phenotype of *gumJ* and *gumE* mutants. However,

the absence of insertions within *gumI* is more difficult to interpret. This gene encodes a mannosyltransferase that participates in the formation of the pentasaccharide repeat units before their translocation and polymerization in the periplasm [93]. Either its disruption also leads to accumulation of toxic intermediates, or its essentiality might be an artefact caused by the potential polar effects that possibly occurred in operons using our mutagenesis approach [27]. Nevertheless, these basic findings concerning the xanthan biosynthesis pathway of *X. hortorum* pv. *vitians* might be helpful for more applied research, as the industrial potential of *X. hortorum* pv. *pelargonii* strains for xanthan production has been investigated [94].

On the other hand, examining the conservation of *X. hortorum* pv. *vitians* LM16734 essential genes revealed a second category of genes whose distribution across the phylogeny was more chaotic (Fig. 3, empty inverted triangles). Although most of these genes were not automatically annotated, manual annotation and analysis of synteny and gene neighbourhood on the MicroScope platform revealed

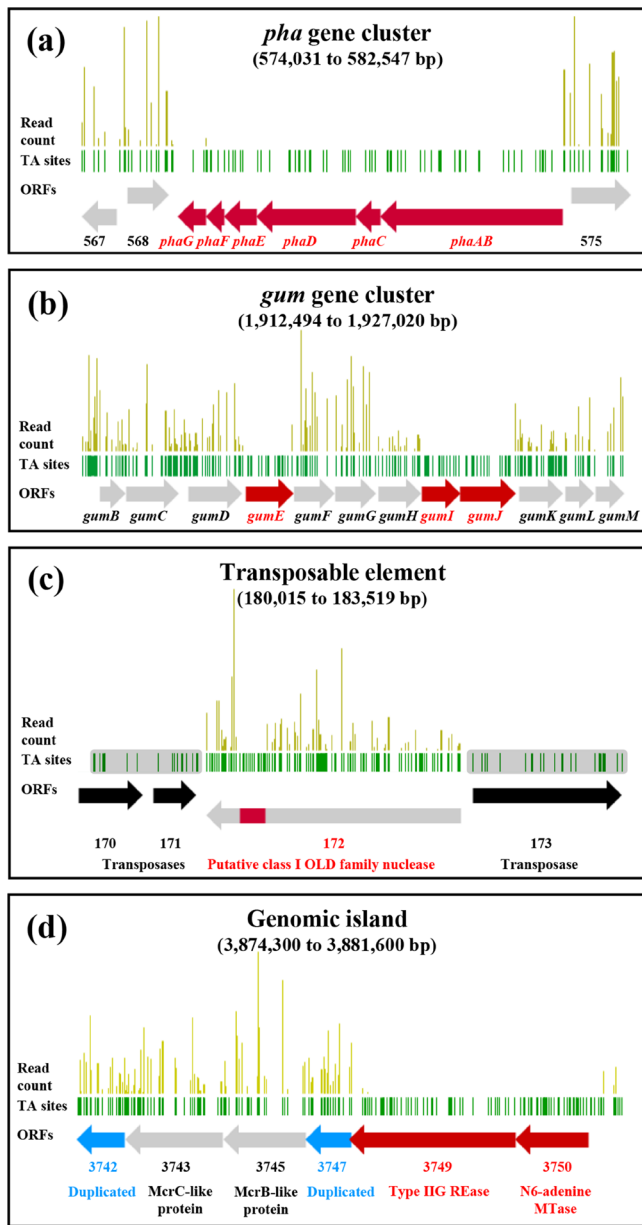


Fig. 4. Schematic representations of gene essentiality within the genomic context. Representations are shown for (a) the *pha* gene cluster (574,031...582,547 bp), (b) the *gum* gene cluster (1,912,494...1,927,020 bp), (c) the transposable element containing the putative OLD family endonuclease XHV734_0172 (180,015...183,519 bp) and (d) the genomic island containing the restriction-modification system composed of restriction endonuclease XHV734_3749 and methyltransferase XHV734_3750 (3,868,330...3,884,140 bp). Essential genes or domains are shown in red, non-essential genes or domains in grey, transposases in black in (c), and duplicated loci in blue in (d). Gene names or simplified loci numbers are indicated at the bottom of each box. Green bars indicate the position of TA sites, and golden bars represent the read count at each TA site. In (c), shadowed TA sites indicate unavailability of read counts because of repeated sequences.

that they were all associated with mobile genetic elements (MGEs), i.e. prophages, transposons, genomic islands, etc. For example, locus XHV734_0172 encodes a putative ATP-dependent endonuclease of the OLD family flanked by transposase genes (Fig. 4c), suggesting transposable-element-mediated horizontal acquisition. The conservation pattern among the *Xanthomonadaceae* implies that it was acquired independently in *X. hortorum* pv. *vitians* and *X. albilineans* (Fig. 3). The absence of this element in strain LM16388 is consistent with the early divergence of this strain from the rest of the pathovar, as we previously reported [39]. According to the classification proposed by Schiltz *et al.* [95], this nuclease belongs to class 1 OLD nucleases, like the canonical *old* gene of phage P2. Exhaustive biological roles of OLD nucleases remain to be unravelled, yet they are known to play a significant role in phage exclusion by protecting lysogenized host genomes against superinfection by other phages, presumably by degrading nucleic acids from newcomers through their ribonuclease and DNA exonuclease activities [95, 96]. Intriguingly, XHV734_0172 was the only locus in our analysis that displayed only one short domain intolerant to insertions (I422 to Y476) (Fig. 4c). A comparison with the recently described structure of OLD nucleases [95] revealed that this essential domain was located in the C-ter Topoisomerase/Primase (Toprim) catalytic domain that bears the nuclease activity, and more precisely starts with the highly conserved metal A-binding aspartate motif D425xD427 (Fig. S3) [97]. Further knowledge of the *in vivo* biological activity of OLD nucleases would be required to understand why this segment of the Toprim domain is essential in *X. hortorum* pv. *vitians*. One possible explanation would be that transposon insertion near catalytic sites generates truncated proteins displaying uncontrolled exonuclease and ribonuclease activities, leading to cell death. Nevertheless, a genome-wide Tn-Seq screening of *Salmonella enterica* also showed that another OLD nuclease was conditionally essential [30], confirming that some OLD nuclease genes behave as ‘essential’ genes whose disruption results in a lethal phenotype. Furthermore, knowledge of the phage exclusion-related genes of *X. hortorum* pv. *vitians* could be of importance in the near future, as phage therapy arises as a promising way to control plant diseases caused by *Xanthomonas* species [98].

We identified other MGE-associated essential features related to restriction-modification (R-M) systems. R-M systems are often considered as primitive immune systems as their biological role is to protect bacterial genomes from foreign DNA integration by recognizing self and non-self DNA according to methylation profiles [99]. A typical R-M system involves a restriction endonuclease (REase) that recognizes and cleaves foreign DNA at specific sites, and a methyltransferase (MTase) that methylates the host DNA at the same specific sites to protect it from restriction. We notably identified an essential R-M system composed of a type-II restriction endonuclease (XHV734_3749) and its cognate N6-adenine methyltransferase (XHV734_3750) (Fig. 3). This system is located on a small genomic island of 7.3 kb also containing an McrBC-like R-M system and two small duplicated unknown

proteins upstream of each R-M system (Fig. 4d). Interestingly, this genomic island is conserved in the few xanthomonads possessing the essential R-M system (Fig. 3), but also strongly homologous to cluster S13 of *Pseudomonas protegens* Cab57, yet with a completely different genomic environment [100]. From a wider perspective, this genomic island is part of a larger variable genomic region located between *moaE* downstream (XHV734_3751) and *dnaX* upstream (XHV734_3720), which seems to have undergone multiple horizontal acquisition events, as indicated by the persistence of several mobility-associated genes (XHV734_3722, 3725, 3734 and 3735). Searching for homologues of XHV734_3749 and 3750 in the restriction enzyme database REBASE [101] yielded a perfect match for an identical R-M system in *X. arboricola* pv. *juglandis* CPBF 1521 (M.Xar1521ORF31530P and Xar1521ORF31540P), with *moaE* downstream and an McrB-like protein-coding gene upstream. However, this genomic island was not detected in the type strain of *X. arboricola* pv. *juglandis* CFBP 2528^T included in our analyses (Fig. 3). This shows some limitations of our taxonomy-based conservation analysis when it comes to MGEs. The presence of this genomic island upstream of the *moa* operon in some of the phylogenetically distant xanthomonads investigated suggests either early acquisition followed by loss events in most strains, or multiple independent site-specific integration events. Nevertheless, it was interesting to observe that both the REase and the cognate MTase were annotated as essential. As R-M systems are often compared to toxin/antitoxin systems because they act as genetic addiction systems [102], we would have expected the sole MTase to be essential. Yet, the similarity with Xar1521ORF31540p and domain analysis using MicroScope revealed that XHV734_3749 is a type-II R-M system, meaning that it possesses both the endonuclease and the methyltransferase domains, and that the presence of a cognate separate MTase is accessory [103]. Thus, its disruption would probably lead to post-segregational killing as the cell REase concentration would become too low to successfully protect bacterial DNA from its own endonuclease activity. Nevertheless, the co-occurrence of two distinct restriction modification systems on a small genomic island raises questions about their biological roles. The type-I R-M system McrBC was originally described as part of the so-called 'Immigration Control Region' in *E. coli* K12 [104] because it plays a role in the protection of the host genome against foreign invading DNA. On the other hand, type-II R-M systems frequently behave like 'selfish' genetic elements promoting their own survival to increase their relative frequency [105, 106]. Observations that type-II R-M systems are often associated with MGEs also suggest that they play an important role in the stabilization of genomic islands [99, 107], a perspective that echoes the essentiality of the type-II R-M system of the genomic island we described.

CONCLUSIONS

In this study, we fully exploited the power of genome-wide Tn-Seq essentiality screening by putting our results in perspective with the insights offered by comparative

genomics, synteny analyses and phylogenomics. Combining these approaches allowed us to separate core essential genes conserved across bacterial phyla from context-dependent essential genes in the essential genome of *X. hortorum* pv. *vitians*. Among these, we identified specific *Xanthomonas* or *Xanthomonadaceae* features that were essential *in vitro*, but also probably play an important role in natural ecological niches. Also, we discovered through systematic synteny and phylogenomic examination that some essential genes were acquired through horizontal gene transfer events, either separately or on genomic islands, and were associated with bacterial defence systems or genetic addiction complexes. Thus, we showed that gene essentiality assessment by Tn-Seq gains significance by systematically examining genome content and gene conservation. Our complete genome sequence and high-density transposon library will reveal useful and reliable tools for the next steps of our research on this important lettuce pathogen, which will aim at discovering *X. hortorum* pv. *vitians* genes associated with virulence and survival *in planta*.

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Author contributions

F.B. supervised the manuscript, funding acquisition and project administration. E.G. also participated in supervision, methodology development and provided some of the resources. S.L. helped to perform experimental investigations and provided some of the resources used. L.M. conceptualized the study, carried out the investigations, data curation, formal analysis, visualization, and wrote the original draft. All four authors contributed to the reviewing and editing of the manuscript.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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