



PhD Thesis in Bioscience engineering

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**Antibacterial secretion systems in plant-pathogenic
xanthomonads: structure, function, and evolution**

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Abstract

Xanthomonas translucens (Xt) is a plant-pathogenic bacterium responsible for bacterial leaf streak (BLS), a disease threatening small-grain cereals worldwide. Among xanthomonads, two distinct contact-dependent systems involved in interbacterial competition have been identified, the *Xanthomonadales*-like type IV (X-T4SS) and type VI (T6SS) secretion systems. These molecular weapons enable bacteria to inject toxic effectors into nearby bacterial cells, thereby killing them or inhibiting their growth. Despite their widespread occurrence, both systems remain largely uncharacterised in the *Xanthomonas* genus, from functional, evolutive and ecological perspectives. This thesis investigates the X-T4SS and T6SSs of *Xanthomonas translucens sensu lato*, aiming to elucidate their contribution to interbacterial competition in cereals. Genomics-informed screening revealed that X-T4SS and T6SS clade i4 loci display mutually exclusive distributions in Xt strains. By combining mutational studies along with fluorescence-based approaches, we demonstrated that both Xt X-T4SS and T6SS-i4 systems play overlapping functions in killing cereal-associated bacteria. Xt T6SS-i4 killing dynamics was further visualised by live confocal imaging. Comparative genetic and phylogenetic analyses support an evolutionary model in which the ancestral X-T4SS evolved first, then underwent degradation and loss in certain lineages, and was subsequently functionally replaced by T6SS-i4, through horizontal transfer. In-depth *in silico* screening and comparative genomic analyses revealed a wide diversity of T6SS effectors within the Xt complex. In particular, across the genus *Xanthomonas*, we found that strains with a non-vascular lifestyle exhibit greater T6SS effector diversity compared to vascular strains from the same species. Bacteriolytic activity was confirmed for all Xt core T6SS effectors. Respective effector-immunity interactions were assessed through structural predictions, and identified cognate immunity proteins were experimentally validated for their protective function. Finally, we showed that Xt T6SS-i4 can impact the spatial organisation of another co-infected Xt strain *in planta*. In conclusion, these findings highlight the X-T4SS and T6SS-i4 as key antibacterial mechanisms contributing to survival and conferring competitive advantage to *Xanthomonas translucens* in cereal phytobiomes.

Preamble

Bacteria live in complex microbial communities, engaging in diverse interactions to survive. Competition for resources and space has driven the evolution of contact-dependent mechanisms for killing bacterial competitors. This interbacterial arms race plays a central role in shaping microbiome composition and has important implications in the field of phytopathology. Innovative biocontrol strategies that exploit beneficial organisms and their competitive potential within phytobiomes to enhance plant health are highly promising, with their development supported by advanced ‘omics’ technologies. Such plant disease biocontrol strategies align with broader global initiatives, including the European One Health Action Plan and the 2030 Agenda for Sustainable Development.

The genus *Xanthomonas* is a large group of plant-associated bacteria causing diseases in more than 350 host plants worldwide. Among those, *Xanthomonas translucens*, the causal agent of bacterial leaf streak disease of cereals, poses a threat in wheat and barley-producing areas. Yet, little is known about how *X. translucens* interacts and competes with other microorganisms, both endophytically and epiphytically, which limits the development of effective control strategies.

This thesis work investigates antibacterial secretion systems in *X. translucens sensu lato*. In particular, two major secretion systems are studied: type IV (X-T4SS) and type VI (T6SS). These nanoweapons have been more broadly described in proteobacteria as delivering toxic effectors into neighbouring bacteria, inhibiting their growth or causing cell death. Despite their prevalence in xanthomonads, their functional, evolutionary, and ecological importance remain poorly characterised. This work aims to clarify how X-T4SS and T6SS contribute to interbacterial competition and support *X. translucens* survival and colonisation in *Poaceae*.

Chapter 1 provides an overview of current knowledge on the *Xanthomonas* X-T4SS and T6SS antibacterial weapons. In parallel to this doctoral research, collaboration with Dr. Ralf Koebnik led to the publication of a study reporting the complete sequencing of three novel species within clade-1 xanthomonads. Accordingly, the introduction begins with a brief overview of recent advances in clade-1, focusing on this publication (see Appendix 1, Peduzzi et al., 2023). Chapter 1 then reviews the *X. translucens* pathosystem and the antibacterial type VI (T6SS) and type IV (X-T4SS) secretion systems in xanthomonads, covering their discovery, structure, distribution, functions, and current knowledge in the *Xanthomonadales* order.

In **Chapter 2**, the distribution of X-T4SS and T6SS-i4 in the *X. translucens* complex makes it an ideal model to study their function and evolutionary history. **Chapter 3** uncovers the yet undescribed diversity of *Xanthomonas* T6SS-i4 effectors, and links it to xanthomonads ecological lifestyles. Finally, **Chapter 4** investigates how X-T4SS

and T6SS-i4 shape competition and survival of *X. translucens* against cereal representative bacteria.

Finally **Chapter 5** discusses the results obtained in the different chapters and gives broader perspectives for X-T4SS and T6SS research, as well as overall conclusions.

Scientific communications

Published papers

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Peduzzi C., Butchacas J., Roman-Reyna V., François N., Delitte M., Jacobs J., Koebnik R. and Bragard C. *Xanthomonas translucens* uses Type VI secretion system to mediate interbacterial killing and leaf community fitness. 2 April 2024. Bacterial Leaf Streak conference. Ohio (USA). [Oral presentation].

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List of acronyms and abbreviations

ANI	Average Nucleotide Identity
att	attachment sites
BLAST	Basic Local Alignment Search Tool
BLS	Bacterial Leaf Streak
bp	base pair
CDI	Contact-Dependent growth Inhibition system
Cdz	Contact-dependent inhibition by glycine zipper proteins
CFBP	Collection Française de Bactéries Phytopathogènes
CFU	Colony Forming Unit
Chlor	Chloramphenicol
CIME	Cis-mobilisable elements
ClpV	Caseinolytic peptidase V
COR	Coronatine
cv.	cultivar
DAP	Diaminopimelic acid
DddA	DS-DNA deaminase toxin A
dDDH	digital DNA-DNA hybridisation
DNA	Deoxyribonucleic acid
dpi	days post inoculation
DUF	Domain of Unknown Function
EAEC	Enteraggregative E. coli
EcFK	Extracytoplasmic function sigma factor
EI	Effector-Immunity
EPPO	European and Mediterranean Plant Protection Organisation
EPS	Extracellular polysaccharide
FAME	Fatty Acid Methyl Ester

FIX	Found in type sIX effectors
GFP	Green Fluorescence Protein
GI	Genomic Island
Gm	Gentamycin
Hcp	Hemolysin-coregulated protein
HGT	Horizontal Gene Transfer
HMM	Hidden Markov Model
hpi	hours post inoculation
HSAF	Heat-Stable Antifungal Factor
ICE	Integrative and Conjugative Element
IcmF	Intracellular multiplication factor
INA	Ice-Nucleating Activity
ipTM	interface pTM score
Km	Kanamycin
LPSN	List of Prokaryotic names with Standing in Nomenclature
MIX	Marker for type sIX effectors
NCBI	National Center for Biotechnology Information
OME	Outer Membrane Exchange
PAAR	Proline-Alanine-Alanine-Arginine repeats
PAMP	Pathogen-Associated Molecular Pattern
pTM	predicted template modelling score
pv.	pathovar
QS	Quorum Sensing
RFP	Red Fluorescence Protein
Rhs	Rearrangement hot spot
rpm	rounds per minute
SEM	Scanning Electron Microscopy
SLC	Species-Level Clade
SNP	Single Nucleotide Polymorphism

Spec	Spectinomycin
SPI-1	Salmonella Pathogenicity Island-1
spp.	species
syn.	synonymous
T1SS	Type I secretion system
T2SS	Type II secretion system
T3SS	Type III secretion system
T4SS	type IV secretion system
T5SS	Type V secretion system
T6E	Type VI effector
T6I	Type VI immunity protein
T6SS	Type VI secretion system
T7SS	Type VII secretion system
Tae	Type VI amidase effectors
Tag	Type six secretion associated genes
Tas	Type VI effector (p)ppApp synthetases
Tc	Tetracycline
Tde	Type VI DNase effectors
Tge	Type VI glycoside hydrolase
Tle	Type VI lipase effector
Tme	Type VI membrane disrupting effectors
Tne	Type VI NADase effector
Tpe	Type VI peptidase effectors
Tre	Type VI ADP-ribosyltransferase effectors
TTR	Transthyretin-like domains
UPB	Unité de Phytopathologie et Bactériologie
var.	variety
VgrG	Valine-glycine repeat protein G
Xg	<i>Xanthomonas graminis</i>

Xoc *Xanthomonas oryzae* pathovar *oryzicola*

Xoo *Xanthomonas oryzae* pathovar *oryzae*

Xt *Xanthomonas translucens*

X-T4SS *Xanthomonadales*-like T4SS

X-Tfe *Xanthomonadales*-like T4SS effector

X-Tfi *Xanthomonadales*-like T4SS immunity protein

Xtt *Xanthomonas translucens* pathovar *translucens*

Xtu *Xanthomonas translucens* pathovar *undulosa*

XVIPCD *Xanthomonas* VirD4-Interacting Proteins Conserved Domains

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Chapter I

Introduction and objectives

Introduction

1.1 The *Xanthomonas translucens* pathosystem

1.1.1 The genus *Xanthomonas*

Lysobacterales (syn. *Xanthomonadales*) is an early-branching order of the Gammaproteobacteria and is itself divided into two families (Bansal et al., 2023). The *Lysobacteraceae* (syn. *Xanthomonadaceae*) family includes, among others, major phytopathogens (*Xanthomonas* and *Xylella*), nosocomial pathogens (*Stenotrophomonas maltophilia*), and non-pathogenic environmental and plant-associated bacteria (*Stenotrophomonas rhizophila*), or gliding predatory bacteria (*Lysobacter*). The *Rhodanobacteraceae* family encompasses genera such as *Rhodanobacter*, *Dyella*, or *Frateuria* (Bayer-Santos, DeMoraes Ceseti, et al., 2019).

The genus *Xanthomonas* currently comprises 39 plant-associated bacterial species, causing diseases in many economically important crops worldwide (An et al., 2020; Parte et al., 2020). These pathogens are known to infect hundreds of different monocot and dicot plants, including economically significant crops such as rice (*Oryza sativa*), tomato (*Solanum lycopersicum*), banana (*Musa* spp.), cassava (*Manihot esculenta*), plum (*Prunus* spp.) or citrus (*Citrus* spp.), as well as widely cultivated ornamental plants like pelargonium (*Pelargonium* spp.), dandelion (*Taraxacum officinale*) or ivy (*Hedera helix*), thriving in a diverse set of ecological niches worldwide.

The name *Xanthomonas* derives from the Greek words *xanthus* (“yellow”) and *monas* (“unit”), referring to their characteristic yellow colonies. Xanthomonads are Gram-negative, straight rod-shaped, measuring $0.4\text{--}0.6 \times 0.8\text{--}2.0 \mu\text{m}$ (Saddler & Bradbury, 2015). They are obligatory aerobic, non-spore-forming, and bear a single polar flagellum (Saddler & Bradbury, 2015). However, studies indicate the occurrence of *Xanthomonas* with 0 or 2 flagella, challenging the traditional view of *Xanthomonas* as strictly unipolar-flagellated bacteria (Darrasse et al., 2013 ; Jacobs et al., 2015 ; Peduzzi et al., 2023). Colonies are typically yellow and mucoid because they produce two key compounds: the yellow pigment xanthomonadin, which protect against light-induced damage, and large amounts of the exopolysaccharide xanthan gum, which increases stress tolerance and promotes attachment to plant surfaces by supporting biofilm formation (Bianco et al., 2016; Poplawsky et al., 2000). Xanthan exhibits high viscosity as well as thermal and pH stability. These properties has enabled its rapid industrial adoption in various fields, including as a food additive

(E415), cosmetics, oil recovery, wastewater treatment, drug delivery systems, and tissue engineering (Kamoun & Kado, 1990; Onofre-Rentería et al., 2024). This biopolymer is obtained by fermentation with *Xanthomonas campestris* and is composed of glucose, mannose, glucuronic acid, pyruvic acid, and acetyl groups (Onofre-Rentería et al., 2024).

Xanthomonads can be phylogenetically divided into two main groups, known as clade-1 and clade-2 (Edmilson Gonc & Rosato, 2002; Ferreira-Tonin et al., 2012; Hauben et al., 1997; Parkinson et al., 2009).

1.1.2 Clade-1 xanthomonads

Clade-1 xanthomonads represent an early-branching lineage within the genus, subdivided into subclade 1A and 1B, and include different plant-associated bacteria (Koebnik et al., 2021). Originally, this clade was mainly composed of pathogens infecting monocotyledonous hosts, such as *Xanthomonas translucens*, *Xanthomonas cerealis* and *Xanthomonas graminis* on *Poaceae*, *Xanthomonas albilineans* and *Xanthomonas sacchari* on sugarcane, or *Xanthomonas hyacinthi* on hyacinths (Bragard et al., 1997; Cohen et al., 2020; Fang et al., 2015; Pieretti et al., 2015). However, recent genome sequencing efforts have uncovered additional diversity within clade-1, leading to the description of novel species (Harrison et al., 2023). An increasing number of non-pathogenic strains as well as strains isolated from dicotyledonous hosts have been documented as falling into clade-1 xanthomonads, listed in Table 1. Species delineation in this clade relies on a 95-96% Average Nucleotide Identity (ANI) threshold and digital DNA-DNA hybridisation (dDDH) values of 70% (McKnight et al., 2025; Peduzzi et al., 2023).

Non-pathogenic *Xanthomonas* strains have received limited attention due to their asymptomatic nature, which makes them more difficult to detect, and their low economic impact (McKnight et al., 2025). However, their study is important as it helps refining species boundaries and shed light on *Xanthomonas* evolution, notably the genetic determinants that underpin the transition from commensalism to pathogenicity (McKnight et al., 2025; Pena et al., 2024). Moreover, their potential coexistence with pathogenic strains on the same hosts could create opportunities for horizontal gene transfer, potentially leading to the emergence of new pathogens. As such, studying non-pathogenic species provides crucial insights into both the mechanisms of plant disease and the evolutionary dynamics of the genus (McKnight et al., 2025).

These genome-based criteria have also helped clarify the taxonomic status of new strains. Some species-level clade (SLC) strains had previously been proposed by Parkinson *et al.*, among which SLC 5 to 7 were phylogenetically positioned within

clade-1 (Parkinson et al., 2009). Three strains were confirmed as belonging to three new species, including *Xanthomonas campestris* pv. *phormiicola* CFBP 8444^T from New Zealand flax, *Xanthomonas* sp. CFBP 8443^T from common bean, and *Xanthomonas* sp. CFBP 8445 from banana. Comparative genomic analyses further demonstrated that strain CFBP 8445 is closely related to strains of *Xanthomonas indica* (Peduzzi et al., 2023).

In-depth genome mining revealed unexpected features in *X. campestris* pv. *phormiicola* strain CFBP 8444^T. Firstly, this pathogen was previously described by its ability to produce coronatine (COR) analogues, a chlorosis-inducing phytotoxin ordinarily produced by several phytopathogenic pseudomonads and *hitherto* unreported in the *Xanthomonas* genus (Tamura et al., 1992; Zhao et al., 2001). COR compounds are known to structurally mimic the plant hormone jasmonoyl-L-isoleucine and affect plant signalling pathways (Bender et al., 1999). Notably, previous work on *Pseudomonas syringae* has highlighted the role of COR in the inhibition of Pathogen-Associated Molecular Pattern (PAMP)-triggered stomatal closure (Melotto et al., 2006). Interestingly, genes related to the biosynthesis of COR-like compounds were identified in *X. campestris* pv. *phormiicola*, suggesting novel virulence mechanisms within *Xanthomonas* (Peduzzi et al., 2023). Secondly, this same strain was found to possess an atypical flagellar gene cluster in addition to the canonical one. This suggests that some clade-1 strains may produce alternative or lateral flagella, challenging the traditional view of *Xanthomonas* as strictly unipolar-flagellated bacteria (Peduzzi et al., 2023).

Since then, many other new species have been discovered extending the list of clade-1 xanthomonads to 14 validly described species in the *List of Prokaryotic names with Standing in Nomenclature* (LPSN), listed in Table 1 (Parte et al., 2020). A new *Xanthomonas surreyensis* species was proposed which, however, belongs to the same species as *Xanthomonas bonasiae* (Gilroy et al., 2021). Sequencing of a strain pathogenic to canna revealed its phylogenetic position within the *X. sacchari* species, highlighting the need to revise the current species description, which currently restricts this taxon to sugarcane (Harrison et al., 2023). New non-pathogenic strains were described belonging to new species, such as *Xanthomonas hawaiiensis* sp. nov. (Chuang et al., 2024) isolated from *Spathiphyllum* and *Colocasia*, *Xanthomonas rydalmensis* sp. nov. (McKnight et al., 2024), *Xanthomonas bundabergensis* sp. nov. (McKnight et al., 2025), and *Xanthomonas tesorerionis* sp. nov. (McKnight et al., 2025) were isolated from *Fragaria x ananassa*, *Xanthomonas medicaginis* sp. nov. (McKnight et al., 2025) was isolated from *Medicago sativa*, with unknown pathogenesis. A novel species, *Xanthomonas protegens* sp. nov. (Rana et al., 2024), was described from healthy rice seeds and appears closely related to the other rice-associated species *Xanthomonas sontii* and *Xanthomonas indica*.

In addition, new species-level clade (SLC) strains have been identified, yet to be formally characterised. From our study, the sequencing of the three novel species

also suggested the existence of at least four additional novel ones isolated from sugarcane, orange or common bean (569; DD13; LMG9002, F4, 3498; LMG8992, NCPPB1128) (Peduzzi et al., 2023). Pena *et al.* sequenced 83 *Xanthomonas* genomes, identifying eight novel species, including four in clade-1 (I: F5; II: F1; IV: F10; VIII: 3307, 3498, F4), all isolated from citrus and rainwater (Pena et al., 2024).

The type III secretion system (T3SS) is a key virulence determinant in most *Xanthomonas* species, enabling the delivery of effectors to modulate plant physiology and immunity to the advantage of the bacterium. Within clade-1, however, Hrp-T3SSs are largely restricted to subclade-1A, where a few members encode a Hrp-T3SS, namely *X. hyacinthi*, *Xanthomonas theicola*, *X. translucens*, *X. graminis* and *X. cerealis* (Koebnik et al., 2021). Interestingly, atypical T3SS gene clusters have also been identified in subclade-1A. Firstly, *X. theicola* encodes both a Hrp-T3SS and a second atypical T3SS closely related to those found in the *Oxalobacteraceae* family (Koebnik et al., 2021). Secondly, *X. albilineans* lacks the Hrp-T3SS but instead carries a *Salmonella* pathogenicity island-1 (SPI-1) T3SS gene cluster, usually associated with animal pathogens and bacterial insect pathogens or symbionts (Merda et al., 2017; Pieretti et al., 2015). Although its role in pathogenicity remains unclear, this system has been well-conserved, suggesting potential relevance in alternative ecological interactions, such as epiphytic survival or insect association (Pieretti et al., 2015). Pathogenicity in *X. albilineans*, however, was shown to be primarily mediated by albicidin, a potent DNA gyrase inhibitor that induces white foliar stripes symptoms and additionally has antagonistic properties (Hashimi et al., 2007). Among xanthomonads, *X. albilineans* stands out by its markedly reduced genome size (~3 Mb), in contrast to the ~5 Mb genomes typical of other xanthomonads, a feature likely linked to its xylem-invading lifestyle, and reminiscent of *Xylella fastidiosa* (Pieretti et al., 2015). Beyond subclade-1A, most clade-1 xanthomonads lack a T3SS, which is unsurprising for non-pathogenic strains given the important role of this system in pathogenicity (Pena et al., 2024). Exceptions do exist within *Xanthomonas* clade-2, such as some commensal *Xanthomonas arboricola* isolates that lack T3SS but carry reduced T3SS repertoires (Merda et al., 2017). This highlights non-pathogenic strains as valuable models for exploring T3SS evolution (Pena et al., 2024). Furthermore, some pathogenic clade-1 members also lack an Hrp-T3SS, suggesting that alternative virulence mechanisms may operate, similarly to *X. albilineans* with albicidin.

Table 1. List of clade-1 *Xanthomonas* species, isolation hosts and pathogenicity. Not validly published names of the List of Prokaryotic names with Standing in Nomenclature (LPSN) list are placed in quotation marks. NS stands for Not Specified and SPI-1 for Salmonella pathogenicity island 1.

Species and type strain	Subclade	Isolation hosts	Monocot/ Dicot	(non)-pathogenic	Presence of T3SS in the type strain	References
"SLC5" CFBP8443 ^T	1A	Common bean	Dicot	NS	No T3SS	Peduzzi <i>et al.</i> , 2023
<i>X. albilineans</i> CFBP2523 ^T	1B	Sugarcane	Monocot	Pathogenic	SPI-1 T3SS	Pieretti <i>et al.</i> , 2015
<i>X. bonasiae</i> CFBP8703 ^T	1A	Ornamental plants (weeping fig)	Dicot	Non-pathogenic	NS	Mafakheri <i>et al.</i> , 2022a
<i>X. bundabergensis</i> DAR80977 ^T	1A	<i>Fragaria × ananassa</i>	Dicot	Non-pathogenic	No T3SS	McKnight <i>et al.</i> , 2025
<i>X. campestris</i> pv. <i>phormiicola</i> , syn. « SLC6 » CFBP8444 ^T	1A	New Zealand flax	Monocot	Pathogenic	No T3SS	Peduzzi <i>et al.</i> , 2023
<i>X. cerealis</i> CFBP2541 ^T	1A	Small-grain cereals, forage grasses, pistachio trees	Monocot and Dicot	Pathogenic	Hrp- T3SS	Tambong <i>et al.</i> , 2024
<i>X. graminis</i> CFBP2053 ^T	1A	Forage grasses	Monocot	Pathogenic	Hrp- T3SS	Tambong <i>et al.</i> , 2024
" <i>X. hawaiiensis</i> " A6251 ^T	1B	<i>Spathiphyllum</i> and <i>Colocasia</i>	Monocot	NS	No T3SS	Chuang <i>et al.</i> , 2024
<i>X. hyacinthi</i> CFBP1156 ^T	1A	<i>Hyacinthus</i> , <i>Scilla</i> , and other related ornamental plant	Monocot	Pathogenic	Hrp- T3SS	Cohen <i>et al.</i> , 2020
" <i>X. indica</i> ", syn. "SLC7", syn. " <i>X. surreyensis</i> " PPL560 ^T	1B	Rice, banana	Monocot	Non-pathogenic	No T3SS	Peduzzi <i>et al.</i> , 2023; Rana <i>et al.</i> , 2024
<i>X. medicaginis</i> DAR35659 ^T	1B	<i>Medicago sativa</i>	Dicot	NS	No T3SS	McKnight <i>et al.</i> , 2025
" <i>X. protegens</i> " PPL118 ^T	1B	Rice	Monocot	Non-pathogenic	No T3SS	Rana <i>et al.</i> , 2024
" <i>X. pseudalbilineans</i> " GPE39 ^T	1B	Sugarcane	Monocot	Pathogenic	No T3SS	Champoiseau <i>et al.</i> , 2006
<i>X. rydalmensis</i> DAR34855 ^T	1B	<i>Fragaria × ananassa</i>	Dicot	Non-pathogenic	No T3SS	McKnight <i>et al.</i> , 2024
<i>X. sacchari</i> CFBP4641 ^T	1B	Sugarcane, canna	Monocot	Pathogenic	No T3SS	Vauterin <i>et al.</i> , 1995
" <i>X. sontii</i> " PPL1 ^T	1B	Rice	Monocot	Non-pathogenic	No T3SS	Bansal <i>et al.</i> , 2021
<i>X. tesoreronis</i> DAR34887 ^T	1A	<i>Fragaria × ananassa</i>	Dicot	Non-pathogenic	No T3SS	McKnight <i>et al.</i> , 2025
<i>X. theicola</i> CFBP4691 ^T	1A	Tea plants	Dicot	Pathogenic	Hrp- T3SS and non-Hrp- T3SS	Koebnik <i>et al.</i> , 2021
<i>X. translucens</i> DSM18974 ^T	1A	Small-grain cereals, pistachio trees, asparagus, wild-rice	Monocot and Dicot	Pathogenic	Hrp- T3SS	Jaenicke <i>et al.</i> , 2016
<i>X. youngii</i> AmX2 ^T	1A	Ornamental plants (amaranth)	Dicot	Non-pathogenic	NS	Mafakheri <i>et al.</i> , 2022a, Mafakheri <i>et al.</i> , 2022b

1.1.3 The “*Xanthomonas translucens*” complex

The *X. translucens* complex exhibits remarkable genomic and ecological diversity among strains, making it an ideal model organism for investigating antibacterial systems in *Xanthomonas*; accordingly, this species complex was selected as the focus of the present thesis.

Xanthomonas translucens sensu stricto is a seed-borne bacterium and the causal agent of bacterial leaf streak disease (BLS) on small grain cereals (Bragard et al., 1997). Widely spread in wheat- and barley-growing areas worldwide, the disease has recently emerged as a growing problem in Northern USA and Asia (Adhikari et al., 2011; Khojasteh et al., 2019). Furthermore, the seed-borne nature of *X. translucens sensu stricto* makes it a pathogen of global concern and limits international germplasm exchange.

1.1.3.1 Refining the pathovar classification through phylogenomics

Although the first evidence of the disease dates back to 1897, it was in 1917 that Jones *et al.* formally identified and isolated the causal agent of bacterial leaf streak on barley (*Hordeum vulgare*) in the USA (Bamberg, 1936; Jones et al., 1917). He and his coworkers named it “*Bacterium translucens*” pertaining to the translucent lesions it causes on leaves. In the following decades, subsequent detections of leaf streak diseases were reported on wheat (*Triticum aestivum*) (E. F. Smith et al., 1919), rye (*Secale cereale*) (C. S. Reddy et al., 1924), various grasses (Wallin J.R., 1994), and on triticale (*Triticum* × *Secale*) (Zillinsky & Borlaug, 1971).

In 1939, Dowson *et al.* created the genus *Xanthomonas* (Dowson, 1939). Despite the broad host range diversity found among xanthomonads, the pathogens exhibit striking phenotypic uniformity, a contrast that long hampered the establishment of a stable taxonomy (Vauterin et al., 2000). Historically, classification of xanthomonads phytopathogens relied on assigning new species to strains isolated from new host plants, known as the “new host-new species” concept (Vauterin et al., 2000). This included the species *X. translucens* responsible for BLS diseases in cereals. Motivated by the phenotypic uniformity of the genus, Dye and Lelliott reclassified *Xanthomonas* species into a single one, *X. campestris*, with the former species re-designated later as pathovars (Dye & Lelliott, 1974; Young et al., 1978). A pathovar is an infraspecific classification unit used to distinguish, within a bacterial species, strains on the basis of their distinct pathogenicity toward one or more plant hosts. As a result, *X. campestris* came to include over 140 pathovars, including those associated with small grain cereals such as pv. *cerealis*, pv. *hordei*, pv. *secalis*, pv. *translucens*, and pv. *undulosa* (Vauterin et al., 1995). However, this classification system based

primarily on host specificity did not reflect the true genomic relationships among strains, as revealed by DNA hybridisation or fatty acid methyl ester (FAME) analyses. To resolve these taxonomic inconsistencies, Vauterin *et al.* proposed the re-establishment of *X. translucens* as a distinct species comprising strains responsible for leaf streak on small grains and some grasses (the “translucens” group; Fig.1A-G) as well as strains causing bacterial wilt on various forage grasses (the “graminis” group; Fig.1H-I) (Vauterin *et al.*, 1992, 1995a). Following this taxonomic revision, Bragard *et al.* confirmed the phytopathological relevance of the *X. translucens* pv. *cerealis*, pv. *translucens*, and pv. *undulosa* as true biological entities, while supporting the taxonomic unification of pv. *translucens* and pv. *hordei* (Bragard *et al.*, 1997).

Besides infecting cereals, new strains of *X. translucens* have been identified as pathogens on other hosts. A first one caused dark water-soaked lesions on stems of ornamental asparagus (*Asparagus virgatus*) in Florida, another has been responsible for pistachio (*Pistacia vera*) dieback in Australia, while a last one caused BLS on cultivated wild rice (*Zizania palustris*) in Minnesota (Curland *et al.*, 2021; Marefat, Ophel-Keller, *et al.*, 2006; Marefat, Scott, *et al.*, 2006; Norman *et al.*, 1997). Isolates from asparagus and wild rice were identified as *X. translucens* pv. *undulosa* and were able to infect wheat. Conversely, *X. translucens* pv. *undulosa* reference strains were shown to cause disease on asparagus and cultivated wild rice, extending the host range for this pathovar (Curland *et al.*, 2021; Rademaker *et al.*, 2006). However, bacteria isolated from pistachios were genetically distinct from other *X. translucens* pathovars and assigned to a new pathovar, *X. translucens* pv. *pistaciae* (Marefat, Scott, *et al.*, 2006). Two genetic groups of pistachio-associated *X. translucens* have been identified: group A, closely related to pvs. *translucens*, *secalis*, and *undulosa*, appears widespread, whereas group B, related to pv. *cerealis*, has been reported from a single growing area (Giblot-Ducray *et al.*, 2009; Marefat, Ophel-Keller, *et al.*, 2006; Marefat, Scott, *et al.*, 2006). These separate lineages suggest that the ability to infect pistachio evolved independently twice, either with the 1930s introduction of pistachio in Australia or via co-evolution within Iranian orchards, the presumed country of *X. translucens* origin (Giblot-Ducray *et al.*, 2009; Goettelmann *et al.*, 2022). Since *X. translucens* pv. *translucens* Australian strains fail to infect pistachio or other *Anacardiaceae*, this host specificity appears unique to the pistachio isolates (Marefat, Scott, *et al.*, 2006). Although both pv. *pistaciae* groups can experimentally infect several *Poaceae* hosts, yet they have never been reported from grasses in pistachio orchards (Giblot-Ducray *et al.*, 2009; Marefat, Scott, *et al.*, 2006). Interestingly, metagenomic profiling of invasive club moss, showing necrotic symptoms, detected *X. translucens* at a high sequence reads abundance (Dang *et al.*, 2019). However, further studies are still required to conclude any potential involvement in a disease and confirm taxonomic status. Currently, full genomic sequences of both *X. translucens* pv. *pistaciae* ICMP16317 (Group B) and CFBP8304

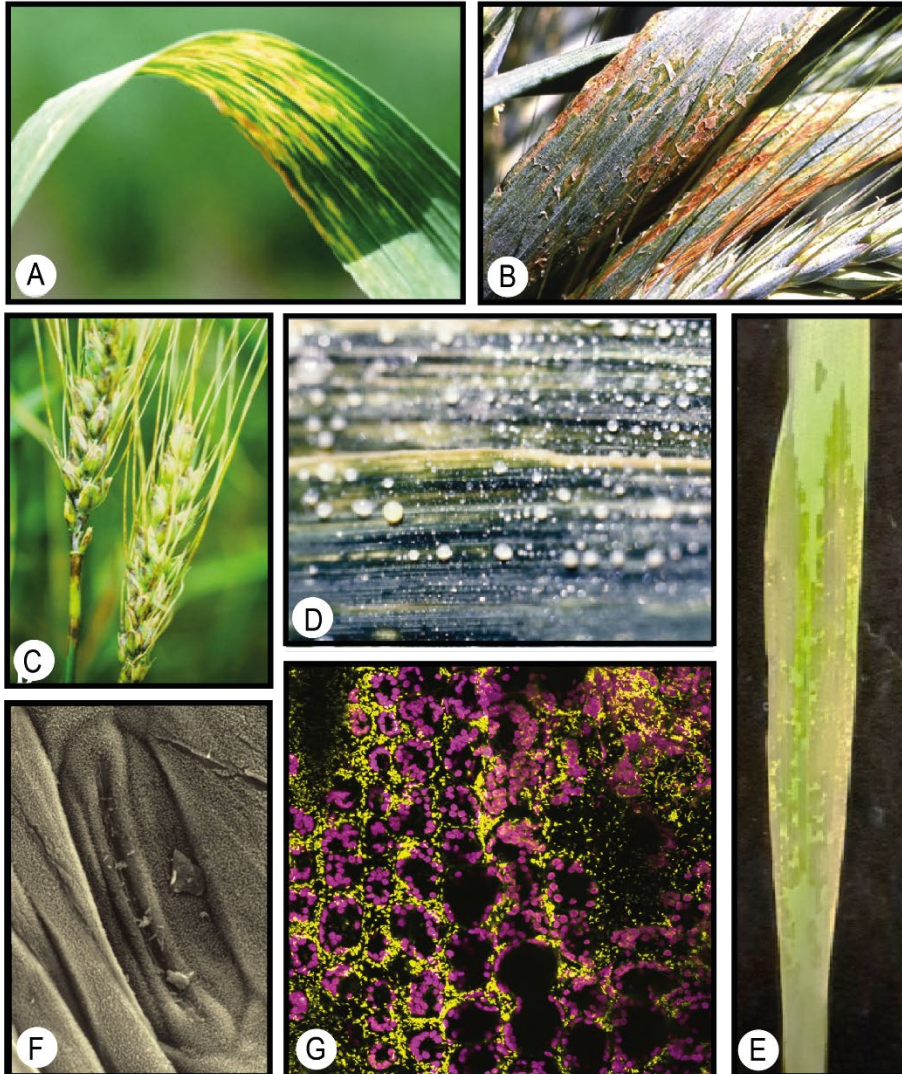
(Group A), as well as the *X. translucens* pv. *undulosa* CIX303 from cultivated wild rice are now publicly available (Curland et al., 2021; Goettelmann et al., 2022).

Thanks to the latest breakthroughs in sequencing technology, a recent study was able to generate high-quality genomes of all previously missing sequenced *X. translucens* pathotypes. This comprehensive genomic dataset enabled a refinement of the pathovar system into a genome-based classification. Average Nucleotide Identity (ANI)-based phylogenetic analyses revealed that the species can be divided into three genetically distinct clades (96% cutoff value), named Xt-I, Xt-II, and Xt-III. To this respect, clade Xt-I, comprises former pathovars *hordei*, *translucens*, *undulosa*, *secalis* and pv. *pistaciae* group A; clade Xt-II, contains pathovar *cerealis* and pv. *pistaciae* group B; and clade Xt-III, contains pathovars *arrhenatheri*, *graminis*, *phlei*, *phleipratensis*, and *poae* (Goettelmann et al., 2022).

In 2024, a study proposed delineating the *X. translucens* species into three separate species containing respectively the following pathovars: ***X. translucens* (Xt)** (including pvs. *translucens* [Xtt] = *hordei*, *pistaciae*, *undulosa* [Xtu] = *secalis*), ***X. cerealis* sp. nov.** (including pvs. *cerealis*, *pistaciae*) and ***X. graminis* sp. nov.** (including pvs. *graminis* [Xg], *arrhenatheri*, *poae*, *phleipratensis* and *phlei*) (Tambong et al., 2024) (Table 2). This new classification has then been accepted by the LPSN (Parte et al., 2020). Genomic analysis showed that pv. *hordei* and pv. *secalis* are over 99% ANI identical to pv. *translucens* and pv. *undulosa* respectively, supporting the retention of *translucens* and *undulosa* and the discontinuation of *hordei* and *secalis* from future use (Tambong et al., 2024).

In the present work, we will therefore refer as the “*Xanthomonas translucens* complex” to designate collectively the three species *X. translucens sensu stricto*, *X. cerealis* and *X. graminis*. Given that the taxonomy has changed very recently, the published article from the second chapter will follow the previous system, treating *X. translucens* as a single species.

Bacterial Leaf Streak and Black chaff disease on cereals



Bacterial Wilt on forage grasses

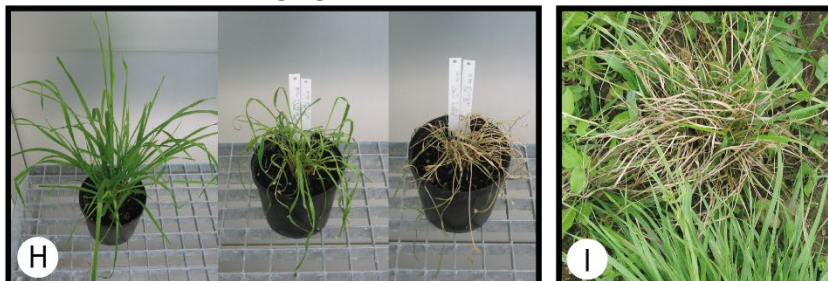


Figure 1: Bacterial leaf streak disease and black chaff on cereals (A-G) and bacterial wilt on forage grasses (H-I) caused by the *X. translucens* complex. (A) Translucid and chlorotic streaks parallel to the leaf midvein (B) Spread of milky exudates, dry down forming thin, almost transparent flakes on leaves (C) Typical black chaff symptoms with discoloration of the peduncle and presence of dark brown streaks along the awns and glumes (D) Triticale leaves exhibiting water-soaking with milky exudates (E) Translucent lesions observed on a barley leaf (F) SEM of *X. translucens* cells entering a stomatal chamber (G) Confocal micrograph of RFP-expressing *Xt* UPB513 (yellow) surrounding barley leaf cells (outlined by pink chloroplasts) and inside substomatal cavity (H-I) Italian ryegrass (*Lolium multiflorum* Lam.) infected with *X. graminis* pv. *graminis* showing bacterial wilt symptoms (H) with disease rating score from left to right: score 1-7-9. Photos from (A,B,C,D) E. Duveiller, C. Bragard and H. Maraite (Duveiller et al., 1997) (E,G) this thesis (F) C. Bragard and (H,I) Courtesy of Dr. Roland Kölliker.

Table 2. Historical pathogens to recent *Xanthomonas translucens* complex classification.

Pathovars	ANI groups (Goettelman, 2022)	LPSN list (Tambong, 2024)	Disease	Hosts
<i>X. translucens</i> pv. <i>undulosa</i> Xtu (syn. <i>X. translucens</i> pv. <i>secalis</i> Xts)			Bacterial leaf streak (BLS) and black chaff on small grain cereals ; Dark water-soaked lesions on asparagus ; Pistachio dieback on pistachio trees	Small grain cereals : Wheat (<i>Triticum aestivum</i>), barley, triticale (<i>Triticum x Secale</i>), rye (<i>Secale cereale</i>), oat (<i>Avena sativa</i>), wild-rice (<i>Zizania palustris</i>) Ornamental asparagus tree fern (<i>Asparagus virgatus</i> ; <i>Liliaceae</i>) Barley (<i>Hordeum vulgare</i>)
<i>X. translucens</i> pv. <i>translucens</i> Xtt (syn. <i>X. translucens</i> pv. <i>hordei</i> Xth)	Xt-I	<i>X. translucens</i>		
<i>X. translucens</i> pv. <i>pistaciae</i> group A				Pistachio trees (<i>Pistacia vera</i> ; <i>Anacardiaceae</i>)
<i>X. translucens</i> pv. <i>cerealis</i> Xtc				Small grain cereals : Wheat, barley, oat, rye, Forage grasses : <i>Agropyron</i> , <i>Bromus</i> , <i>Dactylis</i> , <i>Elymus</i> , and <i>Lolium</i>
<i>X. translucens</i> pv. <i>pistaciae</i> group B	Xt-II	<i>X. cerealis</i>	Bacterial leaf streak (BLS) and black chaff on small grain cereals ; Pistachio dieback on pistachio trees ; Bacterial wilt on forage grasses	Pistachio trees (<i>Pistacia vera</i> ; <i>Anacardiaceae</i>)
<i>X. translucens</i> pv. <i>phlei</i>				<i>Phleum</i>
<i>X. translucens</i> pv. <i>phleipratensis</i> Xtp				
<i>X. translucens</i> pv. <i>poae</i>				<i>Poa</i>
<i>X. translucens</i> pv. <i>arrhenatheri</i> Xta	Xt-III	<i>X. graminis</i>	Bacterial wilt on forage grasses	<i>Arrhenatherum</i>
<i>X. translucens</i> pv. <i>graminis</i> Xtg				<i>Agrostis</i> , <i>Alopecurus</i> , <i>Dactylis</i> , <i>Deschampsia</i> , <i>Festuca</i> , <i>Lolium</i> , <i>Phalaris</i> , <i>Phleum</i> , <i>Poa</i> , <i>Arrhenatherum</i> , <i>Bromus</i> , and <i>Trisetum</i>

1.1.3.2 Geographic distribution

The Fertile Crescent, including the Karacadag Mountains in southeastern Turkey, is recognised as the cradle of wild cereals, where wheat was first domesticated around 10,000 years ago (Eckardt, 2010; Heun et al., 1997). Interestingly, Iranian strains of *X. translucens sensu stricto*, geographically close to the origin of cultivated wheat, display significantly higher genetic diversity than strains from North America, supporting the hypothesis that the Middle East served as the source population for the pathogen's global dissemination (Khojasteh et al., 2019).

From its likely origin in the Fertile Crescent, *X. translucens* has been reported to date in many cereal-growing regions across the continents, spanning North and South America, Asia, Africa, parts of Europe, and Australia (Duveiller et al., 1997). However, the BLS disease remains absent from Western Europe, likely due to a combination of climate, resistant varieties, agricultural practices, and quarantine measures (Duveiller et al., 1997; EPPO, 2025a). *X. translucens* pv. *translucens* was added to the EPPO A2 quarantine list in 1993 (Duveiller, 1994; EPPO, 2025, 2025; Paul & Smith, n.d.). Despite historical reports from Belgium, France, and Spain, the pathogen is currently considered absent in these countries and has only been intercepted in Spain (Duveiller, 1994; EPPO, 2025a). The pathogen is also considered a quarantine organism in Egypt, Morocco, Tunisia, Chile, Jordan, Serbia, Türkiye, and Inter-African Phytosanitary Council (IAPSC) (EPPO, 2025a).

Although often reported sporadically, most records come from warmer cereal-growing areas (Duveiller et al., 1997). In the United States, it was first detected in the Midwest in the early 20th century. BLS has since spread widely, with common outbreaks in the Southeast USA (Kephart et al., 1993; Milus & Mirlohi, 1994; Tubajika et al., 1999) and, more recently, increasing incidence in the Upper Midwest USA (Adhikari et al., 2012; Curland et al., 2018) and western Canada (Arnason, 2021).

In contrast, *X. graminis* has been mainly detected in Western Europe (Switzerland, Germany, Belgium, France, Netherlands, Norway, UK), but also in the USA and New Zealand (Kölliker et al., 2006; Paul & Smith, 1989).

Finally, *X. cerealis* is quite widespread as it has been detected in Argentina, Brazil, Canada, and Japan (Paul & Smith, 1989). Its precise distribution remains less clear due to its symptoms and host range overlapping with the two other species.

1.1.3.3 Lifecycle and dissemination

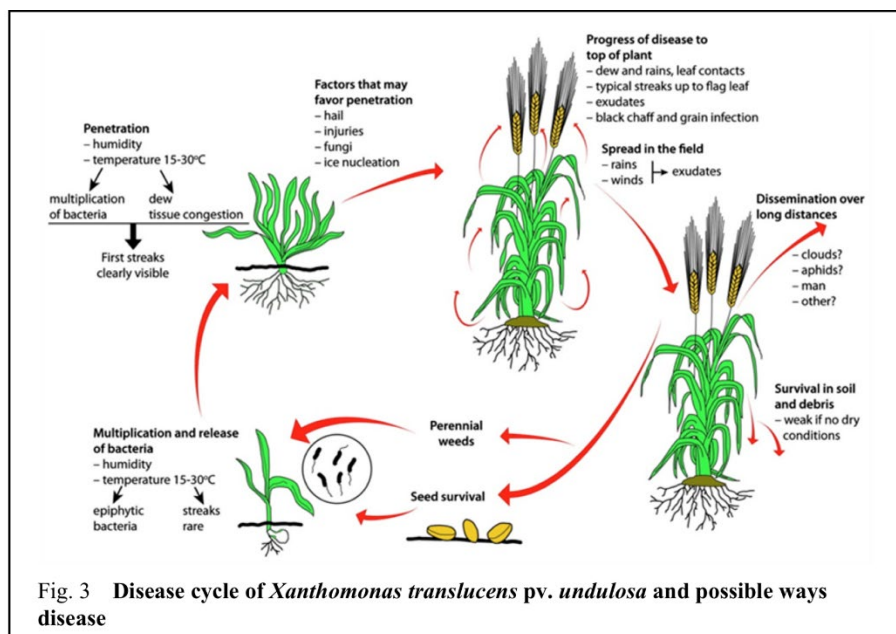


Fig. 3 Disease cycle of *Xanthomonas translucens* pv. *undulosa* and possible ways disease

Figure 2: *X. translucens sensu stricto* lifecycle and possible dissemination routes from (Duveiller et al., 1997). *X. translucens* starts its lifecycle at the seed stage, spreading primarily via contaminated seeds but also locally through rain splash, crop debris, overwintering hosts, and ice-nucleation-mediated long-distance dispersal. The bacterium multiplies epiphytically on leaf surfaces before entering leaves through stomata, hydathodes, or wounds, with vascular and non-vascular strains colonizing distinct tissues. Symptoms begin as water-soaked spots that form streaks and necrotic lesions, potentially affecting flag leaves and panicles, with exudates producing droplets or flakes. Disease outbreaks are favored by warm and humid conditions.

X. translucens lifecycle, depicted in Fig.2, starts and ends at the seed level. The bacterium primarily survives and is transmitted vertically through contaminated seeds but can also be transmitted horizontally within fields via rain splash, crop debris, or overwintering. First the pathogen initiates an epiphytic phase where the bacterium multiplies at the surface of the leaves. During this phase, bacteria are likely to face competition with other cohabiting microorganisms, as well as environmental stressors such as nutrient scarcity, drought, UV radiation, and temperature fluctuations, conditions against which biofilm formation can provide a protective advantage (Alvarez-Martinez et al., 2020). Subject to favourable conditions, it will begin an endophytic phase by penetrating through the stomata

(Fig.1F), hydathodes or wounds on the leaves. The species *X. translucens* includes closely related barley pathogens displaying contrasting tissue specificities, pvs. *undulosa* (non-vascular) and *translucens* (vascular) (Gluck-Thaler et al., 2020). While the non-vascular *X. translucens* pv. *undulosa* remain restricted to the mesophyll parenchyma tissues (Fig.1G) and trigger localised symptoms, the vascular *X. translucens* pv. *translucens* colonise the mesophyll as well as xylem vessel tissues, inducing systemic symptoms. Recent work identified the cell wall-degrading hydrolase CbsA as a key determinant of xanthomonads' vascular lifestyle (Gluck-Thaler et al., 2020). The first symptoms appear as water-soaked spots (Fig.1E), which later coalesce into streaks parallel to the veins of the leaves, then evolving into necrotic streak lesions (Fig.1A), typical of the BLS disease. Under high humidity, yellow bacterial oozes may appear as droplets on the streaks. Those exudates may spread and dry down forming thin, almost transparent flakes on leaves (Duveiller & Bragard, 2017) (Fig.1B,D). The disease might then progress towards the flag leaf and the panicle. At this stage, dark-purple longitudinal streaks on the glumes may develop, referred to as the black chaff disease (Fig.1C).

X. translucens is primarily disseminated through seeds, which serve as the main source of inoculum for both local and long-distance spread by seed transport (Duveiller et al., 1997; Jones et al., 1917). The bacterium was shown to survive on seeds for extended period, up to 63-81 months, with the survival rate influenced by storage conditions, duration, and host susceptibility (Duveiller, 1992; Duveiller et al., 1997; Forster & Schaad, 1990). For instance, the disease has been observed on emerged plants when seed-borne populations were reaching sufficient levels within a range of 3.800-9.300 colony forming unit (CFU) per seed (Milus & Mirlohi, 1995). In a different phase of the lifecycle, at the field level, *X. translucens* spreads locally through rain, dew, and direct contact between plant aerial tissues, with a single infected plant potentially contaminating up to 30 m² during the season (Boosalis, 1951; Sands et al., 1986). Outbreaks are favoured by warm, humid conditions and are more frequent in sprinkler-irrigated fields or during periods of heavy rainfall (Duveiller et al., 1997; Forster et al., 1986). Additionally, *X. translucens* and *X. cerealis* were shown to overwinter on several perennial (quack grass (*Elymus repens*), brome grass (*Bromus* spp.) and timothy grass (*Phleum pratense*)) and winter annual hosts (winter wheat and winter rye) and then infect newly formed tillers (Boosalis, 1951; Wallin J.R., 1994). This overwintering ability provides the pathogen with opportunities to spread to other annual spring cereals (Sapkota et al., 2020). While the bacteria survive for a short period in soil, their persistence is prolonged when associated with crop debris (Boosalis, 1951; Cunfer, 1988; Duveiller, 1994). An unusual phenotype of *X. translucens* is its ice-nucleating activity (INA), which can contribute both to frost injury in plants and to long-distance atmospheric dissemination. INA enables the bacterium to catalyse ice formation within cloud droplets, facilitating dispersal via rain or snow over extended distances (H. K. Kim et

al., 1987; Morris et al., 2004). This mechanism has been confirmed by the isolation of ice-nucleation-active strains from rain in Virginia, highlighting the complexity of BLS epidemiology and the multiple pathways through which the pathogen can spread (Butchacas, 2024; Faylor et al., 2017).

Symptoms are rarely visible at the seedling stage, even from contaminated seeds, and the disease typically appears only late in the season (Bamberg, 1936; Duveiller, 1992). As a result, growers often do not notice infections before heading (Forster et al., 1986). Experimental studies have further demonstrated that yield loss correlates strongly with the infected flag leaf area. For instance, in the USA, 50% infection of the flag leaf was shown to reduce kernel weight by 8–13%, while full infection could lead to losses of 13–34% (Shane W. W. et al., 1987). In Mexico, a nearly linear relationship was observed between infected flag leaf area and yield loss, with up to 20% reduction when 50% of the flag leaf was diseased (Duveiller & Maraite, 1993). These losses are probably linked to reduced photosynthesis, affecting both grain filling and, in some cases, grain number (Duveiller & Maraite, 1993).

X. graminis, causing bacterial wilt on forage grasses, represents a major constraint in temperate grasslands, such as for meadow fescue (*Festuca pratensis*) and Italian ryegrass (*Lolium multiflorum*) (Kölliker et al., 2006). Unlike cereal-infecting seed-borne *X. translucens*, *X. graminis* strains are not transmitted by seed or soil but spread mainly via contaminated mowing tools and are all vascular (Goettelmann et al., 2023). The pathogen enters through stomata or wounds created during mowing and colonises the xylem. Once inside the vascular tissues, the pathogen disrupts the flow of water and nutrients, causing the typical symptoms of leaf and tillers wilting and curling, even leading to plant death (Goettelmann et al., 2023; Kölliker et al., 2006) (Fig.1H-I).

1.1.3.4 Yield loss and management strategies

Accurate estimates of BLS yield losses are limited, since the absence of effective treatments prevents healthy control plots (Duveiller et al., 1997). The impact of BLS on wheat production is highly variable across regions and dependent on cultivar susceptibility and environmental conditions (Sapkota et al., 2020). Nevertheless, reported losses generally average around 10%, but can reach up to 40% in severely affected fields, such as those documented in Idaho (Forster et al., 1986).

Although cultural practices have been proposed to reduce BLS incidence, their efficacy remains poorly documented (Adhikari et al., 2011; Duveiller et al., 1997). Since *X. translucens* exhibit limited survival in plant residues, crop rotation is not considered a reliable control measure (Duveiller et al., 1997; Milus & Mirlohi, 1995). Therefore, the use of pathogen-free seeds is critical, as seeds represent the primary

source of inoculum. Various detection methods, including dilution plating on selective media, seedling infection assays, serological tests, PCR, and loop-mediated isothermal amplification, have been developed to certify clean seeds (Bragard & Verhoyen, 1993; Forster & Schaad, 1985; Langlois et al., 2017; Maes et al., 1996). Chemical or physical seed treatments can reduce bacterial infection, although their effectiveness is inconsistent (Atanasoff & Johnson, 1920; Duveiller et al., 1997; Forster & Schaad, 1990). Very few field studies have tested chemical control, but copper-based compounds such as copper oxychloride and Cobre (Idai Nature Company, Spain) have shown preventive efficacy, reducing disease severity by up to ~75% at 3000 ppm, although excessive use poses environmental and health risks (Shohanipor et al., 2025). Breeding resistant cereal varieties remains the most durable strategy to mitigate BLS (Sapkota et al., 2020). Additionally, biological control offers a sustainable alternative, with microorganisms or their secondary metabolites able to suppress *Xanthomonas* pathogens. Commercial products such as Serenade® OPTI (*Bacillus subtilis* QST 713) and AgriPhage™ (bacteriophage) have been successfully applied against *Xanthomonas* diseases in solanaceous crops and nut trees (Marin et al., 2019). Finally, control strategies against bacterial wilt by *X. graminis* mainly consist of cutting tools disinfection, or most effectively the use of resistant or tolerant cultivars (Kölliker et al., 2006). While the interactions of the *X. translucens* complex with other microorganisms in the cereal phytobiome remain poorly understood, a deeper understanding of the mechanisms that enable their survival is essential for developing innovative disease management strategies.

1.2 Bacteria thrive through an ongoing interbacterial arms race

Recent advances in crop protection increasingly emphasise biocontrol strategies, which rely on the use of living organisms or natural substances, such as macroorganisms, microorganisms, secreted molecules or plant extracts, to regulate phytopathogen populations. Therefore, a deeper understanding of microbial interactions in the phyllosphere is essential. Bacteria live a constant arms race with neighbouring microorganisms occupying the same ecological niche. Bacteria rely on a set of strategies to interact and survive within dynamic and complex polymicrobial communities, such as those found in plants, animal guts or soil (García-Bayona & Comstock, 2018). Inter-bacterial interactions can either be mediated by freely diffusible factors released into the surrounding environment or factors directly delivered into neighbouring target cells by contact (García-Bayona & Comstock, 2018). The struggle for survival has driven bacteria to evolve several contact-dependent mechanisms for bacterial killing.

This includes contact-dependent growth inhibition (CDI) systems, in which a two-partner CdiA/B complex assembles at the cell surface, delivering CdiA toxin upon cell-cell contact, targeting only closely related bacteria (Ruhe et al., 2013). Found in many Gram-positive bacteria, the type VII secretion system (T7SS) was shown to play a key role in intraspecies competition among *Staphylococcus* spp. (Bowman & Palmer, 2021; Ulhuq et al., 2020). The contact-dependent inhibition by glycine zipper proteins (Cdz) is an atypical bacteriocin system, described in *Caulobacter crescentus*, in which the toxin is retained on the surface of producer cells and mediates bacterial killing upon contact (García-Bayona et al., 2017). Another contact-dependent mechanism for delivering toxins has been observed in *Myxococcus xanthus*, referred to as outer membrane exchange (OME) (Sah & Wall, 2020). Widespread amongst Gram-negative bacteria, the type VI secretion system (T6SS) delivers toxic proteinaceous effectors directly into prokaryotic or eukaryotic nearby cells (Hood et al., 2010). Lastly, the *Xanthomonadales*-like T4SS (X-T4SS) was also shown to be involved in bacterial killing against other Gram-negative bacterial species (Sgro et al., 2019; Souza et al., 2015).

1.3 Antibacterial secretion systems in *Xanthomonas*

Xanthomonads rely on multiple secretion systems to adapt to their environment, outcompete nearby microorganisms, and invade plant hosts (Alvarez-Martinez et al., 2020). Secretion systems are multiproteic complexes transporting molecules called effectors from the cytoplasm across bacterial membranes to neighbouring cells or extracellular milieu (Boyer et al., 2009). To date, 11 secretion systems have been described in bacteria, of which only the first six were identified in *Xanthomonas*

(Jamali et al., 2025). While bacteria of the genus *Xanthomonas* are considered major phytopathogens, type I to VI secretion systems (T1SS-T6SS) have been identified among the different *Xanthomonas* species (Alvarez-Martinez et al., 2020). Among these, the T3SS is the most studied for its role in bacterial pathogenesis. T1SS, T2SS and T5SS seem mainly responsible for adhesion and degradation of host cell wall, while T4SS is involved in conjugation and bacterial killing. In addition, to compete with nearby microorganisms, many bacteria deploy T6SS to inject toxic effectors into prokaryotic or eukaryotic target cells.

Among those, both T6SS and X-T4SS have been found within the order *Xanthomonadales* to be involved in outcompeting other bacterial species in a contact-dependent manner through the secretion of toxic effector proteins (Alvarez-Martinez et al., 2020; Bayer-Santos, DeMoraes Ceseti, et al., 2019; Sgro et al., 2019). However, the ecological and evolutionary basis leading xanthomonads to evolve two secretion systems with such similar functions remain unclear. This suggests evolutive advantages conferred by T6SS and X-T4SS systems to the bacterium, leading to the gain or loss of one or the other system.

1.3.1 Type VI secretion system (T6SS)

In 2006, two pioneering studies from the Mekalanos group defined the Type VI Secretion System (T6SS) as a novel bacterial secretion system, distinct from all known classes. The first paper by Pukatzki *et al.* (Pukatzki et al., 2006) demonstrated that the “intracellular multiplication factor (IcmF)-associated homologous protein” cluster in *Vibrio cholerae* was essential for contact-dependent cytotoxicity against amoebae and macrophages and mediated the extracellular secretion of Hemolysin-coregulated protein (Hcp) and Valine-glycine repeat protein G (VgrG) proteins. The second paper by Mougous *et al.* (Mougous et al., 2006) reported that a homologous locus in *Pseudomonas aeruginosa* encoded a protein secretion apparatus that actively secreted Hcp1 during chronic infections in cystic fibrosis patients. Both studies described the T6SS in the context of pathogenesis (Jani & Cotter, 2010).

1.3.1.1 T6SS structure, core and accessory genes

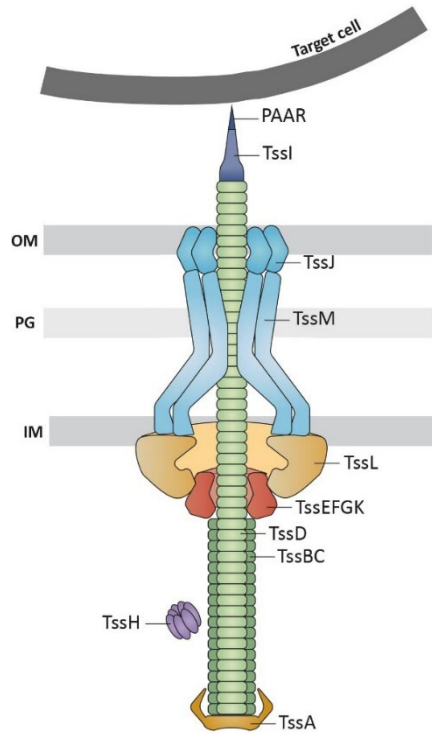
The T6SS is a contractile nanomachine acting as a syringe to inject effector proteins directly into prokaryotic or eukaryotic cells (Alvarez-Martinez et al., 2020; Bayer-Santos, DeMoraes Ceseti, et al., 2019). This multiproteic complex, evolutionarily related to contractile bacteriophage tails, is composed of 13 core structural components (TssA-M, for type six secretion) commonly encoded in gene clusters (Fig.3A). The T6SS is a dynamic apparatus anchored in the bacterial cell envelope and is composed of three main complexes: the cytoplasmic tail, the membrane complex

and the baseplate. The membrane spanning complex is composed of TssJ, TssL and TssM, on which a baseplate, comprising TssE, TssF, TssG and TssK, is docked. The cytoplasmic tail is composed of an inner tube of hexameric Hcp rings stacked on top of one another (Bernal et al., 2018). The tubular edifice is enclosed into a contractile sheath (TssB/C) with helical configuration. TssA interacts with the baseplate to initiate tail polymerisation and stabilises the distal end of the tail.

The tail is finally capped with a trimeric VrgG spike and proline-alanine-alanine-arginine repeats (PAAR)-sharpening tip (Bernal et al., 2018). The recruitment of effectors occurs concomitantly with T6SS assembly, situated either inside the Hcp inner tube or at the tip with VgrG or PAAR (Alvarez-Martinez et al., 2020). Following each sheath contraction, the inner tube Hcp and the spike VgrG-PAAR, are propelled outside of the cell, puncture the membrane of target cells and deliver a cocktail of effectors. After each firing event, the ATPase caseinolytic peptidase V (ClpV) is thought to recycle the contractile sheath TssB-C for the next round, although direct evidence for this is still lacking, while Hcp monomers are lost to the extracellular milieu (Alvarez-Martinez et al., 2020; Cherrak et al., 2018; Costa et al., 2015; Zoued et al., 2014) (Fig.3B).

Beyond core proteins, additional non-conserved accessory proteins (Tag, for Type six secretion associated genes) have been discovered with various functions, such as post-translational regulation (TagE-H) (Mougous et al., 2007), sheath stabilisation (TagA-B and TagJ) (Bernal et al., 2021), or sensing a T6SS-mediated attack (TagQ-T) (Basler et al., 2013; Reglinski et al., 2023). The standardised Tss nomenclature has now been broadly adopted, although traditional names such as Hcp (syn. TssD), VgrG (syn. TssI), and ClpV (syn. TssH) remain commonly employed.

A



B

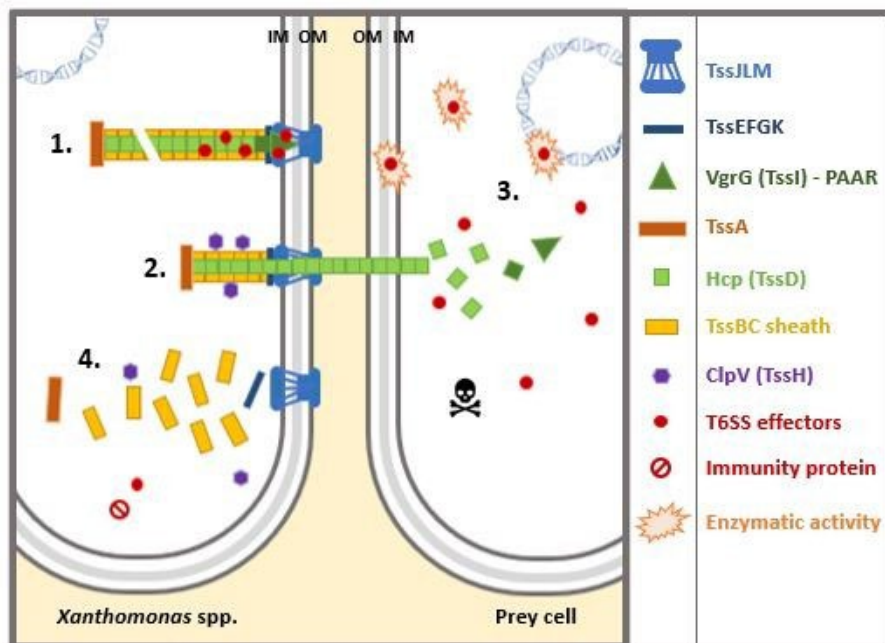


Figure 3: Schematic representation of T6SS structure and bacterial killing activity.

(A) Model of T6SS architecture (TssA-M and PAAR) at the interface of two bacterial cells **(B)** T6SS bacterial killing mechanism (1) Assembly and tail elongation of T6SS in its extended state (2) Sheath contraction and translocation of toxin effector proteins into the targeted cell (3) Enzymatic activity of toxin effectors mainly targeting membranes, cell walls and nucleic acids leading to target cell growth inhibition or death (4) Recycling of membrane base complex for further firing events. OM, PG and IM stand for outer membrane, cell wall made of peptidoglycan, and inner membrane respectively (Inspired from Gallique et al., 2017).

1.3.1.2 T6SS evolutionary origin

Studies on the origins of the T6SS reveal that many of its core components, including the puncturing structure (VgrG, PAAR, Hcp) (Leiman et al., 2009), the contractile sheath (TssBC) (Lossi et al., 2013), and the baseplate (Cherrak et al., 2018), share striking structural similarities with bacteriophage tail structure. However, unlike phages, the T6SS has evolved distinctive features such as its membrane complex sharing homology with the type IVb secretion system (T4bSS) (Allsopp & Bernal, 2023), the ClpV ATPase (Förster et al., 2014), and length regulation dictated by cell width rather than a “tape measure” protein (Santin et al., 2019). Altogether, these findings suggest that T6SS shares common evolutionary origins with phage or even derived from one and has been domesticated to adapt to bacterial needs (Gallegos-Monterrosa & Coulthurst, 2021).

1.3.1.3 T6SS functions in phytobacteria

Although T6SS functions were initially thought to align with phylogenetic groupings of T6SS genes, many evidence now shows that the role of a specific T6SS is primarily determined by the activity of the effectors it secretes (Bernal et al., 2018). For example, *B. thailandensis* E264 T6SS-5 (clade T6SS-i1) involved in host manipulation (Schwarz et al., 2010), phylogenetically cluster with *Pseudomonas syringae* DC3000 T6SS-2 (clade T6SS-i1), which mediates intermicrobial competition (Haapalainen et al., 2012) (see Fig.5). Some T6SS even deliver trans-kingdom effectors, targeting both eukaryotic and prokaryotic cells (Jiang et al., 2014, 2016; Russell et al., 2013; Tan, Yang, et al., 2025). This section broadly describes the impact of T6SS in the plant ecosystem.

Rhizobium-legume symbiosis

T6SS can either play an inhibitory or promoting role in *Rhizobium*-legume symbiosis (Salinero-Lanzarote et al., 2019). Bladergroen *et al.* published the first study in 2003 on a secretion system (later identified as T6SS) in *Rhizobium leguminosarum*, where the so-called “imp locus” impaired nodulation and nitrogen fixation in nonhost pea (Bladergroen et al., 2003). Later studies revealed contrasting results with *Rhizobium etli* T6SS mutants forming smaller nodules compared to the wild type.

Intermicrobial competition

A huge body of literature reports the T6SS role in interbacterial competition, at both inter and intraspecific levels (Ripcke et al., 2023), by inhibiting their growth or causing cell death (Haapalainen et al., 2012). The system also targets eukaryotic microbes by killing fungi (Haapalainen et al., 2012) or protects bacteria from eukaryotic predation such as amoeba (Bayer-Santos et al., 2018; Haapalainen et al., 2012). Intermicrobial competition can also serve ultimate broader ecological role. For instance, T6SS in commensals bacteria, such as *Pseudomonas* sp., can mediate “colonisation resistance” by directly killing or secreting metal chelators, to prevent invasion of phytopathogens, with promising applications in biocontrol (Bernal et al., 2017; Chen et al., 2016). A further example is *Pseudomonas protegens*, which deploys bacterial killing T6SS to invade gut microbiome and kill herbivorous pest insect (Vacheron et al., 2019).

Nutrient uptake

Some T6SS effectors can also be secreted in a contact-independent manner in the proximal environment to take up nutrients, such as metal-scavenging proteins, limiting resources for other competing microbes, such as *Pseudomonas* species, as well as in *Yersinia pseudotuberculosis* and *Burkholderia thailandensis* (Si et al., 2017; T. Wang et al., 2015; X. Yang et al., 2021).

Virulence

Many phyto bacterial T6SSs have an impact on plant virulence, such as *Ralstonia solanacearum* (L. Zhang et al., 2014), or *Pectobacterium atrosepticum* (Mattinen et al., 2007). Some findings point to a direct involvement of T6SS in plant-pathogen interaction, although the precise effectors and mechanisms remain unknown (Gallegos-Monterrosa & Coulthurst, 2021). In *P. atrosepticum*, T6SS mutations attenuated virulence in potato, while filter sterilised potato extracts directly induced T6SS expression (Mattinen et al., 2007). Likewise, in *Pectobacterium wasabiae*, loss of both T6SSs clusters reduced maceration symptoms on potato slices, which were surface sterilised, ruling out intermicrobial competition and instead supporting a direct action of the T6SS on plant cells (Nykyri et al., 2012). Interestingly, in the rice pathogen *Burkholderia glumae*, two T6SS accessory genes, one of which encodes a DUF2169 domain, were shown to be involved in bacterial virulence (N. Kim et al.,

2021). While both proteins play a virulence role at any rice developmental stage (seed, seedling, vegetative stage), the double mutant demonstrated a synergistic effect during the reproductive stage (N. Kim et al., 2021). However, reductions in virulence may also arise indirectly, for example through impaired colonisation, disrupted biofilm formation, limited nutrient acquisition, or reduced competitiveness within the phytobiomes (Alvarez-Martinez et al., 2020). In *R. solanacearum*, bacterial wilt results from biofilm formation that obstructs xylem flow. However, T6SS mutants not only showed reduced virulence, but also produced less biofilm and were less motile, suggesting that T6SS might indirectly influence virulence by modulating these traits, ultimately causing bacterial wilt symptoms (L. Zhang et al., 2014).

Intraspecies social interactions

T6SS also shapes diverse intraspecies social interactions by controlling T6SS immunity gene expression (Gallegos-Monterrosa & Coulthurst, 2021). For example, in *Burkholderia thailandensis*, quorum sensing (QS) controls the expression of effector-immunity modules, but not the core T6SS components (Majerczyk et al., 2016). Within *B. thailandensis* populations, QS-deficient mutants can act as “cheater cells,” gaining a fitness advantage by avoiding the metabolic costs of cooperation, thereby risking outgrowing QS cooperative cells. However, such cheater cells express low levels of immunity proteins making them vulnerable to T6SS attacks from sibling cells in a “policing” mechanism (Majerczyk et al., 2016). Similarly, in *M. xanthus*, a soil-dwelling bacterial predator, T6SS eliminate starving sibling cells, which produce lower levels of immunity proteins, and ultimately maintains a healthy and well-fed population (Troselj et al., 2018; Unni et al., 2022).

1.3.1.4 T6SS classification and genomic organisation

T6SS gene clusters have been divided into four evolutionarily divergent subtypes (T6SSi–iv) (Böck et al., 2017; Boyer et al., 2009; Bröms et al., 2010; Russell et al., 2014). (i) Subtype T6SSi constitutes the vast majority of T6SSs, predominantly found in Proteobacteria including *Xanthomonadales*, it constitutes the most studied canonical form (Boyer et al., 2009). (ii) Subtype T6SSii corresponds to *Francisella* pathogenicity island-like systems (Bröms et al., 2010). (iii) Subtype T6SSiii is specific to *Bacteroidetes* (Russell et al., 2014). (iv) Subtype T6SSiv was discovered in the amoeba symbiont *Amoebophilus asiaticus* (Böck et al., 2017). As the subtypes T6SSii, T6SSiii, and T6SSiv are absent from *Xanthomonadales*, only T6SSi will be considered in the following sections.

Phylogenetic analyses of core gene sequences have revealed that the subtype T6SSi can be further classified into five distinct clades (i1–i5) (Bernal et al., 2018; Boyer et

al., 2009). Beyond their divergent phylogenetic groupings, distinct T6SS clades also differ in terms of genetic organisation, regulatory pathways, number of Hcp, PAAR and VgrG copies, as well as accessory protein content (Bernal et al., 2018). T6SS-i3 is broadly distributed across bacterial genera. However, one T6SS-i3 phylogenetic branch restricted to *Xanthomonadales* was further subdivided into the subclades i3*, i3**, i3*** and i3**** for researchers focusing on this order (Bayer-Santos, DeMoraes Ceseti, et al., 2019; Garin et al., 2024). Comparative genomic studies revealed that T6SSs are widely distributed among the *Xanthomonadales* order and identified representatives from clades and subclades T6SS-i1, i3*, i3**, i3***, i3**** and i4 (Fig.4A). However, the *Xanthomonas* genus only contains T6SS-i3*, i3***, i4 (Alvarez-Martinez et al., 2020; Bayer-Santos, DeMoraes Ceseti, et al., 2019). The genetic organisation of *Xanthomonadales* T6SSs is described below and depicted in Fig.4B and Table 3.

Table 3. *Xanthomonadales* T6SS clades and their respective distinctive features.

T6SS clades and subclades	Distribution within the <i>Xanthomonadales</i> order	Accessory genes	Regulatory genes	Distinctive features
T6SSi1	<i>Stenotrophomonas</i>	<i>tagO, fha</i>	sigma factor σ^{54}	
T6SSi3*	<i>Xanthomonas</i>	<i>tagJ, tagF, fha</i>	<i>ppkA-pppA-fha, tagF, ecfK, pknS</i>	Lacking <i>tssJ</i>
T6SSi3**	<i>Dyella</i>	<i>tagJ, tagF, fha</i>	<i>ppkA-pppA-fha, tagF</i>	Lacking <i>tssJ</i>
T6SSi3***	<i>Xanthomonas, Lysobacter, Luteibacter</i>	<i>tagJ, tagF, fha</i>	<i>ppkA-pppA-fha, tagF</i>	Lacking <i>tssJ</i> and PAAR
T6SSi3****	<i>Stenotrophomonas</i>	Unknown	Unknown	Unknown
	<i>Lysobacter, Stenotrophomonas, Xanthomonas</i>	<i>tagX, tagM, tagN, tagF</i>		Variable regions with multiple VgrG copies
T6SSi4				

Within the *Xanthomonadales*, T6SS-i1 has only been reported in plant-associated *Stenotrophomonas* sp. (Bayer-Santos, DeMoraes Ceseti, et al., 2019). To a broader extent, phylogenetic analyses of T6SS distribution among phytobacteria revealed that *Stenotrophomonas* T6SS-i1 are evolutionarily related to those in *Pseudomonas putida* (Fig.5) (Bayer-Santos, DeMoraes Ceseti, et al., 2019; Bernal et al., 2018). In terms of genetic organisation, T6SS-i1 is close to the *V. cholerae* T6SS. Distinctive features of this clade include the presence of a sigma factor σ^{54} transcriptional regulator, and an accessory *tagO* gene of yet unknown function (Bayer-Santos, DeMoraes Ceseti, et al., 2019; Bernard et al., 2011).

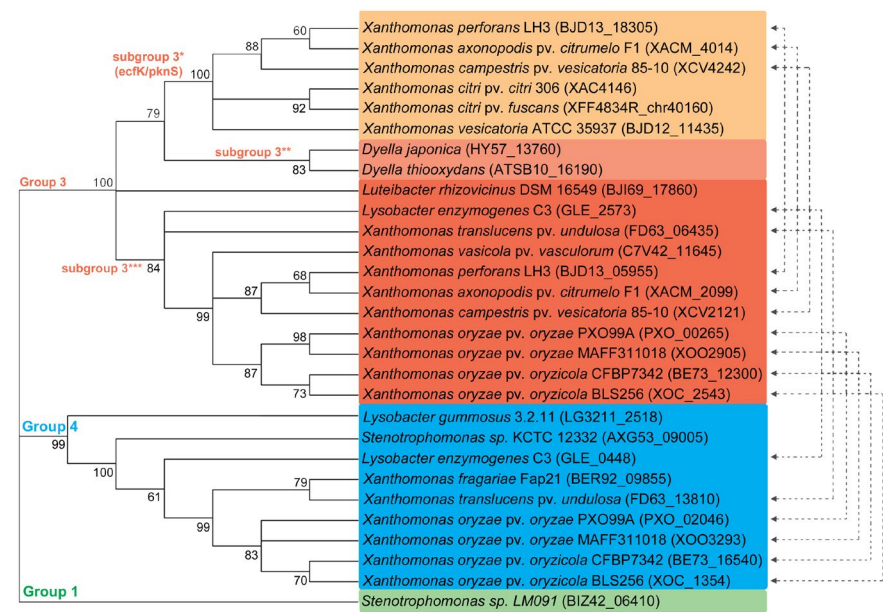
T6SS-i3 is widespread among phytobacteria, with *Burkholderia* T6SS-i3 clustering the closest to the *Xanthomonadales* T6SS-i3 (Bayer-Santos, DeMoraes Ceseti, et al., 2019; Bernal et al., 2018) (Fig.5). Specifically, the T6SS-i3* is found in *Xanthomonas*, i3** in *Dyella*, i3*** in *Xanthomonas*, *Lysobacter* and *Luteibacter* and i3**** in *Stenotrophomonas* genera (Bayer-Santos, DeMoraes Ceseti, et al., 2019; Garin et al., 2024). In *Xanthomonadales*, the genetic architecture of T6SS-i3 consists of two main core gene clusters separated by a variable region, spanning 2.2 to 15.6 kb (Bayer-Santos, DeMoraes Ceseti, et al., 2019). All T6SS-i3 clusters encode the PpkA-PppA-FHA post-translational regulatory pathway, together with its cognate TagF repressor (J. S. Lin et al., 2018; Mougous et al., 2007; Silverman et al., 2011). Additionally, TagI, implicated in the interaction between ClpV and TssB during the sheath disassembly, is also found in association with T6SS-i3 gene clusters (Förster et al., 2014). A notable characteristic of T6SS-i3* is the presence of an additional regulatory cascade, involving the extracytoplasmic function (ECF) sigma factor (EcfK) and its kinase PknS, shown to control T6SS expression in response to amoebal predation (Bayer-Santos et al., 2018). Another distinctive feature of T6SS-i3 clusters is the absence of the core protein TssJ, indicating an alternative pathway for anchoring to the outer membrane, and the absence of PAAR homologs in T6SS-i3*** (Alvarez-Martinez et al., 2020).

Finally, within phytobacteria, *Xanthomonadales* T6SS-i4 are phylogenetically associated with those from *Burkholderia*, *Ralstonia* and *Azoarcus* (Bayer-Santos, DeMoraes Ceseti, et al., 2019; Bernal et al., 2018) (Fig.5). Within the *Xanthomonadales* order, T6SS-i4 was found in *Lysobacter*, *Stenotrophomonas* and *Xanthomonas* (Bayer-Santos, DeMoraes Ceseti, et al., 2019). The genetic architecture of *Xanthomonas* T6SS-i4 is organised in two conserved core gene clusters, interrupted by a large variable region of 40 to 75 kb. These variable regions frequently harbour multiple *vgrG* copies, candidate effector genes and proteins carrying domains of unknown function (DUFs). In only few cases, such as *Xanthomonas fragariae*, the two core gene clusters can be separated by up to 300 kb (Bayer-Santos, DeMoraes Ceseti, et al., 2019). Unique to T6SS-i4 is the presence of TagX, a peptidoglycan hydrolase that facilitates T6SS insertion to the bacterial cell wall during biogenesis (Bayer-Santos, DeMoraes Ceseti, et al., 2019; Weber et al., 2016). Accessory TagM and TagN proteins are consistently associated with this T6SS clade, although their functions are yet to be elucidated (Bayer-Santos, DeMoraes Ceseti, et al., 2019). TagF is also present in T6SS-i4, despite the absence of FHA, and might regulate T6SS through an alternative pathway (Alvarez-Martinez et al., 2020). Interestingly, the *Lysobacter gummosus* T6SS-i4 exhibits a highly divergent genetic organisation compared to the other T6SS-i4s (Bayer-Santos, DeMoraes Ceseti, et al., 2019).

The variation of regulatory and accessory elements among T6SS (sub)clades, together with the occasional presence of the three different T6SS (sub)clades within

a single strain, suggest that each T6SS cluster has specialised in carrying out distinct functions in diverse environments (Boyer et al., 2009).

A



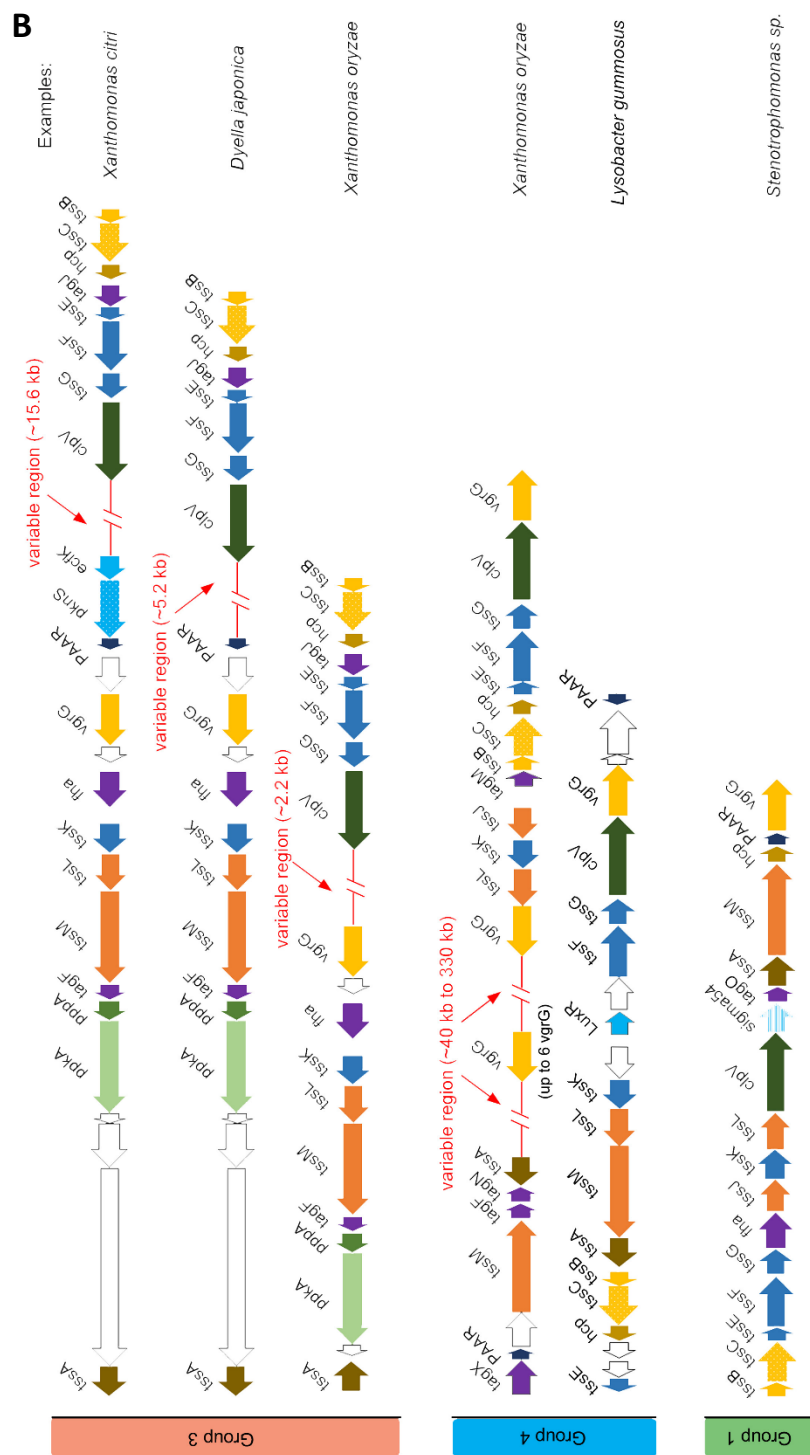
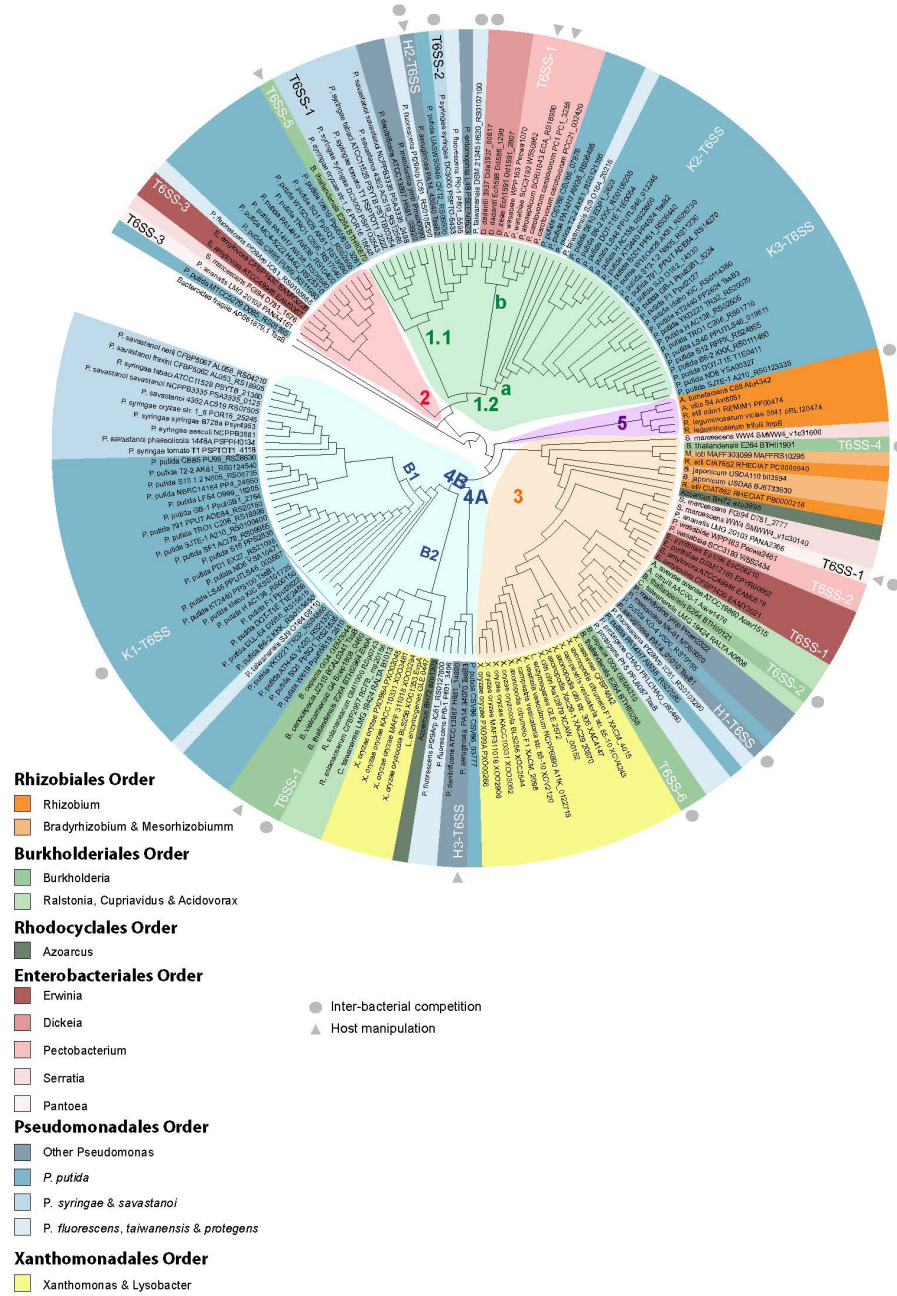


Figure 4: Relationship between phylogeny and T6SS gene content in *Xanthomonadales* **(A)** Phylogenetic distribution of T6SS clusters in *Xanthomonadales*. Left: Maximum-likelihood consensus tree with 1000 bootstrap replicates of T6SS clusters based on aligned TssC (XAC4146) homologs, with 1000 bootstrap replicates, constructed using MEGA 7.0. Groups correspond to T6SS clades and subclades i1, i3 and i4. **(B)** Schematic representation of the different genomic organisations found within T6SS gene clusters clades i1, i3 and i4. The respective size of the variable region is indicated within the T6SS-i3 and i4 clusters. The genes are colour-coded as follows: membrane complex encoded by the *tssJ*, *tssL*, and *tssM* genes (orange), base plate encoded by the *tssE*, *tssF*, *tssG*, and *tssK* genes (dark blue), spike and contractile sheath encoded by *vgrG*, *tssB*, and *tssC* genes (light yellow), inner tube encoded by the *hcp* gene (dark yellow), *tssA* (brown), *PAAR* (very dark blue), the ATPase *clpV* (dark green), regulatory genes (light green and light blue), accessory *tag* genes (purple), and unknown genes (white) (Courtesy of Prof. C. E. Alvarez-Martinez; from Bayer-Santos, DeMoraes Ceseti, et al., 2019).

1.3.1.5 T6SSi distribution among proteobacteria and xanthomonads

Identified in 25% of sequenced proteobacteria, the T6SSi is prevalent across diverse ecosystems, such as in environmental (rain, marine, soil), animal or plant-associated bacteria (Bernal et al., 2018; Unni et al., 2022). Within phytobacteria, all the five T6SS clades i1-i5 are widely represented, with strains containing up to five T6SS clusters (Bernal et al., 2018). Bacterial strains with multiple T6SSs generally harbour phylogenetically distinct clades, supporting independent acquisition by horizontal gene transfer (HGT) (Bernal et al., 2018). The broad distribution of some T6SS clades across genera as shown in Fig.5 (clade i3) suggests that these are particularly prone to HGT, whereas other clades remain restricted to specific genera, suggesting a more ancestral acquisition and subsequent vertical inheritance (clade i4B1 and i1.1; see Fig.5) (Bernal et al., 2018).

A



B



C

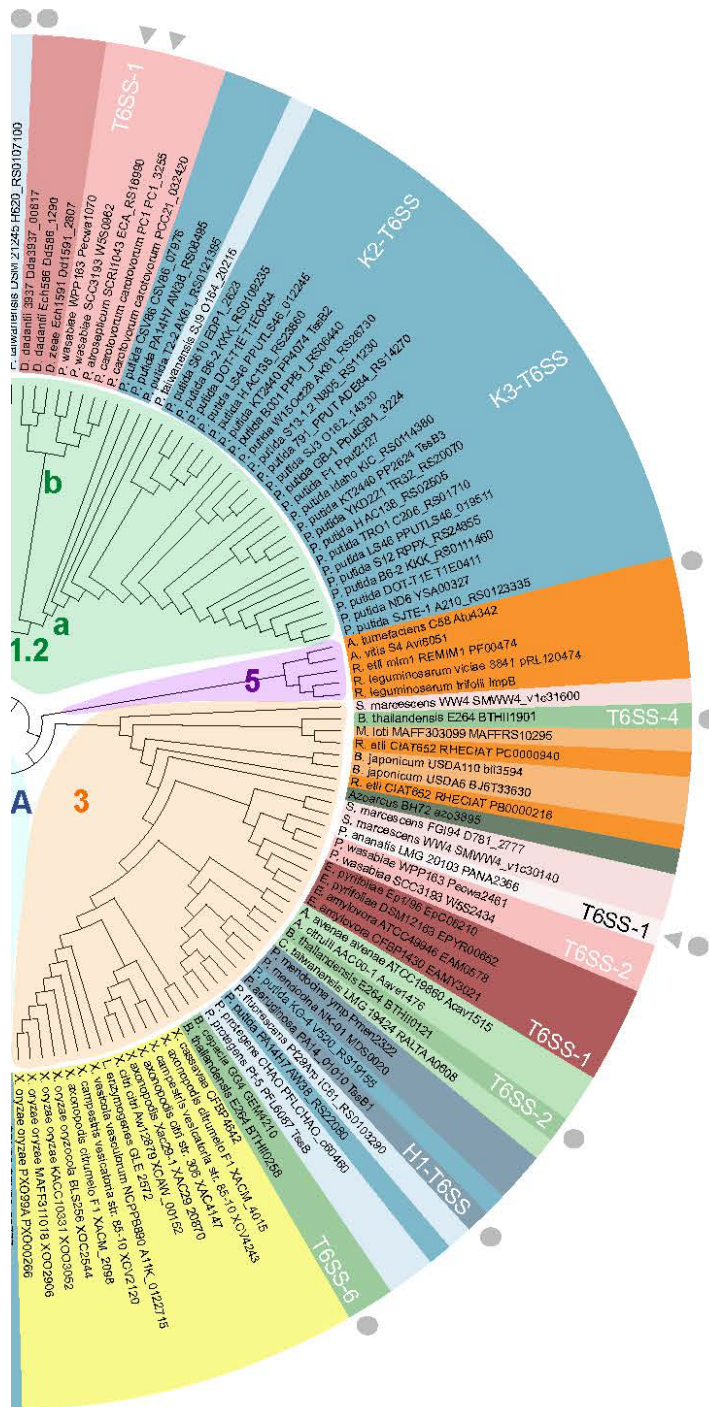


Figure 5: Phylogenetic delineation of T6SS clades among phytobacteria. (A, and zoom B and C) A maximum-likelihood tree was constructed from amino acid sequences of TssB, with 1000 bootstrap replicates using MEGA 6.0. The T6SS classification proposed by Boyer *et al.* (Boyer *et al.*, 2009) is adopted to assign clusters to their respective clades, including clade i1 (green), clade i2 (red), clade i3 (orange), clade i4 (blue), and clade i5 (purple). Additional diversification within these clades is indicated, as subclades 1.1, 1.2a, and 1.2b (green), and for subclades 4A, 4B1, and 4B2 (blue). The subclades i3* and i3*** as proposed by Bayer-Santos *et al.* (Bayer-Santos, DeMoraes Ceseti, *et al.*, 2019) for subclassifying *Xanthomonadales* T6SS-i3 are indicated as well. Experimentally validated functional roles are annotated by symbols, with grey circles standing for interbacterial competition and grey triangles for eukaryotic host manipulation. T6SS names as given in the literature are indicated on the tree adjacent to the corresponding bacterial strains (Courtesy of Prof. P. Bernal; from Bernal *et al.*, 2018).

A comprehensive genomic survey of the *Xanthomonas* genus revealed that 75% of sequenced strains possess at least one T6SS cluster (Liyanapathirana, Wagner, *et al.*, 2022). Some strains harbour two distinct T6SS (sub)clades, including combinations of i3*** and i3*, i3*** and i4 and less commonly i3* and i4. Only the non-pathogenic species *Xanthomonas maliensis* possesses all the three T6SS clusters i3*, i3*** and i4 (Liyanapathirana, Wagner, *et al.*, 2022). Phylogenetic analyses suggest that T6SS-i3* and i3*** were likely inherited from an ancestor of the *Xanthomonas* genus and subsequently lost in some lineages. This same study further proposed that the distribution of T6SS-i4 results from more recent repeated HGT events. They further observed that T6SS-i4 clusters from both *X. graminis* and *Xanthomonas bromi*, and from the three species *Xanthomonas oryzae*, *X. translucens* and *X. cerealis*, grouped phylogenetically together respectively, suggesting shared evolutionary trajectories (Liyanapathirana, Wagner, *et al.*, 2022). Another study focusing on *X. translucens* further observed that T6SS-i3*** is spread across *Xt* ANI clades *Xt*-I and *Xt*-II, while T6SS-i4 is present in *Xt* ANI clade *Xt*-I (only in *pv. undulosa* and some *pv. translucens*), *Xt*-II and a few *Xt*-III strains (Goettelmann *et al.*, 2022).

T6SS is relatively rare in *Stenotrophomonas*, with approximately 8% of sequenced strains carrying at least one cluster, from clades i1, i3, or i4. While only 6% of sequenced *S. maltophilia* species complex contain one or two T6SS clusters from the clades i1, i3 or i4, the few T6SS-carrying strains are spread across the five different continents, and come from various sources, animal, plant, human, environment. By contrast, all the plant-associated *S. rhizophila* strains consistently harbour a T6SS-i4 (Crisan *et al.*, 2023; Garin *et al.*, 2024).

1.3.1.6 Determinants of T6SS presence or absence in bacteria

Although the T6SS is widespread among bacteria, its presence and absence vary at different taxonomic level, even among strains of the same species. However, the proximate causes (e.g., genetic constraints) and ultimate causes (e.g., fitness advantage) driving T6SS distribution remain elusive, despite having important impacts on their ecological fitness (Unni et al., 2022). A review by Unni *et al.* dedicated to this question pointed out various hypotheses. This review considered a bacterium T6SS-positive if it harbours the core T6SS genes, acknowledging that the criteria for defining T6SS presence can be subject to debate (Unni et al., 2022).

Ecological lifestyle

Their first insight is that the T6SS is prevalent among bacteria from various ecological lifestyles, including free-living cells, surface-attached communities, and animal or plant-associated pathogenic, commensal, beneficial or symbiotic bacteria (Bladergroen et al., 2003; Speare et al., 2018; Tang et al., 2016; Unni et al., 2022; Vacheron et al., 2019). Comparative analyses indicate T6SS is less frequent in free-living bacteria and more prevalent in complex microbial communities, particularly within host-associated microbiota (Geller et al., 2021; Kempnich & Sison-Mangus, 2020). This suggests a selective advantage in fierce competitive environments, where bacteria deploy their T6SS, among other functions, to kill competitors (Unni et al., 2022). In line with this and starting from the observation that T6SS is absent in decades-old laboratory *E. coli* strains, a recent study postulated that the absence of microbial competition during extended pure cultivation drove T6SS loss. To test this, the enteroaggregative *E. coli* (EAEC) 17-2 T6SS-positive strain was propagated for 640 generations under controlled competition conditions with either susceptible or immune recipient cells, revealing that T6SS activity was largely abolished by mutations when grown with immune recipients, while it remained active with susceptible cells (Taillefer et al., 2025).

A study focusing on the *Xanthomonas* genus has attempted to link the presence or absence of T6SS to various ecological traits of xanthomonads, yet no consistent correlation has been established. T6SS-i3* is mainly restricted to clade-2 *Xanthomonas* infecting dicotyledonous hosts (Liyanapathirana, Wagner, et al., 2022). However, exceptions exist such as the non-pathogenic *Xanthomonas* strains SI, SS and GW isolated from perennial ryegrass seed (Liyanapathirana, Wagner, et al., 2022). No correlation could be established between T6SS distribution and source of isolation in *Xanthomonas*. This is exemplified by rice-associated strains, among which *X. santonii* lacks T6SS, *X. sp* strain GW carries T6SS-i3*, *X. oryzae* possesses T6SS-i3*** and i4, and *X. maliensis* harbours the three T6SS-i3*, i3*** and i4. Although efforts were made to correlate T6SS-i4 presence with the vascular lifestyle, few counter-examples failed to support this hypothesis (Liyanapathirana, Wagner, et

al., 2022). For instance, both vascular *Xtt* and non-vascular *Xtu*, as well as vascular *Xoo* and non-vascular *Xoc*, all possess T6SS-i4 (Gluck-Thaler et al., 2020; Liyanapathirana, Wagner, et al., 2022). Additionally, all T6SS clusters are completely absent in xylem-limited phytopathogens characterised by reduced genomes, such as *X. albilineans* and all subspecies of *X. fastidiosa* (Bayer-Santos, DeMoraes Ceseti, et al., 2019).

Genomic context

Variation in the genomic context of T6SS clusters affects T6SS mobilisation (Unni et al., 2022). Some T6SS loci are encoded on genomic islands, such as the *Salmonella* pathogenicity islands (SPIs) (Blondel et al., 2009). Differences in GC content between T6SS genes and the core genome further support their horizontal acquisition (Blondel et al., 2009). In *V. cholerae*, the Aux3 cluster provides a striking example, as it encodes a T6SS effector-immunity pair and is flanked by phage-like *att* sites. Experimental evidence shows that in environmental *V. cholerae* strains, Aux3 can be excised and reintegrate via site-specific recombination, whereas in pandemic strains it is fixed due to a truncated integrase gene. Its transfer mechanism in the environment remains unclear but may involve generalised transduction or chitin-induced natural competence (Santoriello et al., 2020). Secondly, some T6SS clusters are located on plasmids, such as in *Pantoea ananatis* or *Rhizobium leguminosarum* (Sánchez-Cañizares et al., 2018; Shyntum et al., 2014). Evidence for plasmid-mediated T6SS transfer was shown in *Campylobacter*, where chromosomal T6SS clusters is highly similar to T6SS in plasmid pCJDM202 (Marasini et al., 2020). Laboratory experiments confirmed that plasmid-mediated T6SS transfer can confer new cytotoxic or virulence traits in recipient strains (Bruto et al., 2017; Marasini et al., 2020). Finally, T6SS genes can also be found on ICEs, so far observed exclusively in *Bacteroidetes* (Coyne et al., 2016). In gut samples, T6SS-encoding ICEs exhibited high nucleotide identity across *Bacteroides* strains and species, indicating recent and repeated gene transfers (García-Bayona et al., 2021).

Additionally, genetic background of recipient cells could also present incompatibilities preventing T6SS acquisition, a phenomenon referred to as “T6SS exclusion” (Unni et al., 2022). Firstly, the transfer of T6SS by conjugation raises questions about the compatibility of the competition and conjugation processes (Peñil-Celis & Garcillán-Barcia, 2019). T6SS antibacterial activity can compromise conjugation by killing either plasmid-carrying donors or non-immune recipients, thereby limiting DNA transfer (Peñil-Celis & Garcillán-Barcia, 2019). For instance, *P. aeruginosa* uses a “tit-for-tat” response, sensing T6SS-mediated exogenous attacks and counterattacking by assembling a T6SS at the precise same location (Basler et al., 2013). This response is triggered by membrane perturbations that activates T6SS expression. Even the contact-dependent T4SS of *E. coli* can trigger these T6SS counterattack, resulting in the killing of the donor cell (Ho et al., 2013). However,

T6SSs are frequently found on conjugative platforms, such as plasmids or a few on ICEs (Peñil-Celis & Garcillán-Barcia, 2019). Interestingly, many plasmid-encoded transcriptional regulators of T6SS have been described, such as AggR (AraC family) in *E. coli* (Dudley et al., 2006; Journet & Cascales, 2016), TF (AraC family) in *Vibrio crassostreae* (Bruto et al., 2017; Piel et al., 2020), or TetR in *Acinetobacter baumannii* (Weber et al., 2015). In the latter, conjugative transfer into the recipient cell depends on TetR-mediated repression of donor T6SS activity (Weber et al., 2015). More research is needed to elucidate how bacteria balance plasmid and ICE dissemination with antibacterial competition. Another incompatibility source is the so-called “ICE exclusion”, a process by which the T6SS-encoding ICE cannot integrate into the genome of the recipient cell because the *attP* integration site is already occupied by another ICE targeting the same *attP* site (Avello et al., 2019). Similarly, “plasmid exclusion” might also prevent T6SS-carrying plasmid acquisition in the recipient cell (Unni et al., 2022).

Geographic separation

Geographic location might also promote or inhibit T6SS acquisition. For example, *V. crassostreae* with T6SS-carrying plasmids were only detected near Brest, north-west France, but not in isolates from Sylt in northern Germany (Bruto et al., 2017). This geographical variation could result either from plasmid not being selected in Sylt, or geographical separation hampering plasmid transfer (Unni et al., 2022). Additionally, T6SS-positive isolates from Brest were found in mixed populations with T6SS-negative strains (Bruto et al., 2017). In mixed populations, T6SS-positive and immune T6SS-negative siblings could potentially cooperate through a division-of-labour strategy, combining competitive killing with energy-efficient growth, as exemplified for T3SS (Sturm et al., 2011; Unni et al., 2022).

T6SS energetic cost

Because the T6SS is composed of a high number of subunits and relies on a dynamic mechanism, the T6SS is assumed to be energetically costly for the cell, which could be a driver of T6SS loss for increased fitness (Taillefer et al., 2023; Unni et al., 2022). However, different strategies have been described that mitigates T6SS cost. ClpV is thought to recycle TssB-C subunits between each firing events, and in *V. cholerae* secreted Hcp, VgrG and effectors can be exchanged via T6SS and reused between adjacent sister cells for new T6SS assembly (Vettiger & Basler, 2016). Beyond recycling, some bacteria can regulate their T6SS expression via tit-for-tat response, to maximise their efficiency, prevent wasteful random T6SS attacks and save energy (Basler et al., 2013). *In vitro* work on *E. coli*, *V. cholerae* and *Bacteroides fragilis* demonstrated that T6SS doesn't entail a significant growth cost to the producing bacteria (Robitaille et al., 2023; Taillefer et al., 2023; C. Zhang et al., 2024). In contrast, *in vivo* studies revealed that T6SS use becomes costly in the presence of resistant competitors, whereas interactions with sensitive strains allow T6SS-

positive cells to offset these costs and maintain a fitness advantage (Robitaille et al., 2023). For *Xanthomonas*, future studies are needed to clarify how xanthomonads balance the energy costs and competitive advantages *in planta*, and on a possible TagF-mediated regulatory mechanism (Bayer-Santos, DeMoraes Ceseti, et al., 2019; Zhu et al., 2020).

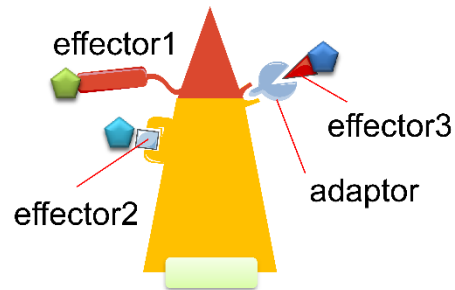
1.3.1.7 T6SS effector loading, delivery, activities and immunity

Effector loading and delivery

T6SS effectors are proteins loaded either inside the central channel of the Hcp inner tube or surface exposed at the tip on the VgrG-PAAR spike and secreted upon T6SS contraction into prokaryotic/ eukaryotic cells or in the proximal environment (Jurénas & Journet, 2021). The T6SS is highly modular, allowing various combinations of VgrG and PAAR proteins to assemble into functional spike (Cianfanelli et al., 2016). As a result, multiple effectors can be delivered simultaneously in a single firing event (Shneider et al., 2013). However, the exact effector composition between each contraction event and the mechanisms of effector selection are still poorly understood.

T6SS can be loaded in two distinct ways: either as “specialised” effectors, when the effector is translationally fused at the C-terminus to one of the three Hcp/VgrG/PAAR core components, or as “cargo” effectors interacting non-covalently with one of those three proteins (Hernandez et al., 2020) (Fig.6A). Additionally, cargo effectors can be assisted by chaperones (syn. adaptors) or structural motifs, which stabilise effectors and Hcp/VgrG/PAAR interactions (Jurénas & Journet, 2021). The lumen of Hcp hexameric ring has a diameter of almost 40 Å, which only allows accommodating small effector proteins (< 20 kDa), while larger proteins, such as phospholipase (60-100 kDa), are usually loaded on the spike (Flaughnatti et al., 2020; Jurénas & Journet, 2021; Mougous et al., 2006). Bacteria translocating T6SS effectors with bacteriolytic activity additionally express immunity proteins to avoid self-intoxication of the secreting cell or attack of kin cells. T6SS effector and immunity genes are usually organised in bicistronic units (Jurénas & Journet, 2021). A multitude of T6SS effector-immunity (EI) gene modules have been described to date.

A



 effector1: Specilized effector containing structure domain including PAAR/hcp/VgrG.

 effector2: Cargo effector by non-covalent interactions with PAAR/ hcp/VgrG.

 effector3: Cargo effector by indirect non-covalent interactions with PAAR/VgrG via adaptor.

 Adaptor: Intermediate bridge of effector and RAAR/VgrG

   Immunity protein

B

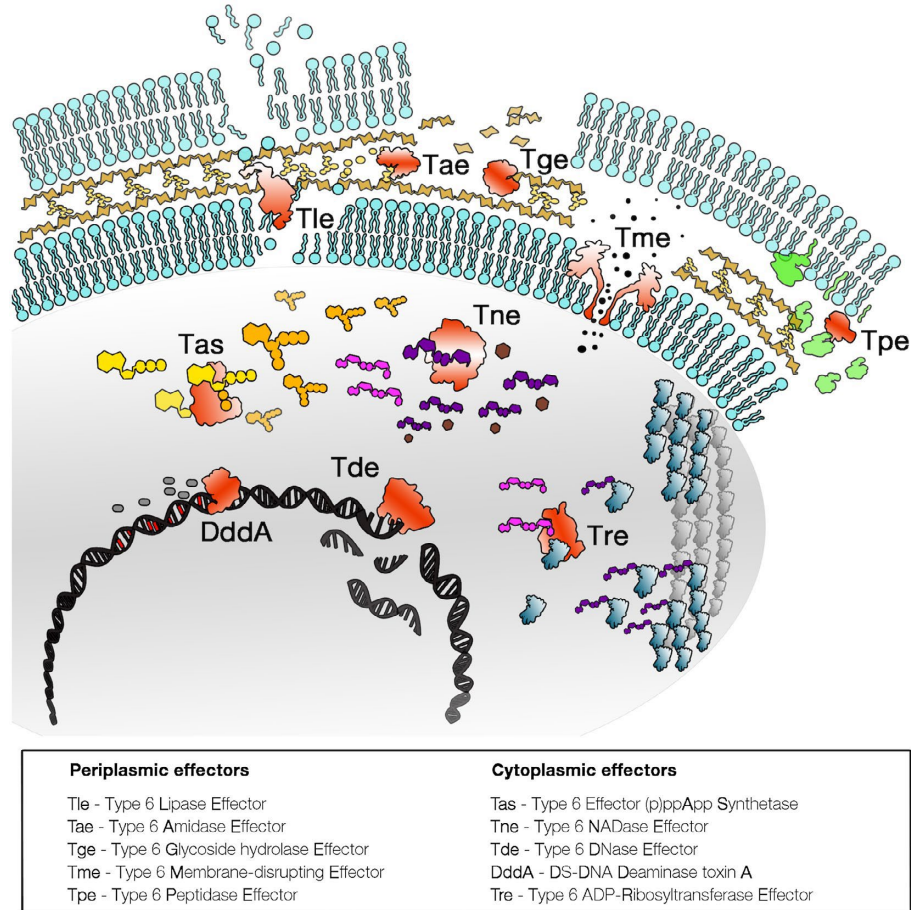


Figure 6: Overview of T6SS effector loading, delivery and antibacterial activities. (A) Representation of the different mode of T6SS effector loading and delivery on Hcp, VgrG or PAAR proteins (From . (B) Antibacterial toxins (red) either acting in the periplasm or cytoplasm (Courtesy of Dr. D. Jurėnas and Dr. L. Journet, from Jurėnas & Journet, 2021).

Chaperones and recruitment motifs

Table 4. Chaperones, motifs, and domains associated with T6SS effector modules.

Name	Domain and/ or Description
T6SS chaperone families	
Eag (Effector associated gene)	DUF1795 DcrB domain; stabilise PAAR-specialised effectors and VgrG interactions
DUF2169-containing chaperone	DUF2169; Dedicated to PAAR-specialised effector
Tec/Tap chaperone	DUF4123; highly conserved central region, C-terminus linked to the effector N-terminus
Tla-3 chaperone	DUF2875; Interacting with Tle3
Motifs and Domains associated to T6SS effector/VgrG/PAAR proteins	
MIX (Marker for type sIX effectors)	N-terminal sequence motifs
FIX (Found in type sIX effectors)	N-terminal sequence motifs
TTR (Transthyretin-like domains)	C-terminus of VgrG/PAAR
DUF2345 domains	C-terminus of VgrG
Rhs (Rearrangement hot spot)	T6SS effectors fused to PAAR or VgrG interacting domain (VIRN) at the N-terminus

Chaperones are usually encoded in tandem with their cognate T6SS effectors. A few chaperone families have been described so far and are listed in Table 4. Eag (for Effector associated gene) family proteins harbour a DcrB (syn. DUF1795) domain stabilising the interaction between adjacently encoded PAAR-specialised effectors and VgrG (Cianfanelli et al., 2016; Quentin et al., 2018). DUF2169-containing chaperones are linked to the secretion of a PAAR-specialised effector (Bondage et al., 2016). Tec/Tap chaperone family members contain a DUF4123 domain, as well as a highly conserved central region, as putative recombination site for generating adaptor chimeras, and a C-terminus which is specifically linked to the N-terminus of their dedicated effectors (Liang et al., 2015; Unterweger et al., 2015). Finally, the Tla-3 chaperone interacting with Tle3 possesses a DUF2875 domain (Berni et al., 2019).

Additionally, a few motifs and domains are commonly found in T6SS effector/VgrG/PAAR proteins and are listed in Table 4. These include smaller domains like MIX (Marker for type sIX effectors) or FIX (Found in type sIX effectors) N-terminal sequence motifs mostly found in T6SS effectors (Jana et al., 2019; Salomon et al., 2014). TTR (Transthyretin-like domains) at the C-terminus of VgrG/PAAR and DUF2345 domains at the C-terminus of VgrG form extensions acting as internal chaperones for recruiting T6SS effectors (Flaunatti et al., 2020). Additional functions were attributed to DUF2345 such as indispensable for T6SS assembly or exerting antibacterial and antifungal activity on its own (Lopez et al., 2020; Storey et al., 2020). Larger structures like Rhs (Rearrangement hot spot) are frequently found in T6SS effector proteins harbouring an additional PAAR or VgrG interacting domain (VIRN) at the N-terminus (Cianfanelli et al., 2016; Pei et al., 2020). These T6SS effectors usually comprise rearrangement hotspot repeats at their N-termini and a nucleolytic toxic domain at their C-termini. Structural analyses suggested that Rhs repeats form a shell structure encapsulating and protecting secreting cells from the C-terminal toxic domain (Alcoforado Diniz & Coulthurst, 2015). Upon secretion, Rhs T6SS effectors undergo two autocleavages at the C and N-terminal ends, releasing the toxin (Kielkopf et al., 2024). Rhs loci were named after

their high propensity for recombination rearrangements, suggesting a mechanism for the diversification of the toxic C-terminal domain (R. J. Lin et al., 1984).

T6SS effectors: antimicrobial and nutrient uptake activities

The T6SS effectors' activities determine the T6SS functions, as described in the previous section, namely subverting eukaryotic host cells, nutrient uptake, antimicrobial activities, etc. Some effectors can target molecules conserved in both prokaryotic and eukaryotic cells, such as cell membrane components, and are called trans-kingdom effectors (Russell et al., 2013). This section will focus essentially on T6SS effectors with antimicrobial and nutrient uptake activities.

Antimicrobial T6SS effectors have been subdivided into two groups according to their activity acting either in the periplasm as lytic toxins (phospholipase, peptidoglycan hydrolase, pore-forming, etc.) or in the cytoplasm (DNases, energy balance, etc.) and are listed in Table 5 (Jurénas & Journet, 2021) (Fig.6B). Periplasmic toxins inducing cell lysis have several advantages in static environment (Jurénas & Journet, 2021). A rapid cell prey lysis prevents the formation of a corpse barrier of accumulated dead cells, which would have blocked attacks towards remaining preys (W. P. J. Smith et al., 2020). Lytic toxins also enable the release of DNA in the environment, facilitating the acquisition of new genetic material by transformation (Borgeaud et al., 2015). By contrast, cytoplasmic non-lytic toxins do not cause rapid cell lysis, but efficiently target vital biological pathways. T6SS toxins can either lead to growth arrest by impacting cell division or complete cell death (Wood et al., 2019).

Table 5. List of periplasmic, cytoplasmic and extracellular space T6SS effectors and their enzymatic activity.

Name	Enzymatic activity
Periplasmic-acting effectors	
type VI lipase effector (Tle)	Hydrolyse the membrane phospholipids - Tle1-4 bear phospholipase A1 (cleavage at sn1 bond) and/or A2 activities (cleavage at sn1 bond) - Tle5 bears phospholipase D activities (cleave at sn3 bond)
Type VI membrane disrupting effectors/ pore-forming effectors (Tme)	Induce membrane depolarisation by inserting into the inner membrane
Type VI glycoside hydrolase (Tge)	Degrade the glycan backbone of peptidoglycan
Type VI amidase effectors/ peptidase (Tae)	degrade the peptide bridges of peptidoglycan
Type VI peptidase effectors/ zinc-dependent metallopeptidase (Tpe)	Broad range of periplasmic and cytoplasmic targets
Cytoplasmic-acting effectors	
Type VI DNase effectors (Tde)	Hydrolyse DNA ; Families: HNH, PoNe, Ntox, Tox-Rease, etc.
DS-DNA deaminase toxin A (DddA)	Induce mutations in dsDNA by converting cytosine to uracil
Type VI NADase effector (Tne)	NAD(P)+ glycohydrolase activity
Type VI effector (p)ppApp synthetases (Tas)	Pyrophosphorylates adenosine nucleotides (ATP, ADP or AMP)
Extracellular space-acting effectors	
Metallophore effectors	Chelating trace metal ions: copper, manganese, zinc, etc.

Among the periplasmic-acting effectors, type VI lipase effector (Tle) hydrolyse the membrane phospholipids and can be classified into five phylogenetic families (Tle1-5) (Russell et al., 2013). Tle1-4 bear phospholipase A1 (cleavage at sn1 bond) and/or A2 activities (cleavage at sn1 bond), while Tle5 bears phospholipase D activities (cleave at sn3 bond) (Flaugnatti et al., 2016). Cells targeted by Tle usually exhibit shrinking, inflation and eventually lysis (Ringel et al., 2017). Some Tle function as trans-kingdom effectors, such as *P. aeruginosa* Tle4 (TlE) disrupting the endoplasmic reticulum (Jiang et al., 2014, 2016). Type VI membrane disrupting effectors (Tme) (syn. pore-forming effectors) induce membrane depolarisation by inserting into the inner membrane, and usually result in growth arrest instead of cell lysis (Fridman et al., 2020). One striking example is VasX in *V. cholerae*, sharing homology with *E. coli* colicins, which works in conjunction with the adaptor VasW and is neutralised by its immunity protein TsiV (Miyata et al., 2013). VasX is required for compromising bacterial competitor membranes, but also for virulence against amoeba (Miyata et al., 2011). T6SS effectors degrading the peptidoglycan include type VI glycoside hydrolase (Tge) targeting the glycan backbone and type VI amidase (syn. peptidase) effectors (Tae) targeting the peptide bridges (Jurénas & Journet, 2021). Tge hydrolyse the N-acetylmuramic acid (NAM)-N-acetylglucosamine (NAG) glycan strands, acting as a muramidase (Whitney et al., 2013). One characterised Tge is the *V. cholerae* VgrG3 harbouring a lysozyme-like domain fused to VgrG (Dong et al., 2013). Tae either display D,L-endopeptidase (Tae1 and Tae4) or D,D-endopeptidase (Tae2-3) activity (Jurénas & Journet, 2021). Finally, type VI peptidase effectors (Tpe; syn. zinc-dependent metallopeptidase) have a broad range of periplasmic (e.g., lipoproteins) and cytoplasmic targets (D. Zhang et al., 2012).

Among the cytoplasmic-acting effectors, type VI DNase effectors (Tde) belong to families, such as HNH, PoNe, Ntox, or Tox-Rease, and hydrolyse DNA without specificity (Jablonska et al., 2017; Jana et al., 2019). Cells attacked by DNase toxins might undergo cell filamentation driven by SOS response (Jurénas & Journet, 2021). DS-DNA deaminase toxin A (DddA) induces mutations in dsDNA by converting cytosine to uracil, which initiates base excision repair leading to a CG>TA mutations (Mok et al., 2020). Type VI NADase effector (Tne) have NAD(P)⁺ glycohydrolase activity, thereby depleting the cells from essential NAD(P) and redox coenzymes, leading to growth inhibition while keeping the cell envelope intact (Whitney et al., 2015). Among type VI effector (p)ppApp synthetases (Tas), Tas1 pyrophosphorylates adenosine nucleotides (ATP, ADP or AMP), intoxicating the cell by ATP depletion and accumulation of (p)ppApp (Ahmad et al., 2019). Among type VI ADP-ribosyltransferase effectors (Tre), Tre1 ribosylates arginine residues of FtsZ bacterial tubulin hampering cell division, causing filamentation and even cell lysis (Ting et al., 2018). Its immunity protein Tri1 has a dual function, which not only binds its cognate toxin Tre1 but also reverses toxin-induced ADP-ribosylation through its hydrolase domain (Ting et al., 2018).

Finally, by secreting metallophore effectors into the extracellular space, the T6SS allows bacteria to compete for trace metals, such as copper, manganese or zinc, without direct cell contact. These captured metals are then imported via TonB-dependent transporters, thereby sustaining growth under scarce conditions (X. Yang et al., 2021).

Immunity and non-immunity-dependent protection for T6SS attacks

Immunity proteins are typically small, single-domain and highly specific molecules, that block the active site of their cognate toxin (Whitney et al., 2013) or switch it into an inactive conformation (Lu et al., 2014). Immunity proteins that neutralise periplasmic-acting toxins are also localised in the periplasm, often as membrane-anchored lipoproteins or periplasmic proteins (Flaughnatti et al., 2016). Some bacteria having lost their T6SS, might still retain orphan immunity genes, independently regulated from the previously present T6SS core genes, allowing protection without the potential cost of maintaining the secretion apparatus (Kirchberger et al., 2017). In *V. cholerae*, successive insertion of novel effector–immunity (E–I) modules can displace ancestral effector genes while conserving immunity genes, which are shifted downstream, and gradually leading to the evolution of long arrays of multiple immunity genes (Kirchberger et al., 2017). In mixed bacterial populations, T6SS producer (T6SS⁺, T6I⁺), resistant (T6SS⁻, T6I⁺) and susceptible (T6SS⁻, T6I⁻) strains are thought to follow rock-paper-scissors dynamics (Robitaille et al., 2023). This game theory relies on the fact that producing T6SS and its immunity proteins carries an energetic cost. As a result, producer cells are outcompeted by the resistant strain that do not produce T6SS. The resistant strain is outcompeted by susceptible cells that does not produce T6SS or carry immunity genes, and the producer outcompetes the susceptible cells that does not carry the immunity genes (X. B. Lin et al., 2024).

The binding of immunity proteins to their effector targets is not instantaneous, which could, in the meantime, trigger fatal cell damage (Hersch, Manera, et al., 2020). In *Vibrio* sp., a WigKR two-component system functions in parallel, repairing the envelope while waiting for the immunity to neutralise the effector (Dörr et al., 2016; Hersch, Watanabe, et al., 2020). Indeed, some immunity-independent T6SS resistance have also been reported. Firstly, intrinsic resistance can occur when the bacterial target of the effector is absent or structurally divergent (Hersch, Watanabe, et al., 2020; Le et al., 2020). Secondly, envelope stress response may sense T6SS damages to the cell and initiate responses, such as “tit-for-tat” T6SS counterattack (Basler et al., 2013), induction of EPS production (Toska et al., 2018), or induction of WigKR repair mechanisms (Hersch, Watanabe, et al., 2020). Thirdly, spatial separation can protect co-existing strains in different ways. Strains can thrive in different host tissues (Speare et al., 2018). Spatial protection by a corpse barrier of dead non-lysed cells at the interface between T6SS killer and susceptible cells can block killers from reaching remaining susceptible cells (W. P. J. Smith et al., 2020). “Dienes lines” are macroscopic boundaries at the intersection between two

unrelated swarming *Proteus* spp. strains and are also the result of spatial separation based on T6SS-dependent killing. (Alteri et al., 2017). Additionally, time-lapse microscopy revealed that T6SS-driven antagonism between *V. cholerae* and *Aeromonas hydrophila* push mixed populations to segregate into distinct single-species clusters, where killing is restricted to boundaries (Wong et al., 2016). Fourthly, access to abundant nutrients might allow bacteria to grow fast, which might outperform the T6SS killing rate (Hersch, Manera, et al., 2020). As discussed previously, additionally, starving cells of *M. xanthus* show lower immunity gene expression and are more susceptible to T6SS killing (Troselj et al., 2018). A fifth immunity-independent protection is using the intrinsic cell capsule as physical armour. The extracellular polysaccharides (EPS) produced by *V. cholerae* act as a protective shield that blocks incoming T6SS attacks while leaving its own secretion system fully functional (Granato et al., 2023; Toska et al., 2018). Despite thicker cell wall, Gram-positive T6SS-dependent killing has been reported. For instance, *A. baumannii* kills *B. subtilis*, *Listeria monocytogenes*, and *Staphylococcus aureus* by secreting a Tse4 bifunctional enzyme with lytic transglycosylase and endopeptidase activities (Le et al., 2021).

1.3.1.8 T6SS functions and effectors within *Xanthomonadales*

This section focusses on the different T6SSs roles experimentally studied within the *Xanthomonadales* order and summarised in Table 6.

Resistance to amoebal predation

Beyond its endophytic phase, *Xanthomonas citri* is found as an epiphyte on leaf surfaces and in soil containing decaying plant material, which are environments shared with predatory amoeba that feed on bacteria (Bayer-Santos et al., 2018). *X. citri* T6SS-i3* was functionally characterised with an anti-eukaryotic function, as required for resistance to amoebal predation (Bayer-Santos et al., 2018). T6SS-i3* was shown to be regulated by a novel signalling cascade, an extracytoplasmic EcfK sigma factor, activated by a serine/threonine kinase PknS (Bayer-Santos et al., 2018). Another study confirmed that *X. citri* T6SS-i3* core genes and its regulator EcfK are specifically induced during epiphytic growth on leaf surfaces, likely in response to environmental signals or protist-associated threats, while remaining at low expression levels inside the plant mesophyll (Ceseti et al., 2019). Although another ECF factor gene, *ecfL*, was also found located near the T6SS cluster, transcriptomic data and predation assays demonstrated that it does not influence T6SS-i3* expression or resistance to bacterivorous amoeba (dos Passos Lima et al., 2022).

Antimicrobial competition

Various reverse genetic studies have shown the role of *Xanthomonadales* T6SS-i4 in bacterial killing through competition assays. The non-vascular *X. oryzae* pv. *oryzicola* T6SS-i4 was first evidenced for killing *E. coli* DH5 α and *Ochrobactrum oryzae*, a rice endophytic bacterium (Zhu et al., 2020). In this study, T6SS-i4 expression was shown to be expressed in all tested conditions, including different media type (NB, XOM3), temperature, and pH (Zhu et al., 2020). An initial study reported that the vascular *X. oryzae* pv. *oryzae* strain PXO99 T6SS-i4 was not required for bacterial competition against *E. coli* DH5 α (Choi et al., 2020). By contrast, a subsequent study challenged these results by showing that T6SS-i4 from this same strain *Xoo* PXO99 is involved in bacterial competition against *E. coli* XL1-Blue (Shao et al., 2023). A third study confirmed the bacterial killing role of *Xoo* PXO99 T6SS-i4 *in planta* through TleA and TleB antibacterial effectors (Tan, Yang, et al., 2025). These contrasting killing results could be due to adaptative mutation, possibly illustrating the work of Taillefer *et al.*, who showed that T6SS activity can be lost after long-term pure cultivation in laboratory (Taillefer et al., 2025). Such discrepancies are not unprecedented, for instance, in *Dickeya solani*, divergent antimicrobial activities were reported by two European laboratories working on the same strain and were ultimately traced back to a single point mutation in a regulatory gene (Brual et al., 2023).

A recent study further predicted the whole T6SS effector repertoire of *Xoo* PXO99A with a total of 21 putative effectors (Tan, Zheng, et al., 2025). They functionally validated a T6SS VasX_N family effector (PXO_00500) with periplasmic toxicity and the cross-protection by two nearby cognate immunity proteins of the T6SS_Burk_exlF superfamily (PXO_RS08595 and PXO_RS08605). They confirmed its secretion through T6SS-i4 via the interactions with upstream encoded VgrG4 and adaptor (PXO_00501) (Tan, Zheng, et al., 2025). Interestingly, another neighbouring Vas_N family effector homolog (PXO_00498) showed host-specific toxicity, by intoxicating *Aeromonas dhakensis*, but not *E. coli*. Similar host-specific toxicities have already been highlighted in *V. cholerae* with TseH being non-toxic in *E. coli*, due to T6SS immunity-independent pathways (Hersch, Watanabe, et al., 2020). They further showed that *Xoo* T6SS-i4 was active against human and plant pathogens, and significantly killed *E. coli* and *R. solanacearum* in competition assays using a *Nicotiana benthamiana* infection model (Tan, Zheng, et al., 2025).

Additionally, *S. rhizophila* outcompetes and reduces seedling transmission of the pathogen *X. campestris* in radish in a T6SS-i4-dependent manner (Garin et al., 2024). *In vitro*, *S. rhizophila* competition against *X. campestris* increased over time from 6h to 24h. However, after 48h, strain *Xcc* 8004 partially escaped inhibition, likely by deploying non-immune defences such as dead cell barriers or EPS production that reduce contact-dependent killing (Garin et al., 2024; Hersch, Manera, et al., 2020; W. P. J. Smith et al., 2020). Competition only occurred on solid and not in liquid media, confirming that *S. rhizophila* T6SS-i4-mediated antagonism relies on contact-

dependent effector injection rather than on contact-independent mechanisms, such as nutrient scavenging (Garin et al., 2024). When co-inoculated on seeds, *S. rhizophila* significantly reduced *X. campestris* populations after 1 day on germinated seeds and on aerial and root parts of seedlings at 5 dpi (Garin et al., 2024). The high occurrence of T6SS in seed-associated bacteria highlights its important ecological role in shaping communities transmitted to seedlings (Garin et al., 2025). Further study on *in vitro* confrontation of *S. rhizophila* wild-type and T6SS mutant against varying synthetic seed bacterial communities showed that the T6SS of *S. rhizophila* CFBP 13503 targets a broad spectrum of competitors such as *Rhizobium*, *Plantibacter*, *Paenibacillus*, *Pantoea*, *Erwinia*, *Massilia*, *Oxalobacteraceae*, *Pseudomonas*, *Stenotrophomonas*, and *Xanthomonas* species (Garin et al., 2025). *In planta*, four strains were impacted by *S. rhizophila* T6SS-i4, belonging to *Pseudomonas*, *Oxalobacteraceae*, *Stenotrophomonas*, and *Pantoea*, only at the early germinated seed stage (Garin et al., 2025). The competition-relatedness theory postulates that competition is stronger between phylogenetically close species sharing similar functions and substrate affinities (Russel et al., 2017). In Garin et al. study, the phylogenetic relatedness to *S. rhizophila* partially accounted for their susceptibility to T6SS (Garin et al., 2025). The opportunistic human pathogen *S. maltophilia* also assembles a functional antibacterial T6SS-i4 (Crisan et al., 2023). Interestingly, the competitive outcome between two bacterial species was shown to vary depending on whether the strains were co-isolated from the same source or obtained from distinct ones (Crisan et al., 2023). This was exemplified by *S. maltophilia* STE00241 competing against *P. aeruginosa* PSA01136 co-isolated from the same patient, but not with *P. aeruginosa* PA14 or PA32 isolated from different sources. In this first case, T6SS-i4 also participates in *S. maltophilia* survival during competition (Crisan et al., 2023).

Finally, *Lysobacter enzymogenes* has been shown to kill filamentous fungi and yeast using its short-range T6SS weapon in the soil microbiome (L. Lin, Shen, et al., 2025). A T6SS effector “Le1893” (D9T17_RS02785; WP_123645987.1) from the RHS family, harbouring PAAR, Rhs and AHH nuclease domains, was identified and participates in the antifungal activity, probably by reducing total DNA content (L. Lin, Shen, et al., 2025). While T6SS is assembled in nutrient-rich environment, *L. enzymogenes* T6SS is still expressed but not assembled in low-nutrient media (M. Yang et al., 2020). However, in these conditions, Hcp is required for full function of the long-range weapon, heat-stable antifungal factor (HSAF) (M. Yang et al., 2020). It has been shown that Hcp is required to bind and protect the CRP-like transcription factor Clp, which ultimately enhances production of HSAF (G. Xu et al., 2018; K. Xu et al., 2021; M. Yang et al., 2020). Hcp probably accumulates in the cytoplasm in the absence of T6SS assembly (L. Lin et al., 2021).

Virulence to plants

The role of T6SS in virulence to plants was also shown by mutation studies with T6SS-i3*** of *X. oryzae* pv. *oryzae* on rice (Choi et al., 2020), T6SS-i3* of *Xanthomonas phaseoli* pv. *manihotis* on cassava (Montenegro Benavides et al., 2021) and T6SS-i3* of *Xanthomonas perforans* on tomato (Prabha Liyanapathirana et al., 2022). Consistently, *Xoo* Δ T6SS-i3*** populations also showed a decrease in survival compared to the wild-type *in planta* (Choi et al., 2020). However, the precise mechanisms by which the T6SS contribute directly or indirectly to virulence remain unknown and deserve further exploration. Recently, one direct T6SS mechanism affecting plant reproduction was uncovered. *Xoo* PXO99A infecting nonhost *Arabidopsis* represses the plant reproduction, by interfering with ovule initiation and reducing the total seed number (J.-T. Yang et al., 2025). This phenotype is driven by the T6SS-i4 effector TleB (J.-T. Yang et al., 2025). Under normal conditions, plant E3 ligase PUB14 stabilises BZR1 to promote ovule initiation. However, during infection with *Xoo* PXO99A, PUB14 instead redirects its activity toward degrading the bacterial effector TleB, weakening stabilisation of BZR1, resulting in lower BZR1 levels and reduced ovule initiation, thereby prioritising defence over reproduction (J.-T. Yang et al., 2025). TleA shares high sequence similarity with TleB, both carrying Tle1 and DUF2235 domains, suggesting potential functional redundancy (Tan, Yang, et al., 2025). This expands the role of *Xoo* T6SS-i4 to both anti-eukaryotic and anti-prokaryotic functions with trans-kingdom effectors (Tan, Yang, et al., 2025; J.-T. Yang et al., 2025). From a broader perspective, if bacteria are to be exploited as biocontrol agents in nonhost plants, such reproductive side effects must be carefully considered (J.-T. Yang et al., 2025).

Role of individual genes on epiphytic survival and virulence to plants

In addition to studying the T6SS as a whole, some studies focused on individual T6SS-i3* genes to understand their versatile roles in the physiology and pathogenicity of the pathogen. Screening of a mariner transposon mutant library of *X. perforans* 91-118 identified a mutant disrupting the core gene TssM of the T6SS-i3* cluster, which caused faster symptom development and greater disease severity compared to the wild type (Prabha Liyanapathirana et al., 2022). Their study further observed that TssM contributes to prolonging asymptomatic epiphytic phase, notably by enhancing osmotolerance, thereby delaying symptoms and reducing overall disease severity, but ensuring pathogen dissemination (Prabha Liyanapathirana et al., 2022). Further transcriptomic analyses by the same group suggested that TssM of T6SS-i3* shapes a complex regulatory cascade by influencing global regulators, such as CsrA, RpoN, or Pho regulon, which in turn coordinate the expression of genes linked to virulence and metabolic functions (Ramamoorthy et al., 2024). It remains unclear whether these observed phenotypes with *tssM* mutants arise from the intrinsic role

of core components, or rather from the loss of a functional T6SS-i3* delivering effectors linked to those phenotypes.

Physiological roles

Other functions have been evidenced as impacted by T6SS-i3* individual genes (TssM or ClpV), including *in vitro* motility (Y. Li et al., 2024; Montenegro Benavides et al., 2021; Ramamoorthy et al., 2024), growth *in vitro* (Montenegro Benavides et al., 2021), biofilm formation (Y. Li et al., 2024), adherence ability (Y. Li et al., 2024), EPS synthesis (Y. Li et al., 2024), sensitivity to environmental stresses (NaCl, SDS, H₂O₂) (Y. Li et al., 2024) and antibiotics resistance (Y. Li et al., 2024). However, for instance, motility attenuation after mutation of ClpV is not due to a non-functional T6SS since other T6SS mutants, such as *tssM*, *hcp* or *vgrG*, showed no motility defects (Montenegro Benavides et al., 2021).

Table 6. List of functions experimentally tested for T6SSs and X-T4SS in *Xanthomonadales*

Strains	T6SS/ X-T4SS presence	Role experimentally tested				Reference
		Bacterial killing/ growth inhibition	Protection against amoeba predation	Fungi killing	Virulence on plant/ human cells	
<i>X. citri</i> pv. <i>citri</i> 306	T6SS-i3*	X No killing activity against <i>E. coli</i>	✓ Regulated by EcdK sigma factor and PknS	X No killing activity against yeast	X No virulence role on sweet orange	Bayer-santos 2018, Cesetti 2019, Lima 2022
	X-T4SS	✓ Killing activity against <i>E. coli</i> and <i>Chromobacterium violaceum</i> , interstrains competition				Souza 2015, Oka 2024
<i>X. citri</i> pv. <i>citri</i> zlm1908	T6SS-i3*				X No virulence role of on citrus leaves	Li 2024
	X-T4SS					
<i>X. oryzae</i> pv. <i>oryzicola</i> GX01	T6SS-i3***	X No killing activity against <i>E. coli</i> DH5α		X No killing activity against yeast	X No virulence role on rice, no difference in HR on tobacco	Zhu 2020, Liao 2019
	T6SS-i4	✓ Killing activity against <i>E. coli</i> and <i>Ochrobactrum oryzae</i>		X No killing activity against yeast	X No virulence role on rice, no difference in HR on tobacco	Zhu 2020, Liao 2019
<i>X. oryzae</i> pv. <i>oryzae</i> PX099	T6SS-i3***	X No killing activity against <i>E. coli</i> DH5α, <i>Burkholderia</i> , <i>Ralstonia</i> X No killing activity against <i>E. coli</i> XL1-Blue and Xcc 8004			✓ Virulence role on rice	Choi 2020, Shao 2022
	T6SS-i4	X No killing activity against <i>E. coli</i> DH5α, <i>Burkholderia</i> , <i>Ralstonia</i> ✓ Killing activity against <i>E. coli</i> XL1-Blue, but not Xcc 8004 ✓ Killing activity against <i>E. coli</i> MG1655 and <i>P. syringae</i> pv. <i>tomato</i> DC3000 in <i>planta</i> , <i>R. solanacearum</i> , <i>E. coli</i> ETEC and DAEC, <i>Citrobacter rodentium</i>			X No virulence role on rice ✓ Supress non-host plant reproduction	Choi 2020, Shao 2022, Yang 2025, Tan 2025a, Tan 2025b
<i>X. phaseoli</i> pv. <i>manihotis</i> CIO151	T6SS-i3* X-T4SS				✓ Virulence role on cassava	Montenegro benavides 2021

Strains	Role experimentally tested					Reference
	T6SS/ X-T4SS presence	Bacterial killing/ growth inhibition	Protection against amoeba predation	Fungi killing	Virulence on plant/ human cells	
<i>X. perforans</i> AL65	T6SS-i3*				✓ TssM decreases virulence on tomato, but increases epiphytic fitness (by higher osmotolerance), and increases dissemination	Liyanapathiranaige 2021, Ramanamorthy 2023
	T6SS-i3***					
	X-T4SS					
	T6SS-i3*				X No virulence role on pepper and tomato	Abendroth 2017
<i>X. euvesicatoria</i> 85-10	T6SS-i3***				X No virulence role on pepper and tomato	Abendroth 2017
	Incomplete X-T4SS (virB1-5 and virB10-11 missing)	X No killing activity against <i>E. coli</i>				Drehkopf 2023
	X-T4SS	✓ Killing activity against <i>E. coli</i> , <i>K. pneumoniae</i> , <i>S. typhi</i> , <i>P. aeruginosa</i> (environmental and clinical isolates), <i>P. mendocina</i> and dueling against <i>X. citri</i> , but not killing activity against <i>P. fluorescens</i> , <i>P. putida</i> , and <i>P. stutzeri</i>				Bayer-santos 2019, Cobe 2024, Nas 2019, Nas 2021
<i>Stenotrophomonas maltophilia</i> K279a						
<i>Stenotrophomonas rhizophila</i> CFBP13503	T6SS-i4	✓ Killing activity against Xcc 8004 <i>in vitro</i> and in germinated seeds and seedlings, and limits its transmission from radish seeds to seedlings ✓ Killing activity role against <i>Enterobacteriales</i> , <i>lysobacteriales</i> , <i>Burkholderiales</i> , <i>Pseudomonadales</i> , and <i>Hyphomicrobiales</i> Competition only on solid media, not in liquid culture				Garin 2023, Garin 2025

Strains	T6SS/ X-T4SS presence	Role experimentally tested				Reference
		Bacterial killing/ growth inhibition	Protection against amoeba predation	Fungi killing	Virulence on plant/ human cells	
<i>Stenotrophomonas maltophilia</i> STEN00241		✓ Killing activity against <i>E. coli</i> DH5α, <i>Burkholderia cenocepacia</i> , but not <i>Staphylococcus aureus</i> <i>in vitro</i>				Crisan 2023
		✓ Killing activity against <i>P. aeruginosa</i> strain PSA01136 co-isolated from the same patient, but not strain from other sources strains PA14, PA32)				
	T6SS-I4	Competition only on solid media, not in liquid culture				
<i>Lysobacter enzymogenes</i> OH11		X No killing activity against <i>E. coli</i> BL21		✓ Killing activity against filamentous fungal pathogens: <i>F. graminearum</i> , <i>F. oxysporum</i> , <i>A. alternata</i> , and <i>C. glaucosporioides</i>		Lin 2024, Yang 2020, Shen 2021, Lin 2025
	T6SS-I4			✓ Killing activity against yeast		
		✓ Killing activity against <i>E. coli</i> and <i>soliborne</i> biocontrol bacterial strains <i>P. fluorescens</i> 2P24 and <i>P. protegens</i> Pf-5 and the plant pathogenic bacterial strain <i>P. carotovorum</i> PccS1)		Non-toxic LtAE effector into <i>P. protegens</i> , leading to enhanced production of the antifungal compound 2,4-DAPG by <i>P. protegens</i> against <i>Trichoderma guizhouense</i>		
<i>Lysobacter enzymogenes</i> C3	X-T4SS	✓ Non-toxic LqQE1 effector for quorum quenching in <i>P. fluorescens</i> , facilitating killing of free living cells				Shen 2021, Liao 2023, Wang 2023
	T6SS-I4					
	T6SS-I3***					
<i>Lysobacter enzymogenes</i> C3				✓ Inhibition of fungal hypha growth and spore germination and growth		Yu 2019
	X-T4SS					

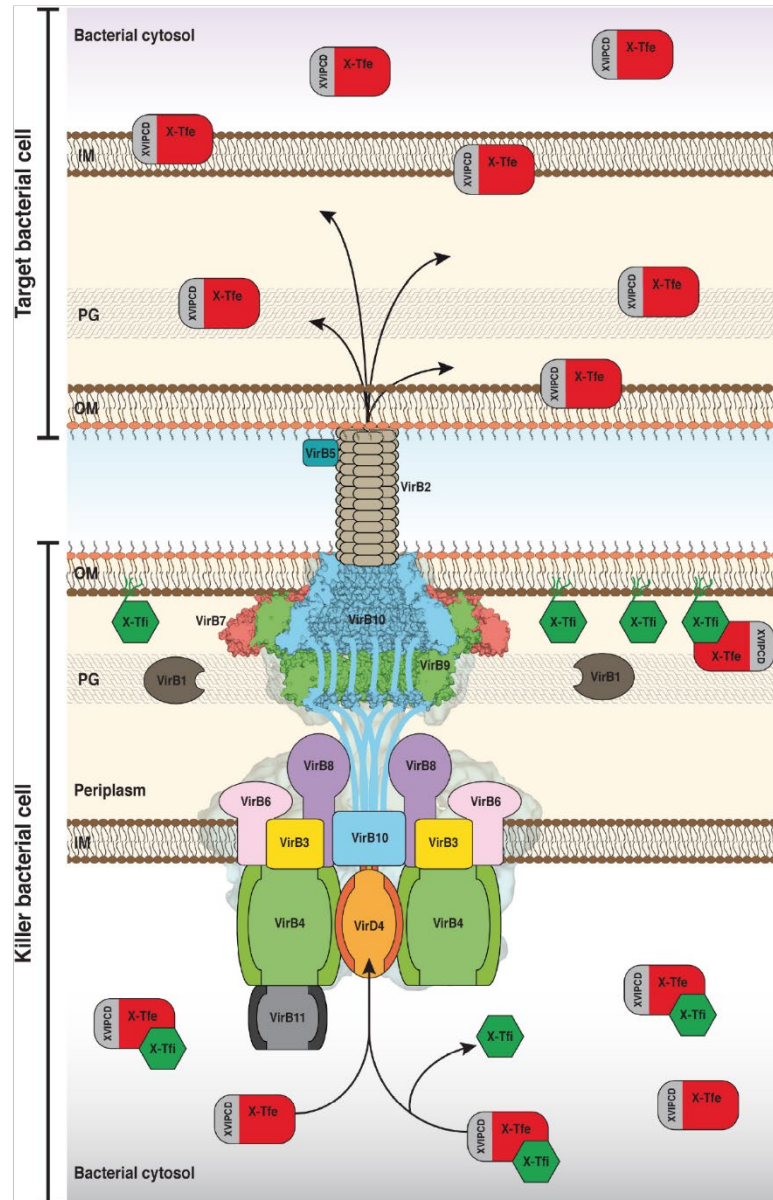
1.3.2 *Xanthomonadales*-like Type IV secretion system (X-T4SS)

The T4SS was first described for its role in conjugation in 1946 by Edward Tatum and Joshua Lederberg (Lederberg & Tatum, 1946). Since then, T4SS has also been shown to be a key virulence factor in many human and plant diseases (Y. G. Li & Christie, 2018), or even as a system for DNA uptake in *Helicobacter pylori* (Hofreuter et al., 2001) or DNA release in *Neisseria gonorrhoeae* (Hamilton et al., 2005). In the field of phytopathology, *Agrobacterium tumefaciens* T4SS is the most widely studied, injecting nucleoprotein complexes into eukaryotic host cells. Interestingly, a new specialised X-T4SS was shown to present antibacterial activities through the secretion of toxic effectors, while exhibiting several structural features that distinguish it from the canonical conjugative T4SS (Souza et al., 2015). First characterised in *X. citri*, several other similar X-T4SSs have since been identified *in silico*, mostly among other members of the *Xanthomonadales* and the system was accordingly called *Xanthomonadales*-like T4SS (X-T4SS) (Sgro et al., 2019; Souza et al., 2015).

1.3.2.1 X-T4SS structure

T4SS are conveniently classified into two classes: Class A systems, which generally comprise the 12 VirB1–11/VirD4 proteins, and Class B systems, which are typically much larger, such as the 27-component Dot/Icm system from *L. pneumophila* (Waksman, 2019). The X-T4SS is composed of 12 structural components (VirB1–VirB11 and VirD4), similarly to class-A T4SSs (Sgro et al., 2018, 2019). It mainly consists of a core complex (VirB7, VirB9 and VirB10) and an inner membrane complex (VirB3, VirB6 and VirB8), anchored in the outer and inner membrane, respectively. The connection between both large subcomplexes is established through VirB10 proteins (Sgro et al., 2018, 2019; Souza et al., 2011). The extracellular pilus is composed of VirB2 and VirB5 subunits, while VirB1 acts as a periplasmic transglycosidase during T4SS biogenesis (Dunger et al., 2016; Sgro et al., 2019). The energy necessary for secretion is supplied by three ATPases VirB4, VirB11 and VirD4. Additionally, VirD4 also serves as a loading site for effectors (Sgro et al., 2019) (Fig. 7A).

A



B

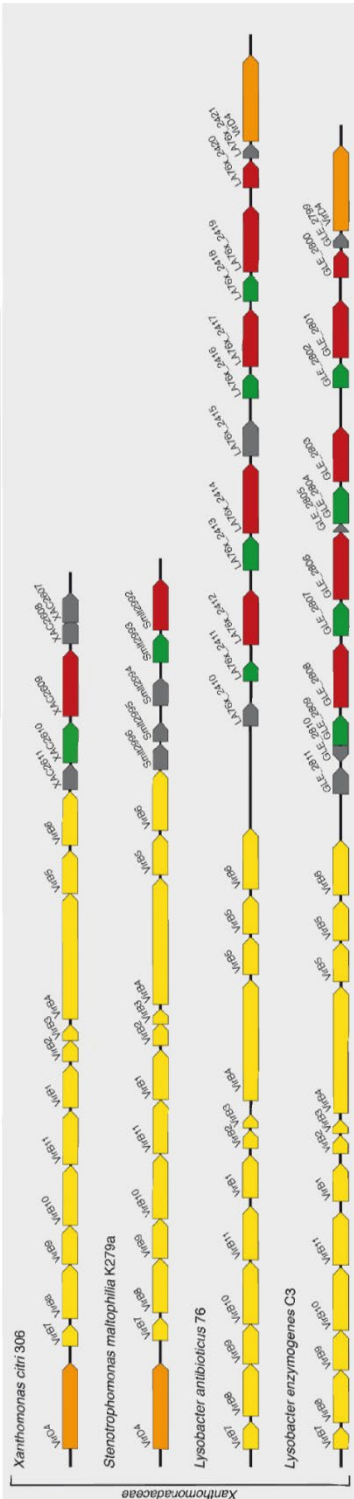


Figure 7 : X-T4SS core components. (A) Model of X-T4SS structure and function at the interface of two bacterial cells. The killer cell carries an X-T4SS, whose architecture is based on the R388 T4SS negative-stain electron microscopy (EM) map and the *X. citri* X-T4SS core complex structure by cryogenic EM (Sgro *et al.*, 2018). Effectors (X-Tfes) bind VirD4 via their XVIPCD domains and are translocated into the target cell leading to growth inhibition or cell death. Cognate immunity proteins (X-Tfis) prevent self-intoxication. (B) Genetic organisation of the *X. citri* X-T4SS gene cluster and homologous loci in other *Xanthomonadaceae* species. X-T4SS core genes are highlighted in yellow (*virB*) and orange (*virD4*), while effectors and their immunity partners are shown in red and green, respectively, neighbouring genes of unknown function are indicated in gray (Courtesy of C. S. Farah; from Sgro *et al.*, 2019).

1.3.2.2 X-T4SS evolutionary origin

Toxin-antitoxin modules are frequently found encoded on plasmids as “addiction systems”. A stable toxin and a short-lived antitoxin act together to ensure vertical plasmid inheritance by making the cell dependent on the plasmid-encoded antitoxin for survival (Harms *et al.*, 2018). The most plausible hypothesis for the origin of the X-T4SS is that it evolved from a conjugative T4SS through the integration of a relaxosome domain within a toxin-antitoxin system which, after interaction with the VirD4 protein, caused the X-T4SS to secrete a toxin instead of transferring the plasmid into the target bacterium (Alvarez-Martinez *et al.*, 2020). However, these toxin-antitoxin modules are widespread among bacteria. Therefore, it would not be surprising that other bactericidal T4SSs, relying on distinct effector recognition motifs, may have independently evolved multiple times across diverse bacterial lineages (Sgro *et al.*, 2019). Indeed, recent research witnessed the role of a novel unrelated type IVB secretion system (T4BSS) in *P. putida* for bacterial killing resulting in biofilm invasion and biocontrol activities (Gordils-Valentin *et al.*, 2024; Purtschert-Montenegro *et al.*, 2022). In *Pectobacterium*, both T4SS and T6SS, evolutionarily distant from the X-T4SS and T6SS of *Xanthomonas*, redundantly secrete effectors to drive bacterial competition (Che *et al.*, 2024). Finally, evidence for another antibacterial T4SS was found in *Bartonella schoenbuchensis* (Harms *et al.*, 2017).

1.3.2.3 X-T4SS functions and effectors within *Xanthomonadales*

This section focusses on the different X-T4SS roles experimentally studied within the *Xanthomonadales* order and summarised in Table 6.

Interbacterial competition

The antibacterial activity of X-T4SS has been demonstrated in a few bacterial species. The citrus pathogen *X. citri* was first shown to kill other Gram-negative bacteria, such as *E. coli* and *Chromobacterium violaceum*, in a X-T4SS dependent manner (Souza et al., 2015). The opportunistic human pathogen *S. maltophilia* was also shown to compete with *E. coli*, *K. pneumoniae*, *S. typhi* and *P. aeruginosa* (Bayer-Santos, Cenens, et al., 2019). Killing of *P. aeruginosa* was not affected by the origin of the isolates, either environmental or clinical (Nas et al., 2019). However, the killing outcome varied among different species, such as killing *P. mendocina*, but not *P. fluorescens*, *P. putida* or *P. stutzeri* (Nas et al., 2019). The bactericidal activity of *S. maltophilia* X-T4SS relies almost entirely on X-tfes TfcA (syn. RS14245) and TfcB (syn. RS14255; glycosyl hydrolase GH-19 chitinase), since deleting both genes abolished killing to the same extent as removing the whole X-T4SS (Nas et al., 2021). However, a GHH-nuclease was also shown to be involved in X-T4SS-dependent killing in this same strain (Cobe et al., 2024). Chlorophenol red- β -D-galactopyranoside (CPRG)-based colorimetric assays and confocal microscopy were used to monitor X-T4SS killing activity of *X. citri* and of *S. maltophilia* in real time. Time-lapse imaging confirmed the contact dependence of the system and highlighted rapid and violent death of target cells by shrinking, which contrasted with the more diverse morphological changes observed for T6SS-mediated killing (cell rounding, blebbing, etc.) (Bayer-Santos, Cenens, et al., 2019; Ho et al., 2013; Souza et al., 2015). A X-T4SS-dependent duelling was also observed between *X. citri* and *S. maltophilia*, which can kill each other, further indicating different X-Tfis repertoires (Bayer-Santos, Cenens, et al., 2019). Moreover, expression of the VirD4 coupling protein from *X. citri* restored functionality in the *S. maltophilia* $\Delta virD4$ mutant, showing that effectors are not strictly species-specific but can be transferred across different bacterial X-T4SS (Bayer-Santos, Cenens, et al., 2019). It is worth noting that the multidrug-resistant *S. maltophilia* is naturally competent, and that X-T4SS-mediated cell lysis may increase opportunities for acquisition of foreign DNA (Bayer-Santos, Cenens, et al., 2019).

Cis-intoxication (syn. self-intoxication, suicide) can be distinguished from *trans*-intoxication, in which a bacterial cell intoxicates another kin cell (syn. fratricide between sister cells) or non-kin cell (interstrains, interspecies). A recent study showed that X-T4SS immunity proteins are dedicated to protecting cells against *cis*-intoxication, while they are not absolutely required to protect against *trans*-intoxication of kin cells (Oka et al., 2024). Monoculture of *X. citri* X-tfi XAC2610 mutants suffered from autolysis and inhibition of biofilm formation in *cis* (Oka et al., 2024). However, *X. citri* lacking immunity genes were still resistant to X-T4SS-

dependent killing by *X. citri* wild-type bacteria, suggesting another mechanism than immunity might be at play in *trans*-intoxication (Oka et al., 2024).

Antifungal activity

Beyond antibacterial activities against soil-borne bacteria (Shen et al., 2021), *L. enzymogenes* X-T4SS also performed antifungal activities, by inhibiting fungal hypha growth and spore germination and growth (L. Lin et al., 2021; Yu et al., 2020). X-T4SS mutants also showed a decrease in extracellular enzyme activity and no HSAF production (Yu et al., 2020).

Signal transduction

The X-T4SS of *L. enzymogenes* also plays a role in signal transduction through the secretion of non-toxic effectors. For instance, the effector LqqE1 is injected into the cytoplasm of *P. fluorescens*, where it interferes with quorum sensing, thereby blocking biofilm formation and ultimately facilitating the killing of free-living bacteria (J. Liao et al., 2023; L. Lin, Shao, et al., 2025). Conversely, other findings suggest that X-T4SS effectors may promote cooperative interactions. In this case, *L. enzymogenes* delivers the LtaE effector into *P. protegens*, where it interacts with the transcriptional repressor PhlF, leading to enhanced production of the antifungal compound 2,4- diacetylphloroglucinol (DAPG) (L. Lin, Shao, et al., 2025; B. Wang et al., 2023).

Virulence

Beyond antibacterial activity, X-T4SS in *S. maltophilia* was shown to reduce apoptosis of epithelial cells, while increasing apoptosis in human macrophages (Nas et al., 2019). To date, pathogenicity assays using X-T4SS mutants among different plant pathogens rules out an involvement of the system in virulence, as exemplified by *X. citri* on citrus, and *X. campestris* on different *Brassicaceae* (He et al., 2007; Souza et al., 2015).

X-T4SS Homeostasis

Additionally, production of X-T4SS by *X. citri* was shown to impose a notable physiological cost to the cell (Cenens et al., 2020). X-T4SS genes appear to be constitutively expressed in culture media (Alvarez-Martinez et al., 2020). X-T4SS homeostasis is controlled by the repressor CsrA, which maintains a constant density of X-T4SS in the cell envelope (Cenens et al., 2020).

1.3.2.4 X-T4SS distribution and genetic organisation

Homologous X-T4SS loci are largely restricted to the *Xanthomonadales* order, including the genera *Xanthomonas*, *Stenotrophomonas*, *Lysobacter*, *Dyella*, *Luteimonas*, *Pseudoxanthomonas*, *Thermomonas*, *Rhodanobacter*, *Luteibacter*, and *Frateuria* (Sgro et al., 2019). Surprisingly, rare additional homologous gene clusters were also found outside *Xanthomonadales*, sometimes restricted to a single strain, like in *Neisseria*, *Variovorax*, *Hydrogenophaga*, *Bacillus*, or *Acinetobacter*, suggesting HGT events, but remain uncharacterised (Sgro et al., 2019). Within the *Xanthomonas* genus, complete X-T4SS have been identified in various species, including clade 1 species, such as *X. translucens*, *X. hyacinthi*, or *X. sacchari*, and clade 2, such as *X. arboricola*, *X. citri*, *X. campestris*, or *X. phaseoli* (Alvarez-Martinez et al., 2020; Sgro et al., 2019). A few clusters were separated into two regions in the genome, as exemplified by *X. campestris* pv. *campestris* ATCC 33913 (Da Silva et al., 2002) or 8004 (Qian et al., 2005). In other strains, the system was incomplete or comprising pseudogenes, such as *X. fragariae* Fap21 or *X. euvesicatoria* 85-10 (Alvarez-Martinez et al., 2020; Drehtkopf et al., 2023; Sgro et al., 2019). The system was totally absent from all screened *X. oryzae* strains (Alvarez-Martinez et al., 2020; Sgro et al., 2019). Although both *Xylella* and *X. albilineans* are restricted to xylem tissues, this cluster is absent in all *Xylella* pathogens but present in *X. albilineans* (Sgro et al., 2019). Interestingly, antibacterial X-T4SS were found in genome-sequenced strains where T6SS-i4 is missing, as observed within the *Xanthomonas* genus and the *S. maltophilia* species (Alvarez-Martinez et al., 2020; Crisan et al., 2023). Furthermore, in *S. maltophilia*, X-T4SS and T6SS-i4 were found to be encoded at distinct genomic locations (Crisan et al., 2023).

In silico analyses demonstrate that all X-T4SS are chromosomally encoded mostly in a single gene cluster composed of the 12 core components (Fig.7B). Additional X-T4SS effectors and immunity proteins are either encoded in the neighbourhood or as orphan genes scattered throughout the genome (Sgro et al., 2019). No hallmarks of DNA conjugation transfer were found in the vicinity of the X-T4SS locus, such as relaxase or DNA origin of transfer (*oriT*) (Alegria et al., 2005). By contrast, some subunits of the *X. citri* X-T4SS exhibit distinctive structural features compared to their homologs in other class-A T4SSs, suggesting a new physiological role distinct from the horizontal transfer of genetic material between bacteria (Sgro et al., 2019). Firstly, VirB7 proteins are unusually large, ranging from 130 to 185 amino acids (~40 aa in other class-A T4SS) due to the acquisition of a globular NO domain at their C-terminus (Souza et al., 2011). This domain is also present in multiple transport systems T2SS (Korotkov et al., 2009), T3SS (Spreter et al., 2009), T4SS (Korotkov et al., 2009; Nakano et al., 2010), T6SS (Leiman et al., 2009), TonB-dependent receptors (Ferguson et al., 2006) or phages (Spagnuolo et al., 2010)) across Gram-negative bacteria, suggesting shared evolutionary pathways. Secondly, another atypical

feature is found in VirB8, ranging from 290 to 370 amino acids in size (<250 aa in other class-A T4SS), which carries unusually long AQGP-rich C-terminal extensions, particularly prevalent in *Xanthomonadales*, but also found in *Hydrogenophaga crassostreae* VirB8, although their biological role has yet to be clarified (Sgro et al., 2019). Fig.7B from Sgro et al. shows the X-T4SS gene cluster organisation diversity among *Xanthomonadales*, *Burkholderiales* and *Neisseriales* (Sgro et al., 2019). Within *Xanthomonadales*, the system is composed of a single operon from *virB7* to *virB6*. The locus is mainly followed downstream by T6SS candidate effector and immunity genes. By contrast, the position of *virD4* is more variable, either found upstream of *virB7* or downstream of candidate effectors-immunity modules (Sgro et al., 2019).

1.3.2.5 X-T4SS effector loading, delivery, activities and immunity

Given that VirD4 coupling protein mediates substrate recognition in T4SSs, the group from C. S. Farah had the insightful idea to exploit it as bait in yeast two-hybrid screens against a genomic library, aiming to identify substrates and better understand X-T4SS function (Alegria et al., 2005). A dozen proteins (later called X-Tfe for *Xanthomonadales*-like T4SS effector) have shown to interact with the VirD4 protein. All these X-Tfes share a common C-terminal domain made of 120 amino acids “*Xanthomonas* VirD4-Interacting Proteins Conserved Domains” (XVIPCD) as a secretion signal required to interact with VirD4 (Alegria et al., 2005). The XVIPCD of X-Tfes binds the central all-alpha domain of VirD4 and consists of two functional regions: a conserved N-terminal region mediating VirD4 interaction, and both termini contributing to effector translocation (Oka et al., 2021). Notably, the N-terminal region loses stability at pH ≤7, a feature that may promote unfolding of X-Tfes and passage through the acidic periplasm (Oka et al., 2021). Recognising XVIPCD as a hallmark motif of X-Tfes opened the way for bioinformatic mining of *X-tfe* genes across diverse bacteria. While the XVIPCD domain is always found at the C-terminus of X-Tfes, the N-terminus is more variable in size and composition. Such predicted N-terminal domains include periplasmic peptidoglycan glycohydrolase, lytic transglycosylase, peptidoglycan peptidase, lipase, or nucleases (Sgro et al., 2019). Many X-Tfes contain N-terminal extensions with no assigned domains, opening opportunities to uncover new functional domain families. The length of some X-Tfes is restricted to the XVIPCD domain, and appear as remaining fragments of ancestral X-Tfes, since they align with longer X-Tfes from other strains (Sgro et al., 2019). The diversity of X-Tfes raises the possibility that multiple effectors could be delivered into the same target cell, exerting complementary bactericidal activities (Sgro et al., 2019).

Additionally, cognate immunity genes, known as *X-tfi*, neutralise antibacterial effectors toxicity. The genes encoding X-Tfis are generally located upstream of their cognate *X-tfe* genes and are likely co-transcribed. X-Tfis are usually localised in the same subcellular compartment as their respective X-Tfes (Jurénas & Journet, 2021). In the case of periplasmic X-Tfes, the cognate inhibitors usually contain an N-terminal signal peptide with a lipobox motif. A conserved cysteine-containing lipobox is a consensus sequence recognized for the modification and processing of lipoprotein precursors (Tibor et al., 1999). Conversely, cytosolic effectors are often paired with immunity proteins that lack such signal sequences (Souza et al., 2015). Interestingly, X-Tfes with both actions in the periplasm and cytoplasm have been discovered. Further sequence analyses suggest that their cognate X-Tfis may be expressed in two forms, one periplasmic and one cytosolic, through alternative translation start sites. This arrangement would enable periplasmic inhibitors to block leaked or exogenous effectors, while cytosolic variants protect against intracellularly active toxins (Sgro et al., 2019). Other effector-immunity pairs have been uncovered. For instance, peptidoglycan degradation of the Gram-positive cell wall was shown *in vitro* using purified X-Tfes XAC2609 and XAC0466 with lysozyme-like and/or peptidoglycan amidase activities (Souza et al., 2015). The cognate immunity protein XAC2610 was shown to inhibit the lysozyme-like activity of XAC2609, which degrades the cell envelope and inhibits biofilm formation (Oka et al., 2024). Other described X-Tfes also include *S. maltophilia* Smlt3024, exhibiting periplasmic growth rate reduction activity of the target cells, and its cognate immunity protein Smlt3025 (Bayer-Santos, Cenens, et al., 2019).

The X-T4SS and T6SS effector-immunity pair repertoires appear to differ on the whole. However, in many respects, X-T4SS effector-immunity modules resemble those of T6SS, sharing similar activity domains but different system-specific secretion signals. This suggests cross-talk between both systems and shared immunity proteins to protect from both systems simultaneously (Bayer-Santos, DeMoraes Ceseti, et al., 2019; Sgro et al., 2019). This is exemplified by the *X. citri* X-T4SS XAC2609-XAC2610 pair highly similar to the *P. aeruginosa* T6SS Tse1-Tsi1 pair with peptidoglycan hydrolase activity (Russell et al., 2011; Souza et al., 2015). Additionally, while no known domain hits were found for *S. maltophilia* X-Tfes Smlt0332, a highly similar sequence to its N-terminus were found fused to VgrG domains in T6SS effectors, including some found in xanthomonads (Sgro et al., 2019). This observation has inspired approaches to discover new pairs of toxins and antitoxins (Shen et al., 2023).

Objectives

More than 100 years after its discovery, bacterial leaf streak caused by *Xanthomonas translucens* persists as an economically important disease of wheat and barley worldwide. Despite increased understanding of the *X. translucens* biology, effective control strategies are still lacking. The study of phytobiomes structure and microbial interactions has received heightened attention recently, especially for their implications in biocontrol. While antagonistic strategies have been extensively characterised in several well-studied plant-associated genera, a similar statement cannot be made for *Xanthomonas*. In particular, *in silico* analyses have revealed the presence of two contact-dependent antibacterial systems, the type IV (X-T4SS) and the type VI (T6SS) secretion systems, in the *X. translucens* complex (Alvarez-Martinez et al., 2020; Bayer-Santos, DeMoraes Ceseti, et al., 2019; Sgro et al., 2019). Both systems have been shown in other bacterial taxa to contribute to interbacterial competition by injecting toxic effectors into rival bacterial cells (Souza et al., 2015; Zhu et al., 2020), promoting survival within their ecological niches. However, their functional, evolutionary and ecological significance in *X. translucens* complex remains unexplored. Therefore, **the main objective of this thesis** is to better understand the competitive strategies of the *X. translucens* complex, including their evolutionary trajectories, effector arsenal diversity, cereal bacterial targets and competition dynamics *in planta*.

The ecological relevance for the evolution of two functionally convergent secretion systems, X-T4SS and T6SS-i4, in xanthomonads remains unclear. Whereas most other *Xanthomonas* species evolved only one antibacterial system between X-T4SS or T6SS-i4, *X. translucens* (recently considered a species complex) stands out by comprising strains with the X-T4SS and other strain with the T6SS-i4 in the same species, making it a valuable model for their study (Alvarez-Martinez et al., 2020; Bayer-Santos, DeMoraes Ceseti, et al., 2019; Sgro et al., 2019). Therefore, **the first aim of this thesis** was to evidence the functional role and explore evolutionary history of both systems in the *X. translucens sensu lato* model. In practical terms, we first explored the distribution of both systems within the *X. translucens* complex. Using mutational and fluorescence-based methods, we tested the role of *X. translucens* complex T6SS (clades i3 and i4) and X-T4SS for competition against the bacterial model *E. coli*, as well as real-time imaging by confocal microscopy. Finally, combined comparative genetic and phylogenetic analyses were used to investigate the evolutionary trajectories of each identified bacterial killing secretion system.

Given the current knowledge gap regarding antibacterial T6SS effectors in xanthomonads, **the second aim of this thesis** was to further describe the T6SS-i4

effector-immunity repertoire of the *X. translucens* complex. The complete genome of 31 T6SS-i4⁺ *X. translucens sensu lato* strains were screened for effectors. We took advantage of the *X. translucens* complex to compare differing ecological lifestyles in closely related strains with their respective T6SS-i4 effector diversity. The experimentally validated antibacterial *Xt* UPB513 T6SS-i4 was further selected for an in-depth characterisation of the bacteriolytic activity of its effectors, by heterologous expression in *E. coli*. Respective effector-immunity pairs were investigated through neutralisation assays and structural predictions.

Given the antibacterial role of *X. translucens* complex X-T4SS and T6SS-i4 against the bacterial model *E. coli*, **the third aim of this thesis** was to explore its killing activity against cereal-associated bacteria. Through pairwise confrontation assays, we compared the X-T4SS and T6SS-i4 ability to kill individual member of a cereal bacterial consortium. We further tested the impact of T6SS-i4 on *Xt* survival against these same bacterial competitors. Given that T6SS incompatibility groups of *Xt* strains with differing effectors repertoires had been observed, we tested the hypothesis that T6SS incompatible *Xt* might kill each other *in vitro* and *in planta*.

Chapter 2 covers the first research objective, presented in the form of a paper entitled “Functional replacement of ancestral antibacterial secretion system in a bacterial plant pathogen”, published in *Nature Ecology and Evolution*. **Chapter 3** addresses the second objective presenting the results of T6SS-i4 effector diversity in the *X. translucens* complex and their respective bacteriolytic activities. **Chapter 4** deals with the third objective showing the *Xt* inter- and intraspecies competitive interactions against individual members of a bacterial cereal community through secretion systems. Finally, **Chapter 5** draws the conclusion and suggests avenues for future studies.

Chapter II

Functional replacement of ancestral antibacterial secretion system in a bacterial plant pathogen

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For accessing supplementary videos

Abstract

Two distinct molecular machineries, T4SS and T6SS, have been found within the order *Xanthomonadales* to be involved in outcompeting other bacterial species through the secretion of toxic effector proteins. However, the ecological and evolutionary basis leading xanthomonads to evolve two secretion systems with such similar functions remain unclear. Here, we show that *Xanthomonas translucens* (*Xt*) lineages have switched from a X-T4SS-mediated to T6SS-i4-mediated bacterial killing strategy. T6SS-i4 was only found in *Xt* strains lacking X-T4SS and conversely, resulting in a patchy distribution of the two nanoweapons along the *Xt* phylogeny. Using genetic and fluorescence-based methods, we demonstrated that X-T4SS and T6SS-i4 are crucial for inter-bacterial competition in *Xt*, but not *Xt* T6SS-i3. Combined comparative genetic and phylogenetic analyses further revealed that the X-T4SS gene clusters have been subject to degradation and had multiple loss events, while T6SS-i4 was inserted through independent gain events. Overall, this research supports the ancestral state of X-T4SS and provides new insights into the mechanisms promoting *Xt* survival within their ecological niches.

Introduction

Bacteria have evolved diverse antibacterial weapons to eliminate competing organisms (Granato et al., 2019). They have adopted none, one or a combination of these competition strategies to thrive within mixed microbial populations, whether in the gut, insect, or plant microbiomes (Booth et al., 2023; Granato et al., 2019). Bacterial secretion systems that deliver toxic effectors directly into neighbouring bacteria via contact-dependent manner appear to be widespread. Their differing structural origins and respective toxin repertoires suggest that each system confers fitness advantages. However, the ecological and evolutionary rationale for adopting one strategy over another remains unclear.

The genus *Xanthomonas* is a large group of Gram-negative plant-associated bacteria causing diseases in more than 350 host plants worldwide (An et al., 2020; Jacques et al., 2016). Hitherto, two distinct families of secretion systems involved in antibacterial competition are found among xanthomonads, namely the T4SS (Souza et al., 2015) and T6SS (Zhu et al., 2020). Although both machineries require physical cell-to-cell contact, they differ markedly in distribution, architecture as well as for effector loading and delivery mechanisms (Bayer-Santos, DeMoraes Ceseti, et al., 2019; Sgro et al., 2019). Although the T4SS and T6SS effector-immunity pair repertoires appear to differ, the redundancy in some toxins sharing similar toxic domains also suggests crosstalk between both systems (Bayer-Santos, DeMoraes Ceseti, et al., 2019; Sgro et al., 2019).

The T6SS is identified in 25% of sequenced proteobacteria and is widely distributed, also among plant-associated pathogens, and symbionts (Boyer et al., 2009). This multiproteic complex is composed of 13 core structural components (TssA-M) and accessory elements (Bernal et al., 2018). The T6SS has a dynamic structure with a

membrane complex anchored in the bacterial envelope and a baseplate. The cytoplasmic tail capped by a spike is enclosed in a contractile sheath. The system releases effectors by puncturing the target cell membrane (Costa et al., 2015; Gallique et al., 2017). Interestingly, T6SS genesis exhibits different origins, with the baseplate and tail subcomplexes structurally related to T-even phages, whereas the membrane complex shares homology with the type IVb secretion system (T4bSS) (Allsopp & Bernal, 2023). In phytobacteria, T6SSs have been classified into five phylogenetic clades (i1-i5) (Bernal et al., 2018). Clade i3 was further subdivided in subclades i3*, i3** and i3*** (Bernal et al., 2018). T6SSs are widely distributed within the *Xanthomonadales* order, with identified representatives from clades and subclades T6SS-i1, i3*, i3**, i3*** and i4 (Alvarez-Martinez et al., 2020; Bayer-Santos, DeMoraes Ceseti, et al., 2019).

A specialised T4SS was also shown to have antibacterial activity via secretion of toxic effectors (Sgro et al., 2019). First characterised in *X. citri*, other chromosomal T4SSs have since been identified *in silico*, mostly among other members of the *Xanthomonadales* (Souza et al., 2015). The system was accordingly called *Xanthomonadales*-like T4SS (Sgro et al., 2019). The X-T4SS is composed of 12 structural components (VirB1–VirB11 and VirD4), similarly to class A T4SSs (Sgro et al., 2019). The secretion machinery mainly consists of the core complex, an inner membrane complex, anchored in the cell envelope, and an extracellular pilus (Sgro et al., 2018, 2019; Souza et al., 2011). The ATPase VirD4 serves as a loading site for effectors (Sgro et al., 2019).

Here we used *Xanthomonas translucens* (*Xt*), a pathogen on small grain cereals and grasses (Bragard et al., 1997; Goettelmann et al., 2022), as a model to investigate the evolution of bacterial killing secretion systems. We demonstrated a distinct distribution pattern of T6SSs and X-T4SS within *Xt*. Strikingly, this correlated with convergent roles of *Xt* X-T4SS and T6SS-i4 for inter-bacterial competition. *Xt* genomic resources have been exploited to evidence functional switches from one competition strategy to another. This work expands our current understanding on how closely related bacteria adapt their competition strategies to survive in competitive environments.

Results

X-T4SS and T6SS-i4 display mutually exclusive distributions

To explore the prevalence and distribution of T6SS and X-T4SS in *X. translucens*, 94 publicly available genome sequences were analysed for the presence of core component's homologs using SecReT6/ SecReT4 HMM search and BLAST tools (Altschul et al., 1990; Bi et al., 2013; J. Zhang et al., 2023). Two clusters of genes encoding for distinct T6SS clades were detected in *Xt* chromosomes. Phylogenetic analysis revealed that these clusters belong to subclade T6SS-i3*** (hereafter referred to as T6SS-i3) and to clade T6SS-i4 (Bernal et al., 2018). Collectively, we observed a heterogeneous distribution of T6SS-i3, T6SS-i4 and X-T4SS gene clusters along the species phylogeny (Fig. 1). In similar analyses, no T6SS-i4 core gene was found in any of the *Xanthomonas* plasmids available at NCBI.

Based on ANI phylogenetic analyses, *Xt* can be classified into three clades: Xt-I, Xt-II and Xt-III (Goettelmann et al., 2022). Xt-I genomic group (small grain cereals pathogens) comprises two sublineages *X. translucens* pv. *undulosa* (*Xtu*) and *X. translucens* pv. *translucens* (*Xtt*). All strains of *Xtu* harboured both T6SS-i3 and T6SS-i4, while *Xtt* strains possess T6SS-i3 and either T6SS-i4 or X-T4SS. The T6SS-i4 system was only found in *Xtt* LIN subgroup K1 (Heiden et al., 2023). Additionally, all X-T4SS from ANI Xt-I group presented at least one pseudogenised gene, most commonly *virD4*. Although there are only five genomes available, all strains belonging to the Xt-II genomic group (small grain cereals and grasses pathogens) contain both T6SS-i3 and T6SS-i4. Finally, T6SS and X-T4SS gene clusters were heterogeneous and varied in their distribution in clade Xt-III (grasses pathogens) with some containing either only T6SS-i4, only X-T4SS, or no T6SSs or X-T4SS cluster at all.

Interestingly, X-T4SS was present when T6SS-i4 was absent and vice versa. We hypothesise that there is a mutual exclusion between T6SS-i4 and X-T4SS, which was found in all *Xt* strain in our analysis. We find a significant negative correlation between both X-T4SS and T6SS-i4 loci (Case 1; posterior mean of X-T4SS = -70.570; MCMC p-value <5e-04 and Case 2; posterior mean of X-T4SS = -11.906; MCMC p-value <5e-04) supporting this hypothesis.

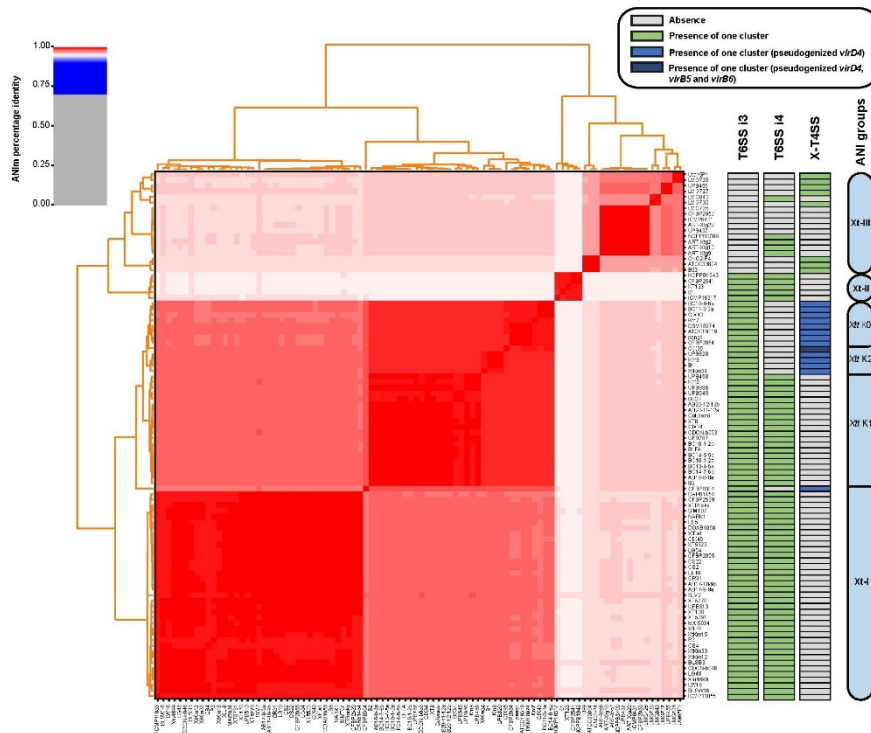


Figure 1: X-T4SS and T6SS-i4 show non-overlapping distribution patterns within *Xt* strains. To the left, the heatmap reflects the Average Nucleotide Identity (ANI) matrix among sequenced 94 strains of the species *Xanthomonas translucens*. Three columns depicted to the right of the heat map summarise, for each strain, the presence or absence of T6SS-i3, T6SS-i4 and X-T4SS gene clusters with the following colour code: absence of gene cluster (gray), presence of gene cluster (green), or presence of gene cluster with pseudogenised genes (dark blue). Presence is based on the presence of the 13 genes (T6SS) and 12 genes (X-T4SS) for complete genomes and at least 11 genes (T6SS) and 9 genes (X-T4SS) for draft genomes. The three ANI clades Xt-I, Xt-II and Xt-III, and three LIN groups within *Xtt*, as described by Goettelmann *et al.* and Heiden *et al.* respectively, are represented on the far right of this figure (Goettelmann *et al.*, 2022; Heiden *et al.*, 2023). Dendrograms were built with single-linkage hierarchical clustering trees based on pairwise identity matrix using Pyani (Pritchard *et al.*, 2015).

***Xt* T6SS-i4 is required for inter-bacterial competition**

Since the distributions of X-T4SS and T6SS-i4 in *Xt* do not overlap, we hypothesised that these systems may play redundant roles in bacterial competition. To experimentally investigate their function, we tested how knocking out each secretion system in *Xt* strains affected their ability to kill the model bacterium *E. coli* DH10B (Durfee *et al.*, 2008).

The strain *X. translucens* UPB513 (ANI group Xt-I) harbours both complete T6SS-i3 and i4 gene clusters. To study T6SS function, we generated single and double mutant

strains for both T6SS clades by deleting independently either the inner tube subunit Hcp protein ($\Delta hcp3$, $\Delta hcp4$ and $\Delta hcp3 \Delta hcp4$), or part of the cluster ($\Delta T6SS-i3$, $\Delta T6SS-i4$, $\Delta T6SS-i3 \Delta T6SS-i4$) (Zhu et al., 2020) (Fig. 2a). Qualitative competition assays were first conducted by spreading *E. coli* DH10B cells on NA plates and dropping patches of each *Xt* UPB513 WT or T6SS mutants. Using GFP-expressing *E. coli* cells, we showed that *E. coli* growth was inhibited by *Xt* UPB513 WT, $\Delta hcp3$ and $\Delta T6SS-i3$, but not by $\Delta hcp4$, $\Delta hcp3 \Delta hcp4$, $\Delta T6SS-i4$ and $\Delta T6SS-i3 \Delta T6SS-i4$ strains (Fig. 2b). Complementing the $\Delta hcp4$ strain with the *hcp4* gene in its original location, *Xt* UPB513 $\Delta hcp4$ *hcp4*_{xtu}, restored the killing activity. The absence of bacterial killing activity in both the single mutant $\Delta hcp4$ and double mutant $\Delta hcp3 \Delta hcp4$ also indicates that Hcp3 cannot functionally replace Hcp4. Quantification of competition between *E. coli* Km⁺ GFP⁺ cells mixed with *Xt* UPB513 WT or T6SS mutants Spec⁺ by antibiotic selection showed that *Xt* UPB513 T6SS-i4 reduces *E. coli* survival by four orders of magnitude, while *Xt* growth remained unaffected (Fig. 2c and Extended Data Fig.1-2). *E. coli* DH10B does not have a T6SS or X-T4SS. Deletions of the T6SS-i3 or T6SS-i4 had no effects on *Xanthomonas* virulence on barley or bacterial growth (Extended Data Fig.3). Further experiments showed that *Xt* UPB513 T6SS-i4 was involved in the competition against *Pantoea agglomerans* UPB1357 and *Pseudomonas sivasensis* UPB1354 (Dubois et al., 2024), two bacterial strains isolated from cereals (Extended Data Fig.4).

Real-time monitoring of T6SS-i4 killing activity confirmed competition assay results. Fig. 2d shows time-lapse series of competition between *Xt* UPB513 RFP⁺ and *E. coli* DH10B GFP⁺ in a 10:1 (*Xt*:*E. coli*) ratio over 4 hours. *Xt* UPB513 WT RFP⁺ cells encircle *E. coli* GFP⁺ cells, leading to rounding and eventual lytic death of the *E. coli* cells. Intriguingly, some *E. coli* bacteria were not killed, but they also did not multiply over the whole 4-hour period, suggesting a bacteriostatic action of some T6SS effectors. In contrast, co-incubation with *Xt* UPB513 $\Delta T6SS-i4$ RFP⁺ did not show any bacterial killing activity, only *E. coli* cells multiplication. The encircling of *E. coli* by *Xt* was observed even with T6SS mutants, indicating that *Xt* could detect and target *E. coli* independently of a functional T6SS. *Xt* lysis events were extremely rare (Supplementary Video 1-16).

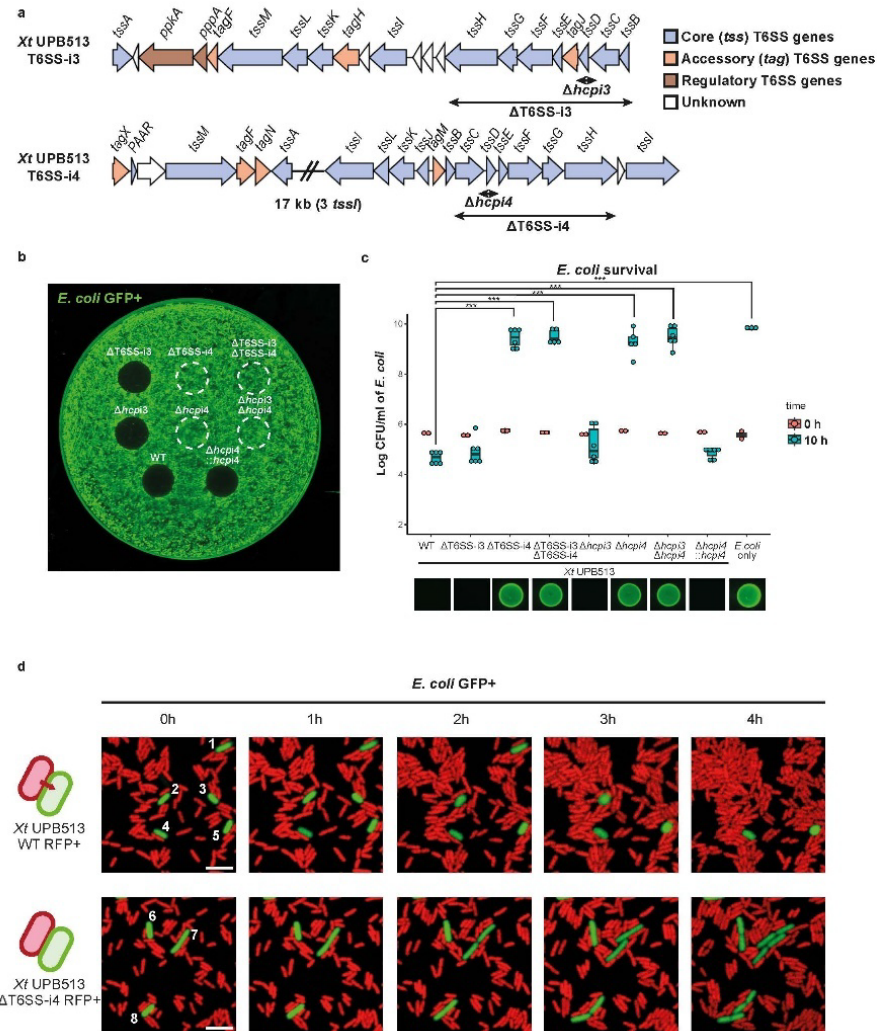


Figure 2: *Xt* inter-bacterial competition relies on T6SS-i4, but not T6SS-i3. (a) Overview of genes deleted in the *Xt* UPB513 T6SS-i3 and i4 gene clusters for the construction of T6SS mutant strains. (b, c, d) Competition assay using *Xt* UPB513 WT, Δ T6SS-i3, Δ T6SS-i4, Δ T6SS-i3 Δ T6SS-i4, Δ hcp i3, Δ hcp i4, Δ hcp i3 Δ hcp i4 and Δ hcp i4 ::hcp i4 as attacker cells and *E. coli* DH10B pNeo-gfp as a target cell (b) Representative qualitative assay plate after 16h of competition on NA using Amersham Typhoon laser scanner (filter Cy2 525BP20) (c) Quantitative assay with competitors mixed in a 10:1 ratio (*Xt*:*E. coli*) for 10h of co-cultivation. At the bottom: Fluorescence images of representative competition patches spotted on NA after 10h of co-cultivation using Amersham Typhoon laser scanner. At the top: Boxplot represents *E. coli* survival log CFU/ml after 0h and 10h of co-cultivation. All boxplots show 1st and 3rd quartiles around the median. Whiskers represent the smallest and largest value no further than 1.5 * IQR (inter-quartile range) from the hinge. Dots indicate independent biological replicates (n = 2 at 0h; n = 6 at 10h). Significance was tested by two-sided Student's

T-test. Significance codes: *** ($P < 0.001$), ** ($P < 0.01$), * ($P < 0.05$), only significant comparisons are shown ($\Delta T6SS-i3$, $P = 1$; $\Delta T6SS-i4$, $P = 1.47 \times 10^{-9}$; $\Delta T6SS-i3 \Delta T6SS-i4$, $P = 3.72 \times 10^{-10}$; $\Delta hcp i3$, $P = 1$; $\Delta hcp i4$, $P = 3.11 \times 10^{-8}$; $\Delta hcp i3 \Delta hcp i4$, $P = 2.13 \times 10^{-9}$; $\Delta hcp i4::hcp i4$, $P = 1$; and *E.coli* only, $P = 1.03 \times 10^{-11}$). **(d)** Time-lapse pictures of *Xt* wild-type (WT) and T6SS-i4 mutant ($\Delta T6SS-i4$), expressing RFP in competition with *E. coli* expressing GFP (1h between frames). Bars represent 4 μ m. Upper panel: *E. coli* cells n°2 and n°5 rounded up; cells n°1, n°2 and n°3 get lysed; cell n°4 shows no cell division. Lower panel: cells n°6, n°7 and n°8 multiply over time. Both mCherry and GFP channels are merged in each image.

***Xt* X-T4SS shows similar bacterial killing activity**

To validate our hypothesis of functional redundancy between T6SS and X-T4SS, we conducted competition assays with *X. translucens* LMG 728 (ANI group *Xt*-III) possessing an intact X-T4SS gene cluster. To test whether X-T4SS is also responsible for inter-bacterial competition, we constructed a X-T4SS mutant lacking ATPase VirD4 protein ($\Delta virD4$) (Bayer-Santos, Cenens, et al., 2019) (Fig.3a). Using similar qualitative and quantitative competition assays as described above, *Xt* LMG 728 WT induced *E. coli* death, whereas *Xt* LMG 728 $\Delta virD4$ did not kill its competitor (Fig. 3b). Killing activity was restored in the mutant when complemented by plasmid pSKX1::*virD4*. In these competition assays, *Xt* LMG728 suppressed *E. coli* viability by four orders of magnitude, but neither *Xt* LMG728 nor the isogenic *virD4* mutant showed any detectable growth defects (Fig.3c and Extended Data Fig.1-2). Additionally, both *Xt* strains grew at comparable rates in liquid medium *in vitro* (Extended Data Fig.3). Similarly to *Xt* UPB513 T6SS-i4, *Xt* LMG728 competitiveness against *Pantoea agglomerans* UPB1357 and *Pseudomonas sivasensis* UPB1354 (Dubois et al., 2024) was X-T4SS dependent (Extended Data Fig.4). Taken together, these results support the hypothesis of functional redundancy between T6SS-i4 and X-T4SS for bacterial killing in *Xt*.

validated X-T4SS involved in bacterial killing were also included (Souza et al., 2015), replacing the species' type strains in those cases. Our results showed that the X-T4SS gene phylogeny was mostly congruent with the species tree (Fig.4). Exceptions have, however, been found for some *Xanthomonas* strains clustering separately from both *Xanthomonas* clade 1 and 2 groups when using other X-T4SS genes than *virD4* to construct the X-T4SS phylogenetic tree (Extended Data Fig.5). To investigate reasons behind these different groupings, we used BLAST and found that some X-T4SS genes had higher sequence similarity to *Stenotrophomonas* X-T4SS genes, indicating X-T4SS inter-genus gene exchange. Finally, we also found X-T4SS in *X. citri* strains from Herbarium specimen spanning a period of more than 120 years in the 19th and 20th centuries (Campos et al., 2023).

In line with our phylogenetic findings, all X-T4SS gene clusters shared the same chromosomal location across all *Xanthomonas* strains in this study. The *virD4* gene is consistently found downstream of the *uvrB* gene, with some genomes also possessing a tRNA^{Val} gene between the *uvrB* and *virD4* genes. On the other border, the neighbouring gene of X-T4SS clusters is mostly the *thrS* gene, although a few in *Xanthomonas* species are affected by genome reorganisations.

The similar X-T4SS and species tree topologies, along with the shared genomic location of X-T4SS in distantly related xanthomonads, provide evidence that X-T4SS was likely acquired prior to the split of *Xanthomonas* clades 1 and 2, which may have occurred approximately 100 to 200 million years ago, and then vertically inherited in the progeny of *Xanthomonas* sp.

rooted and visualised with iTOL. Neighbourhood region associated with each X-T4SS gene cluster are indicated next to the X-T4SS phylogenetic tree. T4SS whose antibacterial effect have been experimentally validated are indicated next to the corresponding strain (LMG 728, 306) (Souza et al., 2015).

Ancestral X-T4SS clusters have been subjected to loss events

To further explore the X-T4SS evolutionary history, we compared X-T4SS clusters and neighbourhood between representative strains of each *Xt* ANI clade, along with the corresponding regions in X-T4SS-lacking strains (Extended Data Fig.6). Comparison of X-T4SS clusters first revealed that all X-T4SS in *Xt* are well conserved in terms of genetic organisation. Interestingly, all *virD4* genes in *Xt* strains from ANI group I were pseudogenised due to frameshift mutations and internal stop codons caused by a nucleotide deletion (Supplementary Fig.1). Accordingly, fragments of the *virB6* gene were found in the corresponding location of X-T4SS-lacking *Xt* strains, indicating these X-T4SS clusters have been in the process of decay or subjected to loss events. Pseudogenes and gene fragments were also common in other *Xanthomonas* species. This indicates a convergent loss of X-T4SS caused by different mechanisms such as IS insertion or indel mutations identified in this study. Additionally, screening for X-T4SS effector XVIPCD motif based on HMM profiles revealed the presence of X-T4SS effectors-immunity pairs in *Xt* strains lacking X-T4SS. Some X-T4SS effectors in X-T4SS-deficient strains have been pseudogenised, while retaining an intact immunity protein, as exemplified by *Xt* UPB513 T6SS-i4⁺ (Extended Data Fig.7).

Horizontal gene transfer drove the evolution of T6SS-i4

To better understand the evolution of T6SS-i4, we conducted similar analyses using the well-conserved sheath subunit *tssC* gene to construct the phylogenetic tree and trace the evolutionary history of T6SS-i4 among *Xanthomonas* species. The T6SS-i4 gene phylogeny was not congruent with the species tree, suggesting that the system experienced independent horizontal gene transfer events during evolution (Fig.5). Specifically, *Xt* ANI groups Xt-I, Xt-II and Xt-III clustered separately. For instance, *X. translucens* pvs. *phleipratensis* and *graminis* (ANI group Xt-III) grouped together with *X. bromi*, a bromegrass pathogen. Phylogenetic analyses were further performed among different *Xt* strains by concatenating T6SS-i4 core genes. Incongruences between the *Xt* species tree and T6SS-i4 phylogenies were also noted within the same *Xt* ANI group, as seen with strains *Xt* LW16 and BLSW16 (Extended Data Fig.8). The incongruence was resolved in the phylogeny without *tssM* gene, where *Xt* LW16 and BLSW16 clustered with other *Xtu* strains, similar to the species tree. A similar pattern was observed for the *tssB* gene with strains XT5523 and CFBP 2055. These instances suggest potential intraspecies T6SS gene exchanges from *Xtt* to *Xtu*.

T6SS-i4 clusters were found at different genomic locations in different *Xanthomonas* lineages. Across all analysed strains, T6SS-i4 was found in seven distinct genomic locations, with *Xt* T6SS-i4 present in four of them (Supplementary Fig.2-3). For each of the four neighbouring regions of *Xt*, we compared T6SS-i4 clusters between representative strains of each *Xt* ANI clade, and the corresponding region in T6SS-i4-

lacking strains (Supplementary Fig.4-7). The comparison showed a conserved genetic organisation, except for a variable region of 13 kb to 64 kb that splits *Xt* T6SS-i4 into two subregions. Some upstream genes were conserved regardless of the neighbourhood type and may regulate the system or represent potential effectors-immunity pairs. Within xanthomonads, two strains in our study had their two T6SS subregions separated by more than 2Mb in distinct regions of the genome, *Xo* NCPPB 4346 and *Xo* PXO99. Interestingly, the type-2 neighbourhood was the most common insertion site for T6SS-i4 in the *Xanthomonas* genus (Fig.5). These results led us to conclude that T6SS-i4 has been horizontally transferred multiple times during xanthomonads evolution.



Figure 5: T6SS-i4 gene phylogeny is not congruent with the species tree. Tanglegram comparing TssC based T6SS-i4 phylogeny (left) with a kSNP3-based species phylogeny (right). The T6SS-i4 tree was inferred by maximum likelihood using IQ-TREE based on encoded amino acid sequences of *tssC* genes aligned by ClustalW. The species pan-genome SNP-based tree was constructed with *Xanthomonas* strain genomes using kSNP3 (kmer of 23bp). Trees are midpoint rooted and visualised with iTOL. Neighbourhood region associated with each T6SS-i4 gene cluster are indicated next to the T6SS-i4 phylogenetic tree. T6SS-i4 whose antibacterial effect have been experimentally validated are indicated next to the corresponding strain name (UPB513, GX01, PXO99) (Shao et al., 2023; Zhu et al., 2020).

ICE mechanisms are involved in T6SS-i4 distribution

To better understand the distribution of T6SS-i4 within the *Xanthomonas* genus, we investigated potential transfer mechanisms. In the type-2 neighbourhood, genes annotated as “prophage integrase” (*intS*) and t-RNA gene were found just upstream of the T6SS-i4 cluster. IslandViewer4 confirmed the presence of genomic islands (GIs) with typical features (Supplementary Fig.8) (Bertelli et al., 2017; Dobrindt et al., 2004; Speare et al., 2018). The GIs containing the T6SS-i4 cluster are 150-200 kb in size, have lower G+C content, which differs from the rest of the genome and include an integrase for insertion and excision, and multiple IS elements. These islands are inserted at a *tRNA^{Gly}* gene and flanked by 16-bp perfect direct repeats (DR) corresponding to attachment core sites of *attL* and *attR*. The same DR sequence was found in related strains lacking the T6SS-i4, corresponding to the *attP* integration site. However, in some T6SS-i4-deficient strains, other mobile genetic elements, including conjugative T4SS genes, were integrated at the same genomic location instead, indicating Integrative and Conjugative Elements (ICEs) insertion (Extended Data Fig.9). Some strains even possess a GI insertion containing the T6SS-i4 cluster followed by a tandem ICE insertion, flanked by similar DRs. This indicates T6SS-i4 gene clusters might be *cis*-mobilisable elements (CIMEs) transferred *in cis* by the ICE to a recipient cell. In some lineages, an IS3 transposase truncated the *intS* gene, potentially fixing the antibacterial trait by preventing GI excision.

X-T4SS and T6SS-i4 effector arsenals in *Xanthomonas*

To compare the effector arsenals of X-T4SS and T6SS-i4, we analysed four pairs of genetically close *Xanthomonas* strains carrying either system. Various candidate T6SS effectors could be identified associated with diverse putative toxic domains, including Tox-URI2 DNase, phospholipase effector Tle1-like, T6SS effector BTH, chitinase and Ntox15 nuclease (see Supplementary Table 1-3). Similarly, many candidates X-T4SS effectors were predicted with potential toxic domains, such as Zeta toxin, fungal lipase and cell wall hydrolases (see Supplementary Table 1-3). Many candidate effectors also showed no match to known domains. Overall, both X-T4SS and T6SS-i4 possess a broad diversity of antibacterial toxin activities.

No T6SS-i4 effectors were detected in the four T6SS-i4 lacking strains. In contrast, X-T4SS effectors were found in both X-T4SS-containing and -lacking strains, though degradation mechanisms (frameshifts, deletions, or IS element insertions) were observed. Strains lacking X-T4SS also had fewer X-T4SS effectors than their

genetically close X-T4SS-containing strain. The X-T4SS effector degradation, along with the exclusive presence of T6SS-i4 effectors in T6SS-i4-possessing strains, support the switch from X-T4SS- to T6SS-i4-mediated bacterial killing strategy in xanthomonads.

Discussion

Antibacterial weapons have arisen multiple times in bacteria, with xanthomonads adopting two competitive strategies depending on their lineage: X-T4SS or T6SS. Here we demonstrate that *Xt* X-T4SS and T6SS-i4 have overlapping bacterial killing activities. Our functional study on both *Xt* T6SS-i4 and X-T4SS aligns with previous findings in the *Xanthomonadales* order. T6SS-i4 has been shown to mediate bacterial competition in *X. oryzae* pvs. *oryzae* (Shao et al., 2023) and *oryzicola* (Zhu et al., 2020), and *Stenotrophomonas rhizophila* (Garin et al., 2024). Similarly, X-T4SS-mediated killing was demonstrated in three genera: *X. citri* (Souza et al., 2015), *Stenotrophomonas maltophilia* (Bayer-Santos, Cenens, et al., 2019), and *Lysobacter enzymogenes* (Shen et al., 2021; Yu et al., 2020), targeting Gram-negative bacteria and/or fungi.

Our results also indicate that X-T4SS and T6SS-i4 have mutually exclusive distributions in xanthomonads. This finding underscores the ecological importance of contact-dependent competition in *Xanthomonas*. Consistent with Alvarez *et al.*, pseudogenised X-T4SS genes suggest that the system lost its function in some strains (Alvarez-Martinez et al., 2020). Interestingly, some strains in this study carried X-T4SS with a mutated *virD4*, while the rest of the X-T4SS cluster genes remained intact. If the absence of VirD4 predicts for a loss of bacterial killing function, it also raises the question of whether the development of a T4SS pilus is still possible (Fronzes et al., 2009). This type of surface structure does not require VirD4 and could suggest a switch to new biological functions (target cell adhesion, cell motility, ...). Finally, phylogenetic and comparative genomic analyses revealed non-orthologous genes displacement of ancestral bacterial killing X-T4SS by a T6SS-i4 competitive strategy in many *Xanthomonas* lineages.

The concept of mutual exclusion applies from the gene level to entire organism communities (Rossi et al., 2015; Soufiane et al., 2011; X. Zhang et al., 2011). The mutual exclusion of X-T4SS and T6SS-i4 could be due to several factors: functional redundancy leading to the replacement of one by the other, energy costs for maintaining both systems, or space constraints for both machineries in the cell envelope. This is especially relevant as both systems appear to be constitutively expressed in culture media (Alvarez-Martinez et al., 2020). X-T4SS homeostasis is controlled by the repressor CsrA, which maintains a constant density of X-T4SS in the cell envelope (Cenens et al., 2020). For T6SS-i4, future studies are needed to clarify how *Xanthomonas* balances the energy costs and competitive advantages, and on a possible TagF-mediated regulatory mechanism (Bayer-Santos, DeMoraes Ceseti, et al., 2019; Zhu et al., 2020). However, even if the loss of a redundant secretion system that is no longer needed could have an evolutionary benefit, it should be kept in

mind that most bacteria use multiple secretion systems and sometimes many copies of the same one. Therefore, the role of energy costs in minimising secretion potential is not yet clear. While this mutual exclusion generally holds true for the *Xanthomonas* genus, exceptions exist, such as *Lysobacter gummosus* 3.2.11 (Bayer-Santos, DeMoraes Ceseti, et al., 2019; Sgro et al., 2019), *L. enzymogenes* C3 (Bayer-Santos, DeMoraes Ceseti, et al., 2019; Sgro et al., 2019), some *X. citri* (e.g. ISO118C5, CFBP 7119, CFBP 6992) and *X. phaseoli* (e.g. NCPPB 1138). In *Pectobacterium*, both T4SS and T6SS, evolutionarily distant from *Xanthomonas*' X-T4SS and T6SS, redundantly secrete effectors to drive bacterial competition (Che et al., 2024).

The loss of ancestral X-T4SS in many *Xanthomonas* species and the acquisition of the T6SS-i4 by HGT only in restricted lineages result from two possible scenarios (Fig.6), either arrival of T6SS-i4 triggered X-T4SS loss, or the presence of X-T4SS prevented the acquisition of T6SS-i4. The decay of X-T4SS occurring independently of T6SS-i4 presence is in favour of the second scenario.

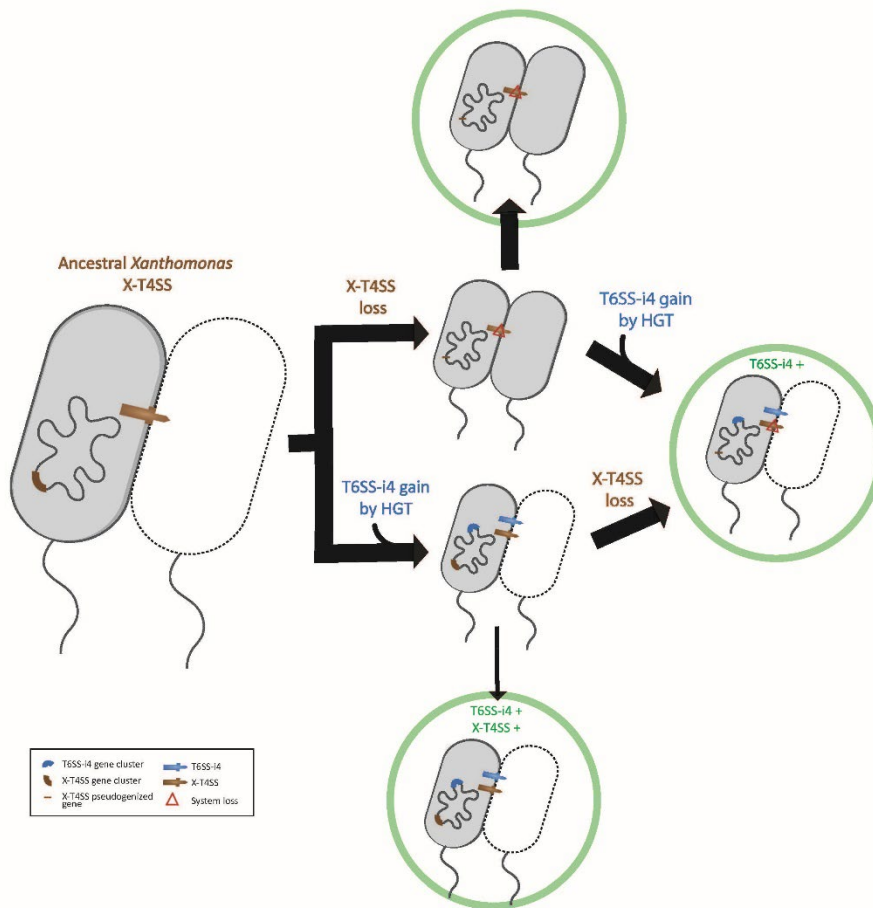


Figure 6: Evolutionary scenarios explaining X-T4SS loss, T6SS-i4 gain and resulting mutual exclusion profiles in xanthomonads. Our competition assays and phylogenetic analyses support an evolutionary model in which the loss of the ancestral X-T4SS and the gain of T6SS-i4 result from two possible scenarios, either by the introduction of T6SS-i4 driving X-T4SS loss, or by the presence of X-T4SS preventing the acquisition of T6SS-i4.

Finally, we propose several ecological reasons for adopting one system over the other. Firstly, each system may provide distinct evolutionary advantages in different microbiome contexts or co-evolving with T6SS-i4 competitors. In line with a recent study, the clustering of both *Xt* (ANI group Xt-III) and *X. bromi* T6SS-i4 suggests HGT events (Liyanapathirana et al., 2022). Occupying similar niches, this shared competitive trait is likely linked to microbiome adaptation. Secondly, the distribution of X-T4SS, restricted to *Xanthomonadales*, differs markedly from the more widespread T6SS in Proteobacteria. The uniqueness of X-T4SS might confer evolutionary advantages due to the seldom occurrence of its effector-immunity sets, while T6SS displays higher diversity in effector-immunity pairs among various Gram-negative bacteria, facilitating transfer and recombination possibilities between effector modules (Kirchberger et al., 2017). At this point, based on sequence comparisons, we have no indication of recruitment of X-T4SS toxins by alternative secretion systems in X-T4SS-lacking strains.

It remains unknown how easily target bacteria can evolve resistance to such contact-dependent bactericidal systems and whether the frequency of X-T4SS- and T6SS-resistant bacteria is different, which could explain the functional replacement of one system by another. Interestingly for T6SS, *in vitro* work on *Escherichia coli*, *Vibrio cholerae* and *Bacteroides fragilis* refute the postulate that T6SS is energetically costly (Robitaille et al., 2023; Taillefer et al., 2023; C. Zhang et al., 2024). In addition, production of X-T4SS by *X. citri* was shown to impose a significant physiological cost to the cell (Cenens et al., 2020). The loss of X-T4SS might result from increased fitness through reduced energy costs or switch to a new biological function with T4SS pilus, while retaining immunity molecules of interest (Unni et al., 2022). Both X-T4SS and T6SS have overlapping roles in bacterial killing, but they may also perform additional functions, such as secreting antifungal effectors like those in *L. enzymogenes* X-T4SS (Shen et al., 2021; Yu et al., 2020), or mediating specific inter- or intra-species competition, which could provide a fitness advantage. T6SS effectors with chitinase domain and X-T4SS effectors with a fungal lipase domain were identified in *Xanthomonas* strains, suggesting potential antifungal activity. It would be worth investigating more attacker and target strain combinations and different environmental conditions, to further support the redundancy of the two systems.

Geographical differences in T6SS availability may also influence its distribution (Unni et al., 2022). Previous studies have examined various ecological characteristics to explain the presence or absence of T6SS in *Xanthomonas*, but no correlation with vascularity, host range, or pathogenic status was found (Liyanapathirana et al., 2022). For instance, both vascular *Xtt* and non-vascular *Xtu*, as well as vascular *Xoo* and non-vascular *Xoc*, all possess T6SS-i4 (Gluck-Thaler et al., 2020).

The type 2 neighbourhood region of T6SS-i4 is a hotspot for ICEs insertion well conserved among xanthomonads, with prior reports in *X. arboricola* pv. *juglandis* CFBP 7179 (Xaj-ICE) (Cesbron et al., 2015) and *X. campestris* CN03 (ICEXca2) (de Assis et al., 2022). Therefore, the turnover between X-T4SS and T6SS-i4 could also be explained by an incompatible genetic background that hinders T6SS-i4 acquisition, such as an ICE exclusion due to unavailable integration sites, or restriction modification systems (Juhas et al., 2008; Unni et al., 2022). The transfer of T6SS by ICE raises questions about the compatibility of competition and conjugation processes (Peñil-Celis & Garcillán-Barcia, 2019). However, T6SSs have been found on various conjugative platforms, including plasmids that regulate T6SS-i4 activity during conjugation, suggesting similar mechanisms may transfer T6SS-i4-encoding CIME-ICE structures (Morgado & Vicente, 2022; Peñil-Celis & Garcillán-Barcia, 2019). To date, T6SS transfer by ICE has only been reported in the *Bacteroides* phylum (García-Bayona et al., 2021). Analyses of other neighbourhoods did not reveal the presence of ICE, indicating alternative transfer mechanisms may be at play.

Our work highlights the role of X-T4SS or T6SS as antibacterial weapons for *Xt*, but also the evolutionary history of two antibacterial mechanisms in bacteria, enhancing our understanding of their ecological role and potential evolutionary trajectories.

Materials and methods

In silico screening for T6SSs and X-T4SS gene clusters

To obtain a comprehensive genomic dataset for assessing the evolution of *Xt* T6SS and X-T4SS, a total of 94 *Xt* genomic sequences were retrieved from the National Center for Biotechnology Information (NCBI) database on January 2023.

T6SSs were predicted by combining both HMM searches and BLAST tools (Altschul et al., 1990; Bi et al., 2013; J. Zhang et al., 2023). T6SS gene clusters were first detected using SecReT6 hmmsearch programme with default parameter values (e-value threshold < 0.0001; BLAST identity threshold for T6SS prediction > 30%; BLAST Ha-value threshold for T6SS-related protein prediction > 0.42), counting a maximum interval between two co-localised T6SS components of 15000 bp and a minimum of three T6SS component proteins for each T6SS region was considered. Since only T6SS clades i3 and i4 were found in SecReT6 results, the 13 core genes from three *Xanthomonas* experimentally-validated T6SS clades i3* (*X. citri* strain 306) (Bayer-Santos et al., 2018), i3*** (*X. oryzae* strain PXO99) (Y. Choi et al., 2020) and i4 (*X. oryzae* strain GX01) (Zhu et al., 2020), were used as baits for further confirming the predicted T6SS loci using TBLASTN (e-value threshold < 0.000001; BLAST identity threshold > 50%). The core genes within each cluster were then carefully manually inspected. X-T4SS predictions were made similarly with SecReT4 and baiting with the 12 core genes from the characterised X-T4SS (*X. citri* strain 306) (Souza et al., 2015).

Prediction of the presence of X-T4SS and T6SS-i4 was then performed by TBLASTN analysis against the entire database of *Xanthomonas* complete and draft genomes

from NCBI. The amino acid sequences of the VirB7 protein (X-T4SS) and the two proteins TssB and TssA (T6SS-i4) were used as queries, since the two left and right regions of T6SS-i4 are often found on different contigs in the case of draft genomes. The type strain of each *Xanthomonas* species or pathovar when applicable was then further screened for the 13 core genes of T6SS-i4 and 12 core genes of X-T4SS and selected for subsequent phylogenetic and comparative genomic analyses.

Bacterial strains and growth conditions

Bacterial strains, plasmids and primers used in this study are listed in Supplementary Tables 4-5. *X. translucens* strains were grown at 28°C with shaking at 220 rounds per minute (rpm) in liquid nutrient broth (NB) or on solid nutrient agar (NA). *E. coli* DH10B and WM3064 strains were grown at 37°C with shaking at 220 rpm in liquid lysogenic broth (LB) or on solid LB agar (1.5%). *Pantoea agglomerans* UPB1357 and *Pseudomonas sivasensis* UPB135424 were grown at 28°C on solid nutrient agar (NA). The antibiotics kanamycin (Km), spectinomycin (Spec), and gentamycin (Gm) were used in a concentration of 50 µg/ml, 40 µg/ml and 10 µg/ml respectively, when appropriate.

DNA manipulations

Total genomic and plasmid DNA were extracted using Wizard® Genomic DNA Purification Kit (Promega) and QIAprep Spin Miniprep Kit (Qiagen). Fasta and faa files of *Xt* UPB513 (RefSeq: NZ_CP096570.1) and *Xt* LMG728 (RefSeq: NZ_CP076250.1) genomes were downloaded from NCBI. Primers were designed using Benchling (<https://benchling.com>). To construct the single deletion mutants *Xtu* UPB513 Δ T6SS-i3 (MZO50_RS16930 - MZO50_RS16895), Δ T6SS-i4 (MZO50_RS09125 - MZO50_RS09150), Δ hcpi3 (MZO50_RS16920) and Δ hcpi4 (MZO50_RS09130), the upstream and downstream regions were amplified using respectively the primers pairs UPB513 T6SSi3 up F/R and T6SSi3 down F/R; T6SSi4 up F/R and T6SSi4 down F/R; Hcp3 up F/R and Hcp3 down F/R ; Hcp4 up F/R and Hcp4 down F/R and purified using QIAquick PCR & Gel Cleanup Kit. Upstream and downstream fragments of each construct were then cloned into the digested pK18mobsacB (Schäfer et al., 1994) at *Hind*III restriction site by Gibson Assembly (New England Biolabs). Each construct was transformed into *Xtu* UPB513 by electroporation at 2,5 kV. The first genomic recombination event was selected on NA + Km and screened for sucrose sensitivity. The second recombination event was selected on NA 10% sucrose and screened for Km sensitivity. Resulting deletion mutants were confirmed by PCR.

Double deletion mutants were generated by introducing pK18mobsacB::*hcpi4* and pK18mobsacB::T6SSi3 in *Xtu* UPB513 Δ hcpi3 and *Xtu* UPB513 Δ T6SS-i4 respectively, using the same method. To complement *Xtu* UPB513 Δ hcpi4, a 1567-bp DNA fragment including the *hcpi4* coding region with its upstream and downstream regions was amplified using primers Hcp4 up F and Hcp4 down R. The amplicon was then cloned into the pK18mobsacB. A similar method of double recombination events was then followed as above.

The *Xt* LMG728 $\Delta virD4$ (KM539_RS13635) strain was generated by amplifying the upstream and downstream regions of the *virD4* gene with the LMG728 VirD4 up F/R and VirD4 down F/R primers and cloning them into pK18mobsacB and then following the same double recombination method, except that the plasmid was delivered into *Xt* by biparental conjugation with *E. coli* strain auxotrophic WM3064. The pSKX1::*virD4* Gm⁺ for complementation was synthesised by GenScript (Piscataway, NJ, U.S.A.) and introduced similarly in *Xt* LMG728 $\Delta virD4$ by conjugation by selection on NA + Gm⁺ and confirmed by PCR.

For generation of RFP-expressing strains, the protocol of Choi *et al.* (2006) was adapted to *Xt* using the mini-Tn7 delivery system to insert single-copy of a gene into *Xt* chromosome at Tn7 attachment (*attTn7*) sites, close to *glmS* gene (K. H. Choi & Schweizer, 2006). This involves the co-transformation of *Xt* strain with the recombinant mini-Tn7 vector and a helper plasmid pTNS3 encoding transposition pathway genes, followed by selection on media supplemented with antibiotics. *Xt* UPB513 WT as well as each T6SS mutant and complemented strains were transformed with Tn7::*mCherry* Spec⁺ by electroporation.

Bacterial competition assays

Xt UPB513 T6SS⁺ and LMG 728 X-T4SS⁺ WT strains and respective mutant strains, as well as *E. coli* DH10B Km⁺ GFP⁺, *P. agglomerans* UPB1357 and *P. sivasensis* UPB135424 Gm⁺ GFP⁺ were grown on solid media and resuspended to OD₆₀₀=0.5 in 10mM MgCl₂. In qualitative competition assays, 100µl of *E. coli* suspension was spread all over an NA plate using glass beads, then 15 µl of *Xt* WT and X-T4SS or T6SS mutant suspensions were dropped on the dried plate. Plates were incubated for 16h at 28°C. In quantitative competition assays, each *Xt* strain was mixed with *E. coli* in a ratio of 10:1 (*Xt*:*E. coli*). 5 µl patches of each mix were dropped on NA plates and incubated for 10h of competition at 28°C. Patches were resuspended in 500 µl of 10mM MgCl₂ and serial dilutions were plated on NA+Km plates for quantifying *E. coli* surviving cells after 0h and 10h. Qualitative and quantitative assays were repeated at least three times with two to six replicates. *Xt* cells growth were quantified once based on antibiotic selection on NA+Spec or colony cell morphology after 0h and 10h with two to six replicates. Qualitative competition assays, quantitative competition patches and dilution plates were scanned for fluorescence using Amersham Typhoon laser scanner (filter Cy2 525BP20).

Phenotypic analyses

Pathogenicity assays were conducted by needleless inoculation of barley cv. Hercule leaves (2 weeks-old) with *Xt* UPB513 WT, Δ T6SS-i3, Δ T6SS-i4 and 10mM MgCl₂ as a negative control. Each strain was grown on NA and resuspended to OD₆₀₀=0.01 in 10 mM MgCl₂. The assay was repeated twice with 8 plants inoculated by treatment. Plants were placed in greenhouse conditions at 28°C and 70% HR. Growth of *Xt* WT, X-T4SS and T6SS mutants was monitored *in vitro* in liquid NB. Initial OD₆₀₀ of *Xt* suspensions were adjusted to 0.001. Each treatment was performed in duplicate,

from which 8 sub-replicates were transferred in 96-well plates and coated with Breathe-Easy® sealing membrane (Diversified Biotech BEM-1). Measurements of OD₅₉₅ were taken every 45 minutes during 72h, with continuous shaking (11Hz, 3mm) at 28°C using a microplate reader (Multiskan FC).

Statistical analysis

Quantitative competition data were computed in *R*. Normality of log-transformed data was tested using Shapiro-Wilk test. Statistical analyses were performed using ANOVA1 and Tukey post-hoc multiple tests for pairwise comparison of means of each group. ANOVA1 with p_value of 0.05 was considered. Significance was tested by Student's T-test and p-value adjustment. Significance codes: *** ($P < 0.001$), ** ($P < 0.01$), * ($P < 0.05$), only significant comparisons are shown.

Mutual exclusivity statistical significance

To confirm mutual exclusion between X-T4SS and T6SS-i4 loci, a series of statistical tests were run. Contingency and frequency tables for the presence and absence of X-T4SS and T6SS-i4 within the 94 *Xt* strains were calculated, considering two possible scenarios: (Case 1) X-T4SS containing pseudogenes is considered as present; (Case 2) X-T4SS containing pseudogenes is considered as absent. Firstly, a one-sided Fisher's exact test was applied to the contingency tables with the alternative hypothesis that true odds ratio is lower than 1. Secondly, Bayesian phylogenetic generalised linear mixed models were run using the *R* package MCMCglmm (v. 2.36) (Hadfield, 2010). UPGMA phylogenetic tree was computed based on ANI distance matrix between the 94 *Xt* strains and used as a random effect. FastANI (v.1.3) (Jain et al., 2018), as implemented in Galaxy (v.23.1.4.dev0) (Jalili et al., 2021), was used to calculate pairwise genome-wide nucleotide identities. A derived distance matrix was used by MEGA (v.11.0.13) (Tamura et al., 2021) to construct a UPGMA phylogenetic tree (Sokal & Michener, 1958). We tested the effect of the presence of X-T4SS loci to explain the presence of T6SS-i4 loci given random distribution across phylogeny by running two MCMCglmm models. A first model (mod_mcmc_0) was run explaining the presence of T6SS-i4 loci as a function of phylogeny as a random effect alone. This first model was then compared to a second model (mod_mcmc_1) that explains the presence of T6SS-i4 loci as a function of phylogeny as a random effect and the presence of X-T4SS loci as a fixed effect. For both case 1 and 2, we chose the model, which minimises the DIC and best explains the response.

Confocal microscopy time-lapse videos

For real-time visualisation of T6SS-i4 killing activity, time-lapse videos were recorded by laser scanning confocal microscopy using an Olympus Life Science FluoView FV3000. *Xt* UPB513 WT and T6SS mutants RFP⁺ Spec⁺ strains, as well as *E. coli* DH10B Km⁺ GFP⁺ were grown on solid media with appropriate antibiotics and resuspended to OD₆₀₀=1 in dH₂O. Bacterial suspensions were mixed in a 10:1 (*Xt*:*E. coli*) ratio. 6 µl of this solution mix was deposited on an agarose pad (25% LB, 1% agarose), then

left to dry. The whole assembly was placed in a humid chamber to prevent the agarose pad from desiccating. Each time-lapse video was started after 1h of incubation at room temperature and taken every 6 minutes for 4h48min with a 60X objective, in which an additional zoom of 2.18X was achieved. We also used the multi-position setting to increase the chance of visualising bacterial killing activity and ZDC control to keep the sample in focus during the length of the time-lapse video and prevent focal drift. In total, 8 time-lapse videos have been produced for each competition assay (n = 8 videos with *Xt* UPB513 WT; n = 8 videos with *Xt* UPB513 Δ T6SS-i4; from multiple positions on the same sample each).

Comparative genetic and phylogenetic analyses

The matrix of average nucleotide identity was calculated using Pyani 0.2.11 (Pritchard et al., 2015) (Supplementary Fig.9) with the option -ANIm, and the ANI-Matrix software Enveomics tool (Rodriguez-R & Konstantinidis, 2016). Visualisation of groups of homologous gene clusters was performed using Clinker v0.0.27 (Gilchrist & Chooi, 2021). To study the evolutionary history of X-T4SS and T6SS-i4, the amino acid sequences of the VirD4 and TssC proteins were extracted for each type strain of selected *Xanthomonas* species. MEGA11 was used for amino acid sequence alignment with ClustalW followed by IQTREE web server (<http://iqtree.cibiv.univie.ac.at/>) for inferring phylogenetic tree using maximum likelihood with default parameters (Tamura et al., 2021; Trifinopoulos et al., 2016). The species phylogenetic tree was then inferred using kSNP3 using kmer of 21 bp (X-T4SS) and 23 bp (T6SS-i4). The VirB8 phylogenetic tree was constructed with the automated multi-locus sequence analysis pipeline (autoMLSA2) 0.8.1 (Davis et al., 2021) using the VirB8 amino acid sequence from *Xcc* 306 as a query. iTOL (<https://itol.embl.de/>) was then used to display and annotate phylogenetic trees (Letunic & Bork, 2024). X-T4SS and T6SS-i4 neighbourhoods were determined using Benchling and Clinker and manually checked in detail. Genomic islands were detected using IslandViewer4 (<https://www.pathogenomics.sfu.ca/islandviewer/>) (Bertelli et al., 2017).

Comparison of X-T4SS and T6SS-i4 effector arsenals

To compare the effector arsenals of X-T4SS and T6SS-i4, we analysed pairs of genetically close *Xanthomonas* strains carrying either system. We selected three pairs of *Xt* (clade 1) and one pair of *Xanthomonas vesicatoria* (*Xv*; clade 2): *Xt* pv. *phlei/phleipratensis* LMG 843 and LMG 730; *Xt* pv. *translucens* UPB886 and DSM 18974; *Xt* pv. *graminis* NCPPB 3709 and NCPPB 3711; and *Xv* BNM214 and LMG 911. To identify X-T4SS effectors, the HMM profile of the C-terminal XVIPCD translocation domain was retrieved from Pfam. For T6SS effectors, different HMM profiles of domains frequently found in T6SS effectors were selected: MIX, FIX, VgrG, PAAR, Rhs, etc., also retrieved from Pfam (see Supplementary Table 3). HMMsearch were conducted against the predicted proteome, as annotated in the NCBI RefSeq files, of each strain. Genes found downstream of a VgrG/Hcp/PAAR-containing protein were further analysed to detect candidate toxic effectors in the vicinity. The resulting candidate effectors were analysed by InterProScan to predict protein domains (Jones et al., 2014) (see Supplementary Table 1-3). They were then

manually analysed to check for pseudogenes, due to frameshifts, early in-frame stop codons or IS elements.

Data availability

All data supporting the findings of this study are available within the paper, its Supplementary Information and as Source Data files at the following URL: https://uclouvain-my.sharepoint.com/:f:/g/personal/claude_bragard_uclouvain_be/EohtJbnyxgNHjtbuLfYqBHABAPnjxE64q-hcez7qNU0jIQ and in **Appendix 2**. Further information and requests for resources and reagents should be directed to Claude Bragard (claude.bragard@uclouvain.be). Bacterial strains and plasmids used in this study can be made available upon request following the signing of a material transfer agreement.

Code availability

Code for the data statistical analyses is available with the data at the following URL: https://uclouvain-my.sharepoint.com/:f:/g/personal/claude_bragard_uclouvain_be/EohtJbnyxgNHjtbuLfYqBHABAPnjxE64q-hcez7qNU0jIQ.

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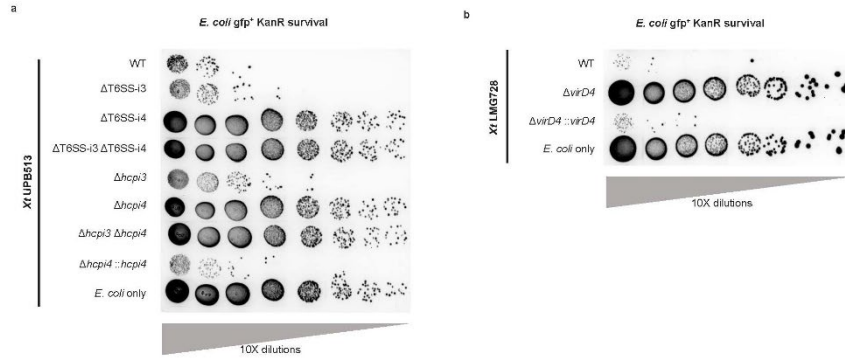
Author contributions

C.P., C.B., R.K. and J.M.J. designed the study and C.B., R.K. and J.M.J. supervised the conducted research. C.P., J.B. and F.N. performed the experiments. C.P. performed bioinformatic analyses. J.B., N.H. and M.V.M. assisted with the experiments and V.R.-R. and R.K. assisted with the bioinformatic analyses. C.P. wrote the manuscript and all authors discussed the results and edited the manuscript.

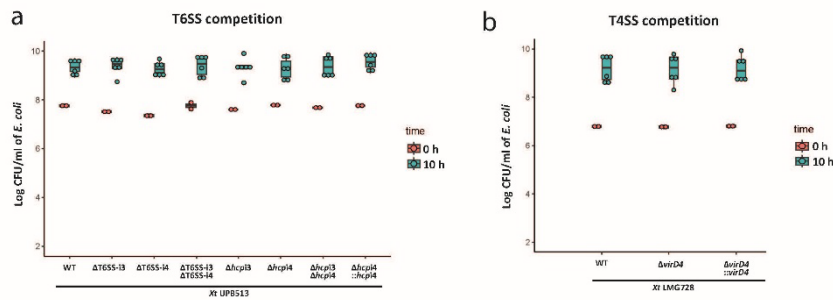
Competing interests

The authors declare no competing interests.

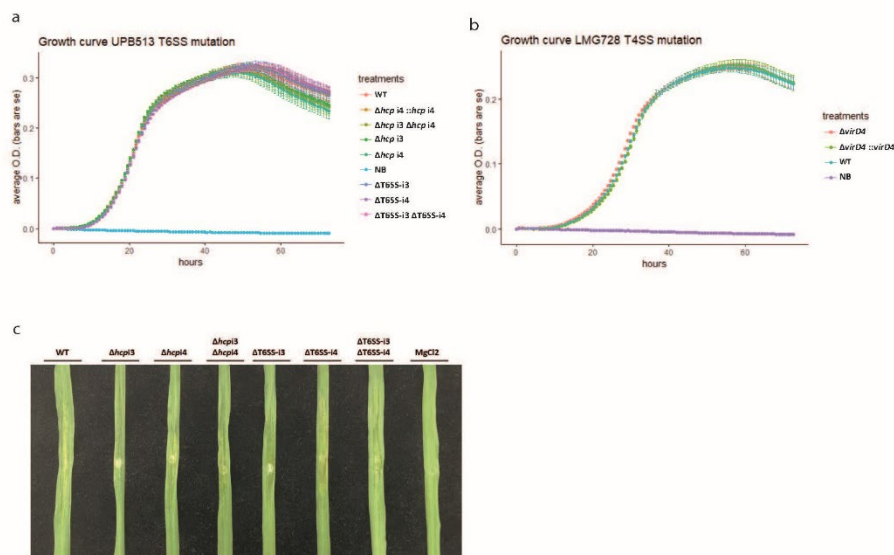
Extended Data Figures



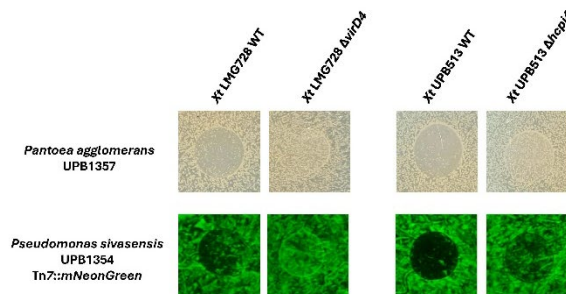
Extended Data Figure 1: *Xt* (a) T6SS-i4 and (b) X-T4SS are required for inter-bacterial competition. Representative NA plates with tenfold dilution series of the quantitative assay showing *E. coli* survival using Amersham Typhoon laser scanner.



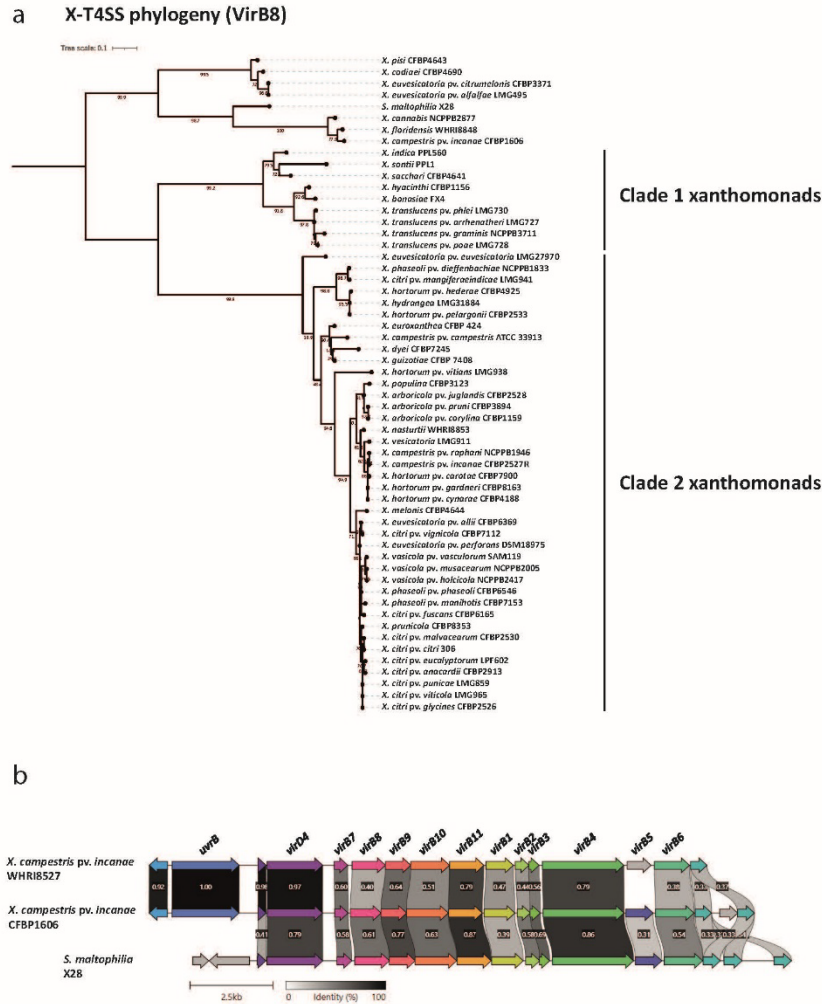
Extended Data Figure 2: *Xt* growth is not impacted by (a) T6SS and (b) X-T4SS mutations during bacterial competition assays against *E. coli*. Quantitative competition assay with *E. coli* mixed in a 10:1 ratio (*Xt*:*E. coli*) for 10h of co-cultivation. Boxplot represents *Xt* growth log CFU/ml after 0h and 10h of co-cultivation. All boxplots show 1st and 3rd quartiles around the median. Whiskers represent the smallest and largest value no further than $1.5 \times IQR$ (inter-quartile range) from the hinge. Dots indicate independent biological replicates ($n = 2$ at 0h; $n = 6$ at 10h). Significance was tested by two-sided Student's T-test. Significance codes: *** ($P < 0.001$), ** ($P < 0.01$), * ($P < 0.05$), only significant comparisons are shown ($\Delta T6SS-i3$: $P = 1$, $\Delta T6SS-i4$: $P = 1$, $\Delta T6SS-i3 \Delta T6SS-i4$: $P = 1$, $\Delta hcpi3$: $P = 1$, $\Delta hcpi4$: $P = 1$, $\Delta hcpi3 \Delta hcpi4$: $P = 1$, and $\Delta hcpi4::hcpi4$: $P = 1$; $\Delta virD4$: $P = 1$, and $\Delta virD4::virD4$: $P = 1$).



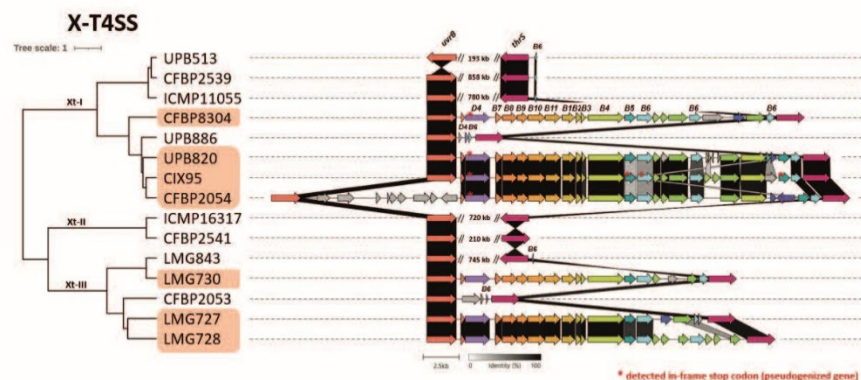
Extended Data Figure 3: Phenotypic characterisation of *Xt* UPB513 T6SS and X-T4SS mutants. (a, b) *In vitro* growth curve of (a) *Xt* UPB513 WT and derived strains mutated in and complemented for T6SS, (b) *Xt* LMG728 WT and derived strains mutants in and complemented for T4SS, in NB medium at 28°C, OD₆₀₀ measured at 45-minute intervals. Dots represent mean and error bars show s.e.m (n = 8 replicates per strain). (c) Barley cv. Hercule leaves infiltrated with *Xtu* UPB513 WT or T6SS-i3 and i4 mutants show water-soaking and necrotic lesions at 6 dpi (n = 8 plants).



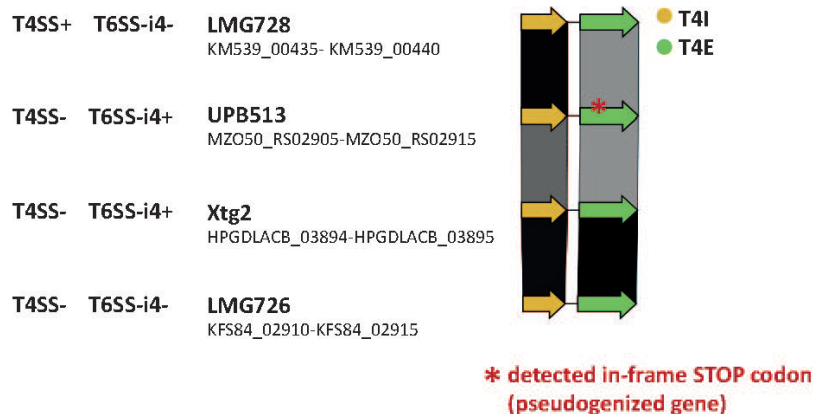
Extended Data Figure 4: *Xt* T6SS-i4 and X-T4SS are required for inter-bacterial competition against *Pseudomonas* sp. and *Pantoea* sp. Representative qualitative competition plate using *Xt* UPB513 WT, $\Delta hcp\ i4$, *Xt* LMG728 WT and $\Delta virD4$ as attacker cells and *Pantoea agglomerans* UPB1357 and *Pseudomonas sivasensis* UPB1354 Tn7::mNeonGreen as a target cells after 16h of competition on NA.



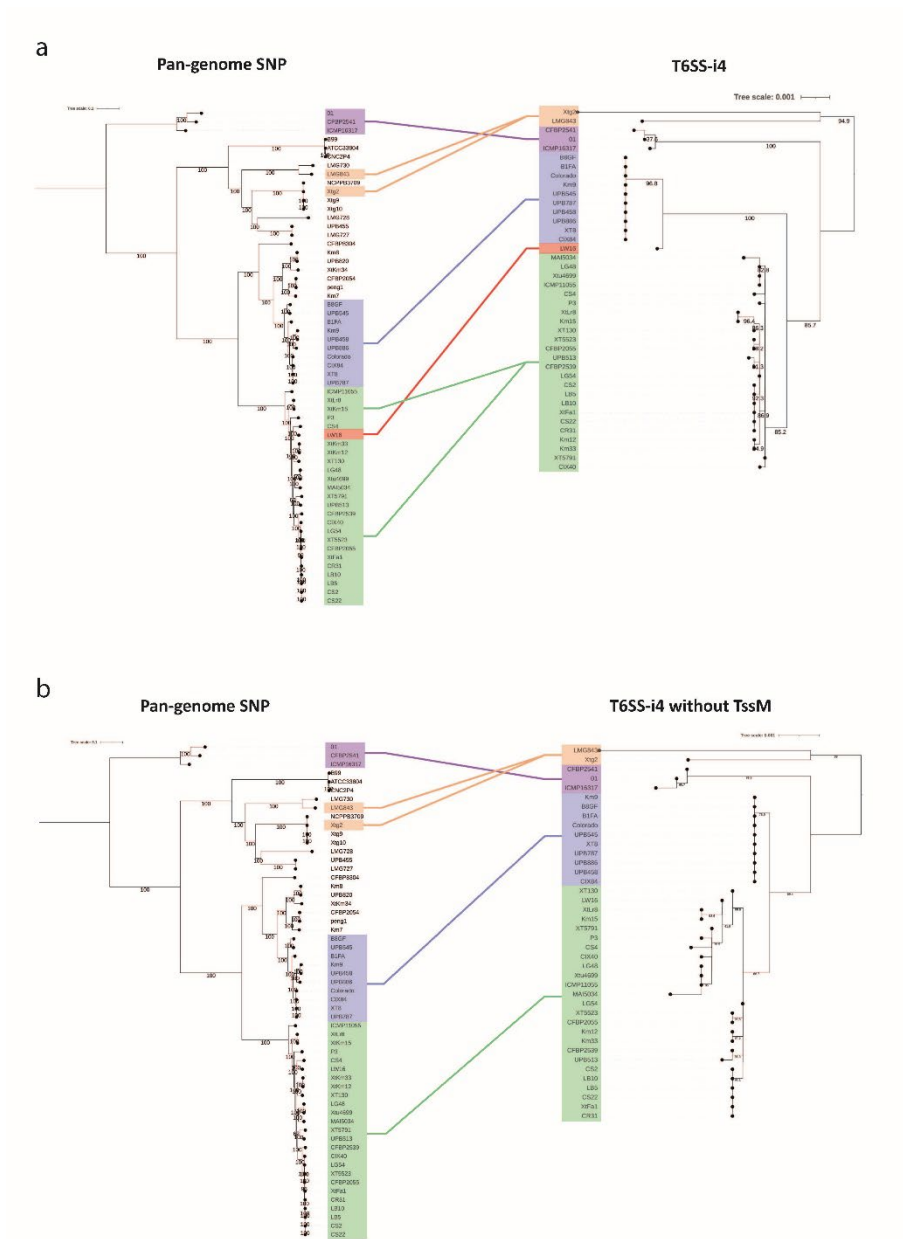
Extended Data Figure 5: VirB8-based phylogeny reveals xanthomonads with atypical X-T4SS sequences. (a) The phylogenetic tree was constructed using autoMLSA2, by extracting and aligning VirB8 amino acid sequences from each type/pathotype strain of each *Xanthomonas* species and pathovar possessing X-T4SS and using IQ-TREE to infer the phylogenetic tree. (b) Comparison of the T4SS gene clusters from *X. campestris* pv. *incanae* and *Stenotrophomonas maltophilia*. Most X-T4SS genes (except for *virD4*) of strain CFBP 1606 share higher sequence identity with the corresponding genes from strain X28 than those strain WHRI 8527.



Extended Data Figure 6: Various decay and loss events occurred in the X-T4SS clusters. The tree on the left side is based on average nucleotide identity (ANI) of *Xt* strains, whereas the right side provides an overview of the organisation of the X-T4SS gene clusters and their neighbouring genes *uvrB* and *thrS*. Letters and numbers above the genes correspond to the *virD* and *virB* genes. Scales for distance and percentage identity between genes are indicated below. Genes are colour-coded according to homology. Pseudogenised genes are highlighted with an asterisk. Fragments of remnant X-T4SS genes are found within the corresponding genomic location in the X-T4SS-lacking strains. The three ANI clades Xt-I, Xt-II and Xt-III are indicated in the tree.

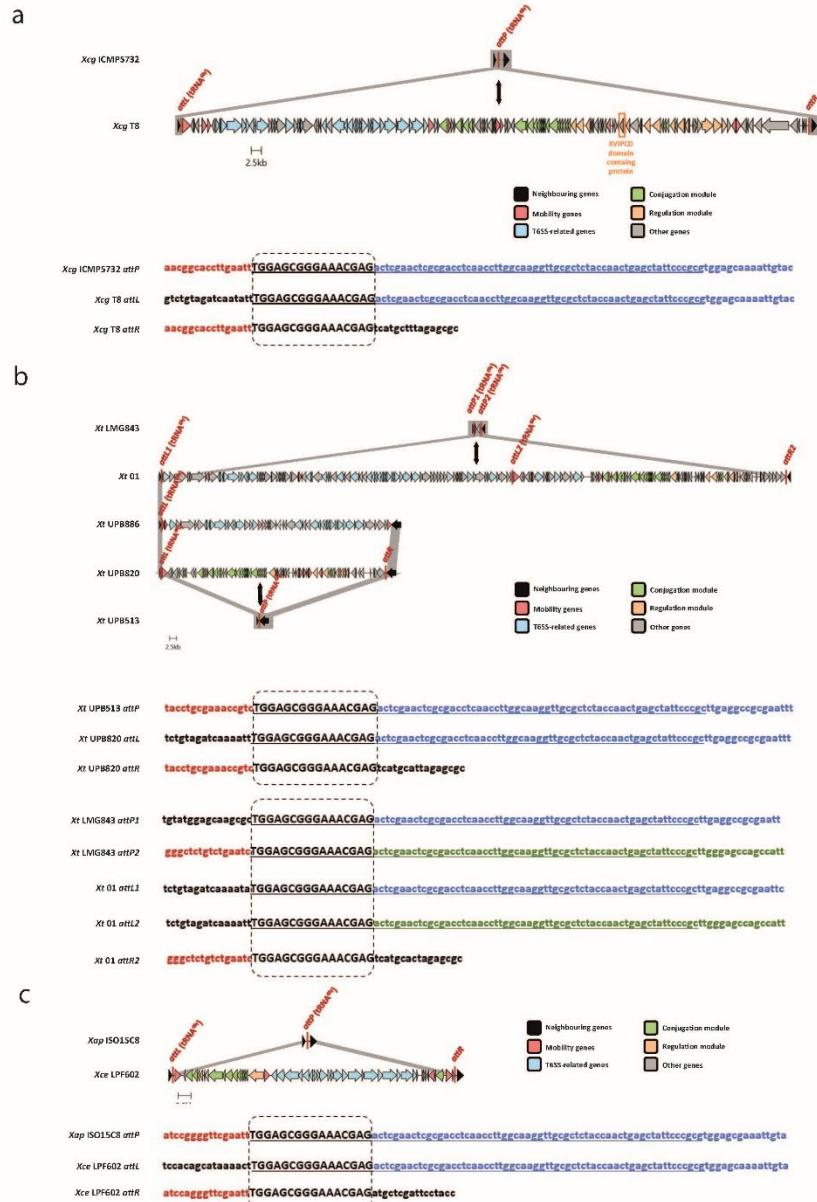


Extended Data Figure 7: X-T4SS-lacking strains possess T4E-T4I pairs. Comparison of candidate X-T4SS effector-immunity pairs across strains possessing either X-T4SS, T6SS-i4, or none of them. The asterisk indicates an in-frame STOP codon.



Extended Data Figure 8: Incongruency between the T6SS-i4 and the pan-genome tree topology of strain *Xt* LW16 is caused by the *tssM* gene. Comparison between species (left) and T6SS-i4 (right) phylogenies. The pan-genome SNP-based tree was constructed using kSNP3 (kmer of 23bp). The T6SS-i4 tree was inferred by maximum likelihood using IQ-TREE based on concatenated alignment of *tssA*, *tssB*, *tssC*, *tssD*, *tssE*, *tssF*, *tssG*, *tssH*, *tssK*, *tssL*, **(a)** with or **(b)** without *tssM* genes. Amino acid sequences were aligned by ClustalW.

The bootstrap supports were estimated based on 1000 replicates. Trees were visualised with iTOL.



Extended Data Figure 9: T6SS-i4 and ICE in type-2 neighbourhood are flanked by *att* sites in strains (a) *Xcg* T8, (b) *Xt* 01, *Xt* UPB886, *Xt* UPB820 and (c) *Xce* LPF602. (Upper panel) Genes corresponding to the same ICE modules are represented in similar colours. Genes with unknown functions are indicated in gray. *attL* and *attR* sites flanking the

GI and *attP* in the T6SS-i4 lacking strain are pointed with red bars. (Lower panel) *attL*, *attR* and *attP* sites sequences are shown, 16-bp direct repeat sequences are boxed with dashed lines and tRNA-Gly sequences are underlined.

Chapter III

Ecology-driven diversification of T6SS effectors in the plant pathogen *Xanthomonas*

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Abstract

Bacteria live a constant arms race with neighbouring microorganisms occupying the same ecological niche. Among the mechanisms supporting survival, the type VI secretion system (T6SS) plays an essential role in interbacterial competition, guided by its effector repertoire. However, what drives the distribution and functional diversity of T6SS effectors among bacteria remains poorly understood. Here, we show that the diversity of T6SS effectors in plant-pathogenic xanthomonads is linked to their ecological lifestyles. Combining *in silico* screening and comparative genomics, we comprehensively describe the diversity of T6SS effectors within the *Poaceae*-infecting *X. translucens* complex. Comparison of xanthomonads with differing ecological lifestyles revealed an enrichment in the T6SS effectors diversity within non-vascular xanthomonads, compared to vascular close relatives. The bacteriolytic activity of the *X. translucens* core T6SS effectome was further experimentally demonstrated. Respective effector-immunity pairs were investigated through functional assays and structural predictions. Overall, this research demonstrates how competition traits are shaped by intrinsic bacterial lifestyles.

Introduction

Bacteria employ different strategies to interact and persist within dynamic polymicrobial communities whether associated with plants, animals, or thriving in environmental niches (Garcia-Bayona L. & Comstock L. E., 2018). Among these, the type VI secretion system (T6SS) provides a competitive advantage by delivering proteinaceous effectors into prokaryotic or eukaryotic cells (Bayer-Santos et al., 2019). The role of a given T6SS is primarily dictated by the activity of the effectors it secretes (Bernal et al., 2018). However, the proximate and ultimate causes driving distribution of T6SSs and their effectors in bacteria remain poorly understood, despite their major ecological implications (Unni et al., 2022).

This multiproteic complex, evolutionarily related to contractile bacteriophage tails, is composed of 13 core structural components (TssA-M, for Type six secretion) commonly encoded in gene clusters (Leiman et al., 2009). Beyond core proteins, T6SS effectors (T6Es) are proteins loaded either inside the central channel of Hcp inner tube or surface exposed at the tip on the VgrG-PAAR spike (Jurénas & Journet, 2021). Acting like a crossbow, the nanotube and spike loaded with effectors are propelled upon contraction, puncturing the membrane of target cells and delivering a cocktail of effectors (Sana et al., 2017). The T6SS is highly modular, allowing various combinations of VgrG and PAAR proteins to assemble a functional spike (Cianfanelli et al., 2016). As a result, multiple effectors can be delivered simultaneously in one firing event (Shneider et al., 2013). T6SS can be loaded in two distinct ways: either as “specialised” effectors, when the effector is translationally fused at the C-terminus to one of the three Hcp/VgrG/PAAR core component, or as “cargo” effectors interacting non-covalently with one these three proteins (Hernandez et al., 2020). Additionally, cargo effectors can be assisted by chaperones (syn. adaptors) or

structural motifs, which stabilise effectors and Hcp/VgrG/PAAR interactions (Jurėnas & Journet, 2021). A few chaperone families have been described so far, containing domains such as DUF1795 (Cianfanelli et al., 2016; Quentin et al., 2018), DUF2169 (Bondage et al., 2016), DUF4123 (Liang et al., 2015; Unterweger et al., 2015), or DUF2875 (Berni et al., 2019). Additionally, a few motifs and domains are commonly found in T6SS effector/VgrG/PAAR. These include smaller domains like MIX (Marker for type sIX effectors) and FIX (Found in type sIX effectors) (Jana et al., 2019; Salomon et al., 2014), or TTR (Transthyretin-like domains) acting as internal chaperone domain (Flaunatti et al., 2020). Additional functions were attributed to DUF2345 such as indispensable for T6SS assembly or exerting antibacterial and antifungal activity on its own (Lopez et al., 2020; Storey et al., 2020). Larger structures like Rhs (Rearrangement hot spot) are frequently found in T6SS effector proteins, harbouring an additional PAAR or VgrG interacting domain (VIRN) at N-terminus (Cianfanelli et al., 2016; Pei et al., 2020). These T6SS effectors usually comprise a nucleic acid toxic domain at their C-terminus. Structural analyses suggested that Rhs repeats form a shell structure encapsulating and protecting secreting cells from the C-terminal toxic domain (Alcoforado Diniz & Coulthurst, 2015). Upon secretion, Rhs T6SS effectors undergo two autocleavages at the C and N-terminal ends, releasing the toxin (Kielkopf et al., 2024). Rhs loci were named after their high propension to recombination rearrangement, suggesting a mechanism for C-terminal toxic domain diversification (R. J. Lin et al., 1984). T6SS effectors with bacteriolytic activity are accompanied by immunity proteins (T6Is) that prevent self-intoxication of the secreting cell or attack of kin cells. T6SS effector and immunity genes are usually organised in bicistronic units (Jurėnas & Journet, 2021) and their gene products usually localise in the same subcellular compartment (Jurėnas & Journet, 2021).

Antimicrobial T6SS effectors have been subdivided into two groups according to their activity acting either in the periplasm as lytic toxins (phospholipases, peptidoglycan hydrolases, pore-forming proteins, etc.) or in the cytoplasm (DNases, enzymes disturbing the energy balance, etc.) (Jurėnas & Journet, 2021). Periplasmic toxins inducing cell lysis have several advantages in static environment, such as prevention of the formation of a corpse barrier of accumulated dead cells (Smith et al., 2020), or release of DNA material in the environment (Borgeaud et al., 2015). By contrast, cytoplasmic non-lytic toxins do not cause rapid cell lysis, but efficiently target vital biological pathways. T6SS toxins can either lead to growth arrest by impacting cell division or complete cell death (Wood et al., 2019). Some T6SS even deliver trans-kingdom effectors, targeting both eukaryotic and prokaryotic cells (Jiang et al., 2014, 2016; Russell et al., 2013; Tan, Yang, et al., 2025).

More than 100 years after its discovery, *Xanthomonas translucens* persist as economically important pathogen of *Poaceae* worldwide, causing bacterial leaf streak and bacterial wilt diseases (Goettelmann et al., 2022). The taxonomy was recently refined using the term *Xanthomonas translucens* complex for designating

collectively the three species *X. translucens sensu stricto*, *X. cerealis* and *X. graminis* (Tambong et al., 2024). While *X. translucens* pv. *translucens* is restricted to barley, *X. translucens* pv. *undulosa* infects a broad range of cereals (Bragard et al., 1997). Similarly, *X. graminis* pv. *graminis* infects a wide host range of forage grasses, whereas *X. phleipratensis* is limited to *Phleum pratense* (Kölliker et al., 2006). *X. cerealis* overlaps the host ranges of both *X. translucens* and *X. graminis* (Bragard et al., 1997). Our group previously demonstrated that T6SS-i4 and X-T4SS of the *X. translucens* complex play overlapping roles in killing of neighbouring bacteria from cereals (Peduzzi et al., 2025). *X. translucens* comprises barley pathogens with contrasting tissue specificity, namely non-vascular pv. *undulosa* (*Xtu*) affects only mesophyll causing localised symptoms, while vascular pv. *translucens* (*Xtt*) also colonises xylem, inducing systemic symptoms. The lifestyle of *Xtt* is linked to the cell wall-degrading hydrolase CbsA (Gluck-Thaler et al., 2020). All *X. graminis* strains are vascular pathogens, entering through stomata or wounds caused by mowing or grazing. These bacteria colonise the xylem, and disrupt water and nutrient flow, causing wilting, leaf and tiller curling, and sometimes plant death (Goettelmann et al., 2023; Kölliker et al., 2006).

Phylogenetic analyses of core gene sequences have revealed that the T6SSi subtype can be classified into five distinct clades (i1–i5) (Bernal et al., 2018; Boyer et al., 2009). T6SS-i3, phylogenetically restricted to *Xanthomonadales*, was further subdivided into the subclades i3*-i3**** (Bayer-Santos et al., 2019; Garin et al., 2024). Comparative genomic analyses identified representatives from clades and subclades T6SS-i3*, i3***, and i4 in the whole *Xanthomonas* genus, but only T6SS-i3*** and i4 were found in the *X. translucens* complex (Alvarez-Martinez et al., 2020; Bayer-Santos et al., 2019). The *Xanthomonadales* T6SS-i4 has been mainly associated with antibacterial effectors and to a lesser extent with antifungal and anti-eukaryotic effectors, while the role of T6SS-i3***-related effector remained unclear (Bayer-Santos et al., 2019; Garin et al., 2024; L. Lin, Shen, et al., 2025; J.-T. Yang et al., 2025). To date, only a few effectors have been experimentally tested. A recent study predicted the T6SS effector repertoire of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) strain PXO99^A with a total of 21 putative effectors (Tan, Zheng, et al., 2025). The phospholipase TleB was found to function as a trans-kingdom effector with dual activity, contributing to both interbacterial competition and host manipulation (Tan, Yang, et al., 2025; J.-T. Yang et al., 2025). They also functionally validated a T6SS VasX_N family effector (PXO_00500) with periplasmic toxicity and its neutralisation by two nearby encoded immunity proteins of the T6SS_Burk_exIF superfamily (PXO_RS08595 and PXO_RS08605) (Tan, Zheng, et al., 2025). In *Lysobacter enzymogenes*, the T6SS effector “Le1893” from the Rhs family, harbouring PAAR, Rhs and AHH nuclease domains, showed antifungal activity probably by reducing total DNA content (L. Lin, Shen, et al., 2025). As such, the field still lacks comprehensive knowledge of *Xanthomonadales* T6SS effector activities and diversity.

Here, we used the *X. translucens* complex to investigate the ecological drivers of T6SS antibacterial effectors diversity, since this group of bacteria includes T6SSi4-positive strains with overlapping host ranges and different lifestyles. We conducted a

comprehensive analysis of the T6SS effector diversity in the model *X. translucens* complex to decipher their ultimate T6SS functions. We further experimentally tested the bacteriolytic activity of a comprehensive set of T6SS effectors found in the reference strain *Xtu* UPB513, a representative strain of the *Xt* T6SS-i4 core effectome, and tested the neutralisation potential of their cognate immunity proteins.

Results

The *X. translucens* complex harbours core and accessory T6SS effectors

To gain insight into the diversity of T6SS-i4 effectors in *X. translucens sensu lato*, we investigated the effector repertoire in the 31 *Xt* T6SS-i4⁺ strains with available complete genomes. We first established a comprehensive list of the three effector-associated T6SS genes based on HMM profile screening of their encoded proteins, Hcp, VgrG and PAAR. While the *hcp* gene was only found in a single copy associated with the T6SS-i4 cluster, genes encoding VgrG or PAAR domains were numerous, either located in the vicinity of the cluster or scattered across the genome (Supplementary Figs.1-2). The ten genes located upstream and downstream of each identified VgrG and PAAR-encoding gene were manually screened for genes of candidate T6SS effectors, adaptors or immunity proteins. Functional domains were further predicted using InterProScan.

We first observed a core effectome, defined as effectors present in at least 95% of *X. translucens sensu lato* strains (Fig.1). This core effectome included a set of four effector domains, namely phospholipase Tle1, ToxREase, Rhs, and VasX domains (Supplementary Figs.3-9). Detected Rhs effector genes each harbour a PAAR domain at their N-terminus, DUF6531, Rhs repeats, and differing toxic domains at their C-terminus. These comprised different nuclease activities, such as Tox-URI-2, SHH, LHH, GIY-YIG domains, or XOO_2897-like deaminase domains. A single PAAR domain-encoding gene was additionally found in all T6SS-i4 clusters. However, homologs were always surrounded downstream by *tagX* and upstream by a gene with no detected domains. It is still unclear whether this upstream gene encodes an effector or corresponds to a new accessory *tag* gene that is associated with the T6SS-i4. In addition to the core effectome, many accessory effectors were uncovered, present in less than 95% of the analysed strains. A phospholipase Tle4 effector is present in the majority of *X. translucens* strains but not in grass-infecting *X. graminis* or *X. cerealis* (Supplementary Fig.10). Another candidate effector with lysozyme-like and M23 peptidase domains, reminiscent of Tae effectors, was found in the whole *X. translucens* complex, except in *Xtt* (Supplementary Fig.11). Additionally, another frequently found accessory effector contained lysozyme-like, chitinase class 1 and calcium-binding domains. The distribution of the other accessory effectors was more heterogeneous, including VRR Nuc (Supplementary Fig.12), PAAR-fused nuclease with DUF2169-containing adaptor (Supplementary Fig.13), FIX_Ntox15, Tle3 (Supplementary Fig.14) or PG binding domains, among others. Diverse Tle1, M23 peptidase and VasX effector variants bearing similar functional domains, respectively, were found, but shared less than 30% sequence identity, and were

therefore subcategorised as distinct effector-immunity (E-I) module groups (from A to B/D/F, see Fig.1 and Supplementary Figs.3-8). Additional analyses confirmed that the trans-kingdom effectors TleA and TleB from *Xoo* PXO99^A share 36.9% and 91.1% sequence identity, respectively, with *Xtu* LW16 Tle1A (Tan, Yang, et al., 2025). Interestingly, long arrays of multiple immunity genes were found next to the *vasX* gene from group A. The VasX_N containing protein from *Xoo* PXO99A shares 37% sequence identity with VasX group B from *X. cerealis* O1 (Tan, Zheng, et al., 2025). Intriguingly, a conserved set of genes corresponding to the Tle1 group B module was found, namely its cognate *vgrG*, chaperone and immunity genes, and a shortened version of the *tle1* effector gene. These were consistently followed by *pilS*, *traD* and *mobH/L* genes, in the middle of the T6SS-i4 variable region. Additionally, only one *vgrG* gene was found to be associated with the *Xt* T6SS-i3*** cluster. A DUF6931-encoding gene was highly conserved between FHA and the corresponding *vgrG* gene in all T6SS-i3 subclades (i3*-i3***). DUF6931 was also found associated with T6SS in alpha and beta-proteobacteria and even fused to FHA domain in *Planctomycetota* according to the InterPro database.

Comparison of the left (next to *tssM*), right (next to *tssH*), and variable regions at the centre of the T6SS-i4 cluster further revealed cognate VgrG/PAAR, effector, chaperone and immunity gene modules that consistently localised together in the genome (Supplementary Figs.3-11, 15). Interestingly, the genomic position of distinct T6SS effector genes in the left-flanking, right-flanking or central variable region of the T6SS gene cluster or as orphans was well conserved. For instance, the Rhs effectors were consistently found to be encoded on the left-flanking region, while *vasX* genes were found in the right-flanking region. All ToxREase genes were consistently found as orphans. Interestingly a series of effector genes were found near *tssL*, the last gene next to the variable region, and were accompanied by *vgrG* genes with highly similar levels of sequence identity. Phylogenetic analyses reveal that these VgrG proteins form one distinct cluster, suggesting a shared evolutionary origin separate from other T6SS effector-associated VgrG proteins (Supplementary Fig.2).

The presence of identical E-I modules assigns strains to a same compatibility group, while differing modules render them T6SS-incompatible. A few strains possessed identical E-I pairs repertoires, such as the strain couples *Xtlr8* and *Km15*, *Km33* and *Km12*, *CFBP2539* and *CFBP2055*, *CO237* and *Xtu4699*, and the three strains *Km9*, *UPB957* and *UPB458*. Interestingly, these compatible groups were always phylogenetically close and usually isolated from close countries or regions. However, the opposite was also observed, as some strains isolated from similar regions showed highly diverging T6SS E-I arsenals, such as *ICMP 11055* and *Km15*, or *CIX207* and *CIX282*.

Among the identified E-I pairs, many effectors were inactivated by insertion of IS elements or replacement by a novel homologous E-I pair variant. This is exemplified by *Xt* CIX282 with a reduced T6SS effector repertoire and a relatively small variable region compared to other phylogenetically close *Xtu* strains.

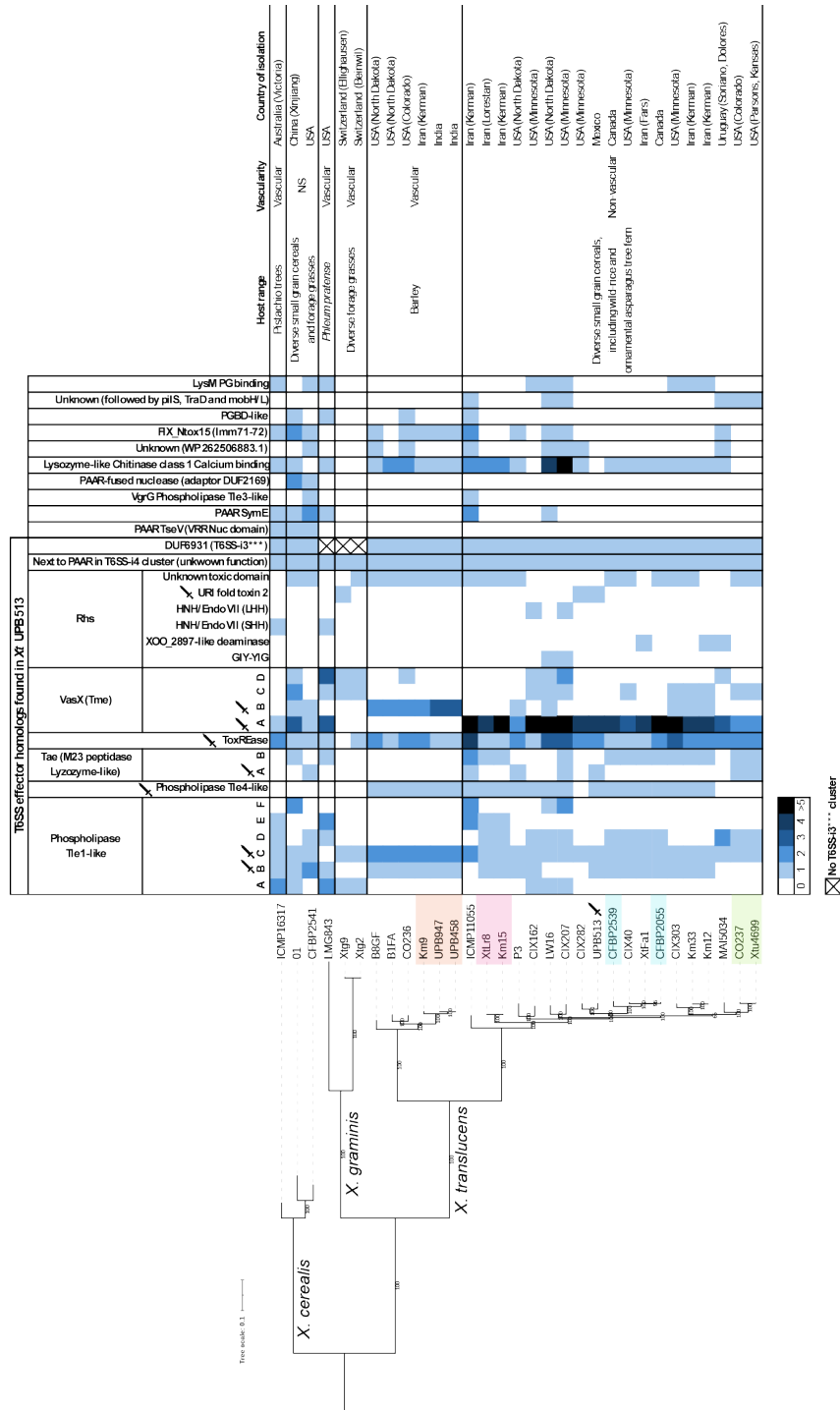


Figure 1: Diversity T6Es in the *X. translucens* complex. A kSNP3-based species phylogenetic tree is shown on the left. Heatmap showing the absence or presence of one or multiple T6E candidates and their subgroupings (30% sequence identity) in each strain. Host range, lifestyle (vascularity) and country of isolation are indicated on the right of the table, respectively. Effector categories that were experimentally validated for their bacteriolytic activity in this study are indicated by the sword symbol next to their name. Strains lacking the T6SS-i3*** cluster are marked with a cross. T6SS compatible strains with identical T6SS effector-immunity repertoire are highlighted in similar colours.

Non-vascular lifestyle drives T6SS effector diversification in xanthomonads

Given the wide diversity of T6SS E–I modules with antibacterial potential among *X. translucens sensu lato* strains, we investigated the drivers of T6SS-i4 and effector distribution. We hypothesised that their repertoires are shaped by differences in ecological lifestyle, exposing them to differing microbial competitive pressures.

T6SS-i4 is found in high occurrence within the *X. translucens* complex, *X. oryzae* and *Xanthomonas fragariae* species, while very few T6SS-i4-positive strains are found in other xanthomonads. We found an enrichment in T6SS effector diversity and quantity within strains with non-vascular lifestyles. Within *X. translucens*, the T6SS effector repertoire in non-vascular *Xtu* was highly heterogeneous, compared to the well-conserved T6SS effector arsenal of vascular *Xtt*. Similarly, low T6SS effectors diversity was found among vascular *X. graminis* pvs. *graminis* and *phleipratensis* strains. In addition, a higher number of T6SS effectors-associated VgrG and PAAR was found among non-vascular *X. oryzae* pv. *oryzicola* (*Xoc*) compared to vascular *Xoo* (Fig.2). The *X. fragariae* species is mainly characterised as non-vascular. All T6SS-i4⁺ strains possessed very close total VgrG and PAAR genes number. Interestingly, while *X. fragariae* YL19 has been described with an unusually vascular lifestyle in the literature, we did not find T6SS-i4 gene cluster in its genome. In *Xanthomonas citri*, *Xanthomonas hortorum*, *Xanthomonas phaseoli*, and *Xanthomonas campestris*, T6SS-i4 is only found in a minority of strains, sometimes only one. Among those, a few strains carry both the X-T4SS and T6SS-i4 systems, such as *X. citri* pv. *glycines* ISO118C5. We therefore studied the repertoire of their T6SS-i4 effectors in all available complete genomes. We found that they all harboured highly reduced T6SS-i4 effector arsenals, restricted to VasX, NTox15 and Tle1 domains, and an additional unknown gene accompanied by a predicted immunity protein in *X. citri* pv. *glycines* 8ra.

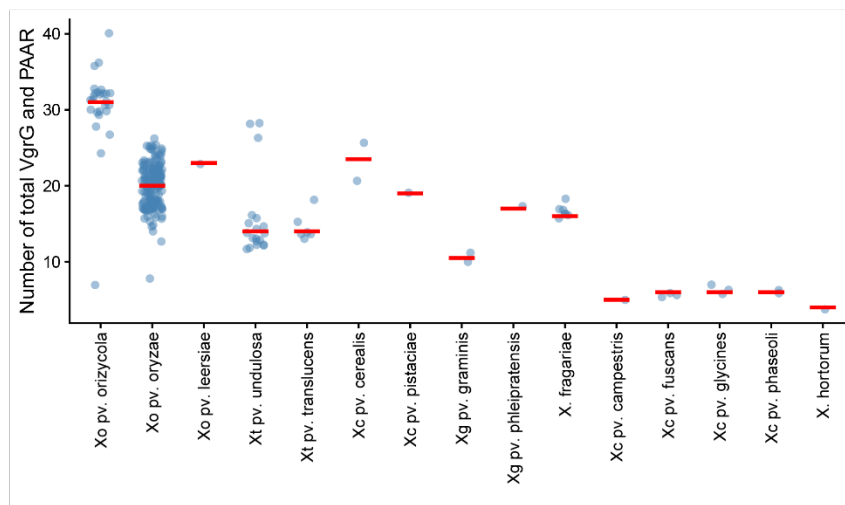


Figure 2: T6SS-i4⁺ xanthomonads and T6SS VgrG and PAAR total repertoires. The total number of VgrG and PAAR genes is depicted for each respective T6SS-i4⁺ *Xanthomonas* species/ pathovar. The mean is indicated by a red bar and each dots represent individual strains.

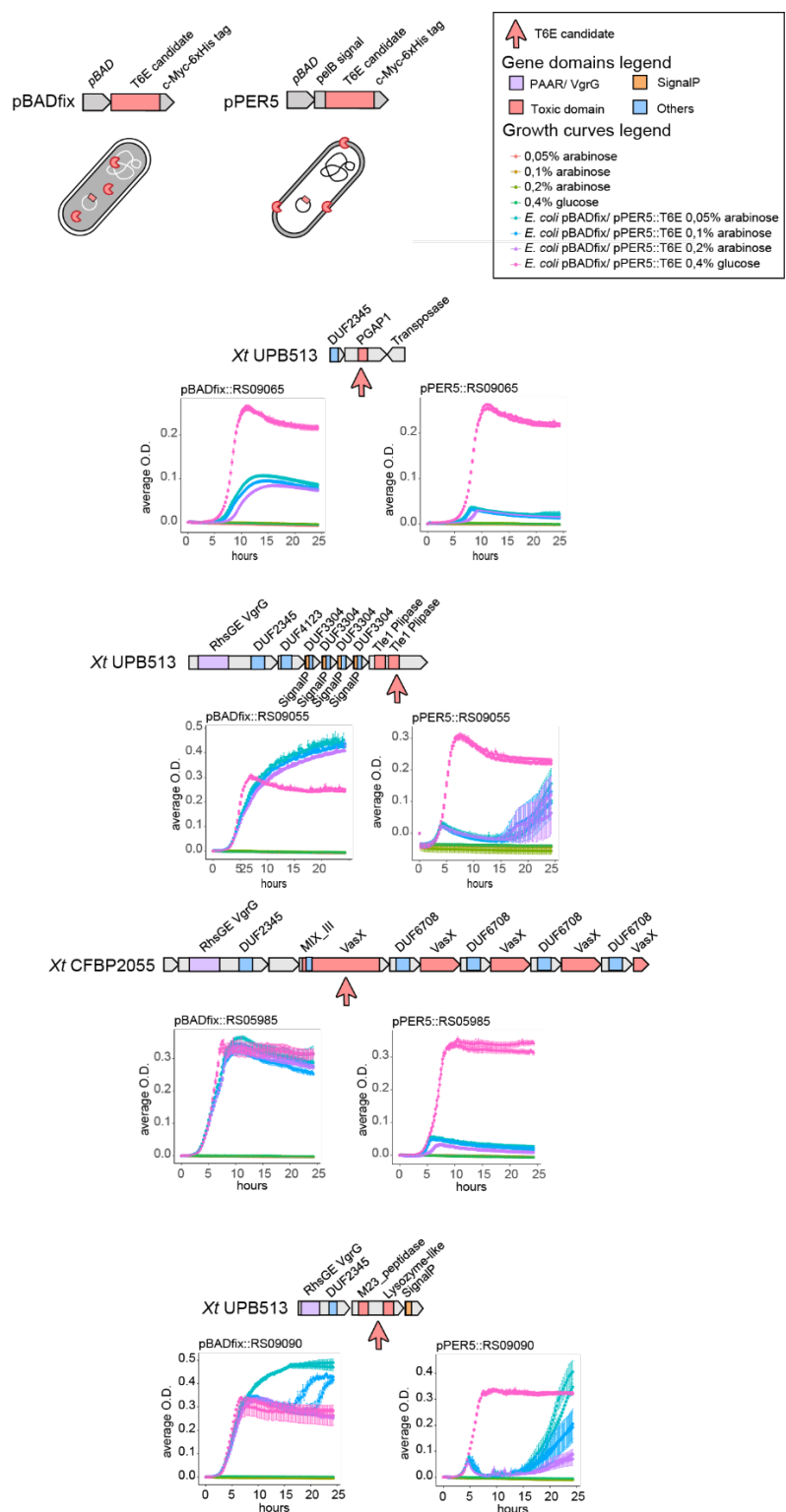
All core effectors of the *X. translucens* complex contribute to bacterial toxicity

The reference strain *Xtu* UPB513 T6SS-i4 is known for its role in bacterial killing. We therefore selected this strain for an in-depth characterisation of the bacteriolytic potential of its T6SS effector arsenal. This strain is representative of the T6SS core effectome of the *X. translucens* complex. From our above findings, a total of eight VgrG- and five PAAR-encoding genes were found in *Xtu* UPB513 (see Supplementary Fig.16). Some of them were either associated with the T6SS-i3*** cluster or pseudogenised by IS elements, or no candidate effector gene was found in their vicinity. These genes were therefore not included in the further analyses. Additionally, one *vgrG* and one PAAR-fused effector gene were associated with the same E-I module. By comparative analyses with other *Xt* T6SS-i4 variable regions, one identified *vgrG* gene was shorter compared to its homologs in other *Xt* strains, and the following candidate effector gene had lost its cognate immunity gene. However, this candidate was still included in the dataset for toxicity testing, bringing the total number of effector candidates to be tested to eight. The predicted functional domains of these proteins included phospholipases Tle1 and Tle4, nuclease, peptidase and membrane-disrupting domains. For VasX, two variants with similar VasX domains sharing 21.6% identity were tested from *Xt* strains CFBP 2055 and CFBP 2541 (see Supplementary Fig.16).

To investigate the toxicity of candidate effectors, two high-copy number vectors with arabinose-inducible promoters were used for heterologous expression in *E. coli*, pBADfix for the synthesis of proteins in the cytoplasm and pPER5 for the delivery of proteins into periplasm thanks to an N-terminally fused PelB signal sequence for secretion. The growth of each *E. coli* transformant was monitored in LB medium

supplemented with kanamycin and either varying concentrations of L-arabinose (0.05%, 0.1%, or 0.2% wt/vol) for expression, or D-glucose (0.4% wt/vol) for repression as a control (Fig.3). Growth of the cultures was monitored for 24h30 at 37°C. We first observed that the growth of *E. coli* wild-type cells was negatively affected when the monosaccharide concentration increased. Growth was lower at 0.2% arabinose and 0.4% glucose concentration than at 0.05% or 0.1% arabinose concentration. However, *E. coli* strains carrying empty plasmids pBADfix or pPER5 showed identical growth as the wild type, ruling out an impact of the plasmid or the antibiotic resistance marker on their growth (Supplementary Fig.17).

Among the effectors, periplasmic expression of the phospholipase (MZO50_RS05440) resulted in substantial toxicity, compared to the cytoplasmic expression, suggestive of an activity in the periplasmic space. Two orphan ToxREase-5 nucleases (MZO50_RS05440 and MZO50_RS14560) were toxic upon delivery to both the cytoplasm and periplasm. Surprisingly, MZO50_RS05440 had a stronger effect in the periplasm. The candidate Rhs nuclease effector bearing a Tox-URI-2 domain (MZO50_RS08935) was similarly toxic in both the cytoplasm and the periplasm, with a notable effect in the latter. Despite a predicted pseudogenised cognate immunity gene, both cytoplasmic and periplasmic expression of the phospholipase Tle4 with a PGAP1 domain (MZO50_RS09065) showed high toxicity. Finally, while both cytoplasmic and periplasmic expression of both VasX variants (KCU58_RS05985 and KHA79_RS11705) showed lower bacterial growth, the periplasmic version was more detrimental. The differing arabinose concentrations only slightly impacted the lag phase of T6SS effector-expressing *E. coli* growth curves, such as observed with the Tle4 effector. The maximal culture density in glucose-supplemented growth curves was slightly higher in bacteria carrying empty vectors than in effector-expressing vectors, probably resulting from leaky expression. These results confirm the bacteriolytic role of all eight tested T6SS core effector candidates.



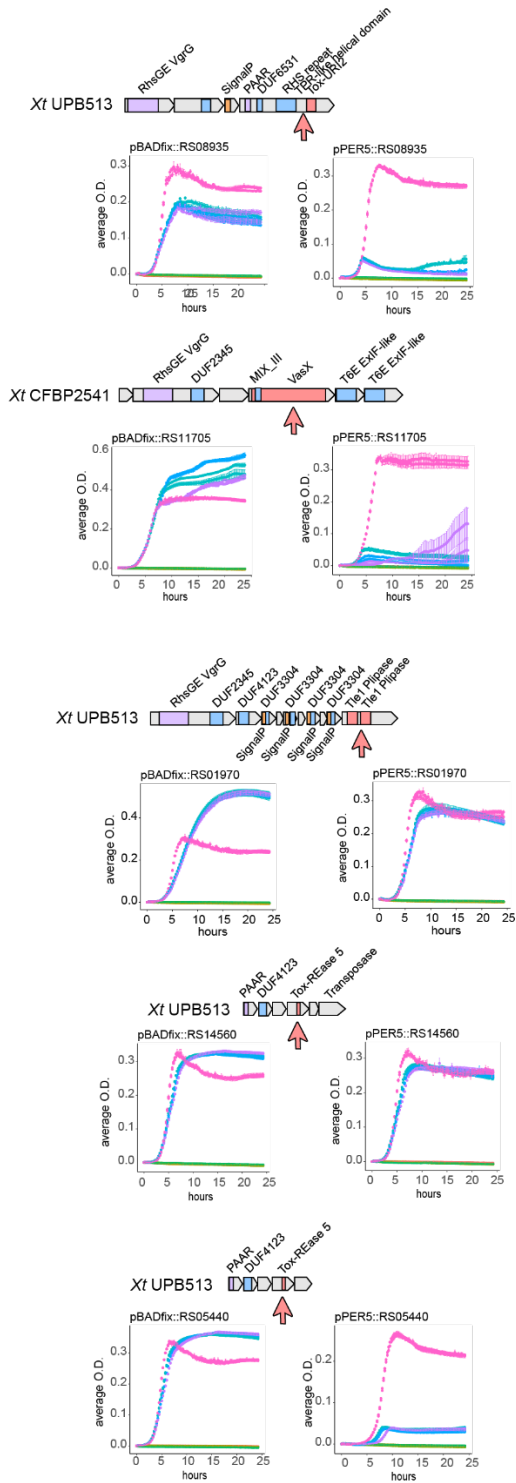


Figure 3: T6E candidates show toxicity when expressed in *E. coli*. Schematic representation of heterologous expression vectors used. Genes that were selected for expression are represented above each graph, and domains are colour coded according to their type: PAAR/ VgrG, toxic domains, signalP, and others. Effector proteins are indicated by red arrows. *E. coli* growth curve in LB at 37°C under the expression (L-arabinose) or repression (D-glucose) of T6E candidates. Two replicates for each treatment (n= 8 repetitions per replicate). Dots represent mean and error bars show s.e.m.

Modelling effector interacting proteins

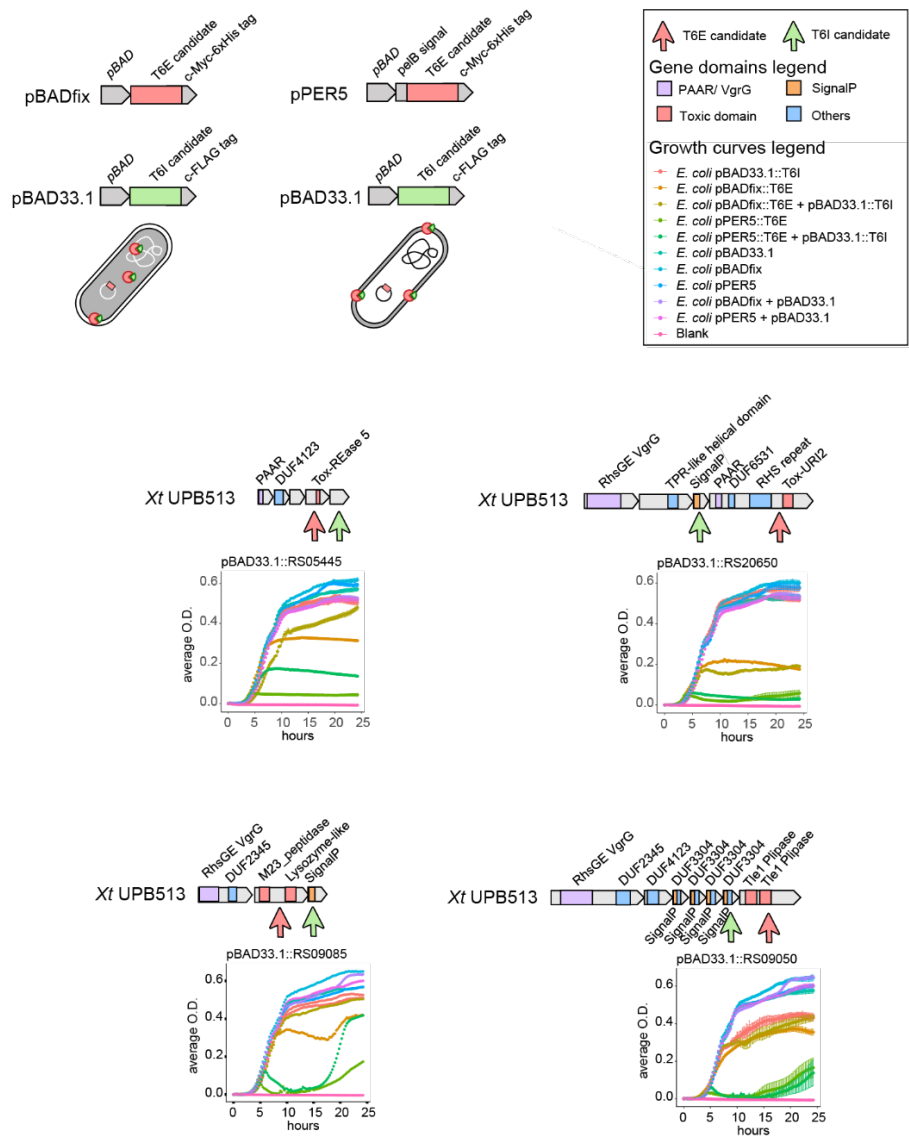
We further aimed at predicting the cognate immunity proteins of the identified anti-prokaryotic effectors. Candidate immunity proteins were selected based on a comparison of the genes adjacent to the effector genes, their typical small size, and the presence of a signal peptide in the case of periplasmic effectors. Both MZO50_RS14560 and MZO50_RS09065 had an IS element insertion in their respective immunity gene and were therefore not included in subsequent analyses. Using AlphaFold we predicted effector-immunity protein interactions (Supplementary Fig.18). The predicted template modelling score (pTM) informs about the quality of the predictions, while the interface pTM score (ipTM) quantifies the confidence of interactions between two given proteins. When pTM exceeds 0.5, the predicted overall fold of the complex can be considered a reasonable approximation of the true structure. ipTM values above 0.8 indicate confident, high-quality predictions, whereas values below 0.6 usually reflect failed predictions. Finally, scores between 0.6 and 0.8 fall into a grey zone where accuracy remains uncertain (Abramson et al., 2024).

AlphaFold predictions with high confidence of tested E-I pairs included protein couples MZO50_RS09035 and MZO50_RS09055 (Tle1B), MZO50_RS09085 and MZO50_RS09090 (M23 peptidase), as well as MZO50_RS05440 (ToxREase) and MZO50_RS05445. The candidate immunity protein array upstream of Tle1 all showed a high ipTM score above 0.75, the highest being the gene directly upstream of the effector. The structure of the whole VgrG, adaptor, immunity and effector protein complex was predicted with a ipTM score of 0.71. While the role of MZO50_RS05435 is unknown, it was confidently predicted in a complex with MZO50_RS05425 (VgrG) and MZO50_RS05430 (adaptor DUF4123). No strong candidate of an immunity protein was found for the nuclease-containing Rhs protein or the nucleolytic toxic domain alone that is thought to be released from the Rhs cage upon post-translational cleavage. However, it is notable that the toxic domain was found to be located encapsulated inside the barrel cage. No clear interaction could be predicted for the other effectors, pending experimental testing.

Cognate immunity neutralises effector antibacterial activities

Following AlphaFold structural predictions, we experimentally characterised the neutralisation potential of these immunity proteins (Fig.4). Given the slight effect of arabinose concentration in the toxicity assays, a concentration of 0.1% arabinose was chosen for the following neutralisation assays. Co-expression of the ToxREase (MZO50_RS05440) with its cognate immunity protein (MZO50_RS05445) partially

neutralised the toxicity, confirming a first E-I pair. Similarly, the toxicity of the M23 peptidase (MZO50_RS09090) was mitigated by co-expression with its immunity protein (MZO50_RS09085) in the periplasmic expression condition and totally abolished in the cytoplasmic expression condition. However, the candidate immunity protein (MZO50_RS20650) failed to antagonise the growth defect caused by the Rhs nuclease (MZO50_RS08935). The candidate immunity protein (MZO50_RS09050) also did not provide immunity against the Tle1 Phospholipase (MZO50_RS09055). Finally, the tested immunity protein (MZO50_RS15745) was toxic when expressed alone and in co-expression with the orphan Tle1 Phospholipase (MZO50_RS15750).



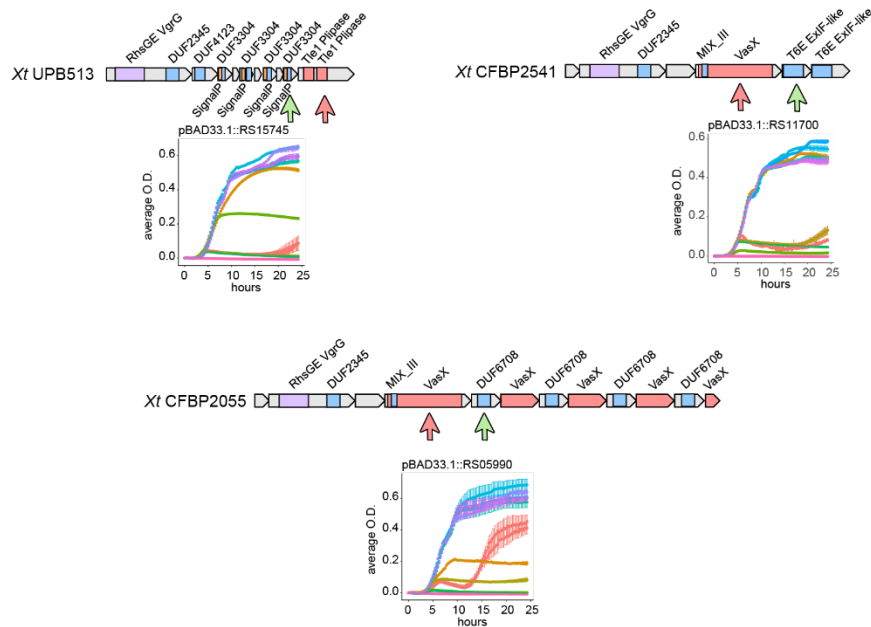


Figure 4: T6I neutralisation assays. Schematic representation of the vectors used for heterologous expression is shown on top. Genes that were selected for expression are represented above each graph, and domains are colour coded according to their type: PAAR/VgrG, toxic domains, signalP, and others. Effector proteins are indicated by red arrows and immunity proteins by green arrows. *E. coli* growth curve of bacterial cultures in LB at 37°C upon L-arabinose-induced expression of T6E-T6I candidates. One replicate for each treatment (n= 8 repetitions per replicate). Dots represent mean and error bars show s.e.m.

Discussion

Bacteria prosper despite a constant battle for resources and space. While the T6SS is widely distributed among bacterial genera, its effectors display remarkable diversity, targeting eukaryotic or prokaryotic cells, or even both. However, the evolutionary and ecological factors shaping the distribution and functional diversification of T6SS effectors across bacterial lineages remain poorly understood. Here, we found evidence that increase in effector diversity in the *X. translucens* complex is at least partially driven by the tissue specificity of the pathogen. Additionally, we provide experimental evidence of bacteriolytic activity for eight effectors of the *X. translucens* complex and the neutralising activity of their cognate immunity proteins.

Our genomic in depth-screening and comparative genomic analyses revealed a core and an accessory effectome within the *X. translucens* complex harbouring the T6SS-i4. Overall predictions highlight toxic domains with potential for prokaryotic and eukaryotic targeting. Homologs of the trans-kingdom effector TleB, which were recently described by Tan *et al.* (Tan, Yang, et al., 2025) and correspond to Tle1A in

this study, were found to be highly dispersed across all *X. translucens sensu lato* strains. We also found homologs of the Tle1, Tle3 and Tle4 phospholipases as classified by Russell *et al.* (Russell *et al.*, 2013). A chaperone with DUF2169 was encoded near a PAAR-fused gene that encode a DNase toxic domain and a *vgrG* gene. Interestingly, in the rice pathogen *Burkholderia glumae*, two T6SS accessory genes, one of which encodes a DUF2169 domain, were shown to be involved in bacterial virulence (Kim *et al.*, 2021).

Additionally, we found homologs of conjugative T4SS genes, including the coupling protein TraD, PilS and MOB_{H/L} family of relaxases, to be encoded within the variable region of some *Xt* T6SS-i4, always localised near the same shortened Tle1B module. In *Vibrio cholerae* it was shown that successive insertion of novel E-I modules can displace ancestral effector genes while conserving immunity genes, which are shifted downstream, thus gradually leading to the evolution of long arrays of multiple immunity genes (Kirchberger *et al.*, 2017). We observed a similar mechanism in the evolution of the VasX modules. In this study, PAAR-related effectors exclusively comprise nucleases, such as the ToxREase, Tox-URI2, VRR-Nuc, Ntox, and SymE domains, which are thought to be active in the cytoplasm. In contrast, VgrG-related effectors are linked to activities in the periplasmic space, such as phospholipases or pore-forming effectors.

In addition to T6SS-i4, *X. translucens* and *X. cerealis* also harbour one T6SS-i3*** cluster, making it unclear whether orphan effectors belong to T6SS-i3*** or to T6SS-i4. However, the presence of T6SS-i4 alone in *X. graminis* allows attribution of all effectors in its repertoire to the clade-i4. In line with Bayer-Santos *et al.* T6SS effector predictions (Bayer-Santos *et al.*, 2019), only one candidate effector was found in the vicinity of *Xt* T6SS-i3*** cluster. However, this putative DUF6931-encoding gene upstream of *vgrG* is present in all T6SS-i3 subclades. Therefore, it is still not clear whether this gene is a new T6SS accessory *tag* gene or an effector gene. In other xanthomonads harbouring T6SS-i3***, only a conserved set of hypothetical proteins harbouring a TIR_2 domain, typical of Toll-like receptors, were identified in a few species (Bayer-Santos *et al.*, 2019). Bacterial proteins with TIR_2 domains act as NADases and interfere with cellular metabolism in prokaryotic and eukaryotic cells (Alvarez-Martinez *et al.*, 2020).

The presence of T6SS compatibility groups within *X. translucens* strains supports the hypothesis that different *Xt* strains may co-exist within the host, or that they compete via alternative mechanisms. It would be interesting to assess whether the immunity proteins of Tle1, M23 peptidases or VasX effector modules confer cross-protection across the different subgroups (A-B/D/F), thereby increasing the number of strains belonging to the same compatibility groups.

In our work, we could also detect rare cases of strains containing both complete X-T4SS and T6SS-i4. We also observed a reduction in the T6SS-i4 effector repertoire in xanthomonads that contain as well the X-T4SS. In the X-T4SS⁺ T6SS-i4⁺ *L. enzymogenes* strain OH11, the T6SS-i4 appears to have antifungal but not

antibacterial activity, whereas the X-T4SS plays a role in interbacterial competition and quorum quenching, but also indirectly favouring other bacteria in its environment to produce the antifungal compound 2,4-DAPG (Liao et al., 2023; L. Lin, Shao, et al., 2025; L. Lin, Shen, et al., 2025; Shen et al., 2021; Wang et al., 2023; M. Yang et al., 2020). Considering that the identified effectors target molecules highly conserved across both prokaryotic and eukaryotic kingdoms, we do not exclude any possible additional antifungal role within identified T6SS effectors.

In light of the general lack of congruence between the distribution of T6SS effectors and species phylogeny, our dataset suggests that T6SS effectors were gained through HGT and lost among lineages numerous times. Some strains had a strongly reduced effectome, which even included the loss of core effectors, such as *Xt* strain CIX282. The loss of T6SS effectors is consistent with the study by Taillefer *et al.* (Taillefer et al., 2025), which showed that T6SS activity can be abolished by mutations when the bacteria are grown alongside immune recipients, while remaining active when grown alongside susceptible cells. By extension, the absence of microbial competition or the presence of immune recipients over an extended period could lead to the loss of T6SS effectors. Additionally, we showed that the Tle4 PGAP-containing effector was still toxic, despite an incomplete module, when expressed in *E. coli*. However, the shortening of its associated VgrG domain and the absence of cognate immunity gene could suggest that the T6SS effector is no longer secreted. Finally, the presence of a pseudogenised immunity gene associated with the ToxREase (MZO50_RS14560) could be explained either by a no longer secreted T6SS effector or a potential cross-protection by the immunity (MZO50_RS05445).

The antimicrobial T6SS effectors repertoire might reflect the pressure of competition with the microbiome that each *Xt* strain has faced. This would make of the T6SS effectome a record of recently and frequently encountered adversaries. We observed an enrichment in the diversity of T6SS effectors in non-vascular *Xtu* and *Xoc*, compared to their vascular relatives. The xylem is considered a nutritionally limited and constrained environment, which likely explains why less diversified communities of microbes can successfully colonise it (Anguita-Maeso et al., 2023; Michl et al., 2023; Zhang et al., 2022). Lower competitive pressure within the xylem compartment could therefore partly explain the reduction of T6SS effector diversity in vascular *Xanthomonas* pathogens. Additionally, the T6SS-i4 gene cluster is widely distributed among *Xtu*, compared to *Xtt*, where it is only present in the *Xtt* LIN subgroup K1 (Peduzzi et al., 2025). We therefore argue that the presence of T6SS-i4 and its effectors should be rather considered as part of a continuum of T6SS effector diversity, rather than as a binary system of presence or absence.

The bacteriolytic activity was confirmed for all the core T6SS-i4 effectors. Our results confirm the study of Tan *et al.* showing that PXO99^A TleA and TleB (Tan, Yang, et al., 2025) (Tle1A in this study) induce toxicity upon periplasmic delivery. Additionally, our results are also compatible with the toxicity of the *Xoo* PXO99^A VasX_N family effector (Tan, Zheng, et al., 2025) (VasX group B in this study), which is active in the periplasmic space. In *L. enzymogenes*, Le1983, an AHH-containing Rhs family toxin exhibits antifungal activities against yeast, for which they further predicted two self-

cleavage sites for toxin release (L. Lin, Shen, et al., 2025). Similar Rhs family proteins were identified with toxic nuclease domains LHH and SHH from the same HNH/ENDO VII superfamily within the *X. translucens* complex. In addition, we could confirm the antibacterial role of Rhs with a Tox-URI2 nuclease domain at the C-terminus.

Surprisingly, some of the tested immunity proteins were toxic when heterologously (over)expressed in *E. coli*. This could be due to unregulated overexpression, which may interfere with certain cellular pathways in a non-native host. Additionally, partial protection of an immunity has previously been observed with a *Burkholderia gladioli* T6SS effector–immunity pair when ectopically expressed from a separate plasmid in *E. coli*, whereas a transcriptionally fused effector–immunity construct restored complete protection (Yadav et al., 2021). This may result from differences in expression levels or protein localisation compared to their native genomic context. Furthermore, all nuclease-containing effectors showed a stronger toxic effect when they were delivered to the periplasm than to the cytoplasm. However, in the case of Rhs, adjacent genes encoding proteins secreted to the periplasm might be clues for inactivation of toxicity in the periplasm by an unknown periplasmic toxic domain.

The diversity of antibacterial effectors of *Xtu* strain UPB513 T6SS-i4 confirms previous confocal microscopy time-lapse studies in which various outcomes were observed for *E. coli* DH10B cells, such as growth inhibition, cell rounding, cell burst, or cell shrinking (Peduzzi et al., 2025). This suggests different effectors with differing activities, such as bacteriostatic or membrane-disrupting effectors, were delivered with each firing event against cells of the same strain. This study provides a comprehensive understanding of the antibacterial effector repertoire of the T6SS in the model bacterium *Xtu* UPB513. This will guide future investigations into key questions, such as the effector composition in each T6SS contraction event and the mechanisms of effector selection.

Materials and methods

Bacterial strains and growth conditions

Bacterial strains, plasmids and primers used in this study are listed in Supplementary Tables 1 and 2. *Xtu* strain UPB513 was grown at 28°C with shaking at 220 rounds per minute (rpm) in liquid nutrient broth (NB) or on solid nutrient agar (NA). *E. coli* DH10B was grown at 37°C with shaking at 220 rpm in liquid lysogenic broth (LB) or on solid LB agar (1.5%) supplemented with glucose (0.4% wt/vol) or L-arabinose (0.05%, 0.1%, or 0.2% wt/vol), when appropriate. The antibiotics kanamycin (Km) and chloramphenicol (Cm) were used in a concentration of 50 µg/ml and 100 µg/ml, respectively, where appropriate.

Plasmid construction

All predicted T6SS effector (T6E) gene of *Xtu* UPB513 were cloned in both vectors, pBADfix (Km^R; for testing cytoplasmic activity) and pPER5 (Km^R; for testing periplasmic activity), for heterologous expression in *E. coli*. Primers for each T6E,

with corresponding vector overlaps, were designed according to the Gibson assembly cloning technique. T6E amplicons and pBADfix/pPER5 amplicons were amplified using Q5 high-fidelity DNA polymerase (New England Biolabs, Ipswich, Massachusetts, USA) and purified with the Monarch® PCR & DNA Cleanup Kit (New England Biolabs). Following amplification of the vector DNA, the restriction enzyme DpnI (New England Biolabs) was used to digest any remaining methylated parental plasmid DNA. T6E amplicons were then cloned into their respective vectors using the GenBuilder Cloning Kit (GenScript, Piscataway, New Jersey, USA). Finally, constructs were transformed into *E. coli* DH10B strains by heat shock and selection on LB agar plates supplemented with 0.4% glucose (wt/vol) and kanamycin. Notably, pBADfix and pPER5-cloned genes encode proteins that are C-terminally tagged with a tandem c-myc-His₆ tag for immunological protein detection. T6E proteins expressed from pPER5 are fused at their N-terminus to a PelB signal sequence for periplasmic protein delivery.

Using the same method, all predicted cognate T6SS immunity (T6I) genes of *Xtu* UPB513 were cloned in vector pBAD33.1 for heterologous expression. Constructs were transformed into *E. coli* DH10B strains by heat shock and selection on LB agar plates supplemented with 0.4% glucose (wt/vol) and chloramphenicol. All pBAD33.1-encoded proteins are C-terminally tagged with a FLAG tag for immunological protein detection. Some hard-to-amplify immunity genes were synthesised and cloned by GenScript due to the high sequence similarity of multiple gene copies in the genome.

Each T6SS effector cloned into pBADfix or pPER5 were then transformed together with their cognate T6SS immunity cloned into pBAD33.1 into *E. coli* DH10B strains and selected on LB agar plates supplemented with 0.4% glucose (wt/vol), kanamycin and chloramphenicol. All constructions were confirmed by PCR and DNA sequencing, for which the plasmid sequencing service from Microsynth AG (Balgach, Switzerland) was used.

T6SS effector-immunity toxicity assays

The growth of *E. coli* cultures expressing T6SS effectors was monitored in LB medium supplemented with kanamycin (50 µg/ml) and varying concentrations of L-arabinose (0.05%, 0.1%, or 0.2% wt/vol for expression) or D-glucose (0.4% wt/vol for repression). The initial OD₅₆₅ of *E. coli* suspensions were adjusted to 0.001. Two replicates for each treatment with eight repetitions each were placed in 96 well-plates covered with a Breathe-Easy® sealing membrane (Sigma-Aldrich, St. Louis, Missouri, USA). Measurements of OD₅₉₅ were taken every 15 minutes during 24h30, with continuous shaking at 37°C using a Multiskan FC microplate reader (ThermoFisher Scientific, Waltham, Massachusetts, USA). Two replicates were established for each treatment, each consisting of eight monitored repetitions. The data from the eight repetitions of each replicate were used to generate one growth curve calculating the mean values and standard errors of mean (s.e.m.).

The same method was used for T6SS effector-immunity neutralisation assays in LB medium containing 0.1% wt/vol of L-arabinose, supplemented with kanamycin (50

µg/ml) and/or chloramphenicol (100 µg/ml). One replicate was established for each treatment, each consisting of eight monitored repetitions. The data from the eight repetitions of each replicate were used to generate one growth curve calculating the mean values and standard errors of mean (s.e.m.).

***In silico* screening for T6SS-i4 effector-immunity arsenals**

To perform a comprehensive screening of all T6SS effector-immunity pairs in the strain *Xtu* UPB513 (RefSeq: NZ_CP096570.1), all VgrG/PAAR/Hcp domains-containing proteins were searched using HMMsearch. Proteins corresponding to the neighbouring genes of the T6SS core genes were analysed using InterProScan and AlphaFold to identify candidate T6SS effectors and immunity proteins.

To explore T6SS effector-immunity diversity in *the Xt* complex, all the 31 *Xt* complete genomic sequences harbouring T6SS-i4 were retrieved from the National Center for Biotechnology Information (NCBI) database on July 2025. The species phylogenetic tree was inferred using kSNP3 using k-mer of 23 bp (Gardner et al., 2015).

For Tle phospholipase, ToxREase and VasX domains, the HMM profiles of these toxic domains were retrieved from InterPro and used in HMMsearch to obtain a comprehensive list of candidate effectors. To cluster the effector candidates, sequences of HMMsearch hits were extracted and aligned using MEGA (v. 12.0.11). Phylogenetic trees were then inferred using the IQTREE web server (Trifinopoulos et al., 2016) (<http://iqtree.cibiv.univie.ac.at/>) using maximum likelihood estimations with default parameters and visualised with iTOL (Letunic & Bork, 2024) (<https://itol.embl.de/>).

For peptidase, PGAP1 and Rhs domain-containing T6SS effectors, HMM profiles were not specific enough for detecting T6SS effector proteins only. Therefore, TBLASTN analyses were conducted using *Xtu* UPB513 T6SS effector sequences as queries (e-value threshold < 0.000001; BLAST identity threshold > 50%).

T6SS modules encoding VgrG/PAAR, chaperones, T6SS effector and immunity proteins were searched and extracted using cblaster (v.1.0) (Gilchrist et al., 2021) at the CAGECAT online platform (<https://cagecat.bioinformatics.nl/>). Sequences of extracted clusters were then aligned and visualised for gene homology groups (minimum alignment sequence identity of 30%) using clinker (v0.0.29) (Gilchrist & Chooi, 2021).

AlphaFold modelling

Three-dimensional structures of proteins or protein complexes, listed in Supplementary Table 3, were predicted using AlphaFold3 at <https://alphafoldserver.com/>.

Data availability

All Supplementary Information supporting the findings of this study are available in **Appendix 3**.

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Chapter IV

Inter- and intraspecies competitive interactions mediated by antibacterial secretion systems in *Xanthomonas translucens*

Author contributions

Chloé Peduzzi, Claude Bragard and Ralf Koebnik designed the study. Claude Bragard and Ralf Koebnik supervised the conducted research. Chloé Peduzzi and Simon Heylens performed the experiments. Chloé Peduzzi performed statistical analyses. Chloé Peduzzi wrote the manuscript, and Claude Bragard and Ralf Koebnik discussed the results and edited the manuscript.

Abstract

The exploration of microbial interactions within plant-associated communities has gained attention recently, particularly for their potential in biocontrol. The *Xanthomonas translucens* (Xt) complex includes bacterial pathogen of cereals that notably rely on secretion systems to compete against surrounding microorganisms. Yet, the ecological dynamics *in planta* and bacterial targets of both antibacterial type IV (X-T4SS) and type VI secretion systems (T6SS) remain poorly known. Here, we show that both X-T4SS and T6SS-i4 mediate interspecies and/or intraspecies competition against bacteria representative of the wheat-associated microbial community, thereby shaping interactions within the phytobiome. Through pairwise confrontation assays, we demonstrate that both systems efficiently kill several Gram-negative competitors, notably *Pantoea* and multiple *Pseudomonas* species, while leaving Gram-positive taxa unaffected. We further reveal that T6SS-i4 also contributes to *X. translucens* survival providing protection against these same *Pantoea* and *Pseudomonas* strains. In addition, *Xt* pv. *undulosa* (Xtu) T6SS-i4 mediates intraspecific competition against *Xt* pv. *translucens* (Xtt), both *in vitro* and *in planta*. Through confocal imaging, we describe the spatial relevance of T6SS-i4 in intraspecific competition of Xtu with Xtt. Together, these findings highlight the dual role of secretion systems in inter- and intraspecific competition, underscoring their importance for the ecological success and pathogenic lifestyle of *X. translucens*.

Introduction

Xanthomonas species cause diseases in more than 400 crops worldwide and thrive across diverse ecological niches (An et al., 2020; Parte et al., 2020). Despite progress in understanding *Xanthomonas* biology, strategies for effective disease control remain limited, highlighting the need for alternative approaches. The exploration of microbial interactions within plant-associated communities has gained attention recently, particularly for their potential in biocontrol. While antagonistic mechanisms are well characterised in several bacterial genera, such as *Pseudomonas*, or *Bacillus*, comparable insights into *Xanthomonas* remain scarce.

Inter-bacterial interactions can either be mediated by freely diffusible factors released into the surrounding environment, or by factors directly delivered into neighbouring target cells in a contact-dependent manner (García-Bayona & Comstock, 2018). Struggle for survival has driven bacteria to evolve diverse contact-dependent mechanisms for bacterial killing (Granato et al., 2019). In the *Xanthomonas* genus, two contact-dependent type IV (X-T4SS) and type VI (T6SS) secretion systems were shown to play key roles in interbacterial competition by injecting toxic effectors into neighbouring cells, as demonstrated in *Xanthomonas oryzae* (Zhu et al., 2020) and *Xanthomonas translucens* (Peduzzi et al., 2025) for T6SS, and *Xanthomonas citri* (Souza et al., 2015) and *Xanthomonas graminis* (Peduzzi et al., 2025) for X-T4SS.

The T6SS is a contractile nanomachine, evolutionarily related to bacteriophage tails, that delivers effector proteins into prokaryotic or eukaryotic cells (Alvarez-Martinez et al., 2020; Bayer-Santos et al., 2019). It is composed of 13 conserved structural proteins (TssA-M, for type six secretion) typically encoded in gene clusters, along with non-conserved accessory Tag proteins (Reglinski et al., 2023). The apparatus comprises a membrane complex (TssJLM), a baseplate (TssEFGK), and a cytoplasmic tail formed by stacked Hcp hexamers encased in a contractile TssB/C sheath, with TssA initiating and stabilising tail polymerisation (Bernal et al., 2018). At its tip, a trimeric Valine-glycine repeat protein G (VgrG) spike sharpened by proline-alanine-alanine-arginine repeats (PAAR) proteins recruits effectors that are loaded either within the Hcp tube or onto the spike for injection into target cells (Jurėnas & Journet, 2021). During firing, sheath contraction propels the Hcp tube and VgrG-PAAR spike outward to puncture neighbouring cells and delivers effectors, after which the ATPase ClpV disassembles and recycles the sheath for subsequent rounds of activity (Alvarez-Martinez et al., 2020; Cherrak et al., 2018; Costa et al., 2015; Zoued et al., 2014). T6SS gene clusters have been divided into four evolutionarily divergent subtypes (T6SSi-iv) (Böck et al., 2017; Boyer et al., 2009; Bröms et al., 2010; Russell et al., 2014). The T6SSi is prevalent across diverse ecosystems, such as in environmental (rain, marine, soil), animal or plant-associated bacteria (Bernal et

al., 2018; Unni et al., 2022). Phylogenetic analyses of core gene sequences have revealed that the subtype T6SSi can be further classified into five distinct clades (i1–i5) (Bernal et al., 2018; Boyer et al., 2009). Within phytobacteria, all the five T6SS clades i1-i5 are widely represented, with strains containing up to five T6SS clusters (Bernal et al., 2018).

The X-T4SS of the *Xanthomonadales* order is a class A type IV secretion system composed of 12 structural components (VirB1–VirB11 and VirD4), forming a core complex anchored in the membranes and associated with an extracellular pilus (Sgro et al., 2019). Effector translocation is energised by the ATPases VirB4, VirB11, and VirD4, the latter also functioning as a coupling protein and loading platform for effectors (Sgro et al., 2019).

Cell resistance to T6SS or X-T4SS killing rely on multiple factors. Both X-T4SS and T6SS-i4 immunity proteins are small, specific molecules that neutralise their cognate effector toxins, and their genes can persist in the genome even without a functional secretion system (Jurénas & Journet, 2021; Kirchberger et al., 2017). Beyond immunity-based protection, bacteria can resist T6SS killing through mechanisms such as envelope stress responses, spatial segregation, or physical barriers like extracellular polysaccharides (Hersch et al., 2020). In *X. citri*, X-T4SS immunity proteins primarily prevent cis-intoxication, as their absence causes autolysis and impaired biofilm formation, whereas resistance to *trans*-intoxication by other cells appears to persist through immunity protein-independent mechanisms (Oka et al., 2024). These complementary strategies highlight the evolutionary complexity of X-T4SS and T6SS-mediated interactions and the multiple layers of defence shaping microbial communities.

Here, we used the model organism *X. translucens sensu lato* to investigate the ecological impact of secretion system-mediated bacterial killing *in planta*. Whereas most other *Xanthomonas* species evolved only one antibacterial system between X-T4SS or T6SS-i4, *X. translucens* (recently considered a species complex) stands out by comprising strains with the X-T4SS and other strain with the T6SS-i4 in the same species, making it a valuable model for their study (Alvarez-Martinez et al., 2020; Bayer-Santos, DeMoraes Ceseti, et al., 2019; Sgro et al., 2019). We provide evidence supporting the inter- and intraspecies competitions using X-T4SS and/or T6SS *in vitro* and *in planta*. Better understanding of competitive traits in the *X. translucens* complex opens new avenues for sustainable disease management.

Results

Killing activity X-T4SS and T6SS of the *Xanthomonas translucens* complex against cereal competitors

The role in bacterial killing of both *X. graminis* (Xg) LMG728 X-T4SS and Xt UPB513 T6SS-i4 was demonstrated in previous work against the bacterial model *E. coli* (Peduzzi et al., 2025). Therefore, we further compared the bacterial killing potential of both systems against a wide range of bacteria representative of cereal phytobiome. To this end, wild-type and mutant strains of both systems, *virD4* for X-T4SS and *hcp* for T6SS-i4, were used. Pairwise qualitative competition assays were conducted at different dilutions against each individual member of the cereal bacterial consortium described by Dubois *et al* (Dubois et al., 2024). Although the consortium initially comprised 16 bacterial strains representative of diverse taxa, four were excluded due to insufficient growth under the assay conditions. From the 12 bacteria tested, five were killed by both X-T4SS and T6SS-i4 (Figure 1). These T6SS-dependently killed bacteria included only Gram-negatives of the gamma-proteobacteria, namely *Pantoea agglomerans*, *Pseudomonas fuscovaginae*, *Pseudomonas sivasensis*, *Pseudomonas lurida*, and *Pseudomonas cichorii*. On the contrary, *Glutamicibacter*, *Microbacterium*, *Staphylococcus*, *Bacillus*, *Sphingobacterium*, *Pedobacter* and *Burkholderia* were not impacted by X-T4SS or T6SS-i4. Interestingly, both systems targeted the same species and strains, which implies a redundant function. However, while the T6SS-i4 *hcp* mutation strongly reduced bacterial killing in pseudomonads compared to the wild type, it did not totally abolish the killing. On the opposite, the X-T4SS *virD4* mutation totally impaired bacterial killing in pseudomonads.

Additionally, we observed that Xg LMG728 X-T4SS and Xt UPB513 T6SS-i4 killing outcome against *Pantoea agglomerans* differed according to the medium's recipe. While killing occurred in NA from Difco brand, it did not in NA from Millipore brand. In addition to slight composition differences, we also noted a stronger hydrophobicity of the second media. However, this medium-dependent killing effect was not observed against the other cereal-associated bacteria.

Figure 1: Cereal representative bacteria killed in a X-T4SS and T6SS-i4 dependent manner. The results of pairwise qualitative competition assays of *Xt* UPB513 WT and $\Delta hcp4$, *Xt* LMG728 WT and $\Delta virD4$ strains against representative cereal bacteria are indicated using a colour code: dark gray for strong bacterial killing activity, light gray for weak bacterial killing activity, and white for no bacterial killing. One strain per species was tested. Respective bacterial class and Gram colorations are indicated.

T6SS impacts *Xt* survival in co-cultures with cereal competitors

Given the role of T6SS-i4 in killing varying bacteria, we additionally tested the opposite hypothesis, namely whether if this system plays a role in *Xt* survival. To this end, we used strains of *Xt* UPB513 WT, *hcp4* mutant and complemented strains, expressing RFP⁺. We screened their fitness when co-cultured with the 16 bacteria that are representative of the wheat-associated microbial community. As a result, we could distinguish three cases, T6SS-independent killing, T6SS-dependent killing or absence of killing of *Xt* UPB513 (Figure 2A-B). Firstly, a strong T6SS-independent killing was observed for *Burkholderia anthina* and *Bacillus subtilis* and a moderate killing for *Glutamicibacter creatinolyticus*, *Sphingobacterium thalpophilum* and *Microbacterium oxydans* against *Xt* UPB513. The T6SS of *Xt* UPB513 was required for its survival in co-cultures with *Pantoea agglomerans*, *Enterobacter kobei*, and all tested *Pseudomonas* species except *Pseudomonas syringae*. Finally, the competition assays performed with *Staphylococcus equorum*, *Sphingomonas albertensis*, *Methylobacterium bullatum* and *Pedobacter foliorum* did not demonstrate an impact on *Xt* UPB513 growth. Further analyses by SeCreT6 prediction revealed the presence of at least one T6SS cluster in each of the *Pseudomonas sivasensis* UPB1354 and *Pantoea agglomerans* UPB1357 strains used in this study.

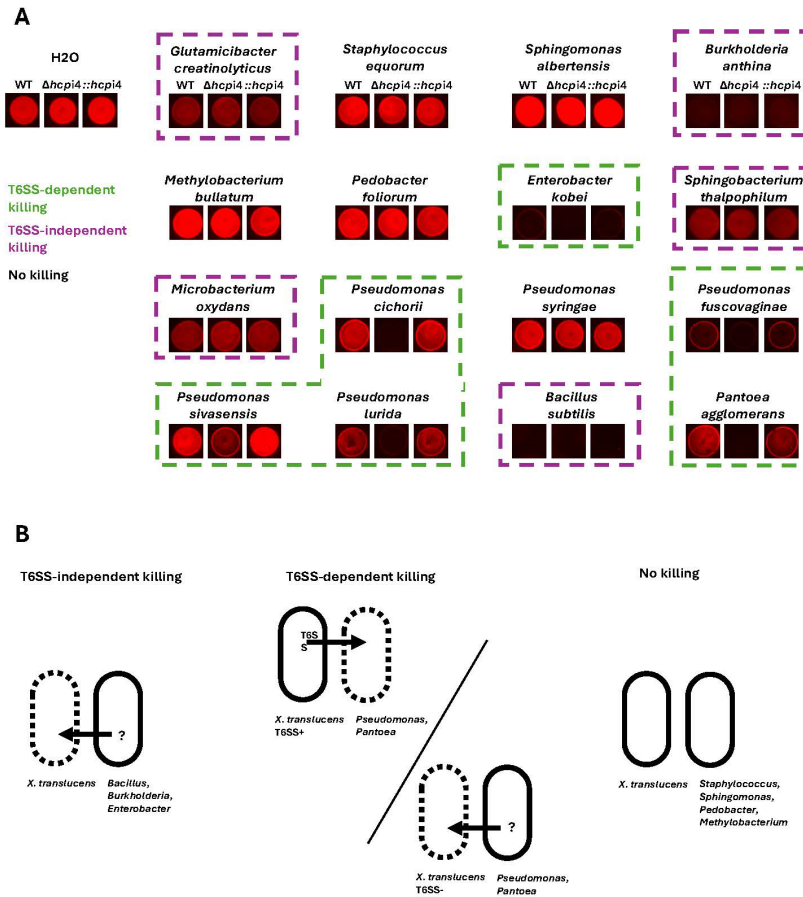


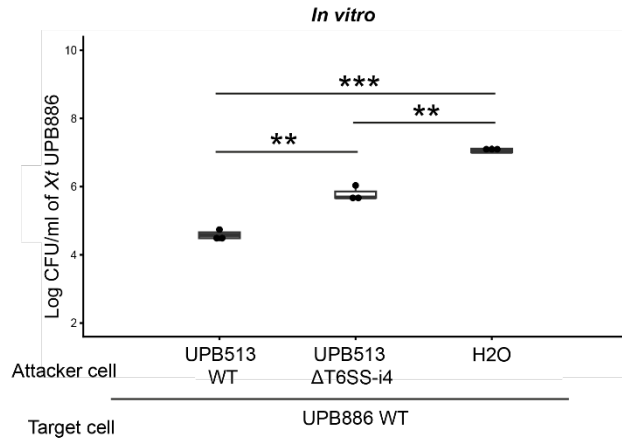
Figure 2: *Xt* survival against cereal representative bacteria. (A) Fluorescence images of representative competition patches spotted on NA after 16h of cocultivation using Amersham Typhoon laser scanner. The three killing outcomes are framed in green for T6SS-dependent killing, pink for T6SS-independent killing, or not framed when no killing was observed. Killing outcomes are highlighted **(B)** Schematic representation of T6SS related different killing and survival *Xt* outcomes: T6SS-independent killing, T6SS-dependent killing and no killing. Each genus for which these interactions have been observed is listed below the relevant case.

***Xt* T6SS-i4 is also involved in intraspecies competition**

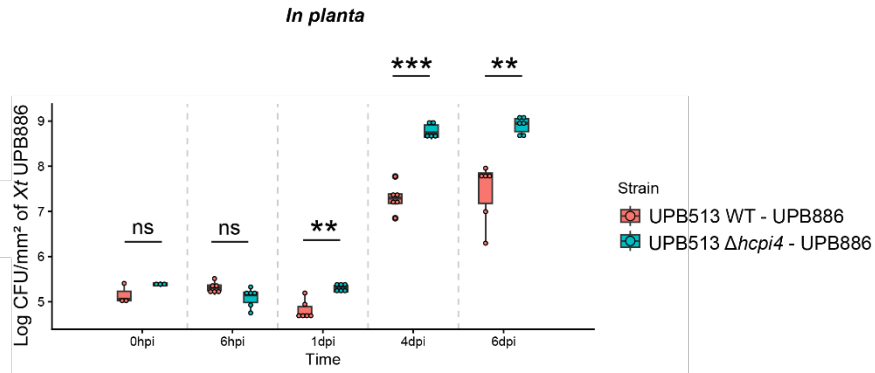
The study of T6SS-i4 effector diversity in our previous work highlighted T6SS compatible and incompatible groups among *Xt* strains. Therefore, we hypothesised that T6SS-i4 might further play a role in intraspecies competition. To test this hypothesis, we chose two closely related *Xt* strains both infecting barley host plants

but exhibiting differing vascular lifestyles. While the non-vascular *Xt pv. undulosa* UPB513 remain restricted to the mesophyll parenchyma tissues and trigger localised symptoms, the vascular *Xt pv. translucens* UPB886 colonise the mesophyll as well as xylem vessel tissues, inducing systemic symptoms (Gluck-Thaler et al., 2020). *Xt* UPB886 also possesses a T6SS-i4 but a differing T6SS effector arsenal compared to *Xt* UPB513. We first tested the ability of *Xt* UPB513 to kill the *Xt* UPB886 wild-type strain based on T6SS-i4 *in vitro*. Results showed that *Xt* UPB513 T6SS-i4 reduced *Xt* UPB886 WT survival by one order of magnitude (Figure 3A). Given those results, we further investigated these competitions *in planta*, including the temporal and spatial dynamics. Barley leaves were directly inoculated using needleless infiltration with mixtures of *Xt* UPB513, either WT or $\Delta hcp4$ mutant RFP⁺ Tc^R, and *Xt* UPB886 GFP⁺ Kan^R in a 10:1 ratio (*Xt* UPB513: *Xt* UPB886). This approach increased opportunities for bacterial contact within the mesophyll tissue. Temporal dynamics were assessed by quantifying the growth of both strains at 0 hpi, 6 hpi, 1 dpi, 4 dpi, and 6 dpi. We did observe significant killing of *Xt* UPB886 by *Xt* UPB513 in a T6SS-i4 dependent manner from 1 day post-infection (dpi), increasing with time at 4 dpi and 6 dpi (Figure 3B). Interestingly, we also noticed a reduction of *Xt* UPB513 $\Delta hcp4$ survival at 4 dpi compared to the wild-type strain (Figure 3C).

A



B



C

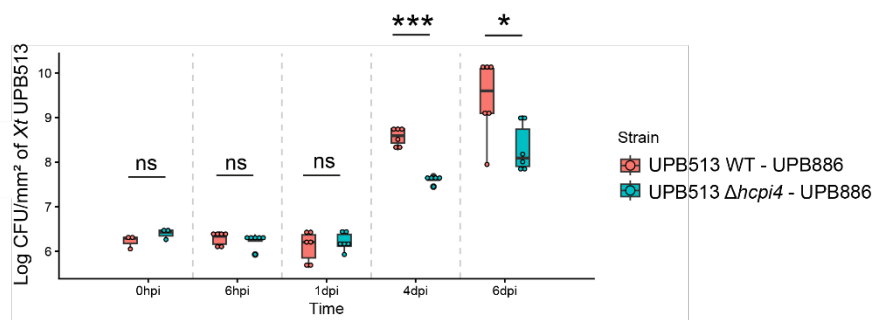


Figure 3: *Xt* T6SS-i4 intraspecies competitions assays *in vitro* and *in planta*. (A) Competitors are mixed in a 50:1 ratio (*Xt* UPB513 : *Xt* UPB886) for 17h of competition on solid NA. (B) *In planta* competition by infiltration in a 10:1 ratio (*Xt* UPB513 : *Xt* UPB886). Boxplot

represents medians (+ IQR, minimum and maximum value) of target cell survival. Dots indicate independent biological replicates ($n =$ three *in vitro*; $n =$ minimum five *in planta*). Significance was tested by two-sided t-test for *in vitro* data and Welch test for *in planta* data. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, only significant comparisons are shown. *In vitro* ($\Delta T6SS$ -WT, $P = 0.004487892$; WT-H₂O, $P = 9.569881 \times 10^{-5}$; $\Delta T6SS$ -H₂O, $P = 0.00240192$); *In planta* (For UPB886 : 0 hpi, $P = 0.2026$; 6 hpi, $P = 0.05319$; 1 dpi, $P = 0.001562$; 4 dpi, $P = 6.604 \times 10^{-6}$; 6 dpi, $P = 0.002236$, For UPB513 : 0 hpi, $P = 0.1957$; 6 hpi, $P = 0.4641$; 1 dpi, $P = 0.5282$; 4 dpi, $P = 2.352 \times 10^{-5}$; 6 dpi, $P = 0.02914$).

We further explored the *in planta* spatial dynamics between both fluorescently tagged bacteria by confocal imaging at 6 dpi, shown in Figure 4. In the mixture with *Xt* UPB513 WT, *Xt* UPB886 appears to survive only in aggregates. In contrast, in the mixture with *Xt* UPB513 *hcpi4* mutant, *Xt* UPB886 is more abundant and is found both in aggregates and as single bacterium, while *Xt* UPB513 appears less abundant compared to the WT strain.

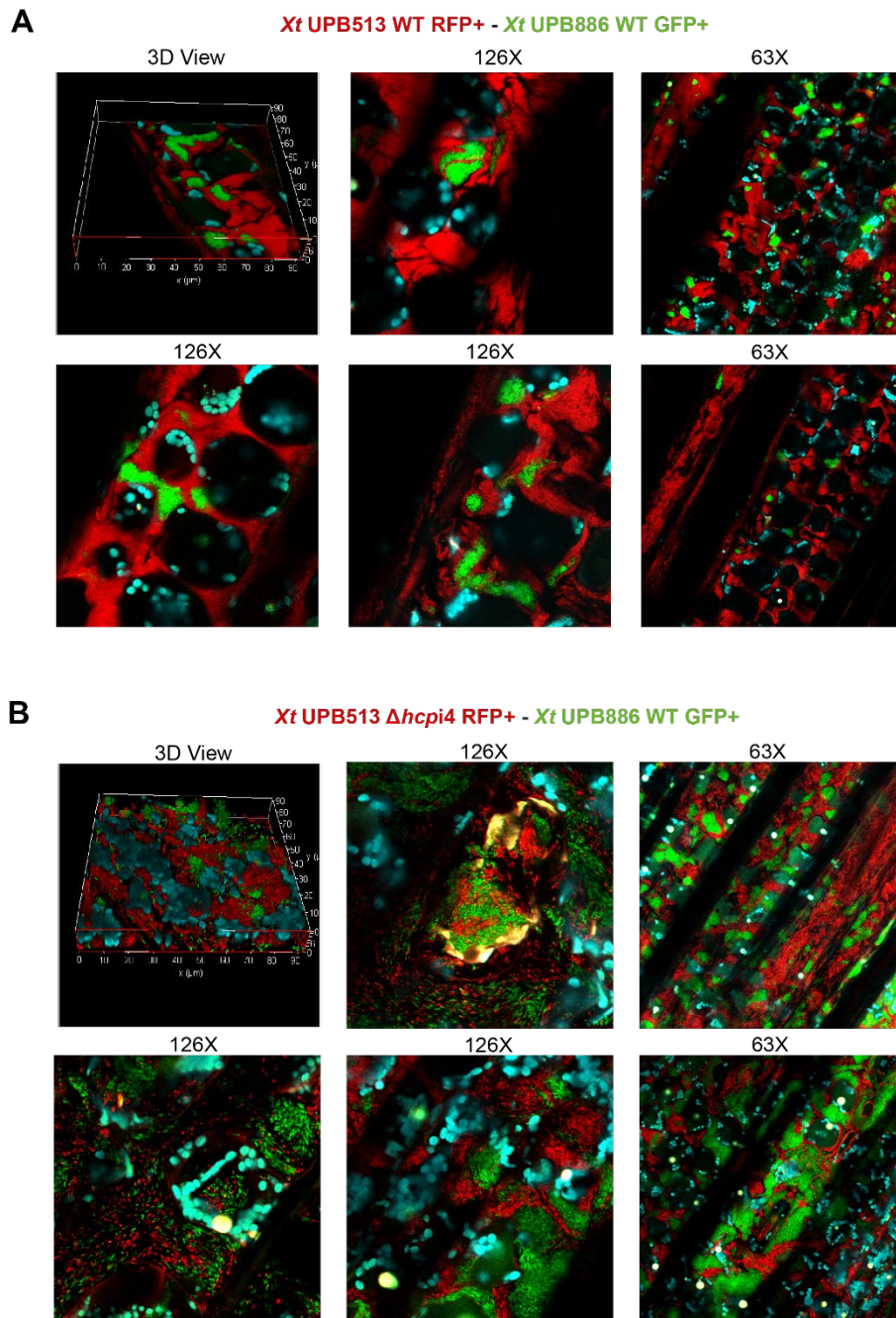


Figure 4: *Xt* T6SS-i4-mediated interactions shape the *in planta* spatial organisation. Confocal images of *Xt* UPB513 (**A**) WT and (**B**) *hcp14* mutant, expressing RFP in competition with *Xt* UPB886 expressing GFP at 6 dpi in a barley leaf. Respective top left images show 3D representations of barley leaf 12.82 μ m layer, with sharpness increased by deconvolution

techniques. Chlorophyll b (blue), RFP (red) and GFP (green) channels are merged in each image. 63X are 244,44 µm large and 126X are 92,22 µm large.

Discussion

The cereal phytobiome is characterised by a broad diversity of bacterial taxa that coexist or compete on shared host surfaces and inside the mesophyll or xylem tissues. However, the contribution of *Xanthomonas* secretion systems X-T4SS and T6SS to such competitive interactions with cereal-associated microbes remained unexplored. Our findings reveal that the Xt X-T4SS and/or T6SS-i4 shapes inter- and intraspecific interactions, while also enhancing its survival in pairwise confrontations with cereal competitors.

Both X-T4SS and T6SS-i4 displayed antibacterial competition against *Pseudomonas* and *Pantoea* species. Interestingly, the observation that deletion of *hcpi4* did not totally abolish bacterial killing against pseudomonads suggests that additional competitive mechanisms may be at play. On the contrary, the T6SS-i4-dependent killing of *Pantoea* was completely impaired in the T6SS mutant, indicating that the deployed killing mechanism is genus-specific. The prediction of T6SS clusters in both the *Pseudomonas* and *Pantoea* genomes, together with the observed Xt T6SS-dependent survival, suggests that T6SS-dependent attacks from *Pseudomonas* and *Pantoea* should not be ruled out. However, the survival even in the absence of a functional T6SS-i4 when co-cultured with *P. syringae* contrasts with other pseudomonads. Additionally, our study aligns with the findings of Garin *et al.*, who observed that *Stenotrophomonas rhizophila* kills *Pseudomonas* and *Pantoea* in a T6SS-i4-dependent manner. However, they also found that the *S. rhizophila* T6SS-i4 had a broader target range, including *Rhizobium* sp., *Paenibacillus* sp., *Plantibacter* sp., *Massilia* sp., *Oxalobacteraceae* sp., *Stenotrophomonas* sp. and *Xanthomonas* sp. While these specific genera were not tested in this study, the narrower target range of Xt T6SS-i4 observed here could have various causes. Firstly, the respective effector repertoires of *S. rhizophila* and Xt UPB513 may differ. Secondly, the qualitative assays may not be sensitive enough to detect small growth inhibition effects. Finally, Silwet L-77 did not affect T6SS-i4 competition on NA plates, but testing other surfaces, such as leaves, could clarify its impact on killing efficiency and bacterial contact *in planta*.

While strong targeted killing of *Pseudomonas* and *Pantoea* by Xt X-T4SS and T6SS-i4 is observed, this result also aligns with findings from studies on the wheat phytobiome. Although seasonal shifts in the plant microbiota are common, the genus *Pseudomonas* is consistently reported as a significant part of the total bacterial diversity on cereals throughout the growing season (Kavamura *et al.*, 2021). A recent study further showed that *Bacillus* accounted for a larger proportion of the diversity on the flag leaf of wheat at BBCH 39 (flag leaf fully unrolled) than at BBCH 81

(ripening), whereas the opposite trend was observed for the genus *Pantoea* more abundant at BBCH 81 than at BBCH 39 (personal communication with Dr. M. Delitte, unpublished data). Interestingly, BLS symptoms generally appear only late in the season at the heading stage, and yield loss is strongly correlated with the extent of flag leaf infection (Bamberg, 1936; Duveiller, 1992; Duveiller & Maraite, 1993; Forster et al., 1986; Shane W. W. et al., 1987). The *in vitro* killing activity of Xt T6SS-i4 and X-T4SS against *Pseudomonas* and *Pantoea* may reflect competitive interactions during key stages of the seed-borne *X. translucens* complex lifecycle, such as in the cereal spicosphere, the wheat spikes niche (Kavamura et al., 2021), or phyllosphere.

Although the strains used in this study were selected to represent a cereal-associated bacterial community, those from collections not originating from cereal field may introduce bias. Future work could therefore explore competition using strains, such as *Pseudomonas* and *Pantoea*, collected from field sites where *X. translucens* infections occur.

Our results also confirm the role of T6SS-i4 in intraspecific competition between strains from T6SS effector incompatible groups. Further studies are needed to explore whether these competitions occur during the epiphytic or endophytic phase in the *Xt* lifecycle. Garin *et al.* observed that competition mainly occurs at early germinated seed stages (Garin et al., 2024, 2025). This is probably due to the competitive advantage that bacteria have when they are still co-located in confined spaces close to the site of inoculation, compared to later plant developmental stages, when the spatial complexity of the plant allows bacteria to avoid contact with each other (Garin et al., 2025).

Overall, the exploration of contact-dependent competitive strategies in the model organism *X. translucens sensu lato* opens the way to new and innovative disease management strategies, by revealing xanthomonads vulnerabilities.

Materials and methods

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Supplementary Table 1. *X. translucens sensu lato* strains and all cereals representative competitors were grown at 28°C on solid nutrient agar (NA). *E. coli* DH10B was grown at 37°C on solid NA. The antibiotics kanamycin (Km) and tetracycline (Tc) were used in a concentration of 50 µg/ml and 5 µg/ml (*Xt*) or 10 µg/ml (*E. coli*), respectively, when appropriate.

Generation of RFP-expressing *Xt*

All mutant strains, *Xt* UPB513 $\Delta hcp4$, *Xt* UPB513 $\Delta T6SS-i4$, *Xg* LMG728 $\Delta virD4$ and the complemented strain *Xt* UPB513 $\Delta hcp4::hcp4$, were generated by pK18mobsacB double recombination events, as described previously in (Peduzzi et al., 2025). For the generation of the RFP-expressing *Xt*, all *Xt* UPB513 WT, *Xt* UPB513 $\Delta hcp4::hcp4$ and *Xt* UPB513 $\Delta hcp4::hcp4$ strains were transformed with pSW002-Pc-mRuby2 Tc^R by electroporation.

Pairwise confrontation assays: *Xt* killing activity

The susceptibility of cereal representative strains to X-T4SS and T6SS-i4 was evaluated. *Xt* UPB513 T6SS⁺ and LMG 728 X-T4SS⁺ WT strains and respective mutant strains, as well as the cereal competitors, were grown on solid media and resuspended to OD₆₀₀=0.5 in dH₂O. *E. coli* DH10B Km^R GFP⁺ was used as a positive control. 100µl of each competitor suspension was spread on an NA plate using glass beads at dilutions 10⁰, 10¹ and 10². Subsequently, 15 µl of suspensions of the *Xt* WT and X-T4SS or T6SS mutant strains were dropped onto the dried plate. Plates were incubated for 16h at 28°C. In quantitative competition assays, each *Xt* strain was mixed with *E. coli* in a ratio of 10:1 (*Xt*:*E. coli*). Qualitative assays were repeated at least three times.

To assess the impact of a wetting agent on T6SS-i4 killing activity, the same method as described before was used, except that 0.05% (v/v) Silwet L-77 was added to each bacterial suspension before plating competition assays with *P. sivasensis* GFP⁺ Km^R and *Xt* WT, X-T4SS or T6SS mutants or complemented strains. Plates were incubated for 16h and scanned for fluorescence using an Amersham Typhoon laser scanner (filter Cy2 525BP20). Qualitative assays with Silwet L-77 were repeated at least three times.

Pairwise confrontation assays: *Xt* survival

The survival of *Xt* co-inoculated with each cereal representative bacterium in a T6SS-i4-dependent or -independent manner was evaluated. The *Xt* UPB513 T6SS⁺ strain and its respective mutant and complemented strains, as well as each cereal competitor, were grown on solid media and resuspended to an OD₆₀₀=0.5 in dH₂O. Each *Xt* strain was mixed with each cereal competitor in 10:1 and 1:1 ratios (*Xt*:competitor). Five-microlitre patches of each mixture were dropped onto NA plates and incubated for 16h at 28°C. Resulting plates were scanned for fluorescence using an Amersham Typhoon laser scanner (filter Cy3 570BP20). Survival assays were repeated at least three times.

In vitro quantitative assays

Xt UPB513 WT, *Xt* UPB513 $\Delta hcp4::hcp4$ and *Xt* UPB513 $\Delta hcp4::hcp4$, as well as *Xt* UPB886 Km^R GFP⁺ bacteria were grown on solid media and resuspended to an

OD₆₀₀=0.5 in dH₂O. Each *Xt* strain was mixed with *Xt* UPB886 in a ratio of 50:1 (*Xt* UPB513:*Xt* UPB886). Five-microlitre patches of each mixture were dropped onto NA plates and incubated for 17h at 28°C. Patches were resuspended in 500 µl of dH₂O and serial dilutions were plated on NA+Km plates for quantifying *Xt* UPB886 surviving cells.

***In planta* quantitative assays**

RFP-expressing *Xt* UPB513 WT and *Xt* UPB513 $\Delta hcp4$ Tc^R bacteria, as well as GFP-expressing *Xt* UPB886 Km^R cells were grown on solid media and resuspended to an OD₆₀₀=0.5 in dH₂O. *Xt* UPB886 was mixed with each *Xt* UPB513 in 10:1 ratio (*Xt* UPB513:*Xt* UPB886). Leaves of ten-day-old barley cv. Morex were inoculated by needleless infiltration. Five plants were inoculated by treatment. The plants were then placed in a greenhouse with a temperature of 28°C and 70% humidity. Six circular leaf punches were harvested by treatment in the inoculated zone at 0hpi, 6hpi, 1dpi, 4dpi and 6dpi. The leaf tissues were resuspended in 500 µl of dH₂O, and serial dilutions were plated onto NA+Km and NA+Tc plates to quantify the surviving cells of *Xt* UPB886 and *Xt* UPB513, respectively.

***In planta* temporal and spatial competition dynamics**

A barley cv. Morex leaf was inoculated by needleless syringe infiltration with *Xt* UPB513 either WT or *hcp4* mutant RFP⁺ and *Xt* UPB886 GFP⁺, at an OD₆₀₀ of 10 and a ratio of 10:1 (*Xt* UPB513:*Xt* UPB886). The plants were transferred to a greenhouse with a temperature of 28°C and 70% humidity. Three to six circular leaf punches were harvested by treatment from inoculated area of the leaf at 0 hpi, 6 hpi, 1 dpi, 4 dpi, and 6 dpi. The leaf tissues were resuspended in 500 µl of dH₂O, and serial dilutions were plated onto NA+Km and NA+Tc plates to quantify the surviving cells of *Xt* UPB886 and *Xt* UPB513, respectively. For confocal imaging, a small square section 0.5 mm wide of the inoculated area of the leaf was cut out and observed with a 63X objective, in which an additional zoom of 2X was achieved.

Acknowledgments

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Supplementary Table 1. Bacterial strains and plasmids used in the present study.

Bacterial strains and plasmids used in the present study			
Strains	Xt ANI group	Genotype or relevant features	Reference or source
Xt UPB513	Xt-I	Wild-type Xt UPB513 (isolation: 1987, <i>Triticum</i> , Mexico)	UPB Lab collection
Xt UPB513 Δ T6SS-i4	Xt-I	Single deletion mutant of genes <i>tssC-H</i> T6SS-i4 in Xt UPB513	Peduzzi <i>et al.</i> , 2025
Xt UPB513 Δ hcp i4	Xt-I	Single deletion mutant of gene <i>hcp</i> of T6SS-i4 in Xt UPB513	Peduzzi <i>et al.</i> , 2025
Xt UPB513 Δ hcp i4::hcp i4	Xt-I	Complementation of <i>hcp</i> T6SS-i4 mutant in Xt UPB513	Peduzzi <i>et al.</i> , 2025
Xt UPB513 RFP ⁺	Xt-I	Wild-type Xt UPB513 (isolation: 1987, <i>Triticum</i> , Mexico) with pSW002- <i>Pc-mRuby2</i> , Tetra ^R	This study
Xt UPB513 Δ hcp i4 RFP ⁺	Xt-I	Single deletion mutant of gene <i>hcp</i> of T6SS-i4 in Xt UPB513 with pSW002- <i>Pc-mRuby2</i> , Tetra ^R	This study
Xt UPB513 Δ hcp i4::hcp i4 RFP ⁺	Xt-I	Complementation of <i>hcp</i> T6SS-i4 mutant in Xt UPB513 with pSW002- <i>Pc-mRuby2</i> , Tetra ^R	This study
Xg LMG728	Xt-III	Wild-type Xt LMG728 (isolation: 1978, <i>Poa trivialis</i> , Switzerland)	BCCM/LMG collection
Xg LMG728 Δ virD4	Xt-III	Single deletion mutant of gene <i>virD4</i> in Xt LMG728	Peduzzi <i>et al.</i> , 2025
Xt UPB886 GFP ⁺	Xt-I	Xt UPB886 carrying the replicative pPneo-gfp, Kan ^R	Gluck-Thaler <i>et al.</i> , 2020
<i>E. coli</i> DH10B GFP ⁺		<i>E. coli</i> DH10B carrying the replicative pPneo-gfp, Kan ^R	Jacobs's Lab collection
Cereal-associated strains			
<i>Pantoea agglomerans</i> UPB1357		Isolated from wheat head, Belgium	Dubois <i>et al.</i> , 2024
<i>Glutamicibacter creatinolyticus</i> UPB 1350		Isolated from field soil	Dubois <i>et al.</i> , 2024
<i>Pseudomonas sivasensis</i> UPB 1354 GFP ⁺		<i>Pseudomonas sivasensis</i> UPB1354 with <i>mNeonGreen</i> gene inserted at <i>att</i> Tn7 site, Gm ^R	Dubois <i>et al.</i> , 2024
<i>Staphylococcus equorum</i> UPB 1351		Isolated from field soil	Dubois <i>et al.</i> , 2024
<i>Sphingomonas albertensis</i> LMG 32139		Isolated from wheat leaf infected with <i>Xanthomonas translucens</i> , Canada	Dubois <i>et al.</i> , 2024
<i>Burkholderia anthina</i> LMG 22950		Isolated from an area under maize monoculture, rhizosphere soil, Netherlands	Dubois <i>et al.</i> , 2024
<i>Methylobacterium bullatum</i> LMG24788		Isolated from greenhouse, moss (<i>Funaria hygrometrica</i>), phyllosphere, Germany	Dubois <i>et al.</i> , 2024
<i>Pedobacter foliorum</i> LMG31463		Isolated from rhizosphere soil, Belgium	Dubois <i>et al.</i> , 2024
<i>Enterobacter kobei</i> UPB 1352		Isolated from field soil	Dubois <i>et al.</i> , 2024
<i>Sphingobacterium thalpophilum</i> UPB 1353		Isolated from field soil	Dubois <i>et al.</i> , 2024
<i>Microbacterium oxydans</i> UPB 460		Isolated from field soil	Dubois <i>et al.</i> , 2024
<i>Pseudomonas cichorii</i> UPB 461		Isolated from field soil	Dubois <i>et al.</i> , 2024
<i>Pseudomonas syringae</i> pv. <i>atrofaciens</i> UPB 463		Isolated from <i>Triticum aestivum</i> , Canada	Dubois <i>et al.</i> , 2024
<i>Pseudomonas fuscovaginae</i> UPB 526		Isolated from <i>Triticum aestivum</i> , Mexico	Dubois <i>et al.</i> , 2024
<i>Pseudomonas lurida</i> UPB 1355		Isolated from wheat leaf, Belgium	Dubois <i>et al.</i> , 2024
<i>Bacillus subtilis</i> UPB 1356		Isolated from wheat leaf, Belgium	Dubois <i>et al.</i> , 2024
Plasmids			
pTNS3		Helper plasmid encoding Tn7 TnsABCD site-specific transposition pathway, Amp ^R	Choi <i>et al.</i> , 2006
pUC18-mini-Tn7-Gm-mNeonGreen		Gm ^R on mini-Tn7, for bacteria GFP tagging	Jacobs's Lab collection
pSW002- <i>Pc-mRuby2</i>		Tetra ^R , for bacteria RFP tagging	Jacobs's Lab collection

Chapter V

Discussion and conclusion

General discussion

Main results

The main objective of this thesis has been to unravel competitive strategies of the *X. translucens* complex in the context of contact-dependent secretion systems, namely X-T4SS and T6SS. In the course of this study, I explored three key aspects of this issue: (i) their antibacterial role and evolutionary trajectories, (ii) the T6SS-i4 effector arsenal diversity, and finally (iii) T6SS cereal bacterial targets and spatial competition dynamics *in planta*.

In this work, we demonstrated that X-T4SS and T6SS-i4 of the *X. translucens* complex have overlapping bacterial killing activities. Screening for both systems within *X. translucens sensu lato* strains revealed that X-T4SS and T6SS-i4 have mutually exclusive distributions in xanthomonads. Phylogenetic and comparative genomic analyses further highlighted non-orthologous genes displacement of ancestral bacterial killing X-T4SS by a T6SS-i4 competitive strategy in many *Xanthomonas* lineages, with X-T4SS decay occurring independently of T6SS-i4 presence. Finally, these analyses further highlighted the type-2 neighbourhood region of T6SS-i4 as a hotspot for ICEs insertion that is well conserved among xanthomonads, suggesting that T6SS transfer occurred by ICEs.

We combined *in silico* screening and comparative genomics and described the diversity of the T6SS core and accessory effectome within the *Poaceae*-infecting *X. translucens* complex. Comparison of xanthomonads differing in ecological lifestyle revealed an enrichment of T6SS effectors diversity within non-vascular xanthomonads, compared to their vascular close relatives. The bacteriolytic activity of the *X. translucens* core T6SS effectome was further experimentally demonstrated. Respective effector-immunity pairs were investigated through neutralisation assays and structural predictions.

Finally, we show that both X-T4SS and T6SS-i4 mediate interspecies and/or intraspecies competition against bacteria that are representative of cereal microbiome, thereby shaping competitive interactions in the phytobiome. Through pairwise confrontation assays, we demonstrate that both systems efficiently kill several Gram-negative competitors, notably *Pantoea* and several *Pseudomonas* species, while leaving Gram-positive taxa unaffected. We further revealed that T6SS-i4 also contributes to *X. translucens* survival by providing protection against these

same *Pantoea* and *Pseudomonas* strains. In addition, *Xtu* T6SS-i4 mediates intraspecific competition against *Xtt*, both *in vitro* and *in planta*.

These studies collectively enhance our understanding of the X-T4SS and T6SS-i4 competitive strategies deployed by the cereal and grass pathogens in the *X. translucens* complex. These results further suggest that, while a T6SS core effectome is maintained between closely related strains, the effector-immunity pair diversity is constantly evolving between those, likely reflecting the arms race pressures experienced within microbial communities. Altogether, this work deepens our knowledge of two mutually exclusive antibacterial systems, shedding light on their ecological roles and evolutionary trajectories. Beyond the genus *Xanthomonas*, the X-T4SS and T6SS systems are found in many environmental bacteria and host-associated bacteria. While this work poises *X. translucens sensu lato* as a promising model for studying antibacterial secretion systems evolution, research on this species complex opens new avenues for research on interbacterial competition in various other fields.

Generation of a collection of X-T4SS and T6SS mutant strains

This work led to the generation of a collection of mutant strains as a promising tool for studying the antibacterial secretion systems, X-T4SS and T6SSs in *Xanthomonas* and their effectors. A collection of mutants of the X-T4SS and T6SS-i4 was generated, including deletions of up to approximately 10 kb of T6SS-i4 *tssC-tssH* genes. It should be noted, however, that certain challenges were encountered during this thesis, such as the inability to obtain a mutant strain for the central region of T6SS-i4 in *Xt* UPB513, either due to the 20 kb large size of this region or requiring further optimization of the upstream and downstream region selected for homologous recombination. This collection of fluorescent and mutant strains opens many opportunities for studying *X. translucens sensu lato* and their interactions within cereal bacterial communities *in planta*. The generation of the *Xt* UPB513 Δ T6SS-i3*** strain provides a tool to further uncover its role in the future. Furthermore, all *Xt* UPB513 effectors and immunity proteins have been cloned into plasmids with C-terminal tags, allowing their purification and further detailed study.

Original methodological approaches in this work

First, this work highlights the search for mutually exclusive gene modules as a powerful methodology for detecting functional relationships between different gene sets in the future. The relevance of this approach is illustrated by recent observations of mutual exclusion between the siderophores lactucheline and pyoverdine, which are thought to be metabolically too costly to maintain simultaneously (Chesneau et al., 2025). Similarly, *Pseudomonas putida* has been reported to harbor an antibacterial T4SS unrelated to the xanthomonad X-T4SS (Purtschert-Montenegro et al., 2022), while antibacterial T6SS have also been described in the same species (Bernal et al., 2017). Applying this approach to such systems offers strong potential in the study of functionally convergent mechanisms. Genome-wide association

studies across closely related strains may help identify mutually exclusive genes and hold great potential for revealing new gene functions beyond the context of competition.

Secondly, this work describes and establishes the basis of a protocol for visualizing T6SS interbacterial competition outcomes *in planta*. These results provide valuable insight to describe the spatial competition dynamics between two closely related strains both harbouring T6SS-i4 with different effector-immunity repertoires. A range of preliminary *in planta* competition assays were conducted on detached leaves, by spray inoculation, by needleless infiltration at low bacterial density, or by leaf clipping, but these methods yielded inconsistent results. The final protocol relied on co-infiltration of both competing strains at high bacterial density, which proved reproducible. The success of this approach is likely due to optimized physical contact between the competing strains. While we do not exclude T6SS activity occur by spray or leaf clipping inoculations, its activity under these conditions was probably too weak to be significantly and consistently observed. One possible avenue for improvement is to combine these approaches with Hcp detection to further enhance detection of T6SS firing events under conditions where the frequency of physical contact between competing strains is reduced at lower bacterial densities.

The role of T6SS-i3*: current insights and future directions**

In the second and third chapter, we did not observe any involvement of *Xt* UPB513 T6SS-i3*** in bacterial killing, virulence on barley, motility or growth *in vitro*. While its role remains an open question, some points are worth highlighting.

1. Regulation of T6SS-i3* expression**

T6SS-i3*** was not expressed under the laboratory conditions tested to date in *Xanthomonas* studies. Only very low expression level was measured under various conditions, namely in NB and XOM3 media, at different temperature and pH, or *in planta* at 24 hpi after syringe infiltration (Choi et al., 2020; Z. X. Liao et al., 2019; Zhu et al., 2020).

It should be noted that all T6SS-i3*-*** systems in *Xanthomonas* encode genes homologous to ppkA-pppA-FHA and TagF regulatory system (Bayer-Santos, DeMoraes Ceseti, et al., 2019). In *P. aeruginosa*, this signalling cascade PpkA-Fha1-PppA together with TagQRST has been proposed to detect environmental stimuli and control assembly or function of the T6SS apparatus post-transcriptionally (Casabona et al., 2013). Indeed, this regulatory pathway in *P. aeruginosa* is required for sensing external T6SS attack and triggering a tit-for-tat response by counter-attacking (Basler et al., 2013).

In xanthomonads, *X. citri* T6SS-i3* is regulated at the transcriptional level via EcfK and PknS, but also probably at the post-translational level given encoded PpkA-Fha1-PppA homologs in its T6SS-i3* cluster (Bayer-Santos et al., 2018). Although this regulatory system has been reported in the tit-for-tat response, the absence of bacterial killing observed with *Xanthomonas* T6SS-i3 to date could imply that other stimuli are perceived for the regulation of this T6SS. The first step would therefore be to identify the conditions for T6SS-i3*** expression, either by detecting Hcp secretion in the extracellular environment or by RT-qPCR.

2. Effectors and T6SS-i3*** role

The role of T6SS-i3*** remains highly controversial in *Xanthomonas*. A role in virulence has been demonstrated for *Xoo* PX099 on rice, but this is not the case for *Xoc* (Choi et al., 2020; Zhu et al., 2020). No role in killing activity against bacteria or yeast, in biofilm and motility, growth in vitro, EPS production, extracellular protease ability, tolerance to osmotic pressure, heavy metal stress, anti-oxidant reactions, or SDS-mediated lysis were observed (Choi et al., 2020; Z. X. Liao et al., 2019).

Regarding T6SS-i3*** effectors, only one candidate effector was found in the vicinity of *X. translucens* T6SS-i3*** cluster (Bayer-Santos, DeMoraes Ceseti, et al., 2019). However, this putative DUF6931-containing protein encoded upstream of *vgrG* is present across all T6SS-i3 subclades. Therefore, it is still not clear whether this gene is a new accessory *tag* gene or an effector gene. In other xanthomonads harbouring T6SS-i3***, only a conserved set of hypothetical proteins harbouring a TIR_2 domain, typical of Toll-like receptors, were identified in a few species, such as *X. euvesicatoria* (Bayer-Santos, DeMoraes Ceseti, et al., 2019). Bacterial proteins with TIR_2 domains act as NADases and interfere with cellular metabolism in prokaryotic and eukaryotic cells (Alvarez-Martinez et al., 2020).

Further exploration of the direct or indirect role played by *Xoo* PX099 T6SS-i3*** on rice, such as comparing its expression *in planta* using different inoculation conditions, would be worth investigating. Other roles involving intercellular communication, such as an impact on quorum sensing, could be interesting avenues to test. Another alternative would be to compare the secretome in the supernatant of wild-type *Xoo* and T6SS-i3*** mutant cells through proteomic analyses to look for effectors.

Evolutionary history of functionally convergent X-T4SS and T6SS-i4

1. Mutual exclusion between X-T4SS and T6SS-i4

Given that X-T4SS degradation occurs independently of the presence of T6SS-i4, the most plausible scenario to explain their mutual exclusion is that, in strains already carrying X-T4SS, acquisition of the functionally overlapping T6SS-i4 cluster provides no additional selective advantage. Consequently, T6SS-i4 would have been acquired only in strains lacking X-T4SS. However, the ecological reasons for adopting T6SS-i4 system over X-T4SS and not the opposite remain open. In the second chapter, I have proposed many research avenues, such as different competitive microbiome pressures, the diversity of X-T4SS and T6SS effector-immunity sets, the occurrence of X-T4SS- and T6SS-resistant bacteria, a physiological cost of X-T4SS to the cell (Cenens et al., 2020), functions versatility of T6SS (Unni et al., 2022), geographical T6SS availability (Unni et al., 2022), vascularity, or even host range (Liyanapathirana, Wagner, et al., 2022). Interestingly, T6SS-i4 is found at high occurrence only in the species *X. oryzae*, *X. translucens* complex and *X. fragariae* (Bayer-Santos, DeMoraes Ceseti, et al., 2019; Liyanapathirana, Wagner, et al., 2022). Furthermore, only a few strains of *X. fragariae* do not possess T6SS-i4. Other T6SS-i4 are sporadically distributed in very few strains of other *Xanthomonas* species (Bayer-Santos, DeMoraes Ceseti, et al., 2019; Liyanapathirana, Wagner, et al., 2022). It would therefore be interesting to compare the lifestyles of these three species and their exceptions to better understand the common advantages of acquiring T6SS-i4 in their lifecycle. Finally, it would be interesting to infer the respective evolutionary dynamics of X-T4SS and T6SS-i4 in greater depth, including most probable gain and loss events using GLOOME, as it was done for T3SS (Teulet et al., 2020).

One way to compare both system efficiency would be to test competition of bacteria with either X-T4SS or T6SS-i4, which possess similar effector toxic domains with respective secreting signals, against a similar target cell. This would allow to compare the killing rates of each system, and highlighting their strengths and weaknesses, and the advantages of possessing one over the other.

2. Drivers of X-T4SS loss

Despite its widespread distribution within *Xanthomonas*, X-T4SS appears to be undergoing degradation in many species. Interestingly, all *virD4* genes in *X. translucens* complex strains from ANI group I were pseudogenised due to frameshift mutations and internal stop codons caused by a nucleotide deletion. Consistent with Alvarez *et al.*, we observed that X-T4SS pseudogenes and remnant gene fragments were also common in other *Xanthomonas* species (Alvarez-Martinez et al., 2020). Exploring the factors underlying the loss of X-T4SS could provide insights into the evolutionary pressures that favored its replacement by T6SS-i4 and explain why T6SS-i4 was preferentially acquired. In this context, it would be interesting to

establish the distribution of X-T4SS in xanthomonads, not only *in silico*, but also of experimentally functional X-T4SS compared to those that are no longer functional due to pseudogenisation. Such analyses may highlight the causes of X-T4SS loss in some strains. It would be worthwhile to test whether, as with T6SS (Taillefer et al., 2025), prolonged absence of microbial competition pressure could drive X-T4SS loss. Similarly, such hypothesis could be tested by propagating a strain with functional X-T4SS⁺ for hundreds of generations under controlled competition conditions with either susceptible or immune recipient cells.

3. *Xtt* subgroups K0, K1 and K2: a promising model for exploring X-T4SS and T6SS-i4 mutual exclusion

Another challenge concerns the different subgroups of *Xtt* as defined by Heiden *et al.*, which evolved distinct antibacterial strategies (Heiden et al., 2023). Subgroup K1 possesses T6SS-i4 with remnant fragments of ancestral X-T4SS. On the opposite, the subgroups K0 and K2 possess X-T4SS with pseudogenised *virD4*, while the rest of the genes in the X-T4SS cluster remained intact, but no T6SS-i4. Despite infecting the same host barley, they have evolved different competitive mechanisms. We could take advantage of this contrast in competition between phylogenetically closely related strains to identify the potential factors driving T6SS-i4 gain in K1 and not the other *Xtt*. Interesting avenues to explore would be differing composition of the biofilm preventing effective deployment of the T6SS, differing cell aggregation creating a physical barrier, co-existence of different *Xtt* strains from different subgroups on a same leaf, differing restriction–modification systems preventing T6SS acquisition, etc. Genome-wide association studies such as using Scoary, could allow to highlight genes significantly present in K1 and absent in K0 and K2, or the opposite. In *Xtt* K0 and K2 subgroups, the pseudogenisation of *virD4* suggests a loss of bacterial killing function but also raises the question of whether the assembly of a T4SS pilus is still possible (Fronzes et al., 2009). This type of surface structure does not require VirD4 and could suggest a shift to new biological functions (adhesion to target cells, cell motility, etc.) (Fronzes et al., 2009). However, exploratory qualitative competition tests have informed us that, while some of *Xtt* K0 and K2 strains have a bacterial killing capacity against *E. coli*, such as *Xtt* UPB820, Km7, Km34, CIX95, others do not, such as *Xtt* CFBP2054 or CIX43. This implies either that X-T4SS is functional in some strains despite a truncated *virD4*, or that there is an alternative competitive mechanism used by these strains. Broader screening of such bacterial killing potential in *Xtt* and the knockout of another *virB* gene in such strains might help elucidate this question. The *virB7* mutants of strains *Xtt* CIX95 and CFBP2054 produced as part of Martin Baufayt's master thesis would be suitable for answering this question (Baufayt, 2022).

4. Rare cases of X-T4SS⁺ and T6SS-i4⁺ strains

During the thesis, we could also detect a few strains containing both complete X-T4SS and T6SS-i4, such as *L. gummosus* 3.2.11 (Bayer-Santos, DeMoraes Ceseti, et al., 2019; Sgro et al., 2019), *L. enzymogenes* C3 (Bayer-Santos, DeMoraes Ceseti, et al., 2019; Sgro et al., 2019), some *X. citri* (e.g., ISO118C5, CFBP 7119, CFBP 6992) and *X. phaseoli* (e.g., NCPPB 1138). A first step would be to confirm the function of these two systems by generating single and double mutant strains. Next, further investigation of the repertoire of effectors associated with the respective X-T4SS and T6SS-i4 within these strains could provide insight into their respective role. Therefore, we could determine, if as observed in *Pectobacterium*, both X-T4SS and T6SS-i4 may redundantly secrete the same effector proteins, potentially encoding both secreting signals, to drive bacterial competition or any other function (Che et al., 2024). Or, conversely, if both systems confer different and complementary functions to the bacteria that encode them both in their genome. Indeed, in *L. enzymogenes*, T6SS-i4 appears to have antifungal but not antibacterial activity, whereas X-T4SS in the same strain plays a role in interbacterial competition and quorum quenching, but also indirectly enhancing other bacteria in its environment to produce the antifungal compound 2,4-DAPG (J. Liao et al., 2023; L. Lin, Shao, et al., 2025; L. Lin, Shen, et al., 2025; Shen et al., 2021; B. Wang et al., 2023; M. Yang et al., 2020). Additionally, it would be worth studying the regulation and expression of X-T4SS and T6SS-i4 present in the same strain during *in planta* infection.

5. *Xtu* XtXJ1 possesses X-T4SS

A new strain of *X. translucens* pv. *undulosa*, XtXJ1, has recently been sequenced and, surprisingly, does not possess T6SS-i4 like all the other phylogenetically closely related *Xtu* strains (Q. Wang et al., 2025). Instead, it possesses X-T4SS. Comparing the neighbouring region of this X-T4SS with those of *Xtt* may help to clarify if it is complete or if *virD4* is also pseudogenised. Additionally, examining its phylogenetic position could clarify if it is congruent with the species tree, implying that the loss of X-T4SS and gain of T6SS-i4 would then be quite recent, or clustering differently, implying X-T4SS reacquisition by HGT. Finally, we could look for evidence of the past presence and loss of any T6SS-i4 in the type-1 neighbouring region of *Xtu* XtXJ1, where all the T6SS-i4 clusters of *Xtu* studied were found, or looking for potential remaining T6SS effectors. This new strain suggests that the diversity of T6SS and X-T4SS in *X. translucens* may be greater than what we currently know and underscores the need for broader, large-scale analyses.

Challenges in X-T4SS and T6SS effector studies and perspectives

1. Construction of effector-free strains

A major challenge in studying X-T4SS and T6SS effectors is their potential complementary and synergistic effects. Indeed, the effect of mutating a single effector gene at a time can be masked by the effect of the remaining effectors, making their study complicated. Further characterisation of effector repertoires could be achieved using multiplex CRISPR-Cas9 based cytosine base editors (Li et al., 2023). This approach could introduce premature stop codon into all candidate effectors, ultimately creating X-T4SS or T6SS effector-free strains with functional X-T4SS or T6SS. Such strains would enable the engineering of bacteria expressing single or selected combinations of effector-immunity pairs, allowing the characterisation of their functions and potential synergistic activities. The foundations of this work have already been established during the master's project of Adrián García Pérez at IRD in Montpellier and represent a strong basis for further investigation (García Pérez, 2023).

2. T6SS effector diversity and bacterial lifestyles

A recent study highlighted the relationship between T6SS gene content and the bacterial lifestyle in *Ralstonia solanacearum* species complex (RSSC). This study demonstrated that two ecologically similar xylem-infecting plant pathogens, RSSC and *Paracidovorax* spp., convergently evolved broad T6SS effector repertoires. Their phylogenetic and genomic context analyses provided evidence that HGT is implicated in diversifying EI repertoires (Aoun et al., 2024). In contrast, our study supports a higher effector diversity in non-vascular *Xanthomonas*. However, both studies suggest that species with similar lifestyles converge towards similar T6SS effectome diversity, probably influenced by specific microbiomes pressure, and plant microenvironments constraints. Another example of relationship between T6SS genes and bacterial lifestyle comes from two RSSC phylotype IV clades that have transitioned to host-restricted lifestyles. Degradation of T6SS core genes and effector-immunity modules in both lineages supports the view that the T6SS is ancestral in the RSSC and has been lost during genome reduction (Aoun et al., 2024). Interestingly, T6SS clusters are also completely absent in xylem-limited *Xanthomonadales* phytopathogens characterised by reduced genomes, such as *X. albilineans* and all subspecies of *X. fastidiosa* (Bayer-Santos, DeMoraes Ceseti, et al., 2019). More broadly, *Xanthomonas* species in which the T6SS is present in nearly all strains, such as *X. fragariae*, display a higher number of effector modules, whereas species with few T6SS-i4-positive strains, such as *X. campestris* or *X. hortorum*, show lower effector modules abundance (see Chapter 3). While there do not appear to be strict rules governing the presence and absence of T6SS and the diversity of their

effector repertoires, there seems to be a continuum, where the bacterial lifestyle appears to play a role in their evolution towards a loss or diversification of effectors.

It would be worth further exploring the link between non-vascularity and diversity of T6SS-i4 effectors. One could compare diversity of the microbial community by metagenomics from which vascular and non-vascular *X. translucens* T6SS-i4⁺ strains have been isolated and correlate this with their respective effector repertoires. Reconstructing the genomes of neighbouring bacteria sharing the same environment would also help to elucidate the diversity and evolution of respective effectors in this arms race, as well as effector resistant-bacterial strains.

3. Exploring novel X-T4SS effectors in *Xanthomonas*

Among xanthomonads, we predicted numerous candidate X-T4SS effectors carrying putative toxic domains, including Zeta toxin, fungal lipases, and cell wall hydrolases. X-T4SS in *Xanthomonadales* has been shown to mediate a variety of activities, such as antibacterial, antifungal, and quorum-quenching functions. However, many effectors identified in the X-T4SS of *Xanthomonas* sp. remain uncharacterized or lack recognisable domains, highlighting a major gap in our understanding. Characterization of these novel effectors could be achieved by combining structural predictions using AlphaFold and Foldseek for protein structure alignments against known protein structure databases, phenotypic analyses of effector activity in cells via TEM or confocal microscopy, and enzymatic assays using purified effectors.

4. Hcp, VgrG or PAAR-associated effector

Figuring out why effectors associate with Hcp, VgrG, or PAAR is interesting because it explains how T6SS selects and delivers its effectors, and it could be used for predicting effector function and possible effector combinations in one firing events, engineering bacteria for biocontrol or microbiome manipulation, and understanding T6SS evolution. In *Xanthomonadales*, only effector modules associated with VgrG or PAAR are observed, and not Hcp (Bayer-Santos, DeMoraes Ceseti, et al., 2019). Furthermore, the activities of PAAR-related effectors are exclusively composed of nucleases, such as ToxREase, Tox-URI2, VRR-Nuc, Ntox, and SymE domains. Meanwhile, effectors related to VgrG all have activities linked to the periplasmic space, such as phospholipases or pore-forming effectors. It would therefore be interesting to study in greater depth how the *Xanthomonas* T6SS-i4 spike punctures and releases the effectors into the target bacterium. But also, to see whether this allows combining complementary cytoplasmic and periplasmic effector activities regardless of the combination of VgrG and PAAR. Future research questions should focus on the active or passive selection of the effector cocktails translocated simultaneously in one firing event to a given target bacterium. However, confocal microscopy time-lapse images show that different outcomes, such as growth inhibition, cell rounding, cell burst, or shrinking, are observed against cells of the same *E. coli* DH10B strain, suggesting different effectors with differing activities are

delivered with each firing event. It would be interesting to see if similar outcomes are observed with other bacterial genera or strains, like *Pseudomonas* sp. or *Pantoea* sp. The rounded-shaped cells resulting from jeopardised cell wall integrity contrasts with observations made by Souza *et al.* with the X-T4SS bacterial killing activity of *X. citri* against *E. coli* (Souza *et al.*, 2015).

T6SS dynamics and heterogeneity in *X. translucens* populations

1. Phenotypic heterogeneity in T6SS⁺ population

Further work is required to investigate whether there is heterogeneity within a *X. translucens* clonal population in the number of assembled X-T4SS or T6SS and in the composition of translocated effectors. In Chapter 2, time-lapse imaging of *X. translucens* T6SS-i4 activity against *E. coli* revealed diverse killing outcomes, including growth inhibition, cell rounding, cell bursting, or cell shrinking. These observations support the hypothesis of phenotypic heterogeneity within the population. However, these distinct killing outcomes may result from differential effector expression levels or from differences in effector loading affinity. New single cell RNA sequencing technologies, as well as the use of reporter genes placed under native effector promoters, could help elucidate variations in effector expression among *X. translucens* cells at a given time point. In addition, using engineered effectors capable of activating a reporter gene in target cells could be used to determine whether specific effectors are actively translocated.

If the latter is true, a key question that would remain is what governs these variations in expression and loading of specific effector cocktails between each T6SS firing event. For instance, is this heterogeneity influenced by the nature of the target cell ? Comparative single-cell analyses using different target species and strains, such as *Pseudomonas* sp. and *Pantoea* sp. could help address this question.

2. T6SS number of structures per cell, localisation and timing

The localisation of T6SS and the average number of T6SS structures per cell have been shown to vary depending on the bacterial species and growth conditions. Multiple structures can coexist within a single cell, with 3–5 structures per cell reported in *V. cholerae* and 1–2 structures per cell in *Acinetobacter baylyi* (Lin *et al.*, 2019). However, in xanthomonads, several questions remain unresolved, including the number of X-T4SS or T6SS structures assembled in a single cell, their localisation within the cell membrane, and the timing between successive firing events. These questions could be addressed using core T6SS components fused to fluorescent proteins and visualised using confocal microscopy.

Translating T6SS insights from *in vitro* to *in planta* contexts

1. Inter- and intraspecies competition on cereals

Wheat-associated microbial communities are influenced by a combination of factors, including farming practices (e.g., irrigation, tilling, fertilization, and crop protection), local soil and environmental conditions, and host selection processes (Kavamura et al., 2021; Delitte et al., 2021). Additionally, seasonal shifts in the plant microbiota are also common (personal communication with Dr. M. Delitte). Only few publications describe the interactions between *X. translucens* and the cereal microbiome. Stromberg *et al.* discovered 13 bacterial epiphytes capable of reducing *X. translucens* leaf-associated population sizes, one of which inhibited *Xtt* *in vitro* through antibiosis (Stromberg et al., 2000). Studies have also examined genetic diversity within and between populations of *X. translucens* at the local, regional, and global scales (Adhikari et al., 2012; Bragard et al., 1995; Curland et al., 2020). Curland *et al.* showed that natural BLS field infections typically consist of composite populations comprising different genetically distinct strains, rather than a single one (Curland et al., 2020). Strains from the three *Xtt* clades were found in each barley field investigated. One *X. cerealis* strain was also isolated from one of these same barley field, and some additional isolates were identified as *Xanthomonas* sp. but not from the *X. translucens* complex (Curland et al., 2020). Similarly, studies on *X. citri* revealed that citrus canker lesions host polyclonal populations (Pruvost et al., 2019).

It would be valuable to investigate whether different *X. translucens* strains that are more or less closely related phylogenetically can coexist on the same cereal leaf naturally in fields and to compare the presence of X-T4SS and T6SS-i4 and their repertoires of effectors. This would provide a better understanding of whether they compete intraspecifically for resources and space. Or, on the contrary, whether in mixed populations, T6SS-positive and immune T6SS-negative siblings could potentially cooperate through a division-of-labour strategy, combining competitive killing with energy-efficient growth, as exemplified for T3SS (Sturm et al., 2011; Unni et al., 2022). Interestingly, the pathogen *X. prunicola* was recently shown to cause bacterial leaf streak in wheat (Martino et al., 2023). It would be interesting to see whether these also coexist with *X. translucens* or whether they compete through X-T4SS or T6SS. Finally, it would be relevant to compare competition between strains of different levels of virulence and the impact on the plant.

2. X-T4SS, T6SS and *X. translucens* lifecycle

Additional work is needed to explore at which stage of *X. translucens* lifecycle, within the plant, X-T4SS and T6SS firing occur most frequently. For instance, is it determined

by plant microenvironments, where cell–cell contacts are most likely, or differential expression of the system and its effectors between different lifecycle steps? Therefore, future research should focus on monitoring the expression of X-T4SS and T6SS-i4, along with their effectors, *in planta* using RT-qPCR throughout the infection process, and combine this with detection of Hcp secretion to confirm system activity.

3. Uncovering non-pathogenic xanthomonads and non-host range for hidden HGT events

Additionally, recent studies highlight the presence of non-pathogenic *Xanthomonas* on similar hosts as some widely studied pathogenic strains, such as non-pathogenic *X. santonii* and pathogenic *X. oryzae* (Bansal et al., 2021; Rana et al., 2024). These bacteria are less well studied due to their lack of symptoms for detection. However, isolating and sequencing them would provide further insight into *in planta* competition and potential horizontal gene transfer of X-T4SS or T6SS effectors, as well as their potential as biocontrol agents. Similarly, studying the host range and non-host plants on which the *X. translucens* complex can survive, such as the discovery of *Xt* CIX303 on cultivated wild rice (Curland et al., 2021), could provide a better understanding of how HGT events and gene exchange could occur between *Xanthomonas* species, including putative X-T4SS or T6SS effector exchanges.

Perspectives

1. Biocontrol agents and X-T4SS/ T6SS for plant protection

Recent advances in crop protection increasingly emphasise biocontrol strategies, which rely on the use of living organisms or natural substances, such as macroorganisms, microorganisms, secreted molecules or plant extracts, to regulate phytopathogen populations. Therefore, a deeper understanding of microbial interactions in the phyllosphere is essential, including competition mediated by antimicrobial X-T4SS and T6SS. In this context, the offensive and defensive mechanisms employed by both the biocontrol agent and the pathogen itself should be considered.

Many bacterial strains have been highlighted as promising biocontrol agents thanks to the action of X-T4SS or T6SS (Yu et al., 2019; Bernal et al., 2017; Garin et al., 2024). This is the case of *P. putida* KT2440, which outcompetes *X. campestris* in a T6SS-dependent manner, both *in vitro* and in *N. benthamiana*, significantly reducing necrotic symptoms on the leaves (Bernal et al., 2017). However, we have seen that the susceptibility of target cells to X-T4SS and T6SS-mediated killing varies between species and even strains. (Crisan et al., 2023). Therefore, it is crucial to select biocontrol strains with effector repertoires for which the targeted pathogen lacks immunity-dependent or -independent resistance mechanisms. However, this approach requires deep knowledge of pathogen immunity genes and defence strategies. Additionally, the effectors arsenal of biocontrol agents should also be relevant to the environmental context. For instance, periplasmic toxins inducing cell lysis are more advantageous in static environment compared to non-lytic ones, for the prevention of the formation of a corpse barrier of accumulated dead cells (Jurénas & Journet, 2021; W. P. J. Smith et al., 2020). Therefore, selection of biocontrol agents translocating non-lytic toxins in a contact-dependent manner might attenuate its potential for killing the pathogen in static environments.

It is also crucial to know whether plant pathogens are themselves equipped with X-T4SS or T6SS, with the risk of counterattacks through “tit-for-tat response” against biocontrol agents. Therefore, evaluating pathogen effector diversity alongside biocontrol agent immunity proteins is required.

Investigating the factors that control the expression of biocontrol agents and pathogens X-T4SS or T6SS, such as infection timing and environmental stimuli, will provide valuable insights. This would enable optimisation of treatment timing and application methods in the field. For instance, since T6SS operates in a contact-dependent manner, adjuvants could alter the likelihood of such contacts.

Finally, all potential side effects of using X-T4SS or T6SS and their effectors in biocontrol should be considered. For instance, *Xo* TleB effector was shown to suppress plant reproduction in non-host plants (J.-T. Yang et al., 2025).

2. Engineering synthetic microbial communities for plant protection

Synthetic microbial communities (SynComs) are artificially assembled microorganisms, taxonomically and functionally defined, to fulfil a specific phenotypic role (Zhang et al., 2025). Emerging approaches consider harnessing the beneficial potential of SynComs to promote plant growth and health. The development of SynCom relies on its stability, resilience, and reproducibility (Xu et al., 2025). However, we have seen that competitive traits, including X-T4SS and T6SS, are key determinants of microbiome composition and dynamics. Therefore, antimicrobial secretion systems represent promising tools for the stabilisation and resilience of microbiomes. From a diagnostic perspective, competitive traits within a microbiome could be used as biomarkers to assess its propensity to remain stable and to evaluate the potential for pathogen establishment. Finally, compared to a single biocontrol agent, SynCom could be designed with a consortium of beneficial strains immune to shared SynCom effectors, while protecting against invasion by pathogens-lacking immunity genes.

3. Microbiomes competitive traits evolution

In this work, we looked at the evolution of antibacterial weapons and the diversification of their effectors between closely related strains. It would be interesting to go further and observe the gain and loss of such bacterial weapons and effector-immunity pairs by experimental evolution in controlled conditions (Taillefer et al., 2025). Such an experiment could reveal how competitive traits in a community evolve when exposed to diverse biotic and abiotic stresses. This includes exposition to a new immune or susceptible strain, impacts of climate change, etc. Indeed, in some bacteria, T6SSs have been shown to be upregulated by elevated temperatures (Gonzalez Moreno et al., 2025). This could highlight how some bacterial lineages become more dominant than others *in planta* and anticipate the emergence of highly competitive pathogens. For instance, we could look at the possibility that other *Xanthomonas* species may evolve towards the loss of X-T4SS and gain of T6SS-i4 in the future.

Such evolution in competitive traits could be monitored by single cell DNA sequencing, highlighting emerging mutations in T6SS core genes or EI modules, as well as single-cell RNA sequencing to directly highlight differences in T6SS and EI gene expression. At the community scale, metatranscriptomics would highlight in which environmental conditions and microbiome compositions, competition weapons are down or upregulated. This would ultimately help understand why some

microbiomes are more stable and resilient than others. These approaches have the advantage of not only relying on culturable bacteria.

4. Influence of plants on T6SS and X-T4SS evolution

Plants may also shape the evolution of T6SS and X-T4SS, by influencing the acquisition, loss and HGT events among bacteria. Plant domestication, cultivar selection, and crop management practices, driven by human activities, can alter plant-associated microbiomes compared with those of wild cultivars. In addition, plant defense responses shape microbiome composition and structure. Together, these factors may modulate the selective pressure for acquiring or maintaining T6SS and X-T4SS within plant-associated bacterial communities. Moreover, the heterogeneous microenvironments created by plant tissues may generate distinct ecological niches in which bacteria carrying specific T6SS or X-T4SS variants have increased fitness and opportunities for HGT. Finally, plant distribution worldwide can influence HGT between xanthomonads. Mixed cropping systems and crop rotation practices may favor HGT among *Xanthomonas* species. For example, rice–wheat production systems cover approximately 24 million hectares of cultivated land in the Asian subtropics, including about 13.5 million hectares in the Indo-Gangetic Plains of South Asia (Bhandari et al., 2002).

5. T6SS as a driver of differentiation

T6SS could be an important driver of *Xanthomonas* pathovar differentiation. The different effector repertoires generate differences in compatibility, with strains that cannot coexist anymore, promoting divergence. Moreover, T6SS core genes and effectors loci are often located on mobile elements, accelerating evolutionary divergence within bacterial populations. Strains with differing T6SS arsenals can also prosper in different microbiome communities, creating ecological separation. Finally, beyond interbacterial competition, some T6SS activities appear to affect bacterial virulence and may drive strains to specialize on different hosts. However, it remains unclear whether T6SS drives niche differentiation or whether adaptation to distinct niches selects for specific T6SS effector repertoires.

6. T6SS as an engineered delivery tool

We have seen that T6SS plays a key role in interbacterial competition. The versatility of T6SS and its widespread distribution among proteobacteria could be exploited to fight pathogenic bacteria in human and veterinary medicine, as well as in phytopathology. As we are approaching a “post-antibiotic era”, such engineered T6SS delivery tools could find applications such as fighting multidrug-resistant bacteria or reshaping the gut microbiota (Jana et al., 2021). Additionally, T6SS was not only shown to target bacteria, but also eukaryotic microorganisms, such as fungi, or protect against amoebal predation, broadening its application potential.

Engineered antibacterial T6SSs have already been developed in a proof-of-concept customised antibacterial toxin delivery platform. These approaches targeted bacteria of choice, either through surface displayed nanobodies for specific adherence to the target cells, or by manipulating the effector repertoires (Jana et al., 2021; Ting et al., 2020).

Engineered antibacterial T6SS has also been developed for bacterial gene editing, as an alternative to transformation and conjugation. In this approach, candidate effectors or VgrG were fused to a Cre recombinase. Cre recombinase is known to remove DNA flanked by loxP sites. Therefore, they engineered a plasmid-based cassette for detecting Cre-mediated recombination in recipient cells. The plasmid carries an ampicillin resistance gene flanked by loxP sites, which prevents translation of a gentamicin resistance gene. Upon successful translocation of the VgrG–Cre fusion protein into recipient cells, Cre-mediated recombination removes the ampicillin resistance cassette, thereby restoring gentamicin resistance (Hersch et al., 2021).

Additionally, human-associated bacteria also use T6SS to target human eukaryotic cells, including epithelial cells, macrophages, etc. (Monjarás Feria et al., 2020). In the long term, T6SS could inspire new targeted delivery systems for therapeutic molecules. Bacteria could be engineered to deliver proteins or drugs directly to tumour or infected cells, mimicking the natural mechanism of T6SS.

Reporter systems have already been developed to monitor effector translocation by Type III, IV and VI secretion systems, such as the TEM-1 Beta-Lactamase Reporter System. In this approach, all candidate effectors were expressed as fusions with TEM β -lactamase. Bacterial strains were then used to translocate these fusion proteins into host cells, which were subsequently loaded with CCF2-AM substrate. Cleavage of the CCF2-AM substrate by the fusion protein within infected cells resulted in the production of blue fluorescence, allowing identification of infected cells (Allombert et al., 2024).

While no T6SS has yet been shown to directly target eukaryotic plant cells, similar approaches could be considered if such activity were to be demonstrated.

7. T6SS and bacteriophages

First of all, it is interesting to note that different contractile injection systems (CISs) have been discovered, including the T6SS and extracellular contractile injection system (eCIS), sharing ancestral and structural similarities to bacteriophages. eCIS comprises tailocin (syn. pyocin in *P. aeruginosa*) targeting prokaryotes, as well as both antifeeding prophages (Afps) and *Photorhabdus* virulence cassettes (PVCs) mainly targeting insect (eukaryotic) cells. Given that phages, T6SS and tailocins share similar target cells, it would be interesting to compare their distribution and target specificities, as well as their respective impact on each other.

Indeed, the impact of T6SS on phages and vice versa has not yet been explored. For example, it would be interesting to see if there are common defence mechanisms of targeted bacteria against T6SS and phages, such as EPS preventing phage adsorption and T6SS attacks. Thus, the pressure exerted by one on a bacterium could indirectly induce resistance to the other.

In addition, by delivering lytic and non-lytic effectors, the T6SS may indirectly eliminate resident prophages that rely on host survival, impact phage genetic material replication or protein synthesis, or potentially impact prophage induction. T6SS activity may also limit phage dissemination by reducing the density of phage host cells, or reshape the spatial organisation of susceptible bacterial cells, leading to separated niches that restrict phage access.

Finally, many temperate phages carry genes to manipulate their bacterial hosts and increase host fitness in a process known as lysogenic conversion (Brüssow et al., 2004). For instance, they may encode genes for resistance against additional phage superinfections. In this context, it would not be surprising that prophages have evolved strategies to protect their hosts from T6SS attacks. It would be interesting to look for prophage encoding immunity proteins. Finally, transduction might help diversification of T6SS effector repertoires. For instance, some effector-immunity modules have been found in genomic context supporting the idea they originate from prophage (Santoriello et al., 2020).

Conclusion

Antibacterial secretion systems play a major role in shaping microbiomes and, in host-associated contexts, ultimately influence host health, growth and development. These anti-prokaryotic machineries deliver effectors into neighbouring bacteria to suppress their growth or cause cell death. A multitude of antibacterial contact-dependent systems have evolved independently in bacteria and spread across bacterial phyla, undergoing continuous diversification.

This thesis explored the competitive strategies deployed by the cereal and grass pathogens *Xanthomonas translucens sensu lato*. More specifically, this work focused on the type IV (X-T4SS) and VI (T6SS) secretion systems, aiming to elucidate their contribution to interbacterial competition in *X. translucens*.

Using mutational and fluorescence-based approaches, we showed that the *X. translucens* X-T4SS and T6SS-i4 systems have overlapping roles in bacterial killing, with T6SS-i4 activity visualised by live confocal imaging. Our comparative genetics and phylogenetic analyses reveal the functional replacement of the ancestral X-T4SS by T6SS-i4 killing strategy in several xanthomonads lineages. We also report on the horizontal gene transfer mechanism of T6SS-i4 by integrative and conjugative elements in *Xanthomonas*, which has so far only been described in *Bacteroidetes*. In-depth *in silico* and comparative genomic analyses revealed a high diversity of T6SS effectors within the *X. translucens* complex, particularly in non-vascular xanthomonads compared to their vascular relatives. All core *X. translucens* T6SS effectors displayed bacteriolytic activity, and some effector–immunity pairs were identified through structural modelling and confirmed experimentally. Finally, *X. translucens* T6SS-i4 was shown to affect the spatial organisation of another co-infecting *X. translucens* strain *in planta*.

Given the significant role in competition and the wide distribution of X-T4SS and T6SS-i4 in *X. translucens sensu lato*, it is essential to consider the pathogen antibacterial weapons when designing and evaluating future disease control strategies. More importantly, continuing to trace the evolutionary history of X-T4SS and T6SS-i4 gain and loss events, as well as the evolution of their effector repertoire, is crucial for informed and precise disease management. Future research should aim to unravel the full scope of X-T4SS and T6SS effector functions, including their potential trans-kingdom impacts on microorganisms and their host physiology. A thorough understanding of these mechanisms is essential before considering their potential applications in plant health.

Finally, our research stresses the need to integrate a competitive dimension when studying the *X. translucens* lifecycle. *X. translucens* infects the seeds, colonises the plant epiphytically and endophytically, where complex microbial communities reside, and its success is tightly linked to its ability to outcompete other microorganisms sharing the same environments.

In conclusion, this work demonstrates the role of *X. translucens sensu lato* X-T4SS and T6SS-i4 as antibacterial secretion systems, integrating functional, structural, and evolutionary perspectives. Beyond advancing fundamental knowledge of interbacterial competition in *Xanthomonas*, it provides a framework for future studies and opens new avenues for the development of innovative disease management strategies.

Bibliography

- Abramson, J., Adler, J., Dunger, J., Evans, R., Green, T., Pritzel, A., Ronneberger, O., Willmore, L., Ballard, A. J., Bambrick, J., Bodenstein, S. W., Evans, D. A., Hung, C. C., O'Neill, M., Reiman, D., Tunyasuvunakool, K., Wu, Z., Žemgulytė, A., Arvaniti, E., ... Jumper, J. M. (2024). Accurate structure prediction of biomolecular interactions with AlphaFold 3. *Nature*, 630(8016), 493–500. <https://doi.org/10.1038/s41586-024-07487-w>
- Adhikari, T. B., Gurung, S., Hansen, J. M., & Bonman, J. M. (2012). Pathogenic and genetic diversity of *Xanthomonas translucens* pv. *undulosa* in North Dakota. *Phytopathology*, 102(4), 390–402. <https://doi.org/10.1094/PHYTO-07-11-0201>
- Adhikari, T. B., Hansen, J. M., Gurung, S., & Bonman, J. M. (2011). Identification of new sources of resistance in winter wheat to multiple strains of *Xanthomonas translucens* pv. *undulosa*. *Plant Disease*, 95(5), 582–588. <https://doi.org/10.1094/PDIS-10-10-0760>
- Ahmad, S., Wang, B., Walker, M. D., Tran, H. K. R., Stogios, P. J., Savchenko, A., Grant, R. A., McArthur, A. G., Laub, M. T., & Whitney, J. C. (2019). An interbacterial toxin inhibits target cell growth by synthesizing (p)ppApp. *Nature*, 575(7784), 674–678. <https://doi.org/10.1038/s41586-019-1735-9>
- Alcoforado Diniz, J., & Coulthurst, S. J. (2015). Intraspecies Competition in *Serratia marcescens* Is Mediated by Type VI-Secreted Rhs Effectors and a Conserved Effector-Associated Accessory Protein. *Journal of Bacteriology*, 197(14), 2350–2360. <https://doi.org/10.1128/JB.05671-11>
- Alegria, M. C., Souza, D. P., Andrade, M. O., Docena, C., Khater, L., Ramos, C. H. I., Da Silva, A. C. R., & Farah, C. S. (2005). Identification of new protein-protein interactions involving the products of the chromosome- and plasmid-encoded type IV secretion loci of the phytopathogen *Xanthomonas axonopodis* pv. *citri*. *Journal of Bacteriology*, 187(7), 2315–2325. <https://doi.org/10.1128/JB.187.7.2315-2325.2005>
- Allombert, J., Vianney, A., Charpentier, X. (2024). Monitoring Effector Translocation with the TEM-1 Beta-Lactamase Reporter System: From Endpoint to Time Course Analysis. In: Journet, L., Cascales, E. (eds) *Bacterial Secretion Systems. Methods in Molecular Biology*, 2715. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-3445-5_35.

- Allsopp, L. P., & Bernal, P. (2023). Killing in the name of: T6SS structure and effector diversity. *Microbiology*, 169(7), 001367. <https://doi.org/10.1099/mic.0.001367>
- Alteri, C. J., Himpsl, S. D., Zhu, K., Hershey, H. L., Musili, N., Miller, J. E., & Mobley, H. L. T. (2017). Subtle variation within conserved effector operon gene products contributes to T6SS-mediated killing and immunity. *PLoS Pathogens*, 13(11), e1006729. <https://doi.org/10.1371/journal.ppat.1006729>
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, 215(3), 403–410. [https://doi.org/10.1016/S0022-2836\(05\)80360-2](https://doi.org/10.1016/S0022-2836(05)80360-2)
- Alvarez-Martinez, C. E., Sgro, G. G., Araujo, G. G., Paiva, M. R. N., Matsuyama, B. Y., Guzzo, C. R., Andrade, M. O., & Farah, C. S. (2020). Secrete or perish: The role of secretion systems in *Xanthomonas* biology. *Computational and Structural Biotechnology Journal*, 19, 279–302. <https://doi.org/10.1016/j.csbj.2020.12.020>
- An, S. Q., Potnis, N., Dow, M., Vorhölter, F. J., He, Y. Q., Becker, A., Teper, D., Li, Y., Wang, N., Bleris, L., & Tang, J. L. (2020). Mechanistic insights into host adaptation, virulence and epidemiology of the phytopathogen *Xanthomonas*. *FEMS Microbiology Reviews*, 44(1), 1–32. <https://doi.org/10.1093/femsre/fuz024>
- Anguita-Maeso, M., Navas-Cortés, J. A., & Landa, B. B. (2023). Insights into the Methodological, Biotic and Abiotic Factors Influencing the Characterization of Xylem-Inhabiting Microbial Communities of Olive Trees. *Plants*, 12(4), 912. <https://doi.org/10.3390/plants12040912>
- Aoun, N., Georgoulis, S. J., Avalos, J. K., Grulla, K. J., Miqueo, K., Tom, C., & Lowe-Power, T. M. (2024). A pangenomic atlas reveals eco-evolutionary dynamics that shape type VI secretion systems in plant-pathogenic *Ralstonia*. *MBio*, 15(10), e00323-24. <https://doi.org/10.1128/mbio.00323-24>
- Arnason, R. (2021). Bacterial leaf streak persists in Alberta. *The Western Producer*. <https://www.producer.com/crops/bacterial-leaf-streak-persists-in-alberta/>
- Atanasoff, D., & Johnson, A. G. (1920). Treatment of cereals seeds by dry heat. *Journal of Agricultural Research*, 18(7), 379–390.
- Avello, M., Davis, K. P., & Grossman, A. D. (2019). Identification, characterization and benefits of an exclusion system in an integrative and conjugative element of *Bacillus subtilis*. *Molecular Microbiology*, 112(4), 1066–1082. <https://doi.org/10.1111/mmi.14359>
- Bamberg, R. H. (1936). Black chaff disease of wheat. *Journal of Agricultural Research*, 52(6), 397.

<https://books.google.com/books?hl=en&lr=&id=jshFAQAAIAAJ&oi=fnd&pg=PA397&ots=v0yOE-ltIE&sig=THohYyYHBwsdpdmGnY2-8z2cGio>

- Bansal, K., Kaur, A., Midha, S., Kumar, S., Korpole, S., & Patil, P. B. (2021). *Xanthomonas sontii* sp. nov., a non-pathogenic bacterium isolated from healthy basmati rice (*Oryza sativa*) seeds from India. *Antonie van Leeuwenhoek*, 114(11), 1935–1947. <https://doi.org/https://doi.org/10.1007/s10482-021-01652-1>
- Bansal, K., Kumar, S., Singh, A., Chaudhary, A., & Patil, P. B. (2023). Redefining the Taxonomic Boundaries of Genus *Xanthomonas*. *Taxonomy*, 3(4), 452–465. <https://doi.org/10.3390/taxonomy3040026>
- Basler, M., Ho, B. T., & Mekalanos, J. J. (2013). Tit-for-tat: Type VI secretion system counterattack during bacterial cell-cell interactions. *Cell*, 152(4), 884–894. <https://doi.org/10.1016/j.cell.2013.01.042>
- Baufayt, M. (2022). *Functional analysis of Xanthomonadales-like type IV secretion system and distribution of Xanthomonadales-like type IV and type VI secretion systems in Xanthomonas translucens*. Université catholique de Louvain.
- Bayer-Santos, E., Cenens, W., Matsuyama, B. Y., Oka, G. U., Di Sessa, G., Del Valle Mininel, I., Alves, T. L., & Farah, C. S. (2019). The opportunistic pathogen *Stenotrophomonas maltophilia* utilizes a type IV secretion system for interbacterial killing. *PLoS Pathogens*, 15(9), e1007651. <https://doi.org/10.1371/journal.ppat.1007651>
- Bayer-Santos, E., DeMoraes Ceseti, L., Farah, C. S., & Alvarez-Martinez, C. E. (2019). Distribution, function and regulation of type 6 secretion systems of *Xanthomonadales*. *Frontiers in Microbiology*, 10, 1635. <https://doi.org/10.3389/fmicb.2019.01635>
- Bayer-Santos, E., Lima, L. dos P., Ceseti, L. de M., Ratagami, C. Y., de Santana, E. S., da Silva, A. M., Farah, C. S., & Alvarez-Martinez, C. E. (2018). *Xanthomonas citri* T6SS mediates resistance to *Dictyostelium* predation and is regulated by an ECF σ factor and cognate Ser/Thr kinase. *Environmental Microbiology*, 20(4), 1562–1575. <https://doi.org/10.1111/1462-2920.14085>
- Bender, C. L., Alarco'n, F., Alarco'n-Chaidez, A., & Gross, D. C. (1999). *Pseudomonas syringae* Phytotoxins: Mode of Action, Regulation, and Biosynthesis by Peptide and Polyketide Synthetases. *Microbiology and Molecular Biology Reviews*, 63(2), 266–292. <https://doi.org/10.1128/mmb.63.2.266-292.1999>
- Bernal, P., Allsopp, L. P., Filloux, A., & Llamas, M. A. (2017). The *Pseudomonas putida* T6SS is a plant warden against phytopathogens. *ISME Journal*, 11(4), 972–987. <https://doi.org/10.1038/ismej.2016.169>

- Bernal, P., Christopher, R., Furniss, D., Fecht, S., Leung, R. C. Y., Spiga, L., Mavridou, D. A. I., & Filloux, A. (2021). A novel stabilization mechanism for the type VI secretion system sheath. *Proceedings of the National Academy of Sciences of the United States of America*, 118(7), e2008500118. <https://doi.org/10.1073/pnas.2008500118/-/DCSupplemental>
- Bernal, P., Llamas, M. A., & Filloux, A. (2018). Type VI secretion systems in plant-associated bacteria. *Environmental Microbiology*, 20(1), 1–15. <https://doi.org/10.1111/1462-2920.13956>
- Bernard, C. S., Brunet, Y. R., Gavioli, M., Llobès, R., & Cascales, E. (2011). Regulation of type VI Secretion gene clusters by σ_{54} and cognate enhancer binding proteins. *Journal of Bacteriology*, 193(9), 2158–2167. <https://doi.org/10.1128/JB.00029-11>
- Berni, B., Soscia, C., Djermoun, S., Ize, B., & Bleves, S. (2019). A type VI secretion system trans-kingdom effector is required for the delivery of a novel antibacterial toxin in *Pseudomonas aeruginosa*. *Frontiers in Microbiology*, 10, 1218. <https://doi.org/10.3389/fmicb.2019.01218>
- Bertelli, C., Laird, M. R., Williams, K. P., Lau, B. Y., Hoad, G., Winsor, G. L., & Brinkman, F. S. L. (2017). IslandViewer 4: Expanded prediction of genomic islands for larger-scale datasets. *Nucleic Acids Research*, 45(W1), W30–W35. <https://doi.org/10.1093/nar/gkx343>
- Bhandari, A. L., Ladha, J. K., Pathak, H., Padre, A. T., Dawe, D., & Gupta, R. K. (2002). Yield and soil nutrient changes in a long-term rice-wheat rotation in India. *Soil Science Society of America Journal*, 66(1), 162–170. <https://doi.org/10.2136/sssaj2002.1620a>
- Bi, D., Liu, L., Tai, C., Deng, Z., Rajakumar, K., & Ou, H. Y. (2013). SecReT4: A web-based bacterial type IV secretion system resource. *Nucleic Acids Research*, 41(D1), D660–D665. <https://doi.org/10.1093/nar/gks1248>
- Bianco, M. I., Toum, L., Yaryura, P. M., Mielnichuk, N., Gudesblat, G. E., Roeschlin, R., Marano, M. R., Ielpi, L., & Vojnov, A. A. (2016). Xanthan pyruvilation is essential for the virulence of *Xanthomonas campestris* pv. *campestris*. *Molecular Plant-Microbe Interactions*, 29(9), 688–699. <https://doi.org/10.1094/MPMI-06-16-0106-R>
- Bladergroen, M. R., Badelt, K., & Spaink, H. P. (2003). Infection-Blocking Genes of a Symbiotic *Rhizobium leguminosarum* Strain That Are Involved in Temperature-Dependent Protein Secretion. *Molecular Plant-Microbe Interactions*, 16(1), 53–64. <https://doi.org/10.1094/MPMI.2003.16.1.53>
- Blondel, C. J., Jiménez, J. C., Contreras, I., & Santiviago, C. A. (2009). Comparative genomic analysis uncovers 3 novel loci encoding type six secretion systems

- differentially distributed in *Salmonella* serotypes. *BMC Genomics*, 10(1), 354. <https://doi.org/10.1186/1471-2164-10-354>
- Böck, D., Medeiros, J. M., Tsao, H. F., Penz, T., Weiss, G. L., Aistleitner, K., Horn, M., & Pilhofer, M. (2017). *In situ* architecture, function, and evolution of a contractile injection system. *Science*, 357(6352), 713–717. <https://doi.org/10.1126/science.aan7904>
- Bondage, D. D., Lin, J. S., Ma, L. S., Kuo, C. H., & Lai, E. M. (2016). VgrG C terminus confers the type VI effector transport specificity and is required for binding with PAAR and adaptor-effector complex. *Proceedings of the National Academy of Sciences of the United States of America*, 113(27), E3931–E3940. <https://doi.org/10.1073/pnas.1600428113>
- Boosalis, M. G. (1951). *The epidemiology of Xanthomonas translucens (JJ and R.) Dowson on cereals and grasses*. University of Minnesota.
- Booth, S. C., Smith, W. P. J., & Foster, K. R. (2023). The evolution of short- and long-range weapons for bacterial competition. *Nature Ecology and Evolution*, 7(12), 2080–2091. <https://doi.org/10.1038/s41559-023-02234-2>
- Borgeaud, S., Metzger, L. C., Scignari, T., & Blokesch, M. (2015). The type VI secretion system of *Vibrio cholerae* fosters horizontal gene transfer. *Science*, 347(6217), 63–67. <https://doi.org/10.1126/SCIENCE.1260064>,
- Bowman, L., & Palmer, T. (2021). The Type VII Secretion System of *Staphylococcus*. *Annual Review of Microbiology*, 75(1), 471–494. <https://doi.org/10.1146/annurev-micro-012721-123600>
- Boyer, F., Fichant, G., Berthod, J., Vandenbrouck, Y., & Attree, I. (2009). Dissecting the bacterial type VI secretion system by a genome wide *in silico* analysis: What can be learned from available microbial genomic resources? *BMC Genomics*, 10, 104. <https://doi.org/10.1186/1471-2164-10-104>
- Bragard, C., Singer, E., Alizadeh, A., Vauterin, L., Maraite, H., & Swings, J. (1997). *Xanthomonas translucens* from Small Grains: Diversity and Phytopathological Relevance. *Phytopathology*, 87(11), 1111–1117. <https://doi.org/10.1094/PHTO.1997.87.11.1111>
- Bragard, C., Verdier, V., & Maraite, H. (1995). Genetic diversity among *Xanthomonas campestris* strains pathogenic for small grains. *Applied and environmental microbiology*, 61(3), 1020-1026. <https://doi.org/10.1128/aem.61.3.1020-1026.1995>
- Bragard, C., & Verhoyen, M. (1993). Monoclonal Antibodies Specific for *Xanthomonas campestris* Bacteria Pathogenic on Wheat and other Small Grains, in Comparison with Polyclonal Antisera. *Journal of Phytopathology*, 139(3), 217–228. <https://doi.org/10.1111/J.1439-0434.1993.TB01420.X>

- Bröms, J. E., Sjöstedt, A., & Lavander, M. (2010). The role of the *Francisella tularensis* pathogenicity island in type VI secretion, intracellular survival, and modulation of host cell signaling. *Frontiers in Microbiology*, 1, 136. <https://doi.org/10.3389/fmicb.2010.00136>
- Brual, T., Effantin, G., Baltenneck, J., Attaiech, L., Grosbois, C., Royer, M., Cigna, J., Faure, D., Hugouvieux-Cotte, N., Pattat, & Gueguen, E. (2023). A natural single nucleotide mutation in the small regulatory RNA ArcZ of *Dickeya solani* switches off the antimicrobial activities against yeast and bacteria. *PLoS Genetics*, 19(4), e1010725. <https://doi.org/10.1371/journal.pgen.1010725>
- Brüssow, H., Canchaya, C., & Hardt, W. D. (2004). Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. *Microbiology and molecular biology reviews*, 68(3), 560-602. <https://doi.org/10.1128/mmbr.68.3.560-602.2004>
- Bruto, M., James, A., Petton, B., Labreuche, Y., Chenivresse, S., Alunno-Bruscia, M., Polz, M. F., & Le Roux, F. (2017). *Vibrio crassostreae*, a benign oyster colonizer turned into a pathogen after plasmid acquisition. *ISME Journal*, 11(4), 1043–1052. <https://doi.org/10.1038/ismej.2016.162>
- Butchacas, J. (2024). *Emerging Threats to Small Grain Production: Exploring the Tissue-Specific Infection Strategies, Population Genetics, and Virulence Mechanisms of Xanthomonas translucens Clade-I, The Causal Agents of Bacterial Leaf Streak on Barley*. The Ohio State University.
- Campos, P. E., Pruvost, O., Boyer, K., Chiroleu, F., Cao, T. T., Gaudeul, M., Baider, C., Utteridge, T. M. A., Becker, N., Rieux, A., & Gagnevin, L. (2023). Herbarium specimen sequencing allows precise dating of *Xanthomonas citri* pv. *citri* diversification history. *Nature Communications*, 14(1), 4306. <https://doi.org/10.1038/s41467-023-39950-z>
- Casabona, M. G., Silverman, J. M., Sall, K. M., Boyer, F., Couté, Y., Poirel, J., Grunwald, D., Mougous, J. D., Elsen, S., & Attree, I. (2013). An ABC transporter and an outer membrane lipoprotein participate in posttranslational activation of type VI secretion in *Pseudomonas aeruginosa*. *Environmental Microbiology*, 15(2), 471–486. <https://doi.org/10.1111/j.1462-2920.2012.02816.x>
- Cenens, W., Andrade, M. O., Llontop, E., Alvarez-Martinez, C. E., Sgro, G. G., & Farah, C. S. (2020). Bactericidal type IV secretion system homeostasis in *Xanthomonas citri*. *PLoS Pathogens*, 16(5), e1008561. <https://doi.org/10.1371/journal.ppat.1008561>
- Cesbron, S., Briand, M., Essakhi, S., Gironde, S., Boureau, T., Manceau, C., Fischer-Le Saux, M., & Jacques, M. A. (2015). Comparative genomics of pathogenic

- and nonpathogenic strains of *Xanthomonas arboricola* unveil molecular and evolutionary events linked to pathoadaptation. *Frontiers in Plant Science*, 6, 1126. <https://doi.org/10.3389/fpls.2015.01126>
- Ceseti, L. M., de Santana, E. S., Ratagami, C. Y., Barreiros, Y., Lima, L. D. P., Dunger, G., Farah, C. S., & Alvarez-Martinez, C. E. (2019). The *Xanthomonas citri* pv. *citri* Type VI Secretion System is Induced During Epiphytic Colonization of Citrus. *Current Microbiology*, 76(10), 1105–1111. <https://doi.org/10.1007/s00284-019-01735-3>
- Champoiseau, P., Daugrois, J. H., Pieretti, I., Cociancich, S., Royer, M., & Rott, P. (2006). High variation in pathogenicity of genetically closely related strains of *Xanthomonas albilineans*, the sugarcane leaf scald pathogen, in Guadeloupe. *Phytopathology*, 96(10), 1081-1091. <https://doi.org/10.1094/PHYTO-96-1081>
- Che, S., Sun, C., Yang, L., Zhou, M., Xia, L., Yan, J., Jiang, M., Wang, J., Wang, H., Zhao, W., Toth, I., Hu, B., Guo, T., & Fan, J. (2024). T6SS and T4SS redundantly secrete effectors to govern the virulence and bacterial competition in *Pectobacterium* PccS1. *Phytopathology*, 114(8), 1926–1939. <https://doi.org/10.1094/PHYTO-11-23-0455-R>
- Chen, W. J., Kuo, T. Y., Hsieh, F. C., Chen, P. Y., Wang, C. S., Shih, Y. L., Lai, Y. M., Liu, J. R., Yang, Y. L., & Shih, M. C. (2016). Involvement of type VI secretion system in secretion of iron chelator pyoverdine in *Pseudomonas taiwanensis*. *Scientific Reports*, 6, 32950. <https://doi.org/10.1038/srep32950>
- Cherrak, Y., Rapisarda, C., Pellarin, R., Bouvier, G., Bardiaux, B., Allain, F., Malosse, C., Rey, M., Chamot-Rooke, J., Cascales, E., Fronzes, R., & Durand, E. (2018). Biogenesis and structure of a type VI secretion baseplate. *Nature Microbiology*, 3(12), 1404–1416. <https://doi.org/10.1038/s41564-018-0260-1>
- Chesneau, G., Noel, A., Bréard, D., Boulanger, A., Briand, M., Bonneau, S., Price, M., Liu, Y., Hendrickson, A., Nielsen, T., Sarniguet, A., Guilet, D., Arkin, A., Lui, L., & Barret, M. (2025). Lactuchelins: New lipopeptide siderophores from *Pseudomonas lactucae* inhibit *Xanthomonas campestris* pv. *campestris* 8004. *BioRxiv*, 2025.02.28.640635. <https://doi.org/10.1101/2025.02.28.640635>
- Choi, K. H., & Schweizer, H. P. (2006). mini-Tn7 insertion in bacteria with single attTn7 sites: Example *Pseudomonas aeruginosa*. *Nature Protocols*, 1(1), 153–161. <https://doi.org/10.1038/nprot.2006.24>
- Choi, Y., Kim, N., Mannaa, M., Kim, H., Park, J., Jung, H., Han, G., Lee, H. H., & Seo, Y. S. (2020). Characterization of type VI secretion system in *Xanthomonas oryzae* pv. *oryzae* and its role in virulence to rice. *The Plant Pathology Journal*, 36(3), 289–296. <https://doi.org/10.5423/PPJ.NT.02.2020.0026>

- Chuang, S. C., Dobhal, S., Alvarez, A. M., & Arif, M. (2024). Three new species, *Xanthomonas hawaiiensis* sp. nov., *Stenotrophomonas aracearum* sp. nov., and *Stenotrophomonas oahuensis* sp. nov., isolated from the *Araceae* family. *Frontiers in Microbiology*, *15*, 1356025. <https://doi.org/10.3389/fmicb.2024.1356025>
- Cianfanelli, F. R., Alcoforado Diniz, J., Guo, M., De Cesare, V., Trost, M., & Coulthurst, S. J. (2016). VgrG and PAAR Proteins Define Distinct Versions of a Functional Type VI Secretion System. *PLoS Pathogens*, *12*(6), e1005735. <https://doi.org/10.1371/journal.ppat.1005735>
- Cobe, B. L., Dey, S., Minasov, G., Inniss, N., Satchell, K. J. F., & Cianciotto, N. P. (2024). Bactericidal effectors of the *Stenotrophomonas maltophilia* type IV secretion system: functional definition of the nuclease TfdA and structural determination of TfcB. *MBio*, *15*(7), e01198-24. <https://doi.org/10.1128/mbio.01198-24>
- Cohen, S. P., Luna, E. K., Lang, J. M., Ziegler, J., Chang, C., Leach, J. E., Le-Saux, M. F., Portier, P., Koebnik, R., & Jacobs, J. M. (2020). High-Quality Genome Resource of *Xanthomonas hyacinthi* Generated via Long-Read Sequencing. *Plant Disease*, *104*(4), 1011–1012. <https://doi.org/10.1094/PDIS-11-19-2393-A>
- Costa, T. R. D., Felisberto-Rodrigues, C., Meir, A., Prevost, M. S., Redzej, A., Trokter, M., & Waksman, G. (2015). Secretion systems in Gram-negative bacteria: structural and mechanistic insights. *Nature Reviews. Microbiology*, *13*(6), 343–359. <https://doi.org/10.1038/NRMICRO3456>
- Coyne, M. J., Roelofs, K. G., & Comstock, L. E. (2016). Type VI secretion systems of human gut *Bacteroidales* segregate into three genetic architectures, two of which are contained on mobile genetic elements. *BMC Genomics*, *17*(1), 58. <https://doi.org/10.1186/s12864-016-2377-z>
- Crisan, C. V., Van Tyne, D., & Goldberg, J. B. (2023). The type VI secretion system of the emerging pathogen *Stenotrophomonas maltophilia* complex has antibacterial properties. *MSphere*, *8*(6), e0058423. <https://doi.org/10.1101/2023.05.30.542968>
- Cunfer, B. (1988). *Bacterial diseases of wheat and their potential importance in tropical regions*. <https://agris.fao.org/search/en/providers/122512/records/6471c88769d6cbfdd4a2d3a8>
- Curland, R. D., Gao, L., Bull, C. T., Vinatzer, B. A., Dill-Macky, R., Van Eck, L., & Ishimaru, C. A. (2018). Genetic diversity and virulence of wheat and barley strains of *Xanthomonas translucens* from the upper midwestern United

- States. *Phytopathology*, 108(4), 443–453. <https://doi.org/10.1094/PHYTO-08-17-0271-R>
- Curland, R. D., Gao, L., Hirsch, C. D., & Ishimaru, C. A. (2020). Localized genetic and phenotypic diversity of *Xanthomonas translucens* associated with bacterial leaf streak on wheat and barley in Minnesota. *Phytopathology*, 110(2), 257–266. <https://doi.org/10.1094/PHYTO-04-19-0134-R>
- Curland, R. D., Hallada, K. R., Ledman, K. E., & Dill-Macky, R. (2021). First Report of Bacterial Leaf Streak Caused by *Xanthomonas translucens* pv. *undulosa* on Cultivated Wild Rice (*Zizania palustris*) in Minnesota. *Plant Disease*, 105(9), 2711. <https://doi.org/10.1094/PDIS>
- Da Silva, A. C. R., Ferro, J. A., Reinach, F. C., Farah, C. S., Furlan, L. R., Quaggio, R. B., Monteiro-Vitorello, C. B., Van Sluys, M. A., Almeida, N. F., Alves, L. M. C., Do Amaral, A. M., Bertolini, M. C., Camargo, L. E. A., Camarotte, G., Cannavan, F., Cardozo, J., Chambergo, F., Ciapina, L. P., Cicarelli, R. M. B., ... Kitajima, J. P. (2002). Comparison of the genomes of two *Xanthomonas* pathogens with differing host specificities. *Nature*, 417(6887), 459–463. <https://doi.org/10.1038/417459a>
- Dang, Z., McLenachan, P. A., Lockhart, P. J., Waipara, N., Er, O., Reynolds, C., & Blanchon, D. (2019). Metagenome profiling identifies potential biocontrol agents for *Selaginella kraussiana* in New Zealand. *Genes*, 10(2), 106. <https://doi.org/10.3390/genes10020106>
- Darrasse, A., Carrere, S., Barbe, V., Boureau, T., Arrieta-Ortiz, M. L., Bonneau, S., ... & Jacques, M. A. (2013). Genome sequence of *Xanthomonas fuscans* subsp. *fuscans* strain 4834-R reveals that flagellar motility is not a general feature of xanthomonads. *BMC genomics*, 14(1), 761. <https://doi.org/10.1186/1471-2164-14-761>
- Davis, E. W., Okrent, R. A., Manning, V. A., & Trippe, K. M. (2021). Unexpected distribution of the 4- formylaminoxyvinylglycine (FVG) biosynthetic pathway in *Pseudomonas* and beyond. *PLoS ONE*, 16(4), e0247348. <https://doi.org/10.1371/journal.pone.0247348>
- de Assis, J. C. S., Gonçalves, O. S., Fernandes, A. S., de Queiroz, M. V., Bazzolli, D. M. S., & Santana, M. F. (2022). Genomic analysis reveals the role of integrative and conjugative elements in plant pathogenic bacteria. *Mobile DNA*, 13(1), 19. <https://doi.org/10.1186/s13100-022-00275-1>
- Declerck, A. (2022). *Kill or be killed : the dangerous Type VI Secretion System game played by Xanthomonas translucens pathovar undulosa*. Université catholique de Louvain.

- Delitte, M., Caulier, S., Bragard, C., & Desoignies, N. (2021). Plant microbiota beyond farming practices: a review. *Frontiers in Sustainable Food Systems*, 5, 624203. <https://doi.org/10.3389/fsufs.2021.624203>
- Delitte, M., Dubois, B., Morandini, L., Bultreys, A., Mahillon, J., Debode, F., & Bragard, C. (2025). Biocontrol-relevant diversity of wheat-associated *Pseudomonas*: prevalence of *P. sivasensis* and identification of the novel species *P. arvensis* sp. nov. *PeerJ*, 13, e20177. <https://doi.org/10.7717/peerj.20177>
- Department of crop sciences university of Illinois at Urbana-Champaign. (1987). *Report on plant disease No.414: Bacterial wilt and decline of turfgrasses*.
- Dobrindt, U., Hochhut, B., Hentschel, U., & Hacker, J. (2004). Genomic islands in pathogenic and environmental microorganisms. *Nature Reviews Microbiology*, 2(5), 414–424. <https://doi.org/10.1038/nrmicro884>
- Dong, T. G., Ho, B. T., Yoder-Himes, D. R., & Mekalanos, J. J. (2013). Identification of T6SS-dependent effector and immunity proteins by Tn-seq in *Vibrio cholerae*. *Proceedings of the National Academy of Sciences of the United States of America*, 110(7), 2623–2628. <https://doi.org/10.1073/pnas.1222783110>
- Dörr, T., Alvarez, L., Delgado, F., Davis, B. M., Cava, F., & Waldor, M. K. (2016). A cell wall damage response mediated by a sensor kinase/response regulator pair enables beta-lactam tolerance. *Proceedings of the National Academy of Sciences of the United States of America*, 113(2), 404–409. <https://doi.org/10.1073/pnas.1520333113>
- dos Passos Lima, L., Pereira, J. B., Flores, A. J. F., Lorenzetti, A. P. R., Boechat, A. L., Pereda, M. C., Gualtieri, S., do Prado, D. F., Rocha, D., de Moraes Ceseti, L., Baldini, R. L., Farah, C. S., Koide, T., Benedetti, C. E., & Alvarez-Martinez, C. E. (2022). An Extracytoplasmic Function Sigma Factor Required for Full Virulence in *Xanthomonas citri* pv. *citri*. *Journal of Bacteriology*, 204(5), e0062421. <https://doi.org/10.1128/jb.00624-21>
- Dowson, W. (1939). On the systematic position and generic names of the Gram-negative bacterial plant pathogens. *Transactions of the British Mycological Society*, 26(1–2), 4–14. <https://doi.org/10.5555/19391101451>
- Drehkopf, S., Scheibner, F., & Büttner, D. (2023). Functional characterization of VirB/VirD4 and Icm/Dot type IV secretion systems from the plant-pathogenic bacterium *Xanthomonas euvesicatoria*. *Frontiers in Cellular and Infection Microbiology*, 13, 1203159. <https://doi.org/10.3389/fcimb.2023.1203159>
- Dubois, B., Delitte, M., Lengrand, S., Bragard, C., Legrève, A., & Debode, F. (2024). PRONAME: a user-friendly pipeline to process long-read nanopore metabarcoding data by generating high-quality consensus sequences.

- Frontiers in Bioinformatics*, 4, 1483255.
<https://doi.org/10.3389/fbinf.2024.1483255>
- Dudley, E. G., Thomson, N. R., Parkhill, J., Morin, N. P., & Nataro, J. P. (2006). Proteomic and microarray characterization of the AggR regulon identifies a pheU pathogenicity island in enteroaggregative *Escherichia coli*. *Molecular Microbiology*, 61(5), 1267–1282. <https://doi.org/10.1111/j.1365-2958.2006.05281.x>
- Dunger, G., Llontop, E., Guzzo, C. R., & Farah, C. S. (2016). The *Xanthomonas* type IV pilus. *Current Opinion in Microbiology*, 30, 88–97.
<https://doi.org/10.1016/j.mib.2016.01.007>
- Durfee, T., Nelson, R., Baldwin, S., Plunkett, G., Burland, V., Mau, B., Petrosino, J. F., Qin, X., Muzny, D. M., Ayele, M., Gibbs, R. A., Csörgo, B., Pósfai, G., Weinstock, G. M., & Blattner, F. R. (2008). The complete genome sequence of *Escherichia coli* DH10B: Insights into the biology of a laboratory workhorse. *Journal of Bacteriology*, 190(7), 2597–2606.
<https://doi.org/10.1128/JB.01695-07>
- Duveiller, E. (1992). *Toward management of bacterial leaf streak of wheat and triticale*. Université Catholique de Louvain.
- Duveiller, E. (1994). Bacterial leaf streak or black chaff of cereals. *EPPO Bulletin*, 24(1), 135–157. <https://doi.org/10.1111/j.1365-2338.1994.tb01057.x>
- Duveiller, E., & Bragard, C. (2017). Detection of *Xanthomonas translucens* in wheat seeds. In U. APS Press (Minnesota (Ed.), *In Detection of Plant-Pathogenic Bacteria in Seed and Other Planting Material* (Second edition, pp. 27–32). The American Phytopathological Society.
- Duveiller, E., Bragard, C., & Maraite, H. E. (1997). Bacterial leaf streak and black chaff caused by *Xanthomonas translucens*. In E. Duveiller, K. Rudolph, & L. Fuzikovsky (Eds.), *The Bacterial Disease of Wheat: Concepts and Methods of Disease Management* (Vol. 25, p. 47). CIMMYT.
- Duveiller, E., & Maraite, H. (1993). Study on yield loss due to *Xanthomonas campestris* pv. *undulosa* in wheat under high rainfall temperate conditions. *Journal of Plant Diseases and Protection*, 100(5), 453–459.
<https://doi.org/https://www.jstor.org/stable/43386798>
- Dye, D. W., & Lelliott, R. A. (1974). Genus II. *Xanthomonas*. In Williams & Wilkins (Ed.), *Bergey's Manual of Determinative Bacteriology* (Vol. 8th, pp. 243–249). R. E. Buchanan and N. E. Gibbons.
- Eckardt, N. A. (2010). Evolution of domesticated bread wheat. *Plant Cell*, 22(4), 993. <https://doi.org/10.1105/tpc.110.220410>

- Edmilson Gonc, B. R., & Rosato, Y. B. (2002). Phylogenetic analysis of *Xanthomonas* species based upon 16S-23S rDNA intergenic spacer sequences. *International Journal of Systematic and Evolutionary Microbiology*, 52(2), 355–361. <https://doi.org/10.1099/ijs.0.01886-0>
- EPPO. (2025a, August 13). *Xanthomonas translucens* pv. *translucens*. EPPO datasheets on pests recommended for regulation. <https://Gd.Eppo.Int/Taxon/XANTTR/Datasheet>.
- EPPO. (2025b, September 13). EPPO A2 List of pests recommended for regulation as quarantine pests. https://Www.Eppo.Int/ACTIVITIES/Plant_quarantine/A2_list.
- Failor, K. C., Schmale, D. G., Vinatzer, B. A., & Monteil, C. L. (2017). Ice nucleation active bacteria in precipitation are genetically diverse and nucleate ice by employing different mechanisms. *ISME Journal*, 11(12), 2740–2753. <https://doi.org/10.1038/ismej.2017.124>
- Fang, Y., Lin, H., Wu, L., Ren, D., Ye, W., Dong, G., Zhu, L., & Guo, L. (2015). Genome sequence of *Xanthomonas sacchari* R1, a biocontrol bacterium isolated from the rice seed. *Journal of Biotechnology*, 206, 77–78. <https://doi.org/10.1016/j.jbiotec.2015.04.014>
- Ferguson, A. D., Amezcua, C. A., Halabi, N. M., Chelliah, Y., Rosen, M. K., Ranganathan, R., & Deisenhofer, J. (2006). Signal transduction pathway of TonB-dependent transporters. *Proceedings of the National Academy of Sciences of the United States of America*, 104(2), 513–518. <https://doi.org/10.1073/pnas.0609887104>
- Ferreira-Tonin, M., Rodrigues-Neto, J., Harakava, R., & Lanza Destéfano, S. A. (2012). Phylogenetic analysis of *Xanthomonas* based on partial *rpoB* gene sequences and species differentiation by PCR-RFLP. *International Journal of Systematic and Evolutionary Microbiology*, 62(6), 1419–1424. <https://doi.org/10.1099/ijs.0.028977-0>
- Flaugnatti, N., Le, T. T. H., Canaan, S., Aschtgen, M. S., Nguyen, V. S., Blangy, S., Kellenberger, C., Roussel, A., Cambillau, C., Cascales, E., & Journet, L. (2016). A phospholipase A1 antibacterial Type VI secretion effector interacts directly with the C-terminal domain of the VgrG spike protein for delivery. *Molecular Microbiology*, 99(6), 1099–1118. <https://doi.org/10.1111/mmi.13292>
- Flaugnatti, N., Rapisarda, C., Rey, M., Beauvois, S. G., Nguyen, V. A., Canaan, S., Durand, E., Chamot-Rooke, J., Cascales, E., Fronzes, R., & Journet, L. (2020). Structural basis for loading and inhibition of a bacterial T6SS phospholipase effector by the VgrG spike. *The EMBO Journal*, 39(11), e104129. <https://doi.org/10.15252/embj.2019104129>

- Förster, A., Planamente, S., Manoli, E., Lossi, N. S., Freemont, P. S., & Filloux, A. (2014). Coevolution of the ATPase ClpV, the sheath proteins TssB and TssC, and the accessory protein TagJ/HsiE1 distinguishes type VI secretion classes. *Journal of Biological Chemistry*, 289(47), 33032–33043. <https://doi.org/10.1074/jbc.M114.600510>
- Forster, R. L., Mihuta-Grimm, L., & Schaad, N. W. (1986). Black chaff of wheat and barley. University of Idaho, Cooperative Extension Service, Agricultural Experiment. In *Current information series* (Vol. 784). University of Idaho, Cooperative Extension Service, Agricultural Experiment Station, College of Agriculture.
- Forster, R. L., & Schaad, N. W. (1985). Evaluation of seed treatments for eradication of *Xanthomonas campestris*. *Phytopathology*, 75(11), 1385.
- Forster, R. L., & Schaad, N. W. (1990). Longevity of *Xanthomonas campestris* pv. *translucens* in wheat seed under two storage conditions. In Akadémiai Kiadó (Ed.), *Proceedings of the 7th International Conference of Plant Pathogenic Bacteria* (pp. 329–331).
- Fridman, C. M., Keppel, K., Gerlic, M., Bosis, E., & Salomon, D. (2020). A comparative genomics methodology reveals a widespread family of membrane-disrupting T6SS effectors. *Nature Communications*, 11(1), 1085. <https://doi.org/10.1038/s41467-020-14951-4>
- Fronzes, R., Christie, P. J., & Waksman, G. (2009). The structural biology of type IV secretion systems. *Nature Reviews Microbiology*, 7(10), 703–714. <https://doi.org/10.1038/nrmicro2218>
- Gallegos-Monterrosa, R., & Coulthurst, S. J. (2021). The ecological impact of a bacterial weapon: Microbial interactions and the Type VI secretion system. *FEMS Microbiology Reviews*, 45(6), fuab033. <https://doi.org/10.1093/femsre/fuab033>
- Gallique, M., Bouteiller, M., & Merieau, A. (2017). The type VI secretion system: A dynamic system for bacterial communication? *Frontiers in Microbiology*, 8, 1454. <https://doi.org/10.3389/FMICB.2017.01454/BIBTEX>
- García-Bayona, L., & Comstock, L. E. (2018). Bacterial antagonism in host-associated microbial communities. *Science*, 361(6408), eaat2456. <https://doi.org/10.1126/science.aat2456>
- García-Bayona, L., Coyne, M. J., & Comstock, L. E. (2021). Mobile Type VI secretion system loci of the gut *Bacteroidales* display extensive intra-ecosystem transfer, multi-species spread and geographical clustering. *PLOS Genetics*, 17(4), e1009541. <https://doi.org/10.1371/JOURNAL.PGEN.1009541>

- Garcia-Bayona, L., Guo, M. S., & Laub, M. T. (2017). Contact-dependent killing by *Caulobacter crescentus* via cell surface-associated, glycine zipper proteins. *Elife*, 6, e24869. <https://doi.org/10.7554/eLife.24869.001>
- García Pérez, A. (2023). *Identification de nouveaux effecteurs du système de sécrétion de type 6 potentiellement impliqués dans la compétition antimicrobienne*. Institut pour la Recherche et le Développement.
- Gardner, S. N., Slezak, T., & Hall, B. G. (2015). kSNP3.0: SNP detection and phylogenetic analysis of genomes without genome alignment or reference genome. *Bioinformatics*, 31(17), 2877–2878. <https://doi.org/10.1093/bioinformatics/btv271>
- Garin, T., Brault, A., Marais, C., Briand, M., Préveaux, A., Bonneau, S., Simonin, M., Barret, M., & Sarniguet, A. (2025). T6SS-mediated competition by *Stenotrophomonas rhizophila* shapes seed-borne bacterial communities and seed-to-seedling transmission dynamics. *MSystems*, 10(8), e0045725. <https://doi.org/10.1128/msystems.00457-25>
- Garin, T., Brin, C., Préveaux, A., Brault, A., Briand, M., Simonin, M., Barret, M., Journet, L., & Sarniguet, A. (2024). The type VI secretion system of *Stenotrophomonas rhizophila* CFBP13503 limits the transmission of *Xanthomonas campestris* pv. *campestris* 8004 from radish seeds to seedlings. *Molecular Plant Pathology*, 25(1), e13412. <https://doi.org/10.1111/MPP.13412>
- Geller, A. M., Zlotkin, D., & Levy, A. (2021). Large-scale discovery of candidate type VI secretion effectors with antibacterial activity. *BioRxiv*, 2021.10.07.463556. <https://doi.org/10.1101/2021.10.07.463556>
- Giblot-Ducray, D., Marefat, A., Gillings, M. R., Parkinson, N. M., Bowman, J. P., Ophel-Keller, K., Taylor, C., Facelli, E., & Scott, E. S. (2009). Proposal of *Xanthomonas translucens* pv. *pistaciae* pv. nov., pathogenic to pistachio (*Pistacia vera*). *Systematic and Applied Microbiology*, 32(8), 549–557. <https://doi.org/10.1016/j.syapm.2009.08.001>
- Gilchrist, C. L. M., Booth, T. J., Van Wersch, B., Van Grieken, L., Medema, M. H., & Chooi, Y. H. (2021). cblaster: a remote search tool for rapid identification and visualization of homologous gene clusters. *Bioinformatics Advances*, 1(1), vbab016. <https://doi.org/10.1093/bioadv/vbab016>
- Gilchrist, C. L. M., & Chooi, Y. H. (2021). clinker & clustermap.js: automatic generation of gene cluster comparison figures. *Bioinformatics*, 37(16), 2473–2475. <https://doi.org/10.1093/BIOINFORMATICS/BTAB007>
- Gilroy, R., Ravi, A., Getino, M., Pursley, I., Horton, D. L., Alikhan, N. F., Baker, D., Gharbi, K., Hall, N., Watson, M., Adriaenssens, E. M., Foster-Nyarko, E., Jarju,

- S., Secka, A., Antonio, M., Oren, A., Chaudhuri, R. R., Ragione, R. La, Hildebrand, F., & Pallen, M. J. (2021). Extensive microbial diversity within the chicken gut microbiome revealed by metagenomics and culture. *PeerJ*, 9, e10941. <https://doi.org/10.7717/peerj.10941>
- Gluck-Thaler, E., Cerutti, A., Perez-Quintero, A. L., Butchacas, J., Roman-Reyna, V., Madhavan, V. N., ..., & Jacobs, J. M. (2020). Repeated gain and loss of a single gene modulates the evolution of vascular plant pathogen lifestyles. *Science Advances*, 6(46), eabc4516. <https://doi.org/10.1126/sciadv.abc4516>
- Goettelmann, F., Koebnik, R., Roman-Reyna, V., Studer, B., & Kölliker, R. (2023). High genomic plasticity and unique features of *Xanthomonas translucens* pv. *graminis* revealed through comparative analysis of complete genome sequences. *BMC Genomics*, 24(1), 741. <https://doi.org/10.1186/s12864-023-09855-8>
- Goettelmann, F., Roman-Reyna, V., Cunnac, S., Jacobs, J. M., Bragard, C., Studer, B., Koebnik, R., & Kölliker, R. (2022). Complete genome assemblies of all *Xanthomonas translucens* pathotype strains reveal three genetically distinct clades. *Frontiers in Microbiology*, 12, 817815. <https://doi.org/10.3389/FMICB.2021.817815>
- Gonzalez Moreno, P. J., & Nishiguchi, M. K. (2025). The Competitive Edge: T6SS-Mediated Interference Competition by Vibrionaceae Across Marine Ecological Niches. *Microorganisms*, 13(6), 1370. <https://doi.org/10.3390/microorganisms13061370>
- Gordils-Valentin, L., Ouyang, H., Qian, L., Hong, J., & Zhu, X. (2024). Conjugative type IV secretion systems enable bacterial antagonism that operates independently of plasmid transfer. *Communications Biology*, 7(1), 499. <https://doi.org/10.1038/s42003-024-06192-8>
- Granato, E. T., Meiller-Legrand, T. A., & Foster, K. R. (2019). The Evolution and Ecology of Bacterial Warfare. *Current Biology*, 29(11), R521–R537. <https://doi.org/10.1016/j.cub.2019.04.024>
- Granato, E. T., Smith, W. P. J., & Foster, K. R. (2023). Collective protection against the type VI secretion system in bacteria. *ISME Journal*, 17(7), 1052–1062. <https://doi.org/10.1038/s41396-023-01401-4>
- Haapalainen, M., Mosorin, H., Dorati, F., Wu, R. F., Roine, E., Taira, S., Nissinen, R., Mattinen, L., Jackson, R., Pirhonen, M., & Lin, N. C. (2012). Hcp2, a secreted protein of the phytopathogen *Pseudomonas syringae* pv. *tomato* DC3000, is required for fitness for competition against bacteria and yeasts. *Journal of Bacteriology*, 194(18), 4810–4822. <https://doi.org/10.1128/JB.00611-12>

- Hadfield, J. D. (2010). MCMC Methods for Multi-Response Generalized Linear Mixed Models: The MCMCglmm R Package. *JSS Journal of Statistical Software*, 33(2), 1–22. <https://doi.org/10.18637/JSS.V033.I02>
- Hamilton, H. L., Domínguez, N. M., Schwartz, K. J., Hackett, K. T., & Dillard, J. P. (2005). *Neisseria gonorrhoeae* secretes chromosomal DNA via a novel type IV secretion system. *Molecular Microbiology*, 55(6), 1704–1721. <https://doi.org/10.1111/j.1365-2958.2005.04521.x>
- Harms, A., Brodersen, D. E., Mitarai, N., & Gerdes, K. (2018). Toxins, Targets, and Triggers: An Overview of Toxin-Antitoxin Biology. *Molecular Cell*, 70(5), 768–784. <https://doi.org/10.1016/j.molcel.2018.01.003>
- Harms, A., Liesch, M., Körner, J., Québatte, M., Engel, P., & Dehio, C. (2017). A bacterial toxin-antitoxin module is the origin of inter-bacterial and inter-kingdom effectors of *Bartonella*. *PLoS Genetics*, 13(10), e1007077. <https://doi.org/10.1371/journal.pgen.1007077>
- Harrison, J., Hussain, R. M. F., Greer, S. F., Ntoukakis, V., Aspin, A., Vicente, J. G., Grant, M., & Studholme, D. J. (2023). Draft genome sequences for ten strains of *Xanthomonas* species that have phylogenomic importance. *Access Microbiology*, 5(7), acmi000532.v3. <https://doi.org/10.1099/acmi.0.000532.v3>
- Hashimi, S. M., Wall, M. K., Smith, A. B., Maxwell, A., & Birch, R. G. (2007). The phytotoxin albicidin is a novel inhibitor of DNA gyrase. *Antimicrobial Agents and Chemotherapy*, 51(1), 181–187. <https://doi.org/10.1128/AAC.00918-06>
- Hauben, L., Vauterin, L., Swings, J., & Moore, E. R. B. (1997). Comparison of 16S ribosomal DNA sequences of all *Xanthomonas* species. *International Journal of Systematic Bacteriology*, 47(2), 328–335. <https://doi.org/10.1099/00207713-47-2-328>,
- He, Y. Q., Zhang, L., Jiang, B. Le, Zhang, Z. C., Xu, R. Q., Tang, D. J., Qin, J., Jiang, W., Zhang, X., Liao, J., Cao, J. R., Zhang, S. S., Wei, M. L., Liang, X. X., Lu, G. T., Feng, J. X., Chen, B., Cheng, J., & Tang, J. L. (2007). Comparative and functional genomics reveals genetic diversity and determinants of host specificity among reference strains and a large collection of Chinese isolates of the phytopathogen *Xanthomonas campestris* pv. *campestris*. *Genome Biology*, 8(10), R218. <https://doi.org/10.1186/gb-2007-8-10-r218>
- Heiden, N., Roman-Reyna, V., Curland, R. D., Dill-Macky, R., & Jacobs, J. M. (2023). Comparative genomics of Minnesotan barley-infecting *Xanthomonas translucens* shows overall genomic similarity but virulence factor diversity. *Phytopathology*, 113(11), 2056–2061. <https://doi.org/10.1094/PHYTO-04-22-0113-SC>

- Hernandez, R. E., Gallegos-Monterrosa, R., & Coulthurst, S. J. (2020). Type VI secretion system effector proteins: Effective weapons for bacterial competitiveness. *Cellular Microbiology*, 22(9), e13241. <https://doi.org/10.1111/cmi.13241>
- Hersch, S. J., Lam, L., & Dong, T. G. (2021). Engineered type six secretion systems deliver active exogenous effectors and cre recombinase. *MBio*, 12(4), 10-1128. <https://doi.org/10.1128/mbio.01115-21>
- Hersch, S. J., Manera, K., & Dong, T. G. (2020). Defending against the Type Six Secretion System: beyond Immunity Genes. *Cell Reports*, 33(2), 108259. <https://doi.org/10.1016/j.celrep.2020.108259>
- Hersch, S. J., Watanabe, N., Stietz, M. S., Manera, K., Kamal, F., Burkinshaw, B., Lam, L., Pun, A., Li, M., Savchenko, A., & Dong, T. G. (2020). Envelope stress responses defend against type six secretion system attacks independently of immunity proteins. *Nature Microbiology*, 5(5), 706–714. <https://doi.org/10.1038/s41564-020-0672-6>
- Heun, M., Schafer-Pregl, R., Klawan, D., Castagna, R., Accerbi, M., Borghi, B., & Salamini, F. (1997). Site of Einkorn Wheat Domestication Identified by DNA Fingerprinting. *Science*, 278(5341), 1312–1314.
- Heylens, S. (2025). *The role of the Type VI secretion system (T6SS) of Xanthomonas translucens: effectors proteins, immunity proteins and interbacterial competition*. Université catholique de Louvain.
- Ho, B. T., Basler, M., & Mekalanos, J. J. (2013). Type 6 Secretion System–Mediated Immunity to Type 4 Secretion System–Mediated Gene Transfer. *Science*, 342(6155), 250–253. <https://doi.org/10.1126/science.1243745>
- Hofreuter, D., Odenbreit, S., & Haas, R. (2001). Natural transformation competence in *Helicobacter pylori* is mediated by the basic components of a type IV secretion system. *Molecular Microbiology*, 41(2), 379–391. <https://doi.org/10.1046/j.1365-2958.2001.02502.x>
- Hood, R. D., Singh, P., Hsu, F. S., Güvener, T., Carl, M. A., Trinidad, R. R. S., Silverman, J. M., Ohlson, B. B., Hicks, K. G., Plemel, R. L., Li, M., Schwarz, S., Wang, W. Y., Merz, A. J., Goodlett, D. R., & Mougous, J. D. (2010). A Type VI Secretion System of *Pseudomonas aeruginosa* Targets a Toxin to Bacteria. *Cell Host and Microbe*, 7(1), 25–37. <https://doi.org/10.1016/j.chom.2009.12.007>
- Jablonska, J., Matelska, D., Steczkiewicz, K., & Ginalski, K. (2017). Systematic classification of the His-Me finger superfamily. *Nucleic Acids Research*, 45(20), 11479–11494. <https://doi.org/10.1093/nar/gkx924>

- Jacobs, J. M., Pesce, C., Lefeuvre, P., & Koebnik, R. (2015). Comparative genomics of a cannabis pathogen reveals insight into the evolution of pathogenicity in *Xanthomonas*. *Frontiers in plant science*, 6, 431. <https://doi.org/10.3389/fpls.2015.00431>
- Jacques, M. A., Arlat, M., Boulanger, A., Boureau, T., Carrère, S., Cesbron, S., Chen, N. W. G., Cociancich, S., Darrasse, A., Denancé, N., Fischer-Le Saux, M., Gagnevin, L., Koebnik, R., Lauber, E., Noël, L. D., Pieretti, I., Portier, P., Pruvost, O., Rieux, A., ... Vernière, C. (2016). Using Ecology, Physiology, and Genomics to Understand Host Specificity in *Xanthomonas*. *Annual Review of Phytopathology*, 54, 163–187. <https://doi.org/10.1146/annurev-phyto-080615-100147>
- Jaenicke, S., Bunk, B., Wibberg, D., Spröer, C., Hersemann, L., Blom, J., ... & Vorhölter, F. J. (2016). Complete genome sequence of the barley pathogen *Xanthomonas translucens* pv. *translucens* DSM 18974T (ATCC 19319T). *Genome Announcements*, 4(6), 10-1128. <https://doi.org/10.1128/genomea.01334-16>
- Jain, C., Rodriguez-R, L. M., Phillippy, A. M., Konstantinidis, K. T., & Aluru, S. (2018). High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. *Nature Communications*, 9(1), 5114. <https://doi.org/10.1038/s41467-018-07641-9>
- Jalili, V., Afgan, E., Gu, Q., Clements, D., Blankenberg, D., Goecks, J., Taylor, J., & Nekrutenko, A. (2021). The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2020 update. *Nucleic Acids Research*, 48(W1), W395–W402. <https://doi.org/10.1093/NAR/GKAA434>
- Jamali, H., Akrami, F., Layeghkhavidaki, H., & Bouakkaz, S. (2025). Bacterial protein secretion systems: Mechanisms, functions, and roles in virulence. *Microbial Pathogenesis*, 206, 107790. <https://doi.org/10.1016/J.MICPATH.2025.107790>
- Jana, B., Fridman, C. M., Bosis, E., & Salomon, D. (2019). A modular effector with a DNase domain and a marker for T6SS substrates. *Nature Communications*, 10(1), 3595. <https://doi.org/10.1038/s41467-019-11546-6>
- Jana, B., Keppel, K., & Salomon, D. (2021). Engineering a customizable antibacterial T6SS-based platform in *Vibrio natriegens*. *EMBO reports*, 22(11), e53681. <https://doi.org/10.15252/embr.202153681>
- Jani, A. J., & Cotter, P. A. (2010). Type VI Secretion: Not just for pathogenesis anymore. *Cell Host and Microbe*, 8(1), 2–6. <https://doi.org/10.1016/j.chom.2010.06.012>
- Jiang, F., Wang, X., Wang, B., Chen, L., Zhao, Z., Waterfield, N. R., Yang, G., & Jin, Q. (2016). The *Pseudomonas aeruginosa* type VI secretion PGAP1-like effector

- induces host autophagy by activating endoplasmic reticulum stress. *Cell Reports*, 16(6), 1502–1509. <https://doi.org/10.1016/j.celrep.2016.07.012>
- Jiang, F., Waterfield, N. R., Yang, J., Yang, G., & Jin, Q. (2014). A *Pseudomonas aeruginosa* type VI secretion phospholipase D effector targets both prokaryotic and eukaryotic cells. *Cell Host and Microbe*, 15(5), 600–610. <https://doi.org/10.1016/j.chom.2014.04.010>
- Jones, L. R., Johnson, A. G., & Reddy, C. (1917). Bacterial-blight of barley. *Journal of Agricultural Research*, 11, 625–643.
- Jones, P., Binns, D., Chang, H. Y., Fraser, M., Li, W., McAnulla, C., McWilliam, H., Maslen, J., Mitchell, A., Nuka, G., Pesseat, S., Quinn, A. F., Sangrador-Vegas, A., Scheremetjew, M., Yong, S. Y., Lopez, R., & Hunter, S. (2014). InterProScan 5: Genome-scale protein function classification. *Bioinformatics*, 30(9), 1236–1240. <https://doi.org/10.1093/bioinformatics/btu031>
- Journet, L., & Cascales, E. (2016). The Type VI Secretion System in *Escherichia coli* and Related Species. *EcoSal Plus*, 7(1), 10.1128/ecosalplus.ESP-0009–2015. <https://doi.org/10.1128/ecosalplus.esp-0009-2015>
- Juhas, M., Crook, D. W., & Hood, D. W. (2008). Type IV secretion systems: Tools of bacterial horizontal gene transfer and virulence. *Cell Microbiol*, 10(12), 2377–2386. <https://doi.org/10.1111/j.1462-5822.2008.01187.x>
- Jurénas, D., & Journet, L. (2021). Activity, delivery, and diversity of Type VI secretion effectors. *Molecular Microbiology*, 115(3), 383–394. <https://doi.org/10.1111/mmi.14648>
- Kamoun, S., & Kado, C. I. (1990). Phenotypic Switching Affecting Chemotaxis, Xanthan Production, and Virulence in *Xanthomonas campestris*. *Applied and Environmental Microbiology*, 56(12), 3855–3860. <https://doi.org/10.1128/aem.56.12.3855-3860.1990>
- Kavamura, V. N., Mendes, R., Bargaz, A., & Mauchline, T. H. (2021). Defining the wheat microbiome: Towards microbiome-facilitated crop production. *Computational and Structural Biotechnology Journal*, 19, 1200–1213. <https://doi.org/10.1016/j.csbj.2021.01.045>
- Kempnich, M. W., & Sison-Mangus, M. P. (2020). Presence and abundance of bacteria with the Type VI secretion system in a coastal environment and in the global oceans. *PLoS One*, 15(12), e0244217. <https://doi.org/10.1371/journal.pone.0244217>
- Kephart, K. D., McKendry, A. L., Tague, D. N., & Berg, J. E. (1993). Winter wheat: 1993 Missouri crop performance. In *Agricultural Experiment Station Special Report 453*. University Archives of the University of Missouri-Columbia. <https://cdm16795.contentdm.oclc.org/digital/collection/agexptstn/id/43399>

- Khojasteh, M., Taghavi, S. M., Khodaygan, P., Hamzehzarghani, H., Chen, G., Bragard, C., Koebnik, R., & Osdaghi, E. (2019). Molecular typing reveals high genetic diversity of *Xanthomonas translucens* strains infecting small-grain cereals in Iran. *Applied and Environmental Microbiology*, 85(20), e01518-19. <https://doi.org/10.1128/AEM.01518-19>
- Kielkopf, C. S., Shneider, M. M., Leiman, P. G., & Taylor, N. M. I. (2024). T6SS-associated Rhs toxin-encapsulating shells: Structural and bioinformatic insights into bacterial weaponry and self-protection. *Structure*, 32(12), 2375-2389.e5. <https://doi.org/10.1016/j.str.2024.10.008>
- Kim, H. K., Orser, C., Lindow, S. E., & Sands, D. C. (1987). *Xanthomonas campestris* pv. *translucens* strains active in ice nucleation. *Plant Disease*, 71, 994–997.
- Kim, N., Han, G., Jung, H., Lee, H. H., Park, J., & Seo, Y. S. (2021). T6SS accessory proteins, including DUF2169 domain-containing protein and pentapeptide repeats protein, contribute to bacterial virulence in T6SS group_5 of *burkholderia glumae* BGR1. *Plants*, 11(1), 34. <https://doi.org/10.3390/plants11010034>
- Kirchberger, P. C., Unterweger, D., Provenzano, D., Pukatzki, S., & Boucher, Y. (2017). Sequential displacement of Type VI Secretion System effector genes leads to evolution of diverse immunity gene arrays in *Vibrio cholerae*. *Scientific Reports*, 7(1), 45133. <https://doi.org/10.1038/srep45133>
- Koebnik, R., Burokiene, D., Bragard, C., Chang, C., Saux, M. F. Le, Kölliker, R., Lang, J. M., Leach, J. E., Luna, E. K., Portier, P., Sagia, A., Ziegler, J., Cohen, S. P., & Jacobs, J. M. (2021). The complete genome sequence of *Xanthomonas theicola*, the causal agent of canker on tea plants, reveals novel secretion systems in clade-1 xanthomonads. *Phytopathology*, 111(4), 611–616. <https://doi.org/10.1094/PHYTO-07-20-0273-SC>
- Kölliker, R., Kraehenbuehl, R., Boller, B., & Widmer, F. (2006). Genetic diversity and pathogenicity of the grass pathogen *Xanthomonas translucens* pv. *graminis*. *Systematic and Applied Microbiology*, 29(2), 109–119. <https://doi.org/10.1016/j.syapm.2005.07.004>
- Korotkov, K. V., Pardon, E., Steyaert, J., & Hol, W. G. J. (2009). Crystal Structure of the N-Terminal Domain of the Secretin GspD from ETEC Determined with the Assistance of a Nanobody. *Structure*, 17(2), 255–265. <https://doi.org/10.1016/j.str.2008.11.011>
- Langlois, P. A., Snelling, J., Hamilton, J. P., Bragard, C., Koebnik, R., Verdier, V., Triplett, L. R., Blom, J., Tisserat, N. A., & Leach, J. E. (2017). Characterization of the *Xanthomonas translucens* complex using draft genomes, comparative genomics, phylogenetic analysis, and diagnostic LAMP assays.

- Phytopathology*, 107(5), 519–527. <https://doi.org/10.1094/PHYTO-08-16-0286-R>
- Le, N.-H., Peters, K., Espaillet, A., Sheldon, J. R., Gray, J., Di Venzio, G., Lopez, J., Djahanschiri, B., Mueller, E. A., Hennon, S. W., Levin, P. A., Ebersberger, I., Skaar, E. P., Cava, F., Vollmer, W., & Feldman, M. F. (2020). Peptidoglycan editing provides immunity to *Acinetobacter baumannii* during bacterial warfare. *Science Advances*, 6(30), eabb5614. <https://doi.org/10.1126/sciadv.abb5614>
- Le, N.-H., Pinedo, V., Lopez, J., Cava, F., & Feldman, M. F. (2021). Killing of Gram-negative and Gram-positive bacteria by a bifunctional cell wall-targeting T6SS effector. *Proceedings of the National Academy of Sciences of the United States of America*, 118(40), e2106555118. <https://doi.org/10.1073/pnas.2106555118>
- Lederberg, J., & Tatum, E. L. (1946). Gene recombination in *Escherichia coli*. *Nature*, 158(4016), 558. <https://doi.org/10.1038/158558A0>
- Leiman, P. G., Basler, M., Ramagopal, U. A., Bonanno, J. B., Sauder, J. M., Pukatzki, S., Burley, S. K., Almo, S. C., & Mekalanos, J. J. (2009). Type VI secretion apparatus and phage tail-associated protein complexes share a common evolutionary origin. *Proceedings of the National Academy of Sciences of the United States of America*, 106(11), 4154–4159. <https://doi.org/https://doi.org/10.1073/pnas.0813360106>
- Letunic, I., & Bork, P. (2024). Interactive Tree of Life (iTOL) v6: recent updates to the phylogenetic tree display and annotation tool. *Nucleic Acids Research*, 52(W1), W78–W82. <https://doi.org/10.1093/NAR/GKAE268>
- Li, C., Wang, L., Cseke, L. J., Vasconcelos, F., Huguet-Tapia, J. C., Gassmann, W., ... & Yang, B. (2023). Efficient CRISPR-Cas9 based cytosine base editors for phytopathogenic bacteria. *Communications Biology*, 6(1), 56. <https://doi.org/10.1038/s42003-023-04451-8>
- Li, Y. G., & Christie, P. J. (2018). The *Agrobacterium* VirB/VirD4 T4SS: Mechanism and architecture defined through in vivo mutagenesis and chimeric systems. *Current Topics in Microbiology and Immunology*, 418, 233–260. https://doi.org/10.1007/82_2018_94
- Li, Y., Wu, Z., Liu, D., Cong, K., Dai, J., Xu, W., Ke, Y., & He, X. (2024). The Role of ClpV in the Physiology and Pathogenicity of *Xanthomonas citri* subsp. *citri* Strain zlm1908. *Microorganisms*, 12(12), 2536. <https://doi.org/10.3390/microorganisms12122536>
- Liang, X., Moore, R., Wilton, M., Wong, M. J. Q., Lam, L., & Dong, T. G. (2015). Identification of divergent type VI secretion effectors using a conserved

- chaperone domain. *Proceedings of the National Academy of Sciences of the United States of America*, 112(29), 9106–9111.
<https://doi.org/10.1073/pnas.1505317112>
- Liao, J., Li, Z., Xiong, D., Shen, D., Wang, L., Lin, L., Shao, X., Liao, L., Li, P., Zhang, L. Q., Wang, H. H., & Qian, G. (2023). Quorum quenching by a type IVA secretion system effector. *ISME Journal*, 17(10), 1564–1577.
<https://doi.org/10.1038/s41396-023-01457-2>
- Liao, Z. X., Ni, Z., Wei, X. L., Chen, L., Li, J. Y., Yu, Y. H., Jiang, W., Jiang, B. Le, He, Y. Q., & Huang, S. (2019). Dual RNA-seq of *Xanthomonas oryzae* pv. *oryzicola* infecting rice reveals novel insights into bacterial-plant interaction. *PLoS ONE*, 14(4), e0215039. <https://doi.org/10.1371/journal.pone.0215039>
- Lin, J. S., Pissaridou, P., Wu, H. H., Tsai, M. D., Filloux, A., & Lai, E. M. (2018). TagF-mediated repression of bacterial type VI secretion systems involves a direct interaction with the cytoplasmic protein Fha. *Journal of Biological Chemistry*, 293(23), 8829–8842. <https://doi.org/10.1074/jbc.RA117.001618>
- Lin, L., Lezan, E., Schmidt, A., & Basler, M. (2019). Abundance of bacterial Type VI secretion system components measured by targeted proteomics. *Nature Communications*, 10, 2584. <https://doi.org/10.1038/s41467-019-10466-9>
- Lin, L., Shao, X., Yang, Y., Murero, A. K., Wang, L., Xu, G., Zhao, Y., Han, S., Su, Z., Xu, K., Yang, M., Liao, J., Li, K., Liu, F., & Qian, G. (2025). *Lysobacter enzymogenes*: a fully armed biocontrol warrior. *Journal of Integrative Agriculture*, 24(1), 23–35. <https://doi.org/10.1016/j.jia.2024.02.021>
- Lin, L., Shen, D., Shao, X., Yang, Y., Li, L., Zhong, C., Jiang, J., Wang, M., & Qian, G. (2025). Soil microbiome bacteria protect plants against filamentous fungal infections via intercellular contacts. *Proceedings of the National Academy of Sciences of the United States of America*, 122(3), e2418766122. <https://doi.org/10.1073/pnas.2418766122>
- Lin, L., Xu, K., Shen, D., Chou, S. H., Gomelsky, M., & Qian, G. (2021). Antifungal weapons of *Lysobacter*, a mighty biocontrol agent. *Environmental Microbiology*, 23(10), 5704–5715. <https://doi.org/10.1111/1462-2920.15674>
- Lin, R. J., Capage, M., & Hill, C. W. (1984). A repetitive DNA sequence, rhs, responsible for duplications within the *Escherichia coli* K-12 chromosome. *Journal of Molecular Biology*, 177(1), 1–18. [https://doi.org/10.1016/0022-2836\(84\)90054-8](https://doi.org/10.1016/0022-2836(84)90054-8)
- Lin, X. B., Liu, T., Schmaltz, R., Ramer-Tait, A. E., Walter, J. W., & Gänzle, M. G. (2024). Competitiveness of reutericyclin producing and nonproducing *Limosilactobacillus reuteri* in food and intestinal ecosystems: a game of rock,

- paper, and scissors? *Letters in Applied Microbiology*, 77(2), ova007.
<https://doi.org/10.1093/lambio/ova007>
- Liyanapathiranaige, P., Jones, J. B., & Potnis, N. (2022). A mutation of a single core gene, *tssM*, of Type VI secretion system of *Xanthomonas perforans* influences virulence, epiphytic survival and transmission during pathogenesis on tomato. *Phytopathology*, 112(4), 752–764. <https://doi.org/10.1094/PHYTO-02-21-0069-R>
- Liyanapathiranaige, P., Wagner, N., Avram, O., Pupko, T., & Potnis, N. (2022). Phylogenetic Distribution and Evolution of Type VI Secretion System in the Genus *Xanthomonas*. *Frontiers in Microbiology*, 13, 840308.
<https://doi.org/10.3389/FMICB.2022.840308>
- Lopez, J., Ly, P. M., & Feldman, M. F. (2020). The tip of the VgrG spike is essential to functional type VI secretion system assembly in *Acinetobacter baumannii*. *MBio*, 11(1), e02761-19. <https://doi.org/10.1128/mBio.02761-19>
- Lossi, N. S., Manoli, E., Förster, A., Dajani, R., Pape, T., Freemont, P., & Filloux, A. (2013). The HsiB1C1 (TssB-TssC) complex of the *Pseudomonas aeruginosa* type VI secretion system forms a bacteriophage tail sheathlike structure. *Journal of Biological Chemistry*, 288(11), 7536–7548.
<https://doi.org/10.1074/jbc.M112.439273>
- Lu, D., Zheng, Y., Liao, N., Wei, L., Xu, B., Liu, X., & Liu, J. (2014). The structural basis of the Tle4-Tli4 complex reveals the self-protection mechanism of H2-T6SS in *Pseudomonas aeruginosa*. *Acta Crystallographica Section D: Biological Crystallography*, 70(12), 3233–3243.
<https://doi.org/10.1107/S1399004714023967>,
- Maes, M., Garbeva, P., & and Kamoen, O. (1996). Recognition and detection in seed of the *Xanthomonas* pathogens that cause cereal leaf streak using rDNA spacer sequences and polymerase chain reaction. *Phytopathology*, 86(1), 63–69. <https://doi.org/10.1094/Phyto-86-63>
- Mafakheri, H., Taghavi, S. M., Zarei, S., Portier, P., Dimkić, I., Koebnik, R., ... & Osdaghi, E. (2022a). *Xanthomonas bonasiae* sp. nov. and *Xanthomonas youngii* sp. nov., isolated from crown gall tissues. *International journal of systematic and evolutionary microbiology*, 72(6), 005418.
<https://doi.org/10.1099/ijsem.0.005418>
- Mafakheri, H., Taghavi, S. M., Zarei, S., Rahimi, T., Hasannezhad, M. S., Portier, P., ... & Osdaghi, E. (2022b). Phenotypic and molecular-phylogenetic analyses reveal distinct features of crown gall-associated *Xanthomonas* strains. *Microbiology spectrum*, 10(1), e00577-21.
<https://doi.org/10.1128/spectrum.00577-21>

- Majerczyk, C., Schneider, E., & Greenberg, E. P. (2016). Quorum sensing control of Type VI secretion factors restricts the proliferation of quorum-sensing mutants. *ELife*, 5, e14712. <https://doi.org/10.7554/eLife.14712.001>
- Marasini, D., Karki, A. B., Bryant, J. M., Sheaff, R. J., & Fakhr, M. K. (2020). Molecular characterization of megaplastids encoding the type VI secretion system in *Campylobacter jejuni* isolated from chicken livers and gizzards. *Scientific Reports*, 10(1), 12514. <https://doi.org/10.1038/s41598-020-69155-z>
- Marefat, A., Ophel-Keller, K., Scott, E. S., & Sedgley, M. (2006). The use of ARMS PCR in detection and identification of xanthomonads associated with pistachio dieback in Australia. *European Journal of Plant Pathology*, 116(1), 57–68. <https://doi.org/10.1007/s10658-006-9038-z>
- Marefat, A., Scott, E. S., Ophel-Keller, K., & Sedgley, M. (2006). Genetic, phenotypic and pathogenic diversity among xanthomonads isolated from pistachio (*Pistacia vera*) in Australia. *Plant Pathology*, 55(5), 639–649. <https://doi.org/10.1111/j.1365-3059.2006.01437.x>
- Marin, V. R., Ferrarezi, J. H., Vieira, G., & Sass, D. C. (2019). Recent advances in the biocontrol of *Xanthomonas* spp. *World Journal of Microbiology and Biotechnology*, 35(5), 72. <https://doi.org/10.1007/s11274-019-2646-5>
- Martino, J. A., Fernández, F. D., Pozzi, E. A., Alberione, E. J., Bainotti, C. T., Márquez, N., Tolocka, P. A., Salines, N., Gomez, D. T., Donaire, G. M., Conci, L. R., & Alemandri, V. M. (2023). First report of *Xanthomonas prunicola* causing bacterial leaf streaks on wheat in Argentina. *Plant Disease*, 107(1), 212. <https://doi.org/10.1094/PDIS-04-22-0886-PDN>
- Mattinen, L., Nissinen, R., Riipi, T., Kalkkinen, N., & Pirhonen, M. (2007). Host-extract induced changes in the secretome of the plant pathogenic bacterium *Pectobacterium atrosepticum*. *Proteomics*, 7(19), 3527–3537. <https://doi.org/10.1002/pmic.200600759>
- McKnight, D. J. E., Wong-Bajracharya, J., Okoh, E. B., Snijders, F., Lidbetter, F., Webster, J., Haughton, M., Darling, A. E., Djordjevic, S. P., Bogema, D. R., & Chapman, T. A. (2024). *Xanthomonas rydalmerensis* sp. nov., a non-pathogenic member of Group 1 *Xanthomonas*. *International Journal of Systematic and Evolutionary Microbiology*, 74(3), 006294. <https://doi.org/10.1099/ijsem.0.006294>
- McKnight, D. J. E., Wong-Bajracharya, J., Okoh, E. B., Snijders, F., Lidbetter, F., Webster, J., Haughton, M., Djordjevic, S. P., Bogema, D. R., & Chapman, T. A. (2025). *Xanthomonas bundabergensis* sp. nov., *Xanthomonas medicaginis* sp. nov. and *Xanthomonas tesorieronis* sp. nov.: three members of group 1

- Xanthomonas*. *International Journal of Systematic and Evolutionary Microbiology*, 75(3), 006686. <https://doi.org/10.1099/ijsem.0.006686>
- Melotto, M., Underwood, W., Koczan, J., Nomura, K., & He, S. Y. (2006). Plant Stomata Function in Innate Immunity against Bacterial Invasion. *Cell*, 126(5), 969–980. <https://doi.org/10.1016/j.cell.2006.06.054>
- Merda, D., Briand, M., Bosis, E., Rousseau, C., Portier, P., Barret, M., Jacques, M. A., & Fischer-Le Saux, M. (2017). Ancestral acquisitions, gene flow and multiple evolutionary trajectories of the type three secretion system and effectors in *Xanthomonas* plant pathogens. *Molecular Ecology*, 26(21), 5939–5952. <https://doi.org/10.1111/mec.14343>
- Michl, K., Berg, G., & Cernava, T. (2023). The microbiome of cereal plants: The current state of knowledge and the potential for future applications. *Environmental Microbiome*, 18(1), 28. <https://doi.org/10.1186/s40793-023-00484-y>
- Milus, E. A., & Mirlohi, A. F. (1994). Use of disease reactions to identify resistance in wheat to bacterial streak. *Plant Disease*, 78, 157–161. <https://doi.org/10.1094/PD-78-0157>
- Milus, E. A., & Mirlohi, A. F. (1995). Survival of *Xanthomonas campestris* pv. *translucens* between successive wheat crops in Arkansas. *Plant Disease*, 79, 263–265. <https://doi.org/10.1094/PD-79-0263>
- Miyata, S. T., Kitaoka, M., Brooks, T. M., McAuley, S. B., & Pukatzki, S. (2011). *Vibrio cholerae* requires the type VI secretion system virulence factor VasX to kill *Dictyostelium discoideum*. *Infection and Immunity*, 79(7), 2941–2949. <https://doi.org/10.1128/IAI.01266-10>
- Miyata, S. T., Unterweger, D., Rudko, S. P., & Pukatzki, S. (2013). Dual Expression Profile of Type VI Secretion System Immunity Genes Protects Pandemic *Vibrio cholerae*. *PLoS Pathogens*, 9(12), e1003752. <https://doi.org/10.1371/journal.ppat.1003752>
- Mok, B. Y., de Moraes, M. H., Zeng, J., Bosch, D. E., Kotrys, A. V., Raguram, A., Hsu, F. S., Radey, M. C., Peterson, S. B., Mootha, V. K., Mougous, J. D., & Liu, D. R. (2020). A bacterial cytidine deaminase toxin enables CRISPR-free mitochondrial base editing. *Nature*, 583(7817), 631–637. <https://doi.org/10.1038/s41586-020-2477-4>
- Monjarás Feria, J., & Valvano, M. A. (2020). An overview of anti-eukaryotic T6SS effectors. *Frontiers in Cellular and Infection Microbiology*, 10, 584751. <https://doi.org/10.3389/fcimb.2020.584751>
- Montenegro Benavides, N. A., Alvarez B, A., Arrieta-Ortiz, M. L., Rodriguez-R, L. M., Botero, D., Tabima, J. F., Castiblanco, L., Trujillo, C., Restrepo, S., & Bernal, A.

- (2021). The type VI secretion system of *Xanthomonas phaseoli* pv. *manihotis* is involved in virulence and *in vitro* motility. *BMC Microbiology*, 21(1), 14. <https://doi.org/10.1186/s12866-020-02066-1>
- Morgado, S., & Vicente, A. C. (2022). Diversity and distribution of Type VI Secretion System gene clusters in bacterial plasmids. *Scientific Reports*, 12(1), 8249. <https://doi.org/10.1038/s41598-022-12382-3>
- Morris, C. E., Georgakopoulos, D. G., & Sands, D. C. (2004). Ice nucleation active bacteria and their potential role in precipitation. *Journal De Physique IV France*, 121, 87–103. <https://doi.org/10.1051/jp4:2004121004>
- Mougous, J. D., Cuff, M. E., Raunser, S., Shen, A., Zhou, M., Gifford, C. A., Goodman, A. L., Joachimiak, G., Ordoñez, C. L., Lory, S., Walz, T., Joachimiak, A., & Mekalanos, J. J. (2006). A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus. *Science*, 312(5779), 1526–1530. <https://doi.org/10.1126/science.1128393>
- Mougous, J. D., Gifford, C. A., Ramsdell, T. L., & Mekalanos, J. J. (2007). Threonine phosphorylation post-translationally regulates protein secretion in *Pseudomonas aeruginosa*. *Nature Cell Biology*, 9(7), 797–803. <https://doi.org/10.1038/ncb1605>
- Nakano, N., Kubori, T., Kinoshita, M., Imada, K., & Nagai, H. (2010). Crystal structure of *Legionella* DotD: Insights into the relationship between type IVB and type II/III secretion systems. *PLoS Pathogens*, 6(10), e1001129. <https://doi.org/10.1371/journal.ppat.1001129>
- Nas, M. Y., Gabell, J., & Cianciotto, N. P. (2021). Effectors of the *Stenotrophomonas maltophilia* type IV secretion system mediate killing of clinical isolates of *Pseudomonas aeruginosa*. *MBio*, 12(3), e0150221. <https://doi.org/10.1128/mBio.01502-21>
- Nas, M. Y., White, R. C., Dumont, A. L., Lopez, A. E., & Cianciotto, N. P. (2019). *Stenotrophomonas maltophilia* Encodes a VirB/VirD4 Type IV Secretion System That Modulates Apoptosis in Human Cells and Promotes Competition against Heterologous Bacteria, Including *Pseudomonas aeruginosa*. *Infection and Immunity*, 87(9), e00457-19. <https://doi.org/10.1128/IAI.00457-19>
- Nikis, F. (2024). *Fight Club: the antagonistic behavior of Xanthomonas translucens secretion systems and its effect on barley*. Université catholique de Louvain.
- Norman, D. J., Yuen, J. M. F., & Hodge, N. C. (1997). New Disease on Ornamental Asparagus Caused by *Xanthomonas campestris* in Florida. *Plant Disease*, 81(8), 847–850. <https://doi.org/10.1094/PDIS.1997.81.8.847>
- Nykyri, J., Niemi, O., Koskinen, P., Nokso-Koivisto, J., Pasanen, M., Broberg, M., Plyusnin, I., Törönen, P., Holm, L., Pirhonen, M., & Palva, E. T. (2012). Revised

- Phylogeny and Novel Horizontally Acquired Virulence Determinants of the Model Soft Rot Phytopathogen *Pectobacterium wasabiae* SCC3193. *PLoS Pathogens*, 8(11), e1003013. <https://doi.org/10.1371/journal.ppat.1003013>
- Oka, G. U., Souza, D. P., Sgro, G. G., Guzzo, C. R., Dunger, G., & Farah, C. S. (2024). *Xanthomonas* immunity proteins protect against the cis-toxic effects of their cognate T4SS effectors. *EMBO Reports*, 25(3), 1436–1452. <https://doi.org/10.1038/s44319-024-00060-6>
- Oka, G. U., Souza, P., Cenens, W., Matsuyama, B. Y., Cardoso, M. V. C., Oliveira, L. C., Da, F., Lima, S., Cuccovia, I. M., Guzzo, C. R., Salinas, R. K., & Farah, C. S. (2021). Structural basis for effector recognition by an antibacterial type IV secretion system. *Proceedings of the National Academy of Sciences of the United States of America*, 119(1), e2112529119. <https://doi.org/10.1073/pnas.2112529119>
- Onofre-Rentería, K., Cabrera-Munguía, D. A., Cobos-Puc, L. E., Claudio-Rizo, J. A., Morales-Oyervides, L., & Oyervides-Muñoz, E. (2024). Xanthan Gum Production and Its Modifications to Obtain Novel Applications: A Review. *Polymers for Advanced Technologies*, 35(11), e70004. <https://doi.org/10.1002/pat.70004>
- Parkinson, N., Cowie, C., Heeney, J., & Stead, D. (2009). Phylogenetic structure of *Xanthomonas* determined by comparison of *gyrB* sequences. *International Journal of Systematic and Evolutionary Microbiology*, 59(2), 264–274. <https://doi.org/10.1099/ijs.0.65825-0>
- Parte, A. C., Carbasse, J. S., Meier-Kolthoff, J. P., Reimer, L. C., & Göker, M. (2020). List of prokaryotic names with standing in nomenclature (LPSN) moves to the DSMZ. *International Journal of Systematic and Evolutionary Microbiology*, 70(11), 5607–5612. <https://doi.org/10.1099/ijsem.0.004332>
- Paul, V. H., & Smith, I. M. (1989). Bacterial pathogens of Gramineae: systematic review and assessment of quarantine status for the EPPO region. *EPPO Bulletin*, 19, 33–42. <https://doi.org/https://doi.org/10.1111/j.1365-2338.1989.tb00126.x>
- Peduzzi, C., Butchacas, J., Nikis, F., Heiden, N., Merfa, M. V., Martin, M., Roman-Reyna, V., Jacobs, J. M., Koebnik, R., & Bragard, C. (2025). Functional replacement of ancestral antibacterial secretion system in a bacterial plant pathogen. *Nature Ecology and Evolution*, 9(8), 1393–1404. <https://doi.org/10.1038/s41559-025-02773-w>
- Peduzzi, C., Sagia, A., Burokiene, D., Nagy, I. K., Saux, M. F. Le, Portier, P., Dereeper, A., Cunnac, S., Roman-Reyna, V., Jacobs, J. M., Bragard, C., & Koebnik, R. (2023). Complete Genome Sequencing of Three Clade-1 *Xanthomonas*

- Reveals Genetic Determinants for a Lateral Flagellin and the Biosynthesis of Coronatine-Like Molecules in *Xanthomonas*. *Phytopathology*, 113(7), 1185–1191. <https://doi.org/10.1094/PHYTO-10-22-0373-SC>
- Pei, T. T., Li, H., Liang, X., Wang, Z. H., Liu, G., Wu, L. L., Kim, H., Xie, Z., Yu, M., Lin, S., Xu, P., & Dong, T. G. (2020). Intramolecular chaperone-mediated secretion of an Rhs effector toxin by a type VI secretion system. *Nature Communications*, 11(1), 1865. <https://doi.org/10.1038/s41467-020-15774-z>
- Pena, M. M., Bhandari, R., Bowers, R. M., Weis, K., Newberry, E., Wagner, N., Pupko, T., Jones, J. B., Woyke, T., Vinatzer, B. A., Jacques, M. A., & Potnis, N. (2024). Genetic and Functional Diversity Help Explain Pathogenic, Weakly Pathogenic, and Commensal Lifestyles in the Genus *Xanthomonas*. *Genome Biology and Evolution*, 16(4), evae074. <https://doi.org/10.1093/gbe/evae074>
- Peñil-Celis, A., & Garcillán-Barcia, M. P. (2019). Crosstalk Between Type VI Secretion System and Mobile Genetic Elements. *Frontiers in Molecular Biosciences*, 6, 126. <https://doi.org/10.3389/FMOLB.2019.00126/BIBTEX>
- Piel, D., Bruto, M., James, A., Labreuche, Y., Lambert, C., Janicot, A., Chenivresse, S., Petton, B., Wegner, K. M., Stoudmann, C., Blokesch, M., & Le Roux, F. (2020). Selection of *Vibrio crassostreae* relies on a plasmid expressing a type 6 secretion system cytotoxic for host immune cells. *Environmental Microbiology*, 22(10), 4198–4211. <https://doi.org/10.1111/1462-2920.14776>
- Pieretti, I., Pesic, A., Petras, D., Royer, M., Süssmuth, R. D., & Cociancich, S. (2015). What makes *Xanthomonas albilineans* unique amongst xanthomonads? *Frontiers in Plant Science*, 6, 289. <https://doi.org/10.3389/fpls.2015.00289>
- Poplawsky, A. R., Urban, S. C., & Chun, W. (2000). Biological role of xanthomonadin pigments in *Xanthomonas campestris* pv. *campestris*. *Applied and Environmental Microbiology*, 66(12), 5123–5127. <https://doi.org/10.1128/AEM.66.12.5123-5127.2000>
- Pritchard, L., Harrington, B., Cock, P., Davey, R., Waters, N., Brankovics, B., & Esen, Ö. (2015). Pyani: Whole-genome classification using Average Nucleotide Identity. *Software, University of Strathclyde*. <https://doi.org/10.5281/ZENODO.594921>
- Pruvost, O., Boyer, K., Ravigné, V., Richard, D., & Vernière, C. (2019). Deciphering how plant pathogenic bacteria disperse and meet: Molecular epidemiology of *Xanthomonas citri* pv. *citri* at microgeographic scales in a tropical area of Asiatic citrus canker endemicity. *Evolutionary Applications*, 12(8), 1523–1538. <https://doi.org/10.1111/eva.12788>
- Pukatzki, S., Ma, A. T., Sturtevant, D., Krastins, B., Sarracino, D., Nelson, W. C., Heidelberg, J. F., & Mekalanos, J. J. (2006). Identification of a conserved

- bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. *Proceedings of the National Academy of Sciences of the United States of America*, 103(5), 1528–1533.
<https://doi.org/10.1073/pnas.0510322103>
- Purtschert-Montenegro, G., Cárcamo-Oyarce, G., Pinto-Carbó, M., Agnoli, K., Bailly, A., & Eberl, L. (2022). *Pseudomonas putida* mediates bacterial killing, biofilm invasion and biocontrol with a type IVB secretion system. *Nature Microbiology*, 7(10), 1547–1557. <https://doi.org/10.1038/s41564-022-01209-6>
- Qian, W., Jia, Y., Ren, S. X., He, Y. Q., Feng, J. X., Lu, L. F., Sun, Q., Ying, G., Tang, D. J., Tang, H., Wu, W., Hao, P., Wang, L., Jiang, B. Le, Zeng, S., Gu, W. Y., Lu, G., Rong, L., Tian, Y., ... He, C. (2005). Comparative and functional genomic analyses of the pathogenicity of phytopathogen *Xanthomonas campestris* pv. *campestris*. *Genome Research*, 15(6), 757–767.
<https://doi.org/10.1101/gr.3378705>
- Quentin, D., Ahmad, S., Shanthamoorthy, P., Mougous, J. D., Whitney, J. C., & Raunser, S. (2018). Mechanism of loading and translocation of type VI secretion system effector Tse6. *Nature Microbiology*, 3(10), 1142–1152.
<https://doi.org/10.1038/s41564-018-0238-z>
- Rademaker, J. L. W., Norman, D. J., Forster, R. L., Louws, F. J., Schultz, M. H., & De Bruijn, F. J. (2006). Classification and identification of *Xanthomonas translucens* isolates, including those pathogenic to ornamental asparagus. *Phytopathology*, 96(8), 876–884. <https://doi.org/10.1094/PHYTO-96-0876>
- Ramamoorthy, S., Pena, M., Ghosh, P., Liao, Y.-Y., Paret, M., Jones, J. B., & Potnis, N. (2024). Transcriptome profiling of type VI secretion system core gene *tssM* mutant of *Xanthomonas perforans* highlights regulators controlling diverse functions ranging from virulence to metabolism. *Microbiology Spectrum*, 12(1), e0285223. <https://doi.org/10.1128/spectrum.02852-23>
- Rana, R., Sharma, A., Madhavan, V. N., Korpole, S., Sonti, R. V., Patel, H. K., & Patil, P. B. (2024). *Xanthomonas protegens* sp. nov., a novel rice seed-associated bacterium, provides in vivo protection against *X. oryzae* pv. *oryzae*, the bacterial leaf blight pathogen. *FEMS Microbiology Letters*, 371, fnae093. <https://doi.org/10.1093/femsle/fnae093>
- Reddy, C. S., Godkin, J., & Johnson, A. G. (1924). Bacterial blight of rye. *Journal of Agricultural Research*, 28, 1039–1040. <https://doi.org/10.5555/19251100268>
- Reglinski, M., Monlezun, L., & Coulthurst, S. J. (2023). The accessory protein TagV is required for full Type VI secretion system activity in *Serratia marcescens*.

- Molecular Microbiology*, 119(3), 326–339.
<https://doi.org/10.1111/mmi.15027>
- Ringel, P. D., Hu, D., & Basler, M. (2017). The Role of Type VI Secretion System Effectors in Target Cell Lysis and Subsequent Horizontal Gene Transfer. *Cell Reports*, 21(13), 3927–3940. <https://doi.org/10.1016/j.celrep.2017.12.020>
- Ripcke, K., Picazo, D. R., Vichare, S., Unterweger, D., Dagan, T., & Hülter, N. F. (2023). T6SS-mediated competitive exclusion among *Pantoea agglomerans* associated with plants. *BioRxiv*, 2023.03.17.533219.
<https://doi.org/10.1101/2023.03.17.533219>
- Robitaille, S., Simmons, E. L., Verster, A. J., McClure, E. A., Royce, D. B., Trus, E., Swartz, K., Schultz, D., Nadell, C. D., & Ross, B. D. (2023). Community composition and the environment modulate the population dynamics of type VI secretion in human gut bacteria. *Nature Ecology and Evolution*, 7(12), 2092–2107. <https://doi.org/10.1038/s41559-023-02230-6>
- Rodriguez-R, L. M., & Konstantinidis, K. T. (2016). The enveomics collection: a toolbox for specialized analyses of microbial genomes and metagenomes. *PeerJ Preprints*, 4, e1900v1. <https://doi.org/10.7287/peerj.preprints.1900v1>
- Rossi, P., Ricci, I., Cappelli, A., Damiani, C., Ulissi, U., Mancini, M. V., Valzano, M., Capone, A., Epis, S., Crotti, E., Chouaia, B., Scuppa, P., Joshi, D., Xi, Z., Mandrioli, M., Sacchi, L., O'Neill, S. L., & Favia, G. (2015). Mutual exclusion of *Asaia* and *Wolbachia* in the reproductive organs of mosquito vectors. *Parasites and Vectors*, 8(1), 278. <https://doi.org/10.1186/S13071-015-0888-0/TABLES/2>
- Ruhe, Z. C., Low, D. A., & Hayes, C. S. (2013). Bacterial contact-dependent growth inhibition. *Trends in Microbiology*, 21(5), 230–237.
<https://doi.org/10.1016/j.tim.2013.02.003>
- Russel, J., Røder, H. L., Madsen, J. S., Burmølle, M., & Sørensen, S. J. (2017). Antagonism correlates with metabolic similarity in diverse bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, 114(40), 10684–10688. <https://doi.org/10.1073/pnas.1706016114>
- Russell, A. B., Hood, R. D., Bui, N. K., Leroux, M., Vollmer, W., & Mougous, J. D. (2011). Type VI secretion delivers bacteriolytic effectors to target cells. *Nature*, 475(7356), 343–349. <https://doi.org/10.1038/nature10244>
- Russell, A. B., Leroux, M., Hathazi, K., Agnello, D. M., Ishikawa, T., Wiggins, P. A., Wai, S. N., & Mougous, J. D. (2013). Diverse type VI secretion phospholipases are functionally plastic antibacterial effectors. *Nature*, 496(7446), 508–512.
<https://doi.org/10.1038/nature12074>

- Russell, A. B., Wexler, A. G., Harding, B. N., Whitney, J. C., Bohn, A. J., Goo, Y. A., Tran, B. Q., Barry, N. A., Zheng, H., Peterson, S. B., Chou, S., Gonen, T., Goodlett, D. R., Goodman, A. L., & Mougous, J. D. (2014). A type VI secretion-related pathway in *Bacteroidetes* mediates interbacterial antagonism. *Cell Host and Microbe*, 16(2), 227–236.
<https://doi.org/10.1016/j.chom.2014.07.007>
- Saddler, G. S., & Bradbury, J. F. (2015). *Xanthomonas*. In *Bergey's Manual of Systematics of Archaea and Bacteria* (pp. 1–53). Wiley.
<https://doi.org/10.1002/9781118960608.gbm01239>
- Sah, G. P., & Wall, D. (2020). Kin recognition and outer membrane exchange (OME) in myxobacteria. *Current Opinion in Microbiology*, 56, 81–88.
<https://doi.org/10.1016/j.mib.2020.07.003>
- Salinero-Lanzarote, A., Pacheco-Moreno, A., Domingo-Serrano, L., Durán, D., Ormeño-Orrillo, E., Martínez-Romero, E., Albareda, M., Palacios, J. M., & Rey, L. (2019). The Type VI secretion system of *Rhizobium etli* Mim1 has a positive effect in symbiosis. *FEMS Microbiology Ecology*, 95(5), fiz054.
<https://doi.org/10.1093/femsec/fiz054>
- Salomon, D., Kinch, L. N., Trudgian, D. C., Guo, X., Klimko, J. A., Grishin, N. V., Mirzaei, H., & Orth, K. (2014). Marker for type VI secretion system effectors. *Proceedings of the National Academy of Sciences of the United States of America*, 111(25), 9271–9276. <https://doi.org/10.1073/pnas.1406110111>
- Sana, T. G., Lugo, K. A., & Monack, D. M. (2017). T6SS: The bacterial ‘fight club’ in the host gut. *PLoS Pathogens*, 13(6), e1006325.
<https://doi.org/10.1371/journal.ppat.1006325>
- Sánchez-Cañizares, C., Jorrín, B., Durán, D., Nadendla, S., Albareda, M., Rubio-Sanz, L., Lanza, M., González-Guerrero, M., Prieto, R. I., Brito, B., Giglio, M. G., Rey, L., Ruiz-Argüeso, T., Palacios, J. M., & Imperial, J. (2018). Genomic diversity in the endosymbiotic bacterium *Rhizobium leguminosarum*. *Genes*, 9(2), 60.
<https://doi.org/10.3390/genes9020060>
- Sands, O. C., Mizrak, G., Hall, V. N., Kim, H. K., Bockelman, H. E., & Golden, M. J. (1986). Seed transmitted bacterial diseases of cereals: epidemiology and control. *Arab Journal of Plant Protection*, 4(2), 125–127.
- Santin, Y. G., Doan, T., Journet, L., & Cascales, E. (2019). Cell Width Dictates Type VI Secretion Tail Length. *Current Biology*, 29(21), 3707–3713.e3.
<https://doi.org/10.1016/j.cub.2019.08.058>
- Santoriello, F. J., Michel, L., Unterweger, D., & Pukatzki, S. (2020). Pandemic *Vibrio cholerae* shuts down site-specific recombination to retain an interbacterial

- defence mechanism. *Nat Commun*, 11(1), 6246.
<https://doi.org/10.1038/s41467-020-20012-7>
- Sapkota, S., Mergoum, M., & Liu, Z. (2020). The translucens group of *Xanthomonas translucens*: Complicated and important pathogens causing bacterial leaf streak on cereals. *Molecular Plant Pathology*, 21(3), 291–302.
<https://doi.org/10.1111/mpp.12909>
- Schäfer, A., Tauch, A., Jäger, W., Kalinowski, J., Thierbach, G., & Pühler, A. (1994). Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene*, 145(1), 69–73.
[https://doi.org/10.1016/0378-1119\(94\)90324-7](https://doi.org/10.1016/0378-1119(94)90324-7)
- Schwarz, S., West, T. E., Boyer, F., Chiang, W. C., Carl, M. A., Hood, R. D., Rohmer, L., Tolker-Nielsen, T., Skerrett, S. J., & Mougous, J. D. (2010). *Burkholderia* type vi secretion systems have distinct roles in eukaryotic and bacterial cell interactions. *PLoS Pathogens*, 6(8), e1001068.
<https://doi.org/10.1371/journal.ppat.1001068>
- Sgro, G. G., Costa, T. R. D., Cenens, W., Souza, D. P., Cassago, A., Coutinho de Oliveira, L., Salinas, R. K., Portugal, R. V., Farah, C. S., & Waksman, G. (2018). Cryo-EM structure of the bacteria-killing type IV secretion system core complex from *Xanthomonas citri*. *Nature Microbiology*, 3(12), 1429–1440.
<https://doi.org/10.1038/s41564-018-0262-z>
- Sgro, G. G., Oka, G. U., Souza, D. P., Cenens, W., Bayer-Santos, E., Matsuyama, B. Y., Bueno, N. F., Dos Santos, T. R., Alvarez-Martinez, C. E., Salinas, R. K., & Farah, C. S. (2019). Bacteria-killing type IV secretion systems. *Frontiers in Microbiology*, 10, 1078. <https://doi.org/10.3389/fmicb.2019.01078>
- Shane W. W., Baumer J. S., & Teng P. S. (1987). Crop losses caused by *Xanthomonas* streak on spring wheat and barley. *Plant Disease*, 71, 927–930.
<https://doi.org/10.1094/PD-71-0927>
- Shao, Y., Tang, G., Huang, Y., Ke, W., Wang, S., Zheng, D., & Ruan, L. (2023). Transcriptional regulator Sar regulates the multiple secretion systems in *Xanthomonas oryzae*. *Molecular Plant Pathology*, 24(1), 16–27.
<https://doi.org/10.1111/MPP.13272>
- Shen, X., Wang, B., Yang, N., Zhang, L., Shen, D., Wu, H., Dong, Y., Niu, B., Chou, S. H., Puopolo, G., Fan, J., & Qian, G. (2021). *Lysobacter enzymogenes* antagonizes soilborne bacteria using the type IV secretion system. *Environmental Microbiology*, 23(8), 4673–4688.
<https://doi.org/10.1111/1462-2920.15662>

- Shen, X., Yang, Z., Li, Z., Xiong, D., Liao, J., He, W., Shen, D., Shao, X., Niu, B., He, Y., Gao, Y. G., & Qian, G. (2023). Identification of atypical T4SS effector proteins mediating bacterial defense. *MLife*, 2(3), 295–307.
<https://doi.org/10.1002/mlf2.12084>
- Shneider, M. M., Buth, S. A., Ho, B. T., Basler, M., Mekalanos, J. J., & Leiman, P. G. (2013). PAAR-repeat proteins sharpen and diversify the type VI secretion system spike. *Nature*, 500(7462), 350–353.
<https://doi.org/10.1038/nature12453>
- Shohanipor, T., Sharifi, R., & Hosseini, S. (2025). Efficacy Assessment of Some Copper Compounds on the Bacterial Leaf Streak Pathogen, *Xanthomonas translucens*. *European Journal of Plant Pathology*, in press.
<https://doi.org/10.1007/s10658-025-03099-w>
- Shyntum, D. Y., Venter, S. N., Moleleki, L. N., Toth, I., & Coutinho, T. A. (2014). Comparative genomics of type VI secretion systems in strains of *Pantoea ananatis* from different environments. *BMC Genomics*, 15(1), 163.
<https://doi.org/10.1186/1471-2164-15-163>
- Si, M., Zhao, C., Burkinshaw, B., Zhang, B., Wei, D., Wang, Y., Dong, T. G., & Shen, X. (2017). Manganese scavenging and oxidative stress response mediated by type VI secretion system in *Burkholderia thailandensis*. *Proceedings of the National Academy of Sciences of the United States of America*, 114(11), E2233–E2242. <https://doi.org/10.1073/pnas.1614902114>
- Silverman, J. M., Austin, L. S., Hsu, F., Hicks, K. G., Hood, R. D., & Mougous, J. D. (2011). Separate inputs modulate phosphorylation-dependent and -independent type VI secretion activation. *Molecular Microbiology*, 82(5), 1277–1290. <https://doi.org/10.1111/j.1365-2958.2011.07889.x>
- Smith, E. F., Jones, L. R., & Reddy, C. S. (1919). The black chaff of wheat. *Science*, 50(1280), 48. <https://doi.org/10.1126/SCIENCE.50.1280.48>,
- Smith, W. P. J., Vettiger, A., Winter, J., Ryser, T., Comstock, L. E., Basler, M., & Foster, K. R. (2020). The evolution of the type VI secretion system as a disintegration weapon. *PLoS Biology*, 18(5), e3000720.
<https://doi.org/10.1371/journal.pbio.3000720>
- Storey, D., McNally, A., Åstrand, M., Santos, J. P. G., Rodriguez-Escudero, I., Elmore, B., Palacios, L., Marshall, H., Hobley, L., Molina, M., Cid, V. J., Salminen, T. A., & Bengoechea, J. A. (2020). *Klebsiella pneumoniae* type VI secretion system-mediated microbial competition is PhoPQ controlled and reactive oxygen species dependent. *PLOS Pathogens*, 16(3), e1007969.
<https://doi.org/10.1371/JOURNAL.PPAT.1007969>

- Stromberg, K. D., Kinkel, L. L., & Leonard, K. J. (2000). Interactions between *Xanthomonas translucens* pv. *translucens*, the causal agent of bacterial leaf streak of wheat, and bacterial epiphytes in the wheat phyllosphere. *Biological Control*, 17(1), 61-72. <https://doi.org/10.1006/bcon.1999.0771>
- Sokal, R., & Michener, C. (1958). A statistical method for evaluating systematic relationships. *University of Kansas Science Bulletin*.
- Soufiane, B., Sirois, M., & Côté, J. C. (2011). Mutually exclusive distribution of the *sap* and *eag* S-layer genes and the *lytB/lytA* cell wall hydrolase genes in *Bacillus thuringiensis*. *Antonie Van Leeuwenhoek*, 100(3), 349–364. <https://doi.org/10.1007/S10482-011-9590-1>
- Souza, D. P., Andrade, M. O., Alvarez-Martinez, C. E., Arantes, G. M., Farah, C. S., & Salinas, R. K. (2011). A component of the *Xanthomonadaceae* type IV secretion system combines a VirB7 motif with a N0 domain found in outer membrane transport proteins. *PLoS Pathogens*, 7(5), e1002031. <https://doi.org/10.1371/journal.ppat.1002031>
- Souza, D. P., Oka, G. U., Alvarez-Martinez, C. E., Bisson-Filho, A. W., Dunger, G., Hobeika, L., Cavalcante, N. S., Alegria, M. C., Barbosa, L. R. S., Salinas, R. K., Guzzo, C. R., & Farah, C. S. (2015). Bacterial killing via a type IV secretion system. *Nature Communications*, 6, 6453. <https://doi.org/10.1038/ncomms7453>
- Spagnuolo, J., Opalka, N., Wen, W. X., Gagic, D., Chabaud, E., Bellini, P., Bennett, M. D., Norris, G. E., Darst, S. A., Russel, M., & Rakonjac, J. (2010). Identification of the gate regions in the primary structure of the secretin pIV. *Molecular Microbiology*, 76(1), 133–150. <https://doi.org/10.1111/j.1365-2958.2010.07085.x>
- Speare, L., Cecere, A. G., Guckes, K. R., Smith, S., Wollenberg, M. S., Mandel, M. J., Miyashiro, T., & Septer, A. N. (2018). Bacterial symbionts use a type VI secretion system to eliminate competitors in their natural host. *Proceedings of the National Academy of Sciences of the United States of America*, 115(36), E8528–E8537. <https://doi.org/10.1073/PNAS.1808302115>
- Spreter, T., Yip, C. K., Sanowar, S., André, I., Kimbrough, T. G., Vuckovic, M., Pfuetzner, R. A., Deng, W., Yu, A. C., Finlay, B. B., Baker, D., Miller, S. I., & Strynadka, N. C. J. (2009). A conserved structural motif mediates formation of the periplasmic rings in the type III secretion system. *Nature Structural and Molecular Biology*, 16(5), 468–476. <https://doi.org/10.1038/nsmb.1603>
- Storey, D., McNally, A., Åstrand, M., Santos, J. P. G., Rodriguez-Escudero, I., Elmore, B., Palacios, L., Marshall, H., Hobley, L., Molina, M., Cid, V. J., Salminen, T. A., & Bengoechea, J. A. (2020). *Klebsiella pneumoniae* type VI secretion system-

- mediated microbial competition is PhoPQ controlled and reactive oxygen species dependent. *PLOS Pathogens*, 16(3), e1007969. <https://doi.org/10.1371/JOURNAL.PPAT.1007969>
- Sturm, A., Heinemann, M., Arnoldini, M., Benecke, A., Ackermann, M., Benz, M., Dormann, J., & Hardt, W. D. (2011). The cost of virulence: Retarded growth of *Salmonella Typhimurium* cells expressing type III secretion system. *PLoS Pathogens*, 7(7), e1002143. <https://doi.org/10.1371/journal.ppat.1002143>
- Taillefer, B., Desjardins, J. B., & Cascales, E. (2025). Molecular bases for the loss of type VI secretion system activity during enteroaggregative *E. coli* experimental evolution. *BioRxiv*, 2025.02.08.637259. <https://doi.org/10.1101/2025.02.08.637259>
- Taillefer, B., Giraud, J. F., & Cascales, E. (2023). No fitness cost entailed by type VI secretion system synthesis, assembly, contraction, or disassembly in enteroaggregative *Escherichia coli*. *Journal of Bacteriology*, 205(12), e0035723. <https://doi.org/10.1128/jb.00357-23>
- Tambong, J. T., Xu, R., Fleitas, M. C., & Kutcher, R. (2024). Taxonogenomic analysis of the *Xanthomonas translucens* complex leads to the descriptions of *Xanthomonas cerealis* sp. nov. and *Xanthomonas graminis* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, 74(9), 006523. <https://doi.org/10.1099/ijsem.0.006523>
- Tamura, K., Stecher, G., & Kumar, S. (2021). MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution*, 38(7), 3022–3027. <https://doi.org/10.1093/MOLBEV/MSAB120>
- Tamura, K., Takikawa, Y., Tsuyumu, S., Goto, M., & Watanabe, M. (1992). Coronatine Production by *Xanthomonas campestris* pv. *phormiicola*. *Japanese Journal of Phytopathology*, 58(2), 276–281. <https://doi.org/10.3186/jjphytopath.58.276>
- Tan, Z.-M., Yang, J.-T., Xiao, Q.-J., Su, J.-T., Wang, Z.-H., Jiang, Y.-T., Liu, J.-S., Pei, T.-T., Liang, X., An, Y., Xue, H.-W., Qin, W.-M., Lin, W.-H., & Dong, T. (2025). A phospholipase effector of the type VI secretion system modulates plant reproduction. *MBio*, 16(9), e01546-25. <https://doi.org/10.1128/mbio.01546-25>
- Tan, Z.-M., Zheng, X., Su, J., Liu, J.-S., Liang, X., Pei, T.-T., & Dong, T. (2025). Dissecting the antibacterial functions of the T6SS-2 cluster in *Xanthomonas oryzae* for environment and plant protection. *Applied and Environmental Microbiology*, 91(10), e0102125. <https://doi.org/10.1128/aem.01021-25>
- Tang, L., Yue, S., Li, G. Y., Li, J., Wang, X. R., Li, S. F., & Mo, Z. L. (2016). Expression, secretion and bactericidal activity of type VI secretion system in *Vibrio*

- anguillarum*. *Archives of Microbiology*, 198(8), 751–760.
<https://doi.org/10.1007/s00203-016-1236-2>
- Teulet, A., Gully, D., Rouy, Z., Camuel, A., Koebnik, R., Giraud, E., & Lassalle, F. (2020). Phylogenetic distribution and evolutionary dynamics of NOD and T3SS genes in the genus *Bradyrhizobium*. *Microbial Genomics*, 6(9), mgen000407.
<https://doi.org/10.1099/mgen.0.000407>
- Tibor, A., Decelle, B. A., & Letesson, J.-J. (1999). Outer Membrane Proteins Omp10, Omp16, and Omp19 of *Brucella* spp. Are Lipoproteins. *Infection and Immunity*, 67(9), 4960–4962. <https://doi.org/10.1128/IAI.67.9.4960-4962.1999>
- Ting, S. Y., Bosch, D. E., Mangiameli, S. M., Radey, M. C., Huang, S., Park, Y. J., Kelly, K. A., Filip, S. K., Goo, Y. A., Eng, J. K., Allaire, M., Veessler, D., Wiggins, P. A., Peterson, S. B., & Mougous, J. D. (2018). Bifunctional Immunity Proteins Protect Bacteria against FtsZ-Targeting ADP-Ribosylating Toxins. *Cell*, 175(5), 1380-1392.e14. <https://doi.org/10.1016/j.cell.2018.09.037>
- Ting, S. Y., Martínez-García, E., Huang, S., Bertolli, S. K., Kelly, K. A., Cutler, K. J., ... & Mougous, J. D. (2020). Targeted depletion of bacteria from mixed populations by programmable adhesion with antagonistic competitor cells. *Cell host & microbe*, 28(2), 313-321.E6.
<https://doi.org/10.1016/j.chom.2020.05.006>
- Toska, J., Ho, B. T., & Mekalanos, J. J. (2018). Exopolysaccharide protects *Vibrio cholerae* from exogenous attacks by the type 6 secretion system. *Proceedings of the National Academy of Sciences of the United States of America*, 115(31), 7997–8002. <https://doi.org/10.1073/pnas.1808469115>
- Trifinopoulos, J., Nguyen, L. T., von Haeseler, A., & Minh, B. Q. (2016). W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. *Nucleic Acids Research*, 44(W1), W232–W235. <https://doi.org/10.1093/NAR/GKW256>
- Troselj, V., Treuner-Lange, A., Søggaard-Andersen, L., & Wall, D. (2018). Physiological Heterogeneity Triggers Sibling Conflict Mediated by the Type VI Secretion System in an Aggregative Multicellular Bacterium. *MBio*, 9(1), e01645-17.
<https://doi.org/10.1128/mBio.01645>
- Tubajika, K. M., Russin, J. S., & Harrison, S. A. (1999). Analysis of Bacterial Leaf Streak Epidemics on Winter Wheat in Louisiana. *Plant Disease*, 83, 541-548.
<https://doi.org/10.1094/PDIS.1999.83.6.541>
- Ulhuq, F. R., Gomes, M. C., Duggan, G. M., Guo, M., Mendonca, C., Buchanan, G., Chalmers, J. D., Cao, Z., Kneuper, H., Murdoch, S., Thomson, S., Strahl, H., Trost, M., Mostowy, S., & Palmer, T. (2020). A membrane-depolarizing toxin substrate of the *Staphylococcus aureus* type VII secretion system mediates

- intraspecies competition. *Proceedings of the National Academy of Sciences of the United States of America*, 117(34), 20836–20847.
<https://doi.org/10.1073/pnas.2006110117>
- Unni, R., Pintor, K. L., Diepold, A., & Unterweger, D. (2022). Presence and absence of type VI secretion systems in bacteria. *Microbiology (Reading)*, 168(4), 001151. <https://doi.org/10.1099/mic.0.001151>
- Unterweger, D., Kostiuk, B., Ötjengerdes, R., Wilton, A., Diaz-Satizabal, L., & Pukatzki, S. (2015). Chimeric adaptor proteins translocate diverse type VI secretion system effectors in *Vibrio cholerae*. *The EMBO Journal*, 34(16), 2198–2210. <https://doi.org/10.15252/embj.201591163>
- Vacheron, J., Péchy-Tarr, M., Brochet, S., Heiman, C. M., Stojiljkovic, M., Maurhofer, M., & Keel, C. (2019). T6SS contributes to gut microbiome invasion and killing of an herbivorous pest insect by plant-beneficial *Pseudomonas protegens*. *ISME Journal*, 13(5), 1318–1329.
<https://doi.org/10.1038/s41396-019-0353-8>
- Vauterin, L., Hoste, B., Kersters, K., & Swings, J. (1995). Reclassification of *Xanthomonas*. *International Journal of Systematic Bacteriology*, 45(3), 472–489. <https://doi.org/10.1099/00207713-45-3-472>
- Vauterin, L., Rademaker, J., & Swings, J. (2000). Synopsis on the taxonomy of the genus *Xanthomonas*. *Phytopathology*, 90(7), 677–682.
<https://doi.org/10.1094/PHTO.2000.90.7.677>
- Vauterin, L., Yang, P., Swings, J., & Kersters, K. (1992). Taxonomy of xanthomonads from cereals and grasses based on SDS-PAGE of proteins, fatty acid analysis and DNA hybridization. *Journal of General Microbiology*, 138(7), 1467–1477.
- Vettiger, A., & Basler, M. (2016). Type VI Secretion System Substrates Are Transferred and Reused among Sister Cells. *Cell*, 167(1), 99–110.e12.
<https://doi.org/10.1016/j.cell.2016.08.023>
- Waksman, G. (2019). From conjugation to T4S systems in Gram-negative bacteria: a mechanistic biology perspective. *EMBO reports*, 20(2), e47012.
<https://doi.org/10.15252/embr.201847012>
- Wallin J.R. (1994). *Parasitism of Xanthomonas translucens*. Iowa State College.
- Wang, B., Zhang, Z., Xu, F., Yang, Z., Li, Z., Shen, D., Wang, L., Wu, H., Li, T., Yan, Q., Wei, Q., Shao, X., & Qian, G. (2023). Soil bacterium manipulates antifungal weapons by sensing intracellular type IVA secretion system effectors of a competitor. *ISME Journal*, 17(12), 2232–2246.
<https://doi.org/10.1038/s41396-023-01533-7>

- Wang, Q., Li, Y., Liu, L., Wang, Y., Yan, J., Shah, S. M. A., Xu, Z., Xu, X., Hu, B., Gao, H., Khojasteh, M., & Chen, G. (2025). Comprehensive Genomic Insights Into Unique TALEs of a *Xanthomonas translucens* pv. *undulosa* Strain From China. *Plant Pathology*, 74(9), 2455–2462. <https://doi.org/10.1111/ppa.70061>
- Wang, T., Si, M., Song, Y., Zhu, W., Gao, F., Wang, Y., Zhang, L., Zhang, W., Wei, G., Luo, Z. Q., & Shen, X. (2015). Type VI Secretion System Transports Zn²⁺ to Combat Multiple Stresses and Host Immunity. *PLoS Pathogens*, 11(7), e1005020. <https://doi.org/10.1371/journal.ppat.1005020>
- Weber, B. S., Hennon, S. W., Wright, M. S., Scott, N. E., de Berardinis, V., Foster, L. J., Ayala, J. A., Adams, M. D., & Feldman, M. F. (2016). Genetic dissection of the type VI secretion system in *Acinetobacter* and identification of a novel peptidoglycan hydrolase, TagX, required for its biogenesis. *MBio*, 7(5), e01253-16. <https://doi.org/10.1128/mBio.01253-16>
- Weber, B. S., Ly, P. M., Irwin, J. N., Pukatzki, S., & Feldman, M. F. (2015). A multidrug resistance plasmid contains the molecular switch for type VI secretion in *Acinetobacter baumannii*. *Proceedings of the National Academy of Sciences of the United States of America*, 112(30), 9442–9447. <https://doi.org/10.1073/pnas.1502966112>
- Whitney, J. C., Chou, S., Russell, A. B., Biboy, J., Gardiner, T. E., Ferrin, M. A., Brittnacher, M., Vollmer, W., & Mougous, J. D. (2013). Identification, structure, and function of a novel type VI secretion peptidoglycan glycoside hydrolase effector-immunity pair. *Journal of Biological Chemistry*, 288(37), 26616–26624. <https://doi.org/10.1074/jbc.M113.488320>
- Whitney, J. C., Quentin, D., Sawai, S., LeRoux, M., Harding, B. N., Ledvina, H. E., Tran, B. Q., Robinson, H., Goo, Y. A., Goodlett, D. R., Raunser, S., & Mougous, J. D. (2015). An Interbacterial NAD(P)⁺ Glycohydrolase Toxin Requires Elongation Factor Tu for Delivery to Target Cells. *Cell*, 163(3), 607–619. <https://doi.org/10.1016/j.cell.2015.09.027>
- Wong, M. J. Q., Liang, X., Smart, M., Tang, L., Moore, R., Ingalls, B., & Dong, T. G. (2016). Microbial herd protection mediated by antagonistic interaction in polymicrobial communities. *Applied and Environmental Microbiology*, 82(23), 6881–6888. <https://doi.org/10.1128/AEM.02210-16>
- Wood, T. E., Howard, S. A., Förster, A., Nolan, L. M., Manoli, E., Bullen, N. P., Yau, H. C. L., Hachani, A., Hayward, R. D., Whitney, J. C., Vollmer, W., Freemont, P. S., & Filloux, A. (2019). The *Pseudomonas aeruginosa* T6SS Delivers a Periplasmic Toxin that Disrupts Bacterial Cell Morphology. *Cell Reports*, 29(1), 187–201.e7. <https://doi.org/10.1016/j.celrep.2019.08.094>

- Xu, G., Han, S., Huo, C., Chin, K. H., Chou, S. H., Gomelsky, M., Qian, G., & Liu, F. (2018). Signaling specificity in the c-di-GMP-dependent network regulating antibiotic synthesis in *Lysobacter*. *Nucleic Acids Research*, 46(18), 9276–9288. <https://doi.org/10.1093/nar/gky803>
- Xu, K., Lin, L., Shen, D., Chou, S. H., & Qian, G. (2021). Clp is a “busy” transcription factor in the bacterial warrior, *Lysobacter enzymogenes*. *Computational and Structural Biotechnology Journal*, 19, 3564–3572. <https://doi.org/10.1016/j.csbj.2021.06.020>
- Xu, X., Dinesen, C., Pioppi, A., Kovács, Á. T., & Lozano-Andrade, C. N. (2025). Composing a microbial symphony: synthetic communities for promoting plant growth. *Trends in Microbiology*, 33(7), 738–751. <https://doi.org/10.1016/j.tim.2025.01.006>
- Yadav, S. K., Magotra, A., Ghosh, S., Krishnan, A., Pradhan, A., Kumar, R., Das, J., Sharma, M., & Jha, G. (2021). Immunity proteins of dual nuclease T6SS effectors function as transcriptional repressors. *EMBO Reports*, 22(6), e51857. <https://doi.org/10.15252/embr.202051857>
- Yang, J.-T., Tan, Z.-M., Jiang, Y.-T., Bai, Y.-X., Zhang, Y.-J., Xue, H.-W., Xu, T.-D., Dong, T., & Lin, W.-H. (2025). Non-adapted bacterial infection suppresses plant reproduction. *Science Advances*, 11(2), eads7738. <https://doi.org/10.1126/sciadv.ads7738>
- Yang, M., Ren, S., Shen, D., Yang, N., Wang, B., Han, S., Shen, X., Chou, S. H., & Qian, G. (2020). An intrinsic mechanism for coordinated production of the contact-dependent and contact-independent weapon systems in a soil bacterium. *PLoS Pathogens*, 16(10), e1008967. <https://doi.org/10.1371/journal.ppat.1008967>
- Yang, X., Liu, H., Zhang, Y., & Shen, X. (2021). Roles of Type VI Secretion System in Transport of Metal Ions. *Frontiers in Microbiology*, 12, 756136. <https://doi.org/10.3389/fmicb.2021.756136>
- Young, J. M., Dye, D. W., Bradbury, J. F., Panagopoulos, C. G., & Robbs, C. F. (1978). A proposed nomenclature and classification for plant pathogenic bacteria. *New Zealand Journal of Agricultural Research*, 21(1), 153–177. <https://doi.org/10.1080/00288233.1978.10427397>
- Yu, M., Zhang, G., Jiang, J., Du, L., & Zhao, Y. (2020). *Lysobacter enzymogenes* Employs Diverse Genes for Inhibiting Hypha Growth and Spore Germination of Soybean Fungal Pathogens. *Phytopathology*, 110(3), 593–602. <https://doi.org/10.1094/PHYTO-09-19-0356-R>
- Zhang, C., Datta, S., Ratcliff, W. C., & Hammer, B. K. (2024). Constitutive expression of the Type VI Secretion System carries no measurable fitness cost in *Vibrio*

- cholerae*. *Ecology and Evolution*, 14(3), e11081.
<https://doi.org/10.1002/ece3.11081>
- Zhang, D., de Souza, R. F., Anantharaman, V., Iyer, L. M., & Aravind, L. (2012). Polymorphic toxin systems: Comprehensive characterization of trafficking modes, processing, mechanisms of action, immunity and ecology using comparative genomics. *Biology Direct*, 7(1), 18. <http://www.biology-direct.com/content/7/1/18>
- Zhang, J., Guan, J., Wang, M., Li, G., Djordjevic, M., Tai, C., Wang, H., Deng, Z., Chen, Z., & Ou, H. Y. (2023). SecReT6 update: a comprehensive resource of bacterial Type VI Secretion Systems. *Science China Life Sciences*, 66(3), 626–634. <https://doi.org/10.1007/s11427-022-2172-x>
- Zhang, L., Xu, J., Xu, J., Zhang, H., He, L., & Feng, J. (2014). TssB is essential for virulence and required for Type VI secretion system in *Ralstonia solanacearum*. *Microbial Pathogenesis*, 74(1), 1–7. <https://doi.org/10.1016/j.micpath.2014.06.006>
- Zhang, L., Zhang, M., Huang, S., Li, L., Gao, Q., Wang, Y., Zhang, S., Huang, S., Yuan, L., Wen, Y., Liu, K., Yu, X., Li, D., Zhang, L., Xu, X., Wei, H., He, P., Zhou, W., Philippot, L., & Ai, C. (2022). A highly conserved core bacterial microbiota with nitrogen-fixation capacity inhabits the xylem sap in maize plants. *Nature Communications*, 13(1), 3361. <https://doi.org/10.1038/s41467-022-31113-w>
- Zhang, X., Kupiec, M., Gophna, U., & Tuller, T. (2011). Analysis of Coevolving Gene Families Using Mutually Exclusive Orthologous Modules. *Genome Biology and Evolution*, 3(1), 413–423. <https://doi.org/10.1093/GBE/EVR030>
- Zhang, Y., Jing, M., Lyu, L., Nie, L., Xu, X., Sun, R., ... & Chu, H. (2025). Principles for Rigorous Design and Application of Synthetic Microbial Communities. *Advanced Science*, e14750. <https://doi.org/10.1002/advs.202514750>
- Zhao, Y. F., Jones, W. T., Sutherland, P., Palmer, D. A., Mitchell, R. E., Reynolds, P. H. S., Damicone, J. P., & Bender, C. L. (2001). Detection of the phytotoxin coronatine by ELISA and localization in infected plant tissue. *Physiological and Molecular Plant Pathology*, 58(6), 247–258. <https://doi.org/10.1006/pmpp.2001.0334>
- Zhu, P. C., Li, Y. M., Yang, X., Zou, H. F., Zhu, X. L., Niu, X. N., Xu, L. H., Jiang, W., Huang, S., Tang, J. L., & He, Y. Q. (2020). Type VI secretion system is not required for virulence on rice but for inter-bacterial competition in *Xanthomonas oryzae* pv. *oryzicola*. *Research in Microbiology*, 171(2), 64–73. <https://doi.org/10.1016/j.resmic.2019.10.004>

Zillinsky, F. J., & Borlaug, N. E. (1971). *Progress in developing triticale as an economic crop*.

<https://repository.cimmyt.org/bitstream/10883/19415/1/1899.pdf>

Zoued, A., Brunet, Y. R., Durand, E., Aschtgen, M. S., Logger, L., Douzi, B., Journet, L., Cambillau, C., & Cascales, E. (2014). Architecture and assembly of the Type VI secretion system. *Biochimica et Biophysica Acta*, 1843(8), 1664–1673.

<https://doi.org/10.1016/j.bbamcr.2014.03.018>

Appendix 1

In parallel of this doctoral research, collaborative work with Dr. Ralf Koebnik led to the publication of a study reporting the complete sequencing of three novel species within clade-1 xanthomonads (Peduzzi et al., 2023). The introduction section therefore takes a closer look at clade-1 recent advances, with a particular focus on the findings of this study, which can be found here in the appendix 1.

Complete genome sequencing of three clade-1 xanthomonads reveals genetic determinants for a lateral flagellin and the biosynthesis of coronatine-like molecules in *Xanthomonas*

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ABSTRACT

Evolutionarily early-branching xanthomonads, also referred to as clade-1 xanthomonads, include major plant pathogens, most of which colonize monocotyledonous plants. Seven species have been validly described, among them the two sugarcane pathogens *Xanthomonas albilineans* and *Xanthomonas sacchari*, and *Xanthomonas translucens*, which infects small-grain cereals, diverse grasses, but also asparagus and pistachio trees. Single-gene sequencing and genomic approaches indicated that this clade likely contains more, yet undescribed species. In this study, we sequenced representative strains of three novel species using long-read sequencing technology. *Xanthomonas campestris* pv. *phormiicola* strain CFBP 8444 causes bacterial streak on New Zealand flax, another monocotyledonous plant. *Xanthomonas* sp. strain CFBP 8443 has been isolated from common bean and *Xanthomonas* sp. strain CFBP 8445 originated from banana. Complete assemblies of the chromosomes confirmed their unique phylogenetic position within clade 1 of *Xanthomonas*. Genome mining revealed novel genetic features, hitherto undescribed in other members of the *Xanthomonas* genus. In strain CFBP 8444, we identified genes related to the synthesis of coronatine-like compounds, a phytotoxin produced by several pseudomonads, which raises interesting questions about the evolution and pathogenicity of this pathogen. In addition, strain CFBP 8444 was found to contain a second, atypical flagellar gene cluster in addition to the canonical flagellar gene cluster. Overall, this research represents an important step toward better understanding the evolutionary history and biology of early-branching xanthomonads.

KEYWORDS: Taxonomy, Evolution, Coronatine, Stomata, Lateral flagellum, Banana, Common bean, New Zealand flax

The Gram-negative bacterial genus *Xanthomonas* constitutes a large group of mostly plant-associated bacteria, collectively causing diseases on hundreds of host plants, including economically important crops and ornamental plants. Thanks to increased genome sequencing efforts, the number of *Xanthomonas* genome sequences has increased tremendously in recent years, including strains of scientifically and/or economically relevant species and pathovars (Mansfield et al. 2012). But also understudied non-model organisms have been systematically sequenced, thus correcting taxonomically mis-assigned strains and leading to description of novel species (Vicente et al. 2017; López et al. 2018; Martins et al. 2020; Bansal et al. 2021; Dia et al. 2021; Mafakheri et al. 2022).

Currently, the List of Prokaryotic names with Standing in Nomenclature (LPSN; <https://www.bacterio.net>, assessed on 8 September 2022) includes 34 validly described *Xanthomonas* species (Parte et al. 2020). Previous work based on partial sequencing of the *gyrB* gene from poorly characterized pathovars and from unidentified xanthomonads, however, suggested the existence of additional species, which were at that time described as species-level clade strains, SLC 1 to SLC 7 (Parkinson et al. 2009). Strains from SLC 1 and 4 have been sequenced since then, corresponding to the proposed species '*Xanthomonas cannabis*' and '*Xanthomonas badrii*', respectively (Zain & Roberts 1977; Jacobs et al. 2015; GenBank accession numbers CP051651 and JSZE00000000).

Xanthomonads are classified into two major groups based on sequence comparisons (Hauben et al. 1997; Gonçalves & Rosato 2002; Parkinson et al. 2007; Ferreira-Tonin et al. 2012). SLC 1 to 4 belong to clade 2, while SLC 5 to 7 belong to clade 1, also known as the clade of early-branching species (Parkinson et al. 2007; 2009). Most of the clade-1 strains have been isolated from monocots, such as asparagus, banana, sugarcane, hyacinths, forage grasses, and small-grain cereals (Jacques et al. 2016). But there are a few exceptions: *Xanthomonas theicola* causes canker on tea plants, *Xanthomonas bonasiae* and *Xanthomonas youngii* were found on canker-symptomatic ornamental plants, and strains of *Xanthomonas translucens* have also been isolated from pistachio trees (Giblot-Ducray et al. 2009; Koebnik et al. 2021; Mafakheri et al. 2022). SLC-5 strain NCPPB 2654 (= CFBP 8443) was isolated from bean (*Phaseolus vulgaris*), the SLC-6 strain NCPPB 2983 (= CFBP 8444, LMG 702) originates from New Zealand flax (*Phormium tenax*), and seven SLC-7 strains had been collected from monocots like banana (*Musa × paradisiaca*), ginger (*Zingiber officinale*) or sugarcane (*Saccharum officinarum*), but also from cotton (*Gossypium* sp.) (Parkinson et al. 2009).

The SLC-6 bacterium *X. campestris* pv. *phormiicola* (Takimoto 1933) Dye 1978 was described as a bacterial pathogen of New Zealand flax (*Phormium tenax* Forst.), causing water-soaked lesions with chlorosis (Takimoto, 1933). The same bacterium was also found to induce hypertrophy on potato tuber tissues at the inoculated site (Tamura et al. 1987). The hypertrophic active substance isolated from the bacterial culture filtrate

produced visible chlorotic lesions on the leaves of New Zealand flax, ryegrass and tobacco plant (Tamura et al. 1992). Biochemical and biophysical parameters (retardation factor on silica gel thin-layer chromatography and ^1H nuclear magnetic resonance [NMR] spectrum) of the purified toxin coincided with those of coronatine, and it was concluded that the toxin produced by *X. campestris* pv. *phormiicola* is coronatine (Tamura et al. 1992). In a parallel study, liquid cultures of *X. campestris* pv. *phormiicola* were found to contain two analogues of coronatine lacking the cyclopropane ring structure, but no trace of coronatine (Mitchell 1991). Using NMR, mass spectroscopy and gas chromatography of hydrolysis products, these two compounds were identified as N-coronafacoyl-L-valine and N-coronafacoyl-L-isoleucine (Mitchell 1991). These were the first two reports of coronatine-like phytotoxins outside of the genus *Pseudomonas*.

To obtain insight into the genetics of coronatine production in *Xanthomonas* and to expand genomic information on clade-1 xanthomonads, we determined the complete genome sequences of the pathotype strain CFBP 8444^{PT} (ICMP 4294^{PT}, LMG 702^{PT}, NCPPB 2983^{PT}), and of the species-level clade strains CFBP 8443 (NCPPB 2654) and CFBP 8445 (NCPPB 1131).

Genome sequencing and assembly

From lyophilized stocks available at CIRM-CFBP (<https://cirm-cfbp.fr/>), single colonies of the three strains were isolated and grown on peptone-sucrose (10 g/liter peptone, 10 g/liter sucrose, 1 g/liter glutamic acid) agar media. Genomic DNA was extracted using the Genomic DNA buffer set and Genomic-tips following the manufacturer's instructions (Qiagen, Valencia, CA, U.S.A.).

Multiplex library preparation in pools of eight strains, including strains CFBP 8443, CFBP 8444, and CFBP 8445, and simultaneous sequencing of eight strains on one SMRTCell was conducted with the PacBio Sequel technology at the GENTYANE genotyping platform (INRAE Clermont-Ferrand, France).

Sequence reads were *de novo* assembled with Flye 2.7 using the "--plasmids --iterations 2" parameters (Kolmogorov et al. 2019), yielding a single chromosome for each of the three strains. Genome sequences were functionally annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP version 6.2) (Li et al. 2021). The clade-1 xanthomonads genome project is available at NCBI as BioProject PRJNA865934. Annotation details are given in Table 1.

Phylogenetic relationships

To clarify the phylogenetic relationships within clade 1, including the three newly sequenced SLC strains, pairwise average nucleotide identities (ANI) were calculated on a Galaxy-implemented version of FastANI (Jain et al. 2018). This analysis included all publicly available genome sequences from clade 1, except for *X. translucens*, where only eight pathotype strains were chosen as representatives of the genetic diversity of this species due to the large number of sequenced *X. translucens* strains (Goettelmann et al. 2022). The evolutionary history was inferred using the UPGMA method (Sneath & Sokal 1973), as implemented in MEGA11 (Stecher et al. 2020; Tamura et al. 2021). This analysis involved 75 genome sequences and confirmed the taxonomic position of the three SLC strains within clade 1 (Figure 1; Supplemental Table 1).

Using a 95% cut-off value as an ANI species delimiter (Goris et al. 2007; Richter & Rosselló-Móra 2009), SLC-5 strain CFBP 8443 was found to be the only representative for this novel species. The SLC-6 strain CFBP 8444 and its clone NCPPB 2983 in the UK strain collection represent a sister species of *X. bonasiae* (Mafakheri et al. 2022). Three nearby strains in the dendrogram, GW, SI, and SS, which were isolated as candidate biocontrol agents from the microbiome of perennial ryegrass (*Lolium perenne*), were found to be phylogenetically close to the species *X. bonasiae* (Li et al. 2020). However, using digital DNA-DNA hybridization (dDDH) as a species delimiter suggests that strains GW, SI, and SS form another sister species of *X. bonasiae* (Supplemental Table 2) (Meier-Kolthoff et al. 2013, 2022). We therefore consider strain CFBP 8444 as the representative of a novel species, corresponding to SLC 6.

The SLC-7 strain CFBP 8445 is a clone of NCPPB 1131, for which a draft genome sequence was published previously (Studholme et al. 2011). These strains clustered with another genetically similar strain, LMG 8989, which was isolated from a *Citrus* plant (Bansal et al. 2020), in contrast to CFBP 8445 and NCPPB 1131, which were isolated from banana. Recently, non-pathogenic strains were isolated from healthy rice seeds. Genome sequencing revealed that two of the strains, PPL560 and PPL568, belong to a novel species, for which the name '*Xanthomonas indica*' was proposed (Rana et al. 2022). ANI and dDDH analyses demonstrate that these two strains belong to the same species-level clade as the four former strains and thus SLC 7 could be renamed '*X. indica*' (Figure 1, Supplemental Tables 1 and 2). Hence, this species-level clade comprises bacteria that can colonize both monocots and dicots. Whether such promiscuity also holds true for SLC 5 and SLC 6 strains awaits the characterization of additional isolates.

This comprehensive comparison of clade-1 strains suggests the existence of at least four additional novel species, as exemplified by strains (origin unknown), DD13 (from sugarcane), LMG 9002 (from orange), F4 (origin unknown), 3498 (origin unknown), LMG 8992 (from orange), and NCPPB 1128 (from common bean) (Figure 1; Supplemental

Tables 1 and 2) (Aritua et al. 2015; Bansal et al. 2020). Notably, for many isolates it is not clear whether they are pathogenic on their host of isolation or on other plants, or if they are regular constituents of the plant's microbiome, perhaps even with protective features. This genome-wide comparison challenges previous views of the phylogenetic relationships within clade-1 and supports recent findings based on comparing 466 core genes (Rana et al. 2022). Comparison of 16S rRNA sequences grouped *X. sacchari* and '*X. sontii*' in one subclade and *X. albilineans*, *X. hyacinthi*, *X. theicola*, and *X. translucens* in another subclade (Hauben et al. 1997; Bansal et al. 2021). In contrast, comparisons based on 16S–23S rDNA intergenic spacer sequences or on a portion of the gyrase B gene suggested that *X. albilineans*, '*X. pseudalbilineans*', *X. sacchari*, and '*X. sontii*' form one subclade and *X. hyacinthi*, *X. theicola*, and *X. translucens* belong to another subclade (Gonçalves and Rosato 2002; Parkinson et al. 2009; Khenfous-Djebari et al. 2019; Koebnik et al. 2021). Our genome-wide ANI comparison clearly separates *X. albilineans* and '*X. pseudalbilineans*' from the rest of the clade-1 strains, which correlates with the reduced genome size of these two species (average 3.7 Mbp for 20 strains) in comparison to the other 55 strains in our comparison (4.9 Mbp) (Pieretti et al. 2009; 2015).

Biosynthesis of coronatine-like compounds in *Xanthomonas*

The phytotoxin coronatine (COR) consists of two moieties, the polyketide coronafacic acid (CFA) and the cyclopropyl amino acid coronamic acid (CMA), which are conjugated by an amide bond. COR and COR-like molecules are produced by several pathovars of *Pseudomonas syringae*, but also some plant-pathogenic enterobacteria and streptomycetes (Bender et al. 1996; Slawiak & Lojkowska 2009; Fyans et al. 2015). In *P. syringae*, COR production is under control of the unconventional two-component system CorRPS, which mediates COR biosynthesis at 18 °C, whereas COR is not detectable at 28 °C (Smirnova et al. 2002). COR and COR-like molecules have been identified as structural mimics of the plant hormone jasmonoyl-L-isoleucine (JA-Ile) (Bender et al. 1999; Bown et al. 2017), which affects plant signaling pathways with importance for plant development and defense (Gimenez-Ibanez et al. 2015). Notably, COR was identified as the virulence factor in *P. syringae* that is responsible for suppressing stomatal innate immune defense (Melotto et al. 2006).

Because *X. campestris* pv. *phormiicola* was reported to synthesize coronatine-like molecules, we searched the three new genome sequences for homologs of the nine *cfa* genes (coronafacic acid biosynthesis), seven *cma* genes (coronamic acid biosynthesis), the *cfl* gene (coupling factor), and the three regulatory *cor* genes from *Pseudomonas syringae* pv. *tomato* strain DC3000 using TBLASTN. Homologs were only detected in strain CFBP 8444 for all nine Cfa sequences (between 66% and 92% sequence identity), with the Cfl sequence (64% sequence identity), and with the three Cor sequences (between 58% and 67% sequence identity). Of the Cma sequences, only CmaA and CmaT

had hits with 32% sequence identity in both cases, which were, however, not associated with coronamic acid biosynthesis.

We then further looked whether related genes are found in other bacteria, using the CFBP 8444 Cfa, Cfl and Cor amino acid sequences as queries. As expected, we found homologs for the whole biosynthetic gene set in species of *Pseudomonas*, in several enterobacteria (e.g. *Brenneria*, *Pectobacterium*, *Dickeya*, *Lonsdalea*), and in *Streptomyces*. However, the regulatory *cor* genes were only found in *Pseudomonas*. Surprisingly, we also identified close homologs of all these genes in two additional strains of *Xanthomonas*, annotated as *X. arboricola* strain 3058 and *X. campestris* strain 3075. According to the BioProject descriptions at NCBI (PRJNA583332, PRJNA583333), these strains originated from a study of plant-associated saprophytic bacteria and their role in plant health and plant-pathogen interactions. However, since these two *Xanthomonas* strains share a genome-wide ANI of 98.7%, they belong to the same species. Based on analyses at the Type Strain Genome Server (<https://tygs.dsmz.de/>) (Meier-Kolthoff & Göker 2019), both strains are predicted to belong to a hitherto undescribed species within clade 2 of *Xanthomonas*.

The regulatory *cor* genes were also identified in the genome sequence of *X. theicola*, strain CFBP 4691. However, none of the *cfa* or *cfl* genes were found. Since the Cor regulatory system serves as a thermosensor in *P. syringae* we speculate that biosynthesis of a COR-like substance is likely controlled in a similar way in *Xanthomonas*, but not in enterobacteria or *Streptomyces*, while other factors might be under thermal control in *X. theicola*.

A novel flagellar gene cluster in *Xanthomonas*

Xanthomonas has been proposed as a name for a group of non-sporing, rod-shaped, uni- or rarely biflagellate or non-motile Gram-negative bacteria, forming abundant yellow, slimy colonies on nutrient agar and potato, and mostly digesting starch and producing acid in lactose but not in salicin (Dowson 1939). Genomics has since confirmed the presence of a canonical gene cluster for flagella biosynthesis in almost all sequenced strains, regardless of the species. Yet, some strains have experienced deletion mutations in the flagellar gene cluster which rendered them non-motile on diagnostic media (Darrasse et al. 2013; Jacobs et al. 2015). All three genomes (Table 1) contained the canonical flagellar gene cluster, which has a very similar genomic organization as the one in clade-2 strains (Supplemental Figure 1).

Surprisingly, strain CFBP 8444 was found to possess a second gene cluster for the possible biosynthesis of alternative or additional flagella (Figure 3). This gene cluster is unique in the genus *Xanthomonas*. The only other *Xanthomonas* that was found to have a similar system, strain XNM01, has been misclassified and belongs to the sister genus *Pseudoxanthomonas* (Gowda et al. 2022). BLASTP searches revealed additional similar

homologs in other strains of *Pseudoxanthomonas*, in one strain of *Pseudomonas*, in several beta proteobacteria (mainly from the order Burkholderiales, but also from Neisseriales, Nitrosomonadales and Rhodocyclales) and in one strain of alpha proteobacteria (*Sphingomonas*). This atypical gene cluster contains a flagellin gene, named *fliC* or *lafA*, that is sometimes annotated as lateral flagellin. Overall, sequence similarity between the predicted CFBP 8444 flagellar proteins and their homologs is not very strong with less than 70% sequence identity for the LafA (FliC) protein and on average 64% sequence identity to the most similar system in strain XNM01.

Vibrio parahaemolyticus is another bacterium that has two types of flagella, which are both associated with a distinct cell types, the swimmer cell and the swarmer cell (McCarter & Silverman 1989). The swimmer cell is a short rod that harbors a single polar flagellum, which is produced when the bacterium is grown in liquid media. On semi-solid media, swarmer cells of *V. parahaemolyticus* are elongated and synthesize, in addition to the polar flagellum, numerous lateral flagella which are responsible for translocation over surfaces. Transition between swimmer and swarmer cells is mediated by contact with surfaces and involves the polar flagellum as a tactile sensor. In addition, iron limitation serves as a second signal that is required for swarmer cell differentiation (McCarter & Silverman 1989).

Mesophilic aeromonads are another group of bacteria that express a polar flagellum in all culture conditions, and certain strains produce lateral flagella on semisolid media or on surfaces (Canals et al. 2006). In addition to motility, lateral flagella of *Aeromonas* species have been found to mediate epithelial cell adherence and biofilm formation (Gavín et al. 2002). Later, homologs of the lateral flagellin were found in many beta proteobacteria, such as *Burkholderia dolosa* (Roux et al. 2018). Notably, a *lafA* deletion mutant was more motile under swimming conditions due to an increase in the number of polar flagella, but was not affected in biofilm formation, host cell invasion, or murine lung colonization or persistence over time. *Shewanella putrefaciens* is another species that possesses two separate flagellar systems (Kühn et al. 2022). Using functional fluorescence tagging it was found that screw thread-like motility is mediated by the primary, polar flagellum, and that the lateral flagella support spreading through constricted environments such as polysaccharide matrices.

Lateral-type flagellins were also found in the alpha proteobacterium *Sphingomonas* (Maruyama et al. 2015). *Sphingomonas* sp. strain A1, originally identified as a non-motile and aflagellate bacterium, possesses two sets of flagellar genes, consisting of 35 and 46 genes, respectively. The smaller set encodes the lateral-type flagellin, whereas the larger set includes two flagellar genes typical for polar flagella (Maruyama et al. 2015). Strain A1 cells became motile when they were cultured on semisolid media, due to the presence of a single flagellum at the cell pole, which surprisingly consisted of both lateral and non-lateral flagellins (Maruyama et al. 2015). Immunogold labeling later

demonstrated that strain A1 produces two types of flagellar filaments, one formed by three flagellins (lateral and non-lateral flagellins) and the other formed only by lateral flagellins (Kobayashi et al. 2016). Interestingly, lateral-type flagellins were found at the proximal end of the chimeric filaments, next to the hook basal body, whereas the polar flagellins were detected further apart from the cell surface (Kobayashi et al. 2016).

Given the low sequence conservation between the CFBP 8444 noncanonical flagellar genes and those in other proteobacteria, it will be interesting to study this phenomenon further. It remains to be explored whether this strain of *Xanthomonas* produces chimeric polar flagella or lateral flagella under certain conditions, and if so, which environmental clues trigger a switch between different types of flagella.

Conclusion

The first complete genome sequence for three undescribed species will stimulate further work on the underexplored clade-1 of xanthomonads. Strikingly, this work revealed the presence of a novel, noncanonical flagellar gene cluster that may produce lateral flagella, challenging the view of *Xanthomonas* as a unipolar flagellated bacterium. This work also presents the genetic basis for the production of COR-like molecules in the genus *Xanthomonas*. Since coronatine has been shown to stimulate re-opening of plant leaves' stomata during the infection process, it will be interesting to study the contribution of these molecules to pathogenicity. Notably, strain CFBP 8444, which synthesizes these COR-like molecules, does not produce type 3 effectors, which in other xanthomonads suppress stomatal immunity (Wang et al. 2021; Liu et al. 2022; Raffeiner et al. 2022).

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References

- Aritua, V., Harrison, J., Sapp, M., Buruchara, R., Smith, J., and Studholme, D. J. 2015. Genome sequencing reveals a new lineage associated with lablab bean and genetic exchange between *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas fuscans* subsp. *fuscans*. *Front. Microbiol.* 6:1080. doi: 10.3389/fmicb.2015.01080
- Bansal, K., Kaur, A., Midha, S., Kumar, S., Korpole, S., and Patil, P. B. 2021. *Xanthomonas sontii* sp. nov., a non-pathogenic bacterium isolated from healthy basmati rice (*Oryza sativa*) seeds from India. *Antonie Van Leeuwenhoek* 114:1935-1947. doi: 10.1007/s10482-021-01652-1
- Bansal, K., Kumar, S., and Patil, P. B. 2020. Phylogenomic insights into diversity and evolution of nonpathogenic *Xanthomonas* strains associated with citrus. *mSphere* 5:e00087-20. doi: 10.1128/mSphere.00087-20
- Bender, C. L., Alarcón-Chaidez, F., and Gross, D. C. 1999. *Pseudomonas syringae* phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. *Microbiol. Mol. Biol. Rev.* 63:266-292. doi: 10.1128/MMBR.63.2.266-292.1999
- Bender, C., Palmer, D., Peñaloza-Vázquez, A., Rangaswamy, V., and Ullrich, M. 1996. Biosynthesis of coronatine, a thermoregulated phytotoxin produced by the phytopathogen *Pseudomonas syringae*. *Arch. Microbiol.* 166: 71-75. doi: 10.1007/s002030050358
- Bown, L., Li, Y., Berru , F., Verhoeven, J. T. P., Dufour, S. C., and Bignell, D. R. D. 2017. Coronafacoyl phytotoxin biosynthesis and evolution in the common scab pathogen *Streptomyces scabiei*. *Appl. Environ. Microbiol.* 83:e01169-17. doi: 10.1128/AEM.01169-17
- Canals, R., Altarriba, M., Vilches, S., Horsburgh, G., Shaw, J. G., Tom s, J. M., and Merino, S. 2006. Analysis of the lateral flagellar gene system of *Aeromonas hydrophila* AH-3. *J. Bacteriol.* 188:852-862. doi: 10.1128/JB.188.3.852-862.2006
- Darrasse, A., Carr re, S., Barbe, V., Boureau, T., Arrieta-Ortiz, M. L., Bonneau, S., Briand, M., Brin, C., Cociancich, S., Durand, K., Fouteau, S., Gagnevin, L., Gu rin, F., Guy, E., Indiana, A., Koebnik, R., Lauber, E., Munoz, A., No l, L. D., Pieretti, I., Poussier, S., Pruvost, O., Rob ne-Soustrade, I., Rott, P., Royer, M., Serres-Giardi, L., Szurek, B., van Sluys, M. A., Verdier, V., Verni re, C., Arlat, M., Manceau, C., and Jacques M. A. 2013. Genome sequence of *Xanthomonas fuscans* subsp. *fuscans* strain

- 4834-R reveals that flagellar motility is not a general feature of xanthomonads. *BMC Genomics* 14:761. doi: 10.1186/1471-2164-14-761
- Dia, N. C., Van Vaerenbergh, J., Van Malderghem, C., Blom, J., Smits, T. H. M., Cottyn, B., and Pothier, J.F. 2021. *Xanthomonas hydrangeae* sp. nov., a novel plant pathogen isolated from *Hydrangea arborescens*. *Int. J. Syst. Evol. Microbiol.*, in press. <https://doi.org/10.1099/ijsem.0.005163>
- Dowson, W. J. 1939. On the systematic position and generic names of the Gram-negative bacterial plant pathogens. *Zentralbl. Bakteriol. Parasitenk. Infektionskr. Hyg. Abt. II* (1939) 100:177-193.
- Ferreira-Tonin, M., Rodrigues-Neto, J., Harakava, R., and Destéfano, S. A. 2012. Phylogenetic analysis of *Xanthomonas* based on partial *rpoB* gene sequences and species differentiation by PCR-RFLP. *Int. J. Syst. Evol. Microbiol.* 62:1419-1424. doi: 10.1099/ijse.0.028977-0
- Fyans, J. K., Altowairish, M. S., Li, Y., and Bignell, D. R. 2015. Characterization of the coronatine-like phytotoxins produced by the common scab pathogen *Streptomyces scabies*. *Mol. Plant Microbe Interact.* 28:443-454. doi: 10.1094/MPMI-09-14-0255-R
- Gavín, R., Rabaan, A. A., Merino, S., Tomás, J. M., Gryllos, I., and Shaw, J. G. 2002. Lateral flagella of *Aeromonas* species are essential for epithelial cell adherence and biofilm formation. *Mol. Microbiol.* 43:383-397. doi: 10.1046/j.1365-2958.2002.02750.x
- Giblot-Ducray, D., Marefat, A., Gillings, M. R., Parkinson, N. M., Bowman, J. P., Ophel-Keller, K., Taylor, C., Facelli, E., and Scott, E. S. 2009. Proposal of *Xanthomonas translucens* pv. *pistaciae* pv. nov., pathogenic to pistachio (*Pistacia vera*). *Syst. Appl. Microbiol.* 32:549-557. doi: 10.1016/j.syapm.2009.08.001
- Gilchrist, C. L. M., and Chooi, Y. H. 2021. Clinker & clustermap.js: Automatic generation of gene cluster comparison figures. *Bioinformatics*, in press. <https://doi.org/10.1093/bioinformatics/btab007>
- Gimenez-Ibanez, S., Boter, M., and Solano, R. 2015. Novel players fine-tune plant trade-offs. *Essays Biochem.* 58:83-100. doi: 10.1042/bse0580083
- Goettelmann, F., Roman-Reyna, V., Cunnac, S., Jacobs, J. M., Bragard, C., Studer, B., Koebnik, R., and Kölliker, R. 2022. Complete genome assemblies of all *Xanthomonas translucens* pathotype strains reveal three genetically distinct clades. *Front. Microbiol.* 12:817815. doi: 10.3389/fmicb.2021.817815

- Gonçalves, E. R., and Rosato, Y. B. 2002. Phylogenetic analysis of *Xanthomonas* species based upon 16S-23S rDNA intergenic spacer sequences. *Int. J. Syst. Evol. Microbiol.* 52:355-361. doi: 10.1099/00207713-52-2-355
- Goris, J., Konstantinidis, K. T., Klappenbach, J. A., Coenye, T., Vandamme, P., and Tiedje, J. M. 2007. DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. *Int. J. Syst. Evol. Microbiol.* 57:81-91. doi: 10.1099/ijs.0.64483-0
- Gowda, K., Ping, D., Mani, M., and Kuehn, S. 2022. Genomic structure predicts metabolite dynamics in microbial communities. *Cell* 185:530-546.e25. doi: 10.1016/j.cell.2021.12.036
- Hauben, L., Vauterin, L., Swings, J., and Moore, E. R. 1997. Comparison of 16S ribosomal DNA sequences of all *Xanthomonas* species. *Int. J. Syst. Bacteriol.* 47:328-335. doi: 10.1099/00207713-47-2-328
- Jacques, M. A., Arlat, M., Boulanger, A., Boureau, T., Carrère, S., Cesbron, S., Chen, N. W., Cociancich, S., Darrasse, A., Denancé, N., Fischer-Le Saux, M., Gagnevin, L., Koebnik, R., Lauber, E., Noël, L. D., Pieretti, I., Portier, P., Pruvost, O., Rieux, A., Robène, I., Royer, M., Szurek, B., Verdier, V., and Vernière, C. 2016. Using ecology, physiology, and genomics to understand host specificity in *Xanthomonas*. *Annu. Rev. Phytopathol.* 54:163-187. doi: 10.1146/annurev-phyto-080615-100147
- Jacobs, J. M., Pesce, C., Lefeuvre, P., and Koebnik, R. 2015. Comparative genomics of a cannabis pathogen reveals insight into the evolution of pathogenicity in *Xanthomonas*. *Front. Plant Sci.* 6:431. doi: 10.3389/fpls.2015.00431
- Jain, C., Rodriguez, R. L., Phillippy, A. M., Konstantinidis, K. T., and Aluru, S. 2018. High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. *Nat. Commun.* 9:5114. doi: 10.1038/s41467-018-07641-9
- Khenfous-Djebari, B., Kerkoud, M., Fischer-Le Saux, M., Benhassine, T., Bouznad, Z., Koebnik, R., and Bragard, C. 2019. Isolation of atypical wheat-associated xanthomonads in Algeria. *Phytopathologia Mediterranea* 58:497-506. doi: 10.14601/Phyto-10855
- Kobayashi, M., Konishi, H., Maruyama, Y., Murata, K., and Hashimoto, W. 2016. Lateral-typed flagellin responsible for formation of a polar flagellum but not of lateral flagella in *Sphingomonas* sp. strain A1. *Microbiology (Reading)* 162:2042-2052. doi: 10.1099/mic.0.000385

- Koebnik, R., Burokiene, D., Bragard, C., Chang, C., Fischer-Le Saux, M., Kölliker, R., Lang, J. M., Leach, J. E., Luna, E. K., Portier, P., Sagia, A., Ziegler, J., Cohen, S. P., and Jacobs, J. M. 2021. The complete genome sequence of *Xanthomonas theicola*, the causal agent of canker on tea plants, reveals novel secretion systems in clade-1 xanthomonads. *Phytopathology* 111:611-616. doi: 10.1094/PHYTO-07-20-0273-SC
- Kolmogorov, M., Yuan, J., Lin, Y., and Pevzner, P. A. Assembly of long, error-prone reads using repeat graphs. *Nat. Biotechnol.* 37:540-546. doi: 10.1038/s41587-019-0072-8
- Kühn, M. J., Edelmann, D. B., and Thormann, K. M. 2022. Polar flagellar wrapping and lateral flagella jointly contribute to *Shewanella putrefaciens* environmental spreading. *Environ. Microbiol.*, in press. <https://doi.org/10.1111/1462-2920.16107>
- Letunic, I., and Bork, P. 2019. Interactive Tree Of Life (iTOL) v4: recent updates and new developments. *Nucleic Acids Res.* 47:W256-W259. doi: 10.1093/nar/gkz239
- Li, T., Mann, R., Sawbridge, T., Kaur, J., Auer, D., and Spangenberg, G. 2020. Novel *Xanthomonas* species from the perennial ryegrass seed microbiome - assessing the bioprotection activity of non-pathogenic relatives of pathogens. *Front. Microbiol.* 11:1991. doi: 10.3389/fmicb.2020.01991
- Li, W., O'Neill, K. R., Haft, D. H., DiCuccio, M., Chetvernin, V., Badretdin, A., Coulouris, G., Chitsaz, F., Derbyshire, M. K., Durkin, A. S., Gonzales, N. R., Gwadz, M., Lanczycki, C. J., Song, J. S., Thanki, N., Wang, J., Yamashita, R. A., Yang, M., Zheng, C., Marchler-Bauer, A., and Thibaud-Nissen, F. 2021. RefSeq: expanding the Prokaryotic Genome Annotation Pipeline reach with protein family model curation. *Nucleic Acids Res.* 49:D1020-D1028. doi: 10.1093/nar/gkaa1105
- Liu, L., Li, Y., Xu, Z., Chen, H., Zhang, J., Manion, B., Liu, F., Zou, L., Fu, Z. Q., and Chen, G. 2022. The *Xanthomonas* type III effector XopAP prevents stomatal closure by interfering with vacuolar acidification. *J. Integr. Plant Biol.*, in press. <https://doi.org/10.1111/jipb.13344>
- López, M. M., Lopez-Soriano, P., Garita-Cambronero, J., Beltrán, C., Taghouti, G., Portier, P., Cubero, J., Fischer-Le Saux, M., and Marco-Noales, E. 2018. *Xanthomonas prunicola* sp. nov., a novel pathogen that affects nectarine (*Prunus persica* var. nectarina) trees. *Int. J. Syst. Evol. Microbiol.* 68:1857-1866. doi: 10.1099/ijsem.0.00274

- Mafakheri, H., Taghavi, S. M., Zarei, S., Portier, P., Dimkić, I., Koebnik, R., Kuzmanović, N., and Osdaghi, E. 2022. *Xanthomonas bonasiae* sp. nov. and *Xanthomonas youngii* sp. nov., isolated from crown gall tissues. Int. J. Syst. Evol. Microbiol., in press. doi: 10.1099/ijsem.0.005418
- Mansfield, J., Genin, S., Magori, S., Citovsky, V., Sriariyanum, M., Ronald, P., Dow, M., Verdier, V., Beer, S. V., Machado, M. A., Toth, I., Salmond, G., and Foster, G. D. 2012. Top 10 plant pathogenic bacteria in molecular plant pathology. Mol. Plant Pathol. 13:614-629. doi: 10.1111/j.1364-3703.2012.00804.x
- Martins, L., Fernandes, C., Blom, J., Dia, N. C., Pothier, J. F., and Tavares, F. 2020. *Xanthomonas euroxanthea* sp. nov., a new xanthomonad species including pathogenic and non-pathogenic strains of walnut. Int. J. Syst. Evol. Microbiol. 70:6024-6031. doi: 10.1099/ijsem.0.004386
- Maruyama, Y., Kobayashi, M., Murata, K., and Hashimoto, W. 2015. Formation of a single polar flagellum by two distinct flagellar gene sets in *Sphingomonas* sp. strain A1. Microbiology (Reading) 161:1552-1560. doi: 10.1099/mic.0.000119
- McCarter, L., and Silverman, M. 1989. Iron regulation of swarmer cell differentiation of *Vibrio parahaemolyticus*. J. Bacteriol. 171:731-736. doi: 10.1128/jb.171.2.731-736.1989
- Meier-Kolthoff, J. P., Auch, A. F., Klenk, H.-P. and Göker, M. 2013. Genome sequence-based species delimitation with confidence intervals and improved distance functions. BMC Bioinformatics 14:60. doi: 10.1186/1471-2105-14-60
- Meier-Kolthoff, J. P., Carbasse, J. S., Peinado-Olarte, R. L., and Göker, M. 2022. TYGS and LPSN: a database tandem for fast and reliable genome-based classification and nomenclature of prokaryotes. Nucleic Acids Res. 50:D801-D807. doi: 10.1093/nar/gkab902
- Meier-Kolthoff, J. P., and Göker, M. 2019. TYGS is an automated high-throughput platform for state-of-the-art genome-based taxonomy. Nat. Commun. 10:2182. doi: 10.1038/s41467-019-10210-3
- Melotto, M., Underwood, W., Koczan, J., Nomura, K., and He, S. Y. 2006. Plant stomata function in innate immunity against bacterial invasion. Cell 126:969-980. doi: 10.1016/j.cell.2006.06.054
- Mitchell, R. E. 1991. Coronatine analogues produced by *Xanthomonas campestris* pv *phormiicola*. Phytochemistry 30:3917-3920. doi: 10.1016/0031-9422(91)83434-M

- Parkinson, N., Aritua, V., Heeney, J., Cowie, C., Bew, J., and Stead, D. 2007. Phylogenetic analysis of *Xanthomonas* species by comparison of partial gyrase B gene sequences. *Int. J. Syst. Evol. Microbiol.* 57:2881-2887. doi: 10.1099/ijms.0.65220-0
- Parkinson, N., Cowie, C., Heeney, J., and Stead, D. 2009. Phylogenetic structure of *Xanthomonas* determined by comparison of *gyrB* sequences. *Int. J. Syst. Evol. Microbiol.* 59:264-274. doi: 10.1099/ijms.0.65825-0
- Parte, A. C., Sardà Carbasse, J., Meier-Kolthoff, J. P., Reimer, L. C., and Göker, M. 2020. List of Prokaryotic names with Standing in Nomenclature (LPSN) moves to the DSMZ. *Int. J. Syst. Evol. Microbiol.* 70:5607-5612. doi: 10.1099/ijsem.0.004332
- Pieretti, I., Cociancich, S., Bolot, S., Carrère, S., Morisset, A., Rott, P., and Royer, M. 2015. Full genome sequence analysis of two isolates reveals a novel *Xanthomonas* species close to the sugarcane pathogen *Xanthomonas albilineans*. *Genes (Basel)* 6:714-733. doi: 10.3390/genes6030714
- Pieretti, I., Royer, M., Barbe, V., Carrere, S., Koebnik, R., Cociancich, S., Couloux, A., Darrasse, A., Gouzy, J., Jacques, M. A., Lauber, E., Manceau, C., Mangenot, S., Poussier, S., Segurens, B., Szurek, B., Verdier, V., Arlat, M., and Rott, P. 2009. The complete genome sequence of *Xanthomonas albilineans* provides new insights into the reductive genome evolution of the xylem-limited *Xanthomonadaceae*. *BMC Genomics* 10:616. doi: 10.1186/1471-2164-10-616
- Raffener, M., Üstün, S., Guerra, T., Spinti, D., Fitzner, M., Sonnewald, S., Baldermann, S., and Börnke, F. 2022. The *Xanthomonas* type-III effector XopS stabilizes CaWRKY40a to regulate defense responses and stomatal immunity in pepper (*Capsicum annuum*). *Plant Cell* 34:1684-1708. doi: 10.1093/plcell/koac032
- Rana, R., Madhavan, V. N., Saroha, T., Bansal, K., Kaur, A., Sonti, R. V., Patel, H. K., and Patil, P. B. 2022. *Xanthomonas indica* sp. nov., a novel member of non-pathogenic *Xanthomonas* community from healthy rice seeds. *Curr. Microbiol.* 79:304. doi: 10.1007/s00284-022-03001-5
- Richter, M., and Rosselló-Móra, R. 2009. Shifting the genomic gold standard for the prokaryotic species definition. *Proc. Natl. Acad. Sci. U. S. A.* 106:19126-19131. doi: 10.1073/pnas.0906412106
- Roux, D., Schaefers, M., Clark, B. S., Weatherholt, M., Renaud, D., Scott, D., LiPuma, J. J., Priebe, G., Gerard, C., and Yoder-Himes, D. R. 2018. A putative lateral flagella of the cystic fibrosis pathogen *Burkholderia dolosa* regulates swimming motility and

- host cytokine production. PLoS One 13:e0189810. doi: 10.1371/journal.pone.0189810
- Slawiak, M., and Lojkowska, E. 2009. Genes responsible for coronatine synthesis in *Pseudomonas syringae* present in the genome of soft rot bacteria. Eur. J. Plant Pathol. 124:353-361. doi: 10.1007/s10658-008-9418-7
- Smirnova, A. V., Wang, L., Rohde, B., Budde, I., Weingart, H., and Ullrich, M. S. 2002. Control of temperature-responsive synthesis of the phytotoxin coronatine in *Pseudomonas syringae* by the unconventional two-component system CorRPS. J. Mol. Microbiol. Biotechnol. 4:191-196.
- Sneath, P. H. A., and Sokal, R. R. 1973. Numerical Taxonomy. Freeman, San Francisco.
- Stecher, G., Tamura, K., and Kumar, S. 2020. Molecular Evolutionary Genetics Analysis (MEGA) for macOS. Mol. Biol. Evol. 37:1237-1239. doi: 10.1093/molbev/msz312
- Studholme, D. J., Wasukira, A., Paszkiewicz, K., Aritua, V., Thwaites, R., Smith, J., and Grant, M. 2011. Draft genome sequences of *Xanthomonas sacchari* and two banana-associated xanthomonads reveal insights into the *Xanthomonas* group 1 clade. Genes (Basel) 2:1050-1065. doi: 10.3390/genes2041050
- Takimoto, S. 1933. Bacterial diseases of New Zealand flax. Byogaichu zasshi 20:774-778.
- Tamura, K., Stecher, G., and Kumar, S. 2021. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. Mol. Biol. Evol. 38:3022-3027. doi: 10.1093/molbev/msab120
- Tamura, K., Takikawa, Y., Tsuyumu, S., Goto, M. and Kijima, T. 1987. Coronatine-like substance produced by *Xanthomonas campestris* pv. *phormiicola*. Ann. Phytopath. Soc. Japan 53:72-73.
- Tamura, K., Takikawa, Y., Tsuyumu, S., Goto, M., and Watanabe, M. 1992. Coronatine production by *Xanthomonas campestris* pv. *phormiicola*. Ann. Phytopath. Soc. Japan 58: 276-281.
- Vicente, J. G., Rothwell, S., Holub, E. B., and Studholme, D. J. 2017. Pathogenic, phenotypic and molecular characterisation of *Xanthomonas nasturtii* sp. nov. and *Xanthomonas floridensis* sp. nov., new species of *Xanthomonas* associated with watercress production in Florida. Int. J. Syst. Evol. Microbiol. 67:3645-3654. doi: 10.1099/ijsem.0.002189

- Wang, S., Li, S., Wang, J., Li, Q., Xin, X. F., Zhou, S., Wang, Y., Li, D., Xu, J., Luo, Z. Q., He, S. Y., and Sun, W. 2021. A bacterial kinase phosphorylates OSK1 to suppress stomatal immunity in rice. *Nat. Commun.* 12:5479. doi: 10.1038/s41467-021-25748-4
- Zain, B. S., and Roberts, R. J. 1977. A new specific endonuclease from *Xanthomonas badrii*. *J. Mol. Biol.* 115:249-255. doi: 10.1016/0022-2836(77)90101-2

Table 1. BioSamples, GenBank accession numbers and annotation details for the clade-1 xanthomonads genome BioProject PRJNA865934.

Strain	CFBP 8443	CFBP 8444	CFBP 8445
Species-level clade	SLC 5	SLC 6	SLC 7
NCBI BioSample	SAMN30147989	SAMN30148166	SAMN30161540
NCBI GenBank accession number (chromosomes)	CP102592	CP102593	CP102594
Coverage	200 x	244 x	208 x
Size (bp)	5,238,480	5,314,063	4,869,984
G+C content (%)	68.96	68.64	69.25
Predicted genes	4,368	4,506	4,192
Predicted CDS	4,303	4,441	4,122
Predicted RNA genes	65	65	70
Predicted pseudogenes	44	72	30

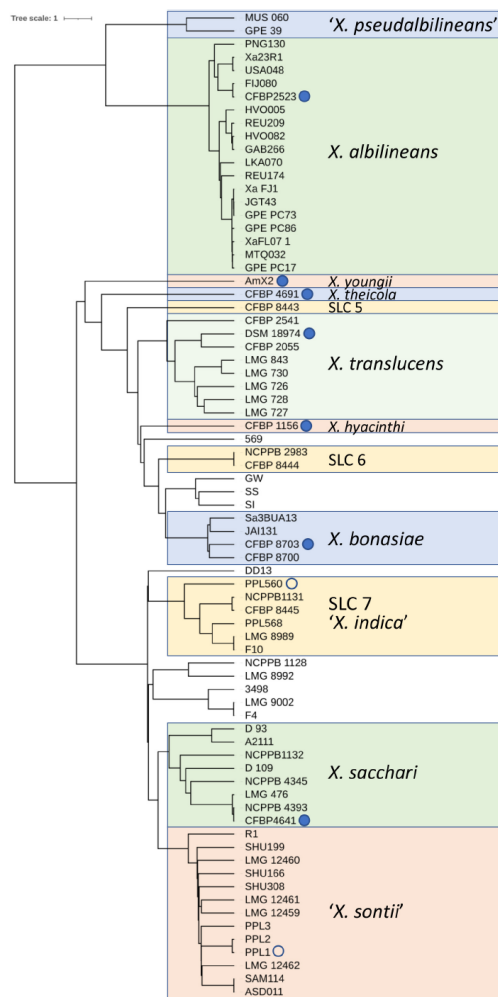


Figure 1. Phylogenetic tree of clade-1 xanthomonads. The evolutionary distances between genome sequences were computed using the FastANI method (Jain et al. 2018) and are in units of percent sequence divergence. The evolutionary history was inferred using the UPGMA method (Sneath & Sokal 1973). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. This analysis involved 75 genome sequences. Evolutionary analyses were conducted in MEGA11 (Stecher et al. 2020; Tamura et al. 2021), and the tree layout was enhanced for better visualization using the iTOL online tool (Letunic & Bork 2019). Names of bacterial strains are indicated next to the branch endpoints. Strains belonging to the same species are boxed with the name of the species given within the colored box. Type strains are labeled with a blue dot, and proposed type strains are labeled with a blue circle. Used genome sequences are listed in Supplemental Table 1.

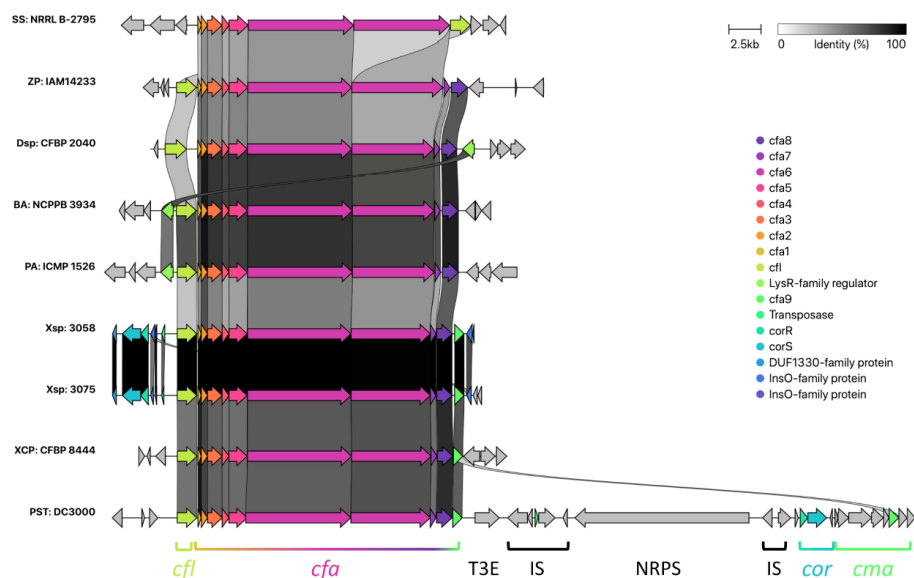


Figure 2. Comparison of bacterial coronatine biosynthesis gene clusters. Corresponding regions, extracted from GenBank files, were compared and visualized with clinker (Gilchrist & Chooi 2021). The following strains and genomes were analyzed (from top to bottom): *Streptomyces scabiei* strain NRRL B-2795 (JAGJBZ010000001), *Zymobacter palmae* strain IAM14233 (AP018933), *Dickeya* sp. strain CFBP 2040 (JAAVXH010000024), *Brenneria alni* strain NCPPB 3934 (MJLZ010000030), *Pectobacterium atrosepticum* strain ICMP 1526 (ALIV010000029), *Xanthomonas* sp. strain 3058 (JACHOF010000019), *Xanthomonas* sp. strain 3075 (JACIFI010000014), *Xanthomonas campestris* pv. *phormiicola* strain CFBP 8444 (CP102593), and *Pseudomonas syringae* pv. *tomato* strain DC3000 (AE016853). Genes and operons in strain DC3000 are labelled at the bottom. T3E = type 3 effector; NRPS = non-ribosomal polyketide synthase; IS = insertion sequence element.

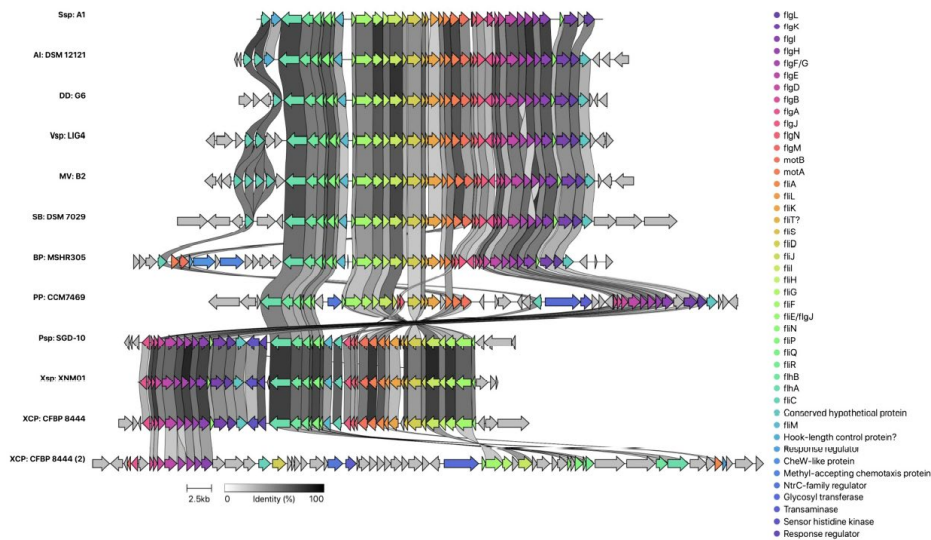


Figure 3. Comparison of flagellar gene clusters. Corresponding regions, extracted from GenBank files, were compared and visualized with clinker (Gilchrist & Chooi 2021). The following strains and genomes were analyzed (from top to bottom): *Sphingomonas* sp. strain A1 (LC043068), *Azoarcus indigenus* strain DSM 12121 (SNVV01000001), *Dechloromonas denitrificans* strain G6 (CP075186), *Vogesella* sp. strain LIG4 (LT607802), *Massilia violaceinigra* strain B2 (CP024608), *Schlegelella brevitalea* strain DSM 7029 (CP011371), *Pseudomonas panipatensis* strain CCM 7469 (FNDS01000003), *Pseudoxanthomonas* sp. strain SGD-10 (RRYL01000001), *Xanthomonas* sp. strain XNM01 (JACVAP010000008), and *Xanthomonas campestris* pv. *phormiicola* strain CFBP 8444 (CP102593). Strain CFBP 8444 contains two flagellar gene clusters, with the novel cluster above the canonical cluster (2), which is shown at the bottom.

e-Xtra Supplemental Table 1. Heatmap of pairwise genome-wide average nucleotide identities (ANI) between clade-1 xanthomonads. Genome sequences were downloaded from GenBank (accession numbers given in row 3) and pairwise ANI were calculated on a Galaxy-implemented version of FastANI (Jain et al. 2018). This analysis included all publicly available genome sequences from clade 1, except for *X. translucens*, where only eight pathotype strains were chosen as representatives of the genetic diversity of this species (Goettelmann et al. 2022). Names of strains are given in the first column and in the second row. The first row indicates the respective *Xanthomonas* species. SLC, species-level clade (Parkinson et al. 2009).

e-Xtra Supplemental Table 2. Pairwise genome-wide average nucleotide identities (ANI) and digital DNA-DNA hybridization (dDDH) values of selected clade-1 xanthomonads.

Pairwise average nucleotide identities (ANI) were calculated on a Galaxy-implemented version of FastANI (Jain et al. 2018). ANI values are given in the upper triangle of the comparison matrix, in blue color. Digital DNA-DNA hybridization values were calculated at the TYGS/GGDC platform

(<https://ggdc.dsmz.de>) (Meier-Kolthoff et al. 2013, 2022). GLM-based DDH estimate, as estimated by the recommended formula 2 (identities / HSP length), are given in the lower triangle of the comparison matrix, in red color. Cells with values that are considered to indicate that the two compared genomes belong to strains of the same species are highlighted in green. Cells with values that are in the 'grey zone' of species delineation are in light green. GenBank accession numbers given in row 3.

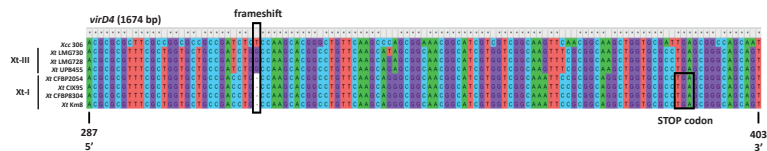
e-Xtra Supplemental Figure 1. Comparison of canonical flagellar gene clusters.

Corresponding regions, extracted from GenBank files, were compared and visualized with clinker (Gilchrist & Chooi 2021). The following strains and genomes were analyzed (from top to bottom): *Xanthomonas* sp. strain CFBP 8443 (CP102592), *Xanthomonas campestris* pv. *phormiicola* strain CFBP 8444 (CP102593), *Xanthomonas* sp. strain CFBP 8445 (CP102594), and *Xanthomonas euvesicatoria* pv. *euvesicatoria* strain 85-10 (AM039952).

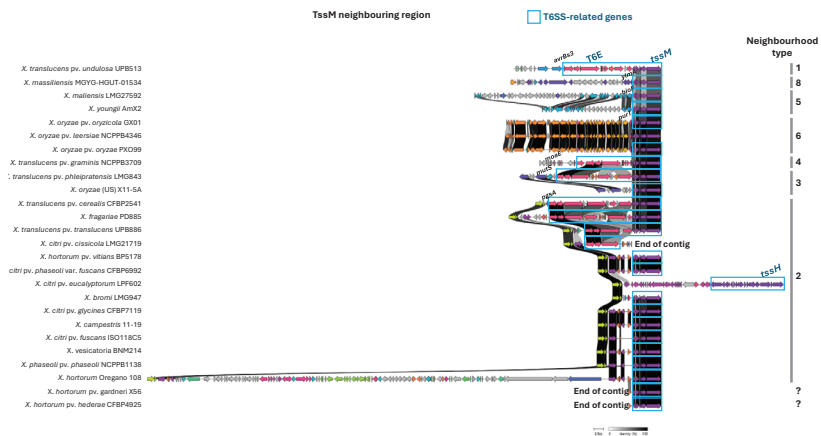
Appendix 2

Supplementary material pertaining to Chapter 2 can be found here in Appendix 2.

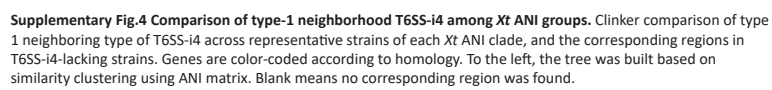
Supplementary Information



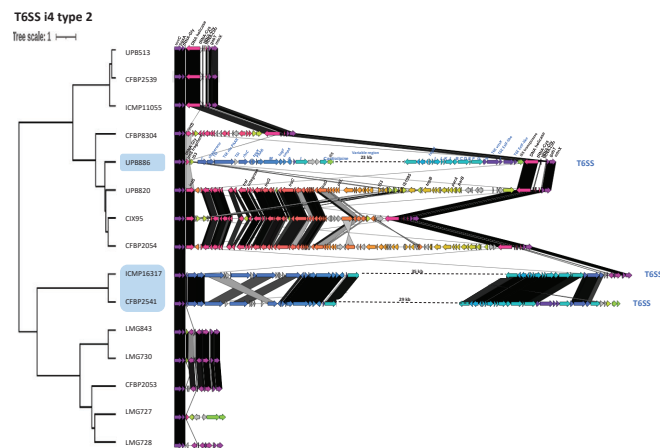
Supplementary Fig.1 Nucleotide alignment of *virD4* sequences. The alignment shows a single base pair deletion in members of the Xt-I ANI group, resulting in a premature stop codon on the mRNA.



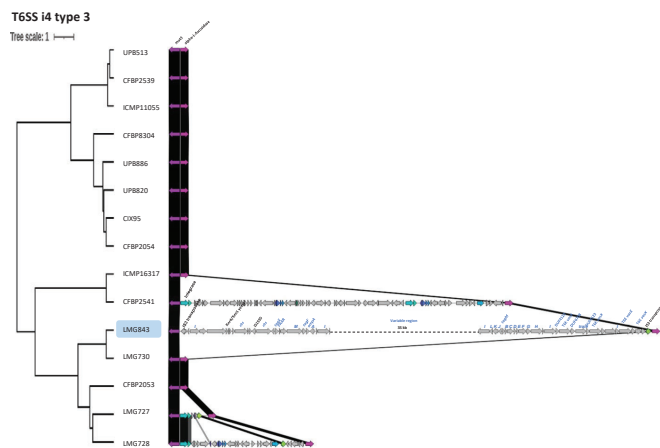
Supplementary Fig.2 TssM neighboring region and respective neighborhood types. Clinker comparison of neighboring regions near *tssM* gene from T6SS-i4 across the selected type and pathotype *Xanthomonas* strains harboring T6SS-i4. T6SS-related genes are framed in blue. Genes are color-coded according to homology.



Supplementary Fig.4 Comparison of type-1 neighborhood T6SS-i4 among Xt ANI groups. Clinker comparison of type 1 neighboring type of T6SS-i4 across representative strains of each Xt ANI clade, and the corresponding regions in T6SS-i4-lacking strains. Genes are color-coded according to homology. To the left, the tree was built based on similarity clustering using ANI matrix. Blank means no corresponding region was found.

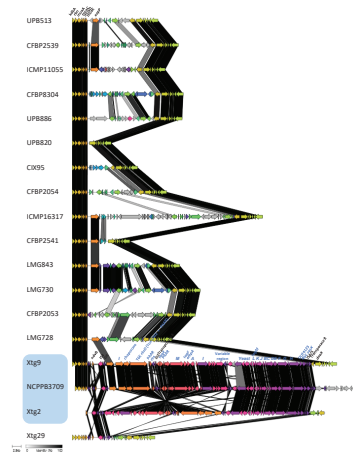


Supplementary Fig.5 Comparison of type-2 neighborhood T6SS-i4 among *Xt* ANI groups. Clinker comparison of type 2 neighboring type of T6SS-i4 across representative strains of each *Xt* ANI clade, and the corresponding regions in T6SS-i4-lacking strains. Genes are color-coded according to homology. To the left, the tree was built based on similarity clustering using ANI matrix.

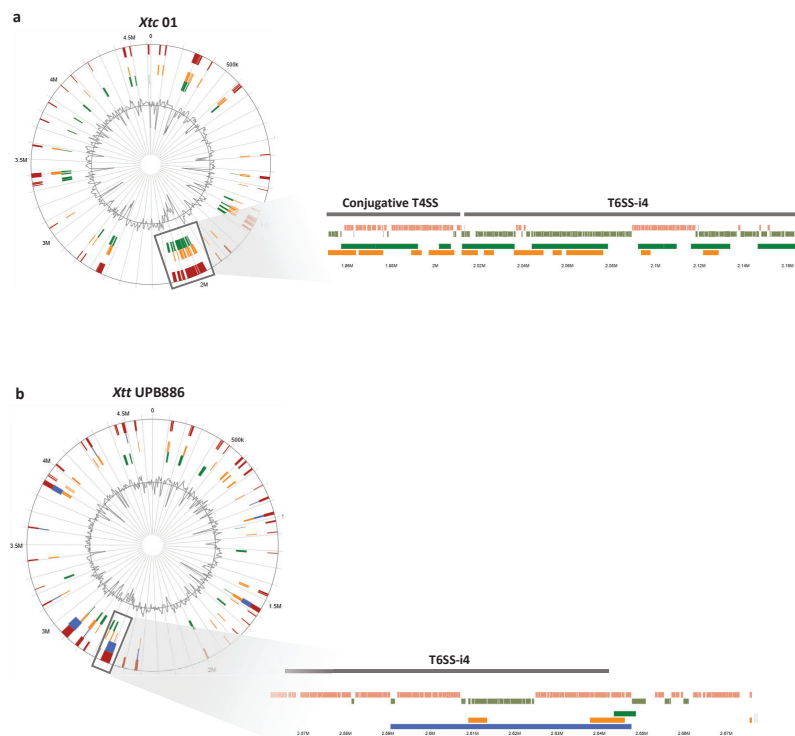


Supplementary Fig.6 Comparison of type-3 neighborhood T6SS-i4 among *Xt* ANI groups. Clinker comparison of type 3 neighboring type of T6SS-i4 across representative strains of each *Xt* ANI clade, and the corresponding regions in T6SS-i4-lacking strains. Genes are color-coded according to homology. To the left, the tree was built based on similarity clustering using ANI matrix.

T6SS i4 type 4



Supplementary Fig.7 Comparison of type-4 neighborhood T6SS-i4 among *Xt* ANI groups. Clinker comparison of type 4 neighboring type of T6SS-i4 across representative strains of each *Xt* ANI clade, and the corresponding regions in T6SS-i4-lacking strains. Genes are color-coded according to homology.



Supplementary Fig.8 Genomic islands in (a) *Xtc* strain 01 and (b) *Xtt* strain UPB886. Genome sequences were scrutinized for genomic islands using IslandViewer4. (Left) Circular representation of genome with the predicted T6SS-i4 GI shown in grey box. (Right) Linear map of the detected GI including conjugative T4SS and/or T6SS-i4 gene clusters.

[illegible]

Supplementary Fig.9 Xt ANI matrix

Supplementary Table 1. T6SS-i4 effector arsenals in xanthomonads. Similar proteins are highlighted by similar background colors. NF = Not found

Species	Strain	Secretion system	YggG/ PAAR GenBank acc. no. <i>(Predicted effector domains by InterPro)</i>	T6SS effector GenBank acc. no. <i>(Predicted effector domains by InterPro)</i>	Remarks
<i>X. translucens</i> pv. <i>graminis</i>	NCPP93709	No T6SS effector			
<i>X. translucens</i> pv. <i>graminis</i>	NCPP93709	T6SS-14	WP_074375434.1 (YggG)	WP_081374346.1 (PAAR_4 - DUF6331; YD repeat - RNS repeat-associated core domain - Tss-L302 domain)	
<i>X. translucens</i> pv. <i>graminis</i>	NCPP93709		WP_074375705.1 (YggG - DUF 2345)	WP_141134432.1 (Toxin VacA - Mtx_H)	
<i>X. translucens</i> pv. <i>graminis</i>	NCPP93709		WP_053113825.1 (YggG - DUF 2345)	NF	YggG pseudogenized
<i>X. translucens</i> pv. <i>graminis</i>	NCPP93709		WP_053113825.1 (YggG - DUF 2345)	WP_020599869.3 (Toxin VacA - Mtx_H)	
<i>X. translucens</i> pv. <i>graminis</i>	NCPP93709		WP_245202050.1 (YggG - DUF 2345)	WP_020599869.3 (Phosphotransfer effector Tss1-like)	
<i>X. translucens</i> pv. <i>phila</i>	LMG170	No T6SS effector			
<i>X. translucens</i> pv. <i>phlogorotensis</i>	LMG843	T6SS-14	WP_237653385.1 (YggG)	WP_237653387.1 (PAAR_4 - DUF6331; YD repeat - RNS repeat-associated core domain; RNS/Toxin YD repeat-associated toxin with a Tss-L302 domain)	
<i>X. translucens</i> pv. <i>phlogorotensis</i>	LMG843		WP_237653407.1 (YggG - DUF 2345)	WP_237653409.1 (Toxin VacA - Mtx_H)	
<i>X. translucens</i> pv. <i>phlogorotensis</i>	LMG843		WP_237653384.1 (YggG - DUF 2345)	WP_237653386.3 (Phosphotransfer effector Tss1-like)	
<i>X. translucens</i> pv. <i>phlogorotensis</i>	LMG843		WP_237653382.1 (YggG - DUF 2345; PGSD-like)	WP_237653381.1 (PGSD-like)	
<i>X. translucens</i> pv. <i>phlogorotensis</i>	LMG843		WP_237653387.1 (YggG - DUF 2345)	WP_237653389.1 (Phosphotransfer effector Tss1-like)	
<i>X. translucens</i> pv. <i>phlogorotensis</i>	LMG843		WP_237653386.1 (YggG - DUF 2345)	WP_237653379.1 (SoxH domain)	
<i>X. translucens</i> pv. <i>phlogorotensis</i>	LMG843		WP_237653425.1 (YggG - DUF 2345)	WP_237653416.1 (Toxin VacA - Mtx_H)	
<i>X. translucens</i> pv. <i>phlogorotensis</i>	LMG843		WP_237653768.1 (YggG - DUF 2345)	WP_237653431.1 (Toxin VacA - Mtx_H)	
<i>X. translucens</i> pv. <i>phlogorotensis</i>	LMG843		WP_237653747.1 (YggG - DUF 2345)	WP_237653376.1 (Chitinase class I)	
<i>X. translucens</i> pv. <i>phlogorotensis</i>	LMG843		WP_237653746.1 (YggG - DUF 2345)	WP_237653372.1 (M23_xylofuranase, lysozyme-like)	
<i>X. translucens</i> pv. <i>phlogorotensis</i>	LMG843		WP_083472089.1 (PAAR - CT_2)	NF	
<i>X. translucens</i> pv. <i>phlogorotensis</i>	LMG843		WP_237651687.1 (YggG - DUF 2345)	WP_237651659.1 (Toxin VacA - Mtx_H)	
<i>X. translucens</i> pv. <i>phlogorotensis</i>	LMG843		WP_237651848.1 (YggG)	WP_237651851.1 (Phosphotransfer effector Tss1-like)	
<i>X. translucens</i> pv. <i>phlogorotensis</i>	LMG843		WP_237651835.1 (YggG - DUF 2345)	WP_237651838.1 (Phosphotransfer effector Tss1-like)	
<i>X. translucens</i> pv. <i>phlogorotensis</i>	LMG843		WP_402176844.1 (YggG - DUF 2345)	NF	
<i>X. translucens</i> pv. <i>phlogorotensis</i>	LMG843		WP_237652907.1 (PAAR - CT_2)	WP_237652508.1 (T6SS Tss3 phosphotransfer effector domain - Antibacterial effector protein Tss3_C-terminal domain)	
<i>X. translucens</i> pv. <i>phlogorotensis</i>	LMG843		WP_237652414.1 (PAAR - CT_2)	WP_237652413.1 (Toxin VacA - Mtx_H)	
<i>X. translucens</i> pv. <i>translucens</i>	DS112014	T6SS-13	WP_054367648.1 (YggG)	WP_054367650.1 (DUF6331)	
<i>X. translucens</i> pv. <i>translucens</i>	UP8886	T6SS-14	WP_054369322.1 (YggG)	WP_153476826.1 (PAAR_4 - DUF6331; YD repeat - RNS repeat-associated core domain)	
<i>X. translucens</i> pv. <i>translucens</i>	UP8886		WP_054363071.1 (YggG)	WP_153476808.1 (PAAR_4 - DUF6331; YD repeat - RNS repeat-associated core domain)	
<i>X. translucens</i> pv. <i>translucens</i>	UP8886		WP_054360919.1 (YggG - DUF 2345)	WP_081087381.1 (Toxin VacA - Mtx_H)	T6E pseudogenized
<i>X. translucens</i> pv. <i>translucens</i>	UP8886		WP_153476803.1 (YggG - DUF 2345)	WP_274680781.1 (Toxin VacA - Mtx_H)	
<i>X. translucens</i> pv. <i>translucens</i>	UP8886		WP_235405050.1 (YggG - DUF 2345)	WP_153476802.1 (Chitinase class I)	
<i>X. translucens</i> pv. <i>translucens</i>	UP8886		WP_235405049.1 (YggG - DUF 2345)	WP_043404384.1 (PRX_NucleoX_NUC_DUF4122_RNA-like)	
<i>X. translucens</i> pv. <i>translucens</i>	UP8886		WP_235405044.1 (YggG - DUF 2345)	WP_128809992.1 (PSA7-like alpha/beta domain)	
<i>X. translucens</i> pv. <i>translucens</i>	UP8886		WP_235405036.1 (YggG - DUF 2345)	WP_043404384.1 (Phosphotransfer effector Tss1-like)	
<i>X. translucens</i> pv. <i>translucens</i>	UP8886	Orphan	WP_082746027.1 (PAAR - CT_2)	WP_043404384.1 (Toxin VacA - Mtx_H)	
<i>X. translucens</i> pv. <i>translucens</i>	UP8886		WP_153476753.1 (YggG - DUF 2345)	WP_054369322.1 (Phosphotransfer effector Tss1-like)	
<i>X. vesicatoria</i>	LMG911	T6SS-13	WP_005993965.1 (PAAR_2)	NF	
<i>X. vesicatoria</i>	LMG911	T6SS-13	WP_005993968.1 (YggG)	WP_005993970.1 (DUF6331)	
<i>X. vesicatoria</i>	BM4214		WP_005993969.1 (YggG)	WP_005993970.1 (DUF6331)	
<i>X. vesicatoria</i>	BM4214		WP_005993965.1 (PAAR_2)	NF	
<i>X. vesicatoria</i>	BM4214		WP_046811288.1 (YggG - DUF 2345)	WP_043404384.1 (PRX_NucleoX_NUC_DUF4122_RNA-like)	
<i>X. vesicatoria</i>	BM4214	T6SS-14	WP_04503433.1 (YggG)	WP_250577931.1 (Toxin VacA - Mtx_H)	YggG and T6E pseudogenized
<i>X. vesicatoria</i>	BM4214		WP_005992213.1 (PAAR - CT_2)	NF	
<i>X. vesicatoria</i>	BM4214		WP_03140245.1 (YggG - DUF 2345)	WP_020599843.1 (Phosphotransfer effector Tss1-like)	
<i>X. vesicatoria</i>	BM4214	NF	NF	WP_277937997.1 (Toxin VacA - Mtx_H)	

Supplementary Table 2. X-T4SS effector arsenals in xanthomonads. Similar proteins are highlighted by similar background colors.

Species	Strain	Secretion system	Effector GenBank acc. no.	Predicted effector domains (InterPro)	Remarks	Group
<i>X. translucens</i> pv. <i>graminis</i>	NCPPB 3711	X-T4SS	WP_175402592.1	X-Tfes, XVPICD		1
<i>X. translucens</i> pv. <i>graminis</i>	NCPPB 3711	X-T4SS	WP_284566863.1	Peptidoglycan binding-like; X-Tfes, XVPICD	Absent in NCPPB 3709	2
<i>X. translucens</i> pv. <i>graminis</i>	NCPPB 3711	X-T4SS	WP_284566866.1	Peptidoglycan binding-like; X-Tfes, XVPICD	Absent in NCPPB 3709	2
<i>X. translucens</i> pv. <i>graminis</i>	NCPPB 3711	X-T4SS	WP_284567054.1	X-Tfes, XVPICD	Pseudogenized in NCPPB 3709 (frameshift)	3
<i>X. translucens</i> pv. <i>graminis</i>	NCPPB 3711	X-T4SS	WP_284567126.1	RTX calcium-binding nonapeptide repeat; X-Tfes, XVPICD	Absent in NCPPB 3709	
<i>X. translucens</i> pv. <i>graminis</i>	NCPPB 3711	X-T4SS	WP_284567851.1	X-Tfes, XVPICD	Absent in NCPPB 3709	
<i>X. translucens</i> pv. <i>graminis</i>	NCPPB 3711	X-T4SS	WP_284567950.1	X-Tfes, XVPICD	Pseudogenized in NCPPB 3709 (frameshift)	4
<i>X. translucens</i> pv. <i>graminis</i>	NCPPB 3711	X-T4SS	WP_284567994.1	X-Tfes, XVPICD		5
<i>X. translucens</i> pv. <i>graminis</i>	NCPPB 3711	X-T4SS	WP_284567997.1	X-Tfes, XVPICD	Absent in NCPPB 3709	
<i>X. translucens</i> pv. <i>graminis</i>	NCPPB 3711	X-T4SS	WP_284568060.1	X-Tfes, XVPICD	Absent in NCPPB 3709	
<i>X. translucens</i> pv. <i>graminis</i>	NCPPB 3711	X-T4SS	WP_284568104.1	X-Tfes, XVPICD		6
<i>X. translucens</i> pv. <i>graminis</i>	NCPPB 3711	X-T4SS	WP_284568425.1	Peptidoglycan binding-like; X-Tfes, XVPICD		7
<i>X. translucens</i> pv. <i>graminis</i>	NCPPB 3711	X-T4SS	WP_284568595.1	zeta toxin domain; X-Tfes, XVPICD	Pseudogenized in NCPPB 3709 (large deletion, frameshift)	
<i>X. translucens</i> pv. <i>graminis</i>	NCPPB 3711	X-T4SS	WP_284569112.1	X-Tfes, XVPICD		8
<i>X. translucens</i> pv. <i>graminis</i>	NCPPB 3709	X-T4SS	WP_009480763.1	X-Tfes, XVPICD		6
<i>X. translucens</i> pv. <i>graminis</i>	NCPPB 3709	X-T4SS	WP_009580757.1	X-Tfes, XVPICD		8
<i>X. translucens</i> pv. <i>graminis</i>	NCPPB 3709	X-T4SS	WP_009605644.1	Peptidoglycan binding-like; X-Tfes, XVPICD		7
<i>X. translucens</i> pv. <i>graminis</i>	NCPPB 3709	X-T4SS	WP_009610391.1	X-Tfes, XVPICD		5
<i>X. translucens</i> pv. <i>graminis</i>	NCPPB 3709	X-T4SS	WP_039955302.1	X-Tfes, XVPICD		1
<i>X. translucens</i> pv. <i>phlei</i>	LMG 730	X-T4SS	WP_053838135.1	X-Tfes, XVPICD	Absent in LMG 843	4
<i>X. translucens</i> pv. <i>phlei</i>	LMG 730	X-T4SS	WP_053838249.1	X-Tfes, XVPICD	Absent in LMG 843	
<i>X. translucens</i> pv. <i>phlei</i>	LMG 730	X-T4SS	WP_053838971.1	X-Tfes, XVPICD		5
<i>X. translucens</i> pv. <i>phlei</i>	LMG 730	X-T4SS	WP_186008173.1	X-Tfes, XVPICD		1
<i>X. translucens</i> pv. <i>phlei</i>	LMG 730	X-T4SS	WP_237651283.1	X-Tfes, XVPICD		8
<i>X. translucens</i> pv. <i>phlei</i>	LMG 730	X-T4SS	WP_237651294.1	Peptidoglycan binding-like; X-Tfes, XVPICD		2
<i>X. translucens</i> pv. <i>phlegmatensis</i>	LMG 843	X-T4SS	WP_237651798.1	Peptidoglycan binding-like; X-Tfes, XVPICD		8
<i>X. translucens</i> pv. <i>phlegmatensis</i>	LMG 843	X-T4SS	WP_237651914.1	X-Tfes, XVPICD		1
<i>X. translucens</i> pv. <i>phlegmatensis</i>	LMG 843	X-T4SS	WP_237652053.1	X-Tfes, XVPICD	No homologue found in LMG 730	3
<i>X. translucens</i> pv. <i>phlegmatensis</i>	LMG 843	X-T4SS	WP_237652690.1	X-Tfes, XVPICD		5
<i>X. translucens</i> pv. <i>phlegmatensis</i>	LMG 843	X-T4SS	WP_237653823.1	X-Tfes, XVPICD		1
<i>X. translucens</i> pv. <i>translucens</i>	DSM 18974	X-T4SS	WP_003476263.1	Mtbq1-like; Alpha/Beta hydrolase fold; X-Tfes, XVPICD		8
<i>X. translucens</i> pv. <i>translucens</i>	DSM 18974	X-T4SS	WP_003476434.1	X-Tfes, XVPICD		8
<i>X. translucens</i> pv. <i>translucens</i>	DSM 18974	X-T4SS	WP_003480679.1	Peptidoglycan binding-like; X-Tfes, XVPICD		7
<i>X. translucens</i> pv. <i>translucens</i>	DSM 18974	X-T4SS	WP_003480683.1	Type VI secretion system spike protein VspG3-like; C-terminal; Peptidoglycan binding-like; X-Tfes, XVPICD		9
<i>X. translucens</i> pv. <i>translucens</i>	DSM 18974	X-T4SS	WP_038231763.1	X-Tfes, XVPICD		1
<i>X. translucens</i> pv. <i>translucens</i>	DSM 18974	X-T4SS	WP_060747839.1	X-Tfes, XVPICD		6
<i>X. translucens</i> pv. <i>translucens</i>	DSM 18974	X-T4SS	WP_060748376.1	X-Tfes, XVPICD		6
<i>X. translucens</i> pv. <i>translucens</i>	DSM 18974	X-T4SS	WP_229017421.1	Peptidoglycan binding-like; X-Tfes, XVPICD	Absent in UPB886	2
<i>X. translucens</i> pv. <i>translucens</i>	DSM 18974	X-T4SS	WP_229017423.1	Peptidoglycan binding-like; X-Tfes, XVPICD	Absent in UPB886	2
<i>X. translucens</i> pv. <i>translucens</i>	UPB886	X-T4SS	WP_060476434.1	X-Tfes, XVPICD		8
<i>X. translucens</i> pv. <i>translucens</i>	UPB886	X-T4SS	WP_064521759.1	Peptidoglycan binding-like; X-Tfes, XVPICD		7
<i>X. translucens</i> pv. <i>translucens</i>	UPB886	X-T4SS	WP_064521760.1	Type VI secretion system spike protein VspG3-like; C-terminal; Peptidoglycan binding-like; X-Tfes, XVPICD		9
<i>X. translucens</i> pv. <i>translucens</i>	UPB886	X-T4SS	WP_153476721.1	Mtbq3-like; Alpha/Beta hydrolase fold; X-Tfes, XVPICD		3
<i>X. translucens</i> pv. <i>translucens</i>	UPB886	X-T4SS	WP_193566008.1	X-Tfes, XVPICD		1
<i>X. translucens</i> pv. <i>translucens</i>	UPB886	X-T4SS	WP_233700394.1	X-Tfes, XVPICD		6
<i>X. translucens</i> pv. <i>translucens</i>	UPB886	X-T4SS	WP_233700395.1	X-Tfes, XVPICD		6
<i>X. vesicatoria</i>	LMG 911	X-T4SS	WP_005889148.1	X-Tfes, XVPICD		1 (XV09)
<i>X. vesicatoria</i>	LMG 911	X-T4SS	WP_005889865.1	X-Tfes, XVPICD		10 (XV16)
<i>X. vesicatoria</i>	LMG 911	X-T4SS	WP_005898080.1	X-Tfes, XVPICD		11 (XV12)
<i>X. vesicatoria</i>	LMG 911	X-T4SS	WP_005992945.1	Peptidoglycan binding-like; X-Tfes, XVPICD	Absent in BNM214	XV14
<i>X. vesicatoria</i>	LMG 911	X-T4SS	WP_005994591.1	X-Tfes, XVPICD		12 (XV07)
<i>X. vesicatoria</i>	LMG 911	X-T4SS	WP_005994245.1	X-Tfes, XVPICD		10 (XV02)
<i>X. vesicatoria</i>	LMG 911	X-T4SS	WP_005996740.1	X-Tfes, XVPICD		XV08
<i>X. vesicatoria</i>	LMG 911	X-T4SS	WP_005997363.1	Rungal lipase-type domain; X-Tfes, XVPICD		13 (XV11)
<i>X. vesicatoria</i>	LMG 911	X-T4SS	WP_005997375.1	X-Tfes, XVPICD	Absent in BNM214	XV17
<i>X. vesicatoria</i>	LMG 911	X-T4SS	WP_006450474.1	Peptidoglycan binding-like; X-Tfes, XVPICD		7 (XV10)
<i>X. vesicatoria</i>	LMG 911	X-T4SS	WP_042828490.1	X-Tfes, XVPICD	Pseudogenized in BNM214 (frameshift)	6 (XV06)
<i>X. vesicatoria</i>	LMG 911	X-T4SS	WP_042828809.1	Mtbq1-like; Alpha/Beta hydrolase fold; X-Tfes, XVPICD		3 (XV13)
<i>X. vesicatoria</i>	LMG 911	X-T4SS	WP_228869957.1	X-Tfes, XVPICD	Absent in BNM214	XV15
<i>X. vesicatoria</i>	BNM214	X-T4SS	WP_005996740.1	X-Tfes, XVPICD		XV08
<i>X. vesicatoria</i>	BNM214	X-T4SS	WP_039420002.1	Mtbq1-like; Alpha/Beta hydrolase fold; X-Tfes, XVPICD		3 (XV13)
<i>X. vesicatoria</i>	BNM214	X-T4SS	WP_039420329.1	X-Tfes, XVPICD		12 (XV07)
<i>X. vesicatoria</i>	BNM214	X-T4SS	WP_039421230.1	X-Tfes, XVPICD		1 (XV09)
<i>X. vesicatoria</i>	BNM214	X-T4SS	WP_039424956.1	X-Tfes, XVPICD		10 (XV02)
<i>X. vesicatoria</i>	BNM214	X-T4SS	WP_03942475.1	X-Tfes, XVPICD		11 (XV12)
<i>X. vesicatoria</i>	BNM214	X-T4SS	WP_039425231.1	Rungal lipase-type domain; X-Tfes, XVPICD		13 (XV11)
<i>X. vesicatoria</i>	BNM214	X-T4SS	WP_039425674.1	Peptidoglycan binding-like; X-Tfes, XVPICD		7 (XV10)
<i>X. vesicatoria</i>	BNM214	X-T4SS	WP_052118662.1	X-Tfes, XVPICD		10 (XV04)
<i>X. vesicatoria</i>	BNM214	X-T4SS	WP_331238671.1	X-Tfes, XVPICD	Homolog found in LMG 911 but not annotated in NZ_CP018725	14 (XV05)

Supplementary Table 3. List of domains found in X-T4SS and T6SS-i4 effectors in the present study

Gene	Accession	Start codon	Stop codon	Name	RefSeq	Start position	Stop position	Accession	Chromosome	Start name	Name	Chromosome	Start name	Name
Oran	PF00077	ATG	TAA	Apoptosis-inducing factor 1	PF00077	105	345	U00001	1	105	Apoptosis-inducing factor 1	1	105	Apoptosis-inducing factor 1
Oran	PF00081	ATG	TAA	Fungal toxin gene 1	PF00081	105	345	U00001	1	105	Fungal toxin gene 1	1	105	Fungal toxin gene 1
Oran	PF00082	ATG	TAA	Fungal toxin gene 2	PF00082	105	345	U00001	1	105	Fungal toxin gene 2	1	105	Fungal toxin gene 2
Oran	PF00083	ATG	TAA	Fungal toxin gene 3	PF00083	105	345	U00001	1	105	Fungal toxin gene 3	1	105	Fungal toxin gene 3
Oran	PF00084	ATG	TAA	Fungal toxin gene 4	PF00084	105	345	U00001	1	105	Fungal toxin gene 4	1	105	Fungal toxin gene 4
Oran	PF00085	ATG	TAA	Fungal toxin gene 5	PF00085	105	345	U00001	1	105	Fungal toxin gene 5	1	105	Fungal toxin gene 5
Oran	PF00086	ATG	TAA	Fungal toxin gene 6	PF00086	105	345	U00001	1	105	Fungal toxin gene 6	1	105	Fungal toxin gene 6
Oran	PF00087	ATG	TAA	Fungal toxin gene 7	PF00087	105	345	U00001	1	105	Fungal toxin gene 7	1	105	Fungal toxin gene 7
Oran	PF00088	ATG	TAA	Fungal toxin gene 8	PF00088	105	345	U00001	1	105	Fungal toxin gene 8	1	105	Fungal toxin gene 8
Oran	PF00089	ATG	TAA	Fungal toxin gene 9	PF00089	105	345	U00001	1	105	Fungal toxin gene 9	1	105	Fungal toxin gene 9
Oran	PF00090	ATG	TAA	Fungal toxin gene 10	PF00090	105	345	U00001	1	105	Fungal toxin gene 10	1	105	Fungal toxin gene 10
Oran	PF00091	ATG	TAA	Fungal toxin gene 11	PF00091	105	345	U00001	1	105	Fungal toxin gene 11	1	105	Fungal toxin gene 11
Oran	PF00092	ATG	TAA	Fungal toxin gene 12	PF00092	105	345	U00001	1	105	Fungal toxin gene 12	1	105	Fungal toxin gene 12
Oran	PF00093	ATG	TAA	Fungal toxin gene 13	PF00093	105	345	U00001	1	105	Fungal toxin gene 13	1	105	Fungal toxin gene 13
Oran	PF00094	ATG	TAA	Fungal toxin gene 14	PF00094	105	345	U00001	1	105	Fungal toxin gene 14	1	105	Fungal toxin gene 14
Oran	PF00095	ATG	TAA	Fungal toxin gene 15	PF00095	105	345	U00001	1	105	Fungal toxin gene 15	1	105	Fungal toxin gene 15
Oran	PF00096	ATG	TAA	Fungal toxin gene 16	PF00096	105	345	U00001	1	105	Fungal toxin gene 16	1	105	Fungal toxin gene 16
Oran	PF00097	ATG	TAA	Fungal toxin gene 17	PF00097	105	345	U00001	1	105	Fungal toxin gene 17	1	105	Fungal toxin gene 17
Oran	PF00098	ATG	TAA	Fungal toxin gene 18	PF00098	105	345	U00001	1	105	Fungal toxin gene 18	1	105	Fungal toxin gene 18
Oran	PF00099	ATG	TAA	Fungal toxin gene 19	PF00099	105	345	U00001	1	105	Fungal toxin gene 19	1	105	Fungal toxin gene 19
Oran	PF00100	ATG	TAA	Fungal toxin gene 20	PF00100	105	345	U00001	1	105	Fungal toxin gene 20	1	105	Fungal toxin gene 20
Oran	PF00101	ATG	TAA	Fungal toxin gene 21	PF00101	105	345	U00001	1	105	Fungal toxin gene 21	1	105	Fungal toxin gene 21
Oran	PF00102	ATG	TAA	Fungal toxin gene 22	PF00102	105	345	U00001	1	105	Fungal toxin gene 22	1	105	Fungal toxin gene 22
Oran	PF00103	ATG	TAA	Fungal toxin gene 23	PF00103	105	345	U00001	1	105	Fungal toxin gene 23	1	105	Fungal toxin gene 23
Oran	PF00104	ATG	TAA	Fungal toxin gene 24	PF00104	105	345	U00001	1	105	Fungal toxin gene 24	1	105	Fungal toxin gene 24
Oran	PF00105	ATG	TAA	Fungal toxin gene 25	PF00105	105	345	U00001	1	105	Fungal toxin gene 25	1	105	Fungal toxin gene 25
Oran	PF00106	ATG	TAA	Fungal toxin gene 26	PF00106	105	345	U00001	1	105	Fungal toxin gene 26	1	105	Fungal toxin gene 26
Oran	PF00107	ATG	TAA	Fungal toxin gene 27	PF00107	105	345	U00001	1	105	Fungal toxin gene 27	1	105	Fungal toxin gene 27
Oran	PF00108	ATG	TAA	Fungal toxin gene 28	PF00108	105	345	U00001	1	105	Fungal toxin gene 28	1	105	Fungal toxin gene 28
Oran	PF00109	ATG	TAA	Fungal toxin gene 29	PF00109	105	345	U00001	1	105	Fungal toxin gene 29	1	105	Fungal toxin gene 29
Oran	PF00110	ATG	TAA	Fungal toxin gene 30	PF00110	105	345	U00001	1	105	Fungal toxin gene 30	1	105	Fungal toxin gene 30
Oran	PF00111	ATG	TAA	Fungal toxin gene 31	PF00111	105	345	U00001	1	105	Fungal toxin gene 31	1	105	Fungal toxin gene 31
Oran	PF00112	ATG	TAA	Fungal toxin gene 32	PF00112	105	345	U00001	1	105	Fungal toxin gene 32	1	105	Fungal toxin gene 32
Oran	PF00113	ATG	TAA	Fungal toxin gene 33	PF00113	105	345	U00001	1	105	Fungal toxin gene 33	1	105	Fungal toxin gene 33
Oran	PF00114	ATG	TAA	Fungal toxin gene 34	PF00114	105	345	U00001	1	105	Fungal toxin gene 34	1	105	Fungal toxin gene 34
Oran	PF00115	ATG	TAA	Fungal toxin gene 35	PF00115	105	345	U00001	1	105	Fungal toxin gene 35	1	105	Fungal toxin gene 35
Oran	PF00116	ATG	TAA	Fungal toxin gene 36	PF00116	105	345	U00001	1	105	Fungal toxin gene 36	1	105	Fungal toxin gene 36
Oran	PF00117	ATG	TAA	Fungal toxin gene 37	PF00117	105	345	U00001	1	105	Fungal toxin gene 37	1	105	Fungal toxin gene 37
Oran	PF00118	ATG	TAA	Fungal toxin gene 38	PF00118	105	345	U00001	1	105	Fungal toxin gene 38	1	105	Fungal toxin gene 38
Oran	PF00119	ATG	TAA	Fungal toxin gene 39	PF00119	105	345	U00001	1	105	Fungal toxin gene 39	1	105	Fungal toxin gene 39
Oran	PF00120	ATG	TAA	Fungal toxin gene 40	PF00120	105	345	U00001	1	105	Fungal toxin gene 40	1	105	Fungal toxin gene 40
Oran	PF00121	ATG	TAA	Fungal toxin gene 41	PF00121	105	345	U00001	1	105	Fungal toxin gene 41	1	105	Fungal toxin gene 41
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Oran	PF00129	ATG	TAA	Fungal toxin gene 49	PF00129	105	345	U00001	1	105	Fungal toxin gene 49	1	105	Fungal toxin gene 49
Oran	PF00130	ATG	TAA	Fungal toxin gene 50	PF00130	105	345	U00001	1	105	Fungal toxin gene 50	1	105	Fungal toxin gene 50
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Oran	PF00148	ATG	TAA	Fungal toxin gene 68	PF00148	105	345	U00001	1	105	Fungal toxin gene 68	1	105	Fungal toxin gene 68
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Oran	PF00150	ATG	TAA	Fungal toxin gene 70	PF00150	105	345	U00001	1	105	Fungal toxin gene 70	1	105	Fungal toxin gene 70
Oran	PF00151	ATG	TAA	Fungal toxin gene 71	PF00151	105	345	U00001	1	105	Fungal toxin gene 71	1	105	Fungal toxin gene 71
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Oran	PF00160	ATG	TAA	Fungal toxin gene 80	PF00160	105	345	U00001	1	105	Fungal toxin gene 80	1	105	Fungal toxin gene 80
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Oran	PF00163	ATG	TAA	Fungal toxin gene 83	PF00163	105	345	U00001	1	105	Fungal toxin gene 83	1	105	Fungal toxin gene 83
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Oran	PF00167	ATG	TAA	Fungal toxin gene 87	PF00167	105	345	U00001	1	105	Fungal toxin gene 87	1	105	Fungal toxin gene 87
Oran	PF00168	ATG	TAA	Fungal toxin gene 88	PF00168	105	345	U00001	1	105	Fungal toxin gene 88	1	105	Fungal toxin gene 88
Oran	PF00169	ATG	TAA	Fungal toxin gene 89	PF00169	105	345	U00001	1	105	Fungal toxin gene 89	1	105	Fungal toxin gene 89
Oran	PF00170	ATG	TAA	Fungal toxin gene 90	PF00170	105	345	U00001	1	105	Fungal toxin gene 90	1	105	Fungal toxin gene 90
Oran	PF00171	ATG	TAA	Fungal toxin gene 91	PF00171	105	345	U00001	1	105	Fungal toxin gene 91</			

Supplementary Table 4. Bacterial strains and plasmids used in the present study

Bacterial strains and plasmids used in the present study			
Strains	Xt ANI group	Genotype or relevant features	Reference or source
Xt UPB513	Xt-I	Wild-type Xt UPB513 (isolation: 1987, Tlaxiaco, Mexico)	UPB Lab collection
Xt UPB513 AT65S-13	Xt-I	Single deletion mutant of genes <i>tsuA</i> -H T65S-13 in XtU UPB513	This study
Xt UPB513 AT65S-14	Xt-I	Single deletion mutant of genes <i>tsuA</i> -H T65S-14 in XtU UPB513	This study
Xt UPB513 AT65S-13 AT65S-14	Xt-I	Double deletion mutant of genes <i>tsuA</i> -H T65S-13 and <i>tsuA</i> -H T65S-14 in XtU UPB513	This study
Xt UPB513 Δ hcp13	Xt-I	Single deletion mutant of gene <i>hcp</i> of T65S-13 in XtU UPB513	This study
Xt UPB513 Δ hcp14	Xt-I	Single deletion mutant of gene <i>hcp</i> of T65S-14 in XtU UPB513	This study
Xt UPB513 Δ hcp14 Δ hcp14	Xt-I	Double deletion mutant of genes <i>hcp</i> of T65S-13 and <i>hcp</i> of T65S-14 in XtU UPB513	This study
Xt UPB513 Δ hcp14 Δ hcp14	Xt-I	Complementation of <i>hcp</i> T65S-14 mutant in XtU UPB513	This study
Xt UPB513 RFP ⁺	Xt-I	Wild-type Xt UPB513 (isolation: 1987, Tlaxiaco, Mexico) with mCherry gene inserted at attTn7 site, <i>Spe</i> R	This study
Xt UPB513 AT65S-13 RFP ⁺	Xt-I	Single deletion mutant of genes <i>tsuA</i> -H T65S-13 in XtU UPB513 with mCherry gene inserted at attTn7 site, <i>Spe</i> R	This study
Xt UPB513 AT65S-14 RFP ⁺	Xt-I	Single deletion mutant of genes <i>tsuA</i> -H T65S-14 in XtU UPB513 with mCherry gene inserted at attTn7 site, <i>Spe</i> R	This study
Xt UPB513 AT65S-13 AT65S-14 RFP ⁺	Xt-I	Double deletion mutant of genes <i>tsuA</i> -H T65S-13 and <i>tsuA</i> -H T65S-14 in XtU UPB513 with mCherry gene inserted at attTn7 site, <i>Spe</i> R	This study
Xt UPB513 Δ hcp13 RFP ⁺	Xt-I	Single deletion mutant of gene <i>hcp</i> of T65S-13 in XtU UPB513 with mCherry gene inserted at attTn7 site, <i>Spe</i> R	This study
Xt UPB513 Δ hcp14 RFP ⁺	Xt-I	Single deletion mutant of gene <i>hcp</i> of T65S-14 in XtU UPB513 with mCherry gene inserted at attTn7 site, <i>Spe</i> R	This study
Xt UPB513 Δ hcp13 Δ hcp14 RFP ⁺	Xt-I	Double deletion mutant of genes <i>hcp</i> of T65S-13 and <i>hcp</i> of T65S-14 in XtU UPB513 with mCherry gene inserted at attTn7 site, <i>Spe</i> R	This study
Xt UPB513 Δ hcp14 Δ hcp14 RFP ⁺	Xt-I	Complementation of <i>hcp</i> T65S-14 mutant in XtU UPB513 with mCherry gene inserted at attTn7 site, <i>Spe</i> R	This study
Xt LMG728	Xt-III	Wild-type Xt LMG728 (isolation: 1978, Pova Isolates, Switzerland)	BCCM/LMG collection
Xt LMG728 Δ wiD4	Xt-III	Single deletion mutant of gene <i>wiD4</i> in Xt LMG728	This study
Xt LMG728 Δ wiD4 Δ wiD4	Xt-III	Complementation of <i>wiD4</i> mutant in Xt LMG728, using <i>pXCK1</i> : <i>wiD4</i> , <i>Gm</i> R	This study
E. coli DH10B GFP ⁺		E. coli DH10B carrying the replicative <i>pRhe</i> -gfp ₊ , Kan ^R	Jacobs's Lab collection
P. agglomerans		Pantoea agglomerans UPB1357	Dubois et al., 2004
P. siamensis, GFP ⁺		<i>Parusomonas siamensis</i> UPB1354 with mNeonGreen gene inserted at attTn7 site, <i>Gm</i> R	Dubois et al., 2004
Plasmids			
pK18mobsacB		Suicide plasmid used for gene knock-out by two homologous recombination events, Kan ^R , Suc ^R	Schäffer et al., 1994
pK18mobsacB AT65S-13 UPB513		Recombinant pK18mobsacB plasmid used for creating XtU UPB513 AT65S-13 mutant strain, Kan ^R , Suc ^R	This study
pK18mobsacB AT65S-14 UPB513		Recombinant pK18mobsacB plasmid used for creating XtU UPB513 AT65S-14 mutant strain, Kan ^R , Suc ^R	This study
pK18mobsacB Δ hcp13 UPB513		Recombinant pK18mobsacB plasmid used for creating XtU UPB513 Δ hcp13 mutant strain, Kan ^R , Suc ^R	This study
pK18mobsacB Δ hcp14 UPB513		Recombinant pK18mobsacB plasmid used for creating XtU UPB513 Δ hcp14 mutant strain, Kan ^R , Suc ^R	This study
pK18mobsacB Δ hcp14 Δ hcp14 UPB513		Recombinant pK18mobsacB plasmid used for creating XtU UPB513 Δ hcp14 Δ hcp14 complemented strain, Kan ^R , Suc ^R	This study
pK18mobsacB Δ wiD4 LM728		Recombinant pK18mobsacB plasmid used for creating Xt LMG728 Δ wiD4 mutant strain, Kan ^R , Suc ^R	This study
pK18mobsacB Δ wiD4 LM728		Recombinant pK18mobsacB plasmid used for creating Xt LMG728 Δ wiD4 Δ wiD4 complemented strain, <i>Gm</i> ^R	This study
pN33		Helper plasmid encoding Tn7 TransABC site-specific transposition pathway, <i>Amp</i> ^R	Choi et al., 2006
pUC8-miwi-Tn7-Gm-mNeonGreen		<i>Gm</i> ^R on miwi-Tn7, for bacteria GFP tagging	Jacobs's Lab collection
pUC8-miwi-Tn7-Spec-mCherry		<i>Spec</i> ^R on miwi-Tn7, for bacteria RFP tagging	Jacobs's Lab collection

Supplementary Table 5. Primers used in the present study

[illegible]

Supplementary videos 1-8

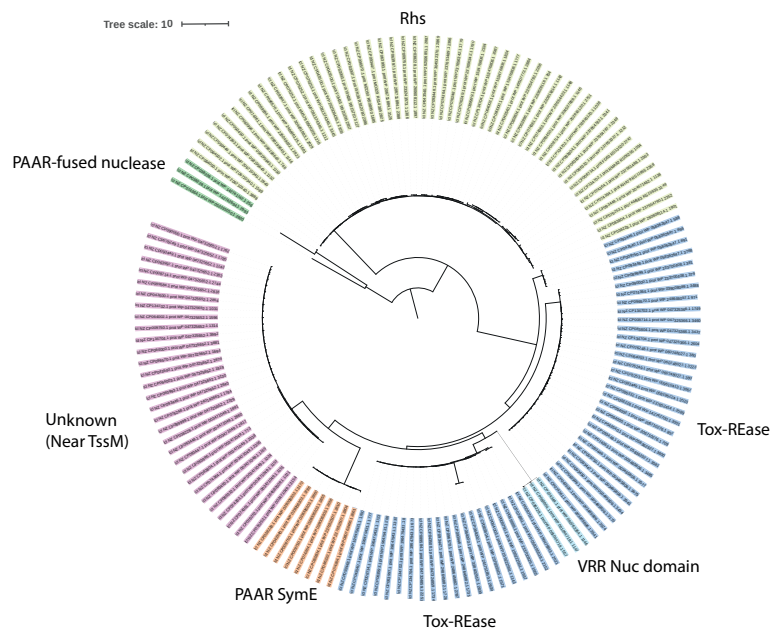
Real-time monitoring of T6SS bacterial killing activity in *Xanthomonas translucens* WT. Time-lapse videos of Xt UPB513 wild-type (WT), expressing RFP in competition with *E. coli* DH10B expressing GFP, mixed in a 10:1 (Xt:*E. coli*) ratio, on solid agarose pad (25% LB). Bar represents 10 μ m showed on the right, while magnification and time are indicated on the left. Pictures were taken every 6 minutes for 4h48min.

Supplementary videos 9-16

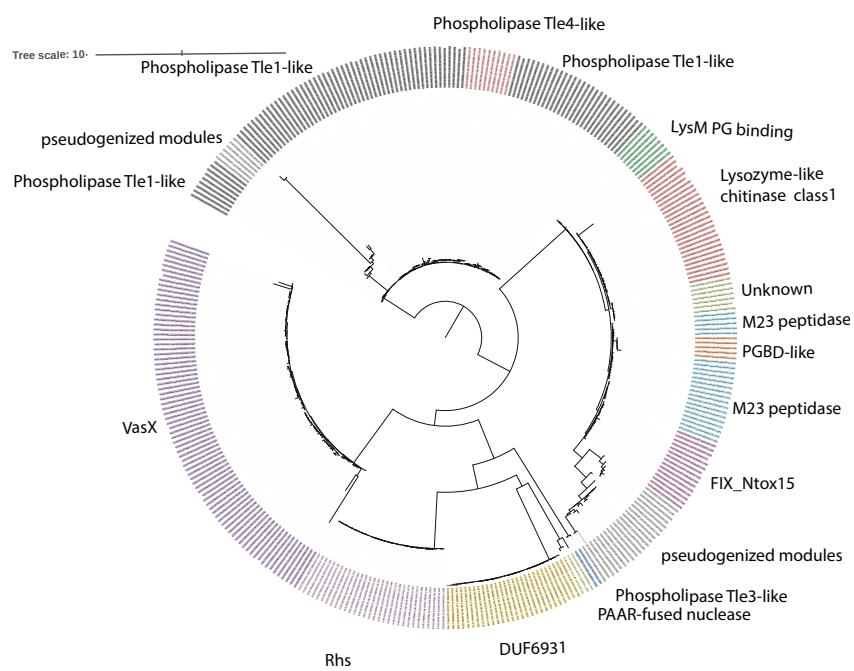
Real-time monitoring of T6SS bacterial killing activity in *Xanthomonas translucens* T6SS-i4 mutant. Time-lapse videos of Xt UPB513 Δ T6SS-i4, expressing RFP in competition with *E. coli* DH10B expressing GFP, mixed in a 10:1 (Xt:*E. coli*) ratio, on solid agarose pad (25% LB). Bar represents 10 μ m showed on the right, while magnification and time are indicated on the left. Pictures were taken every 6 minutes for 4h48min.

Appendix 3

Supplementary material pertaining to Chapter 3 can be found in Appendix 3.

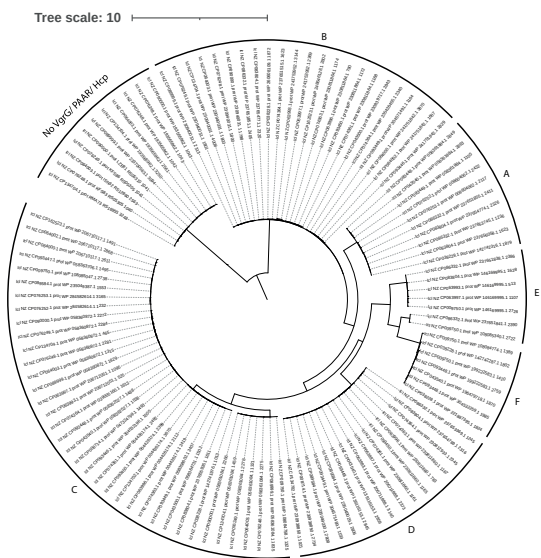


Supplementary Fig.1 Diversity of PAAR domains-containing proteins in the *X. translucens* complex. Phylogenetic trees is built based on CustalW alignment of amino acid sequences and inference of the tree midpoint rooted using IQTREE.

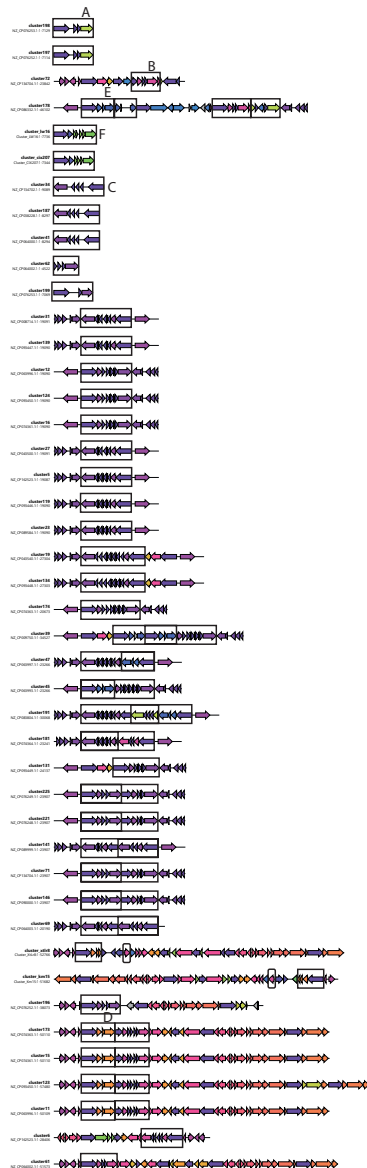


Supplementary Fig.2 Diversity of VgrG domains-containing proteins in the *X. translucens* complex. Phylogenetic trees are built based on ClustalW alignment of amino acid sequences and inference of the tree midpoint rooted using IQTREE.

Supplementary information



Supplementary Fig.3 Phylogenetic tree of Tle phospholipase domain containing proteins in *Xanthomonas translucens*

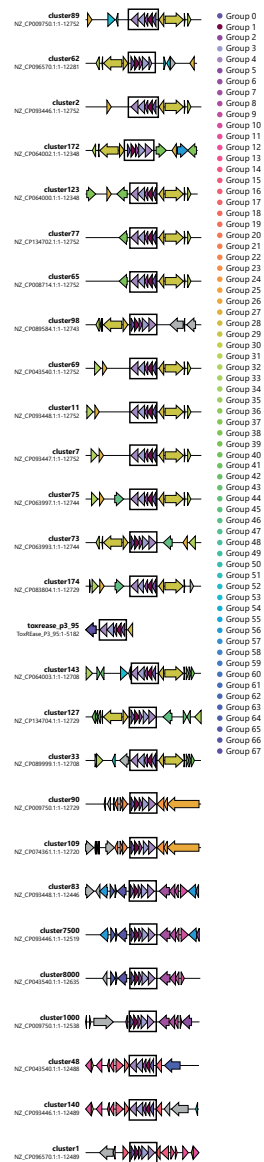


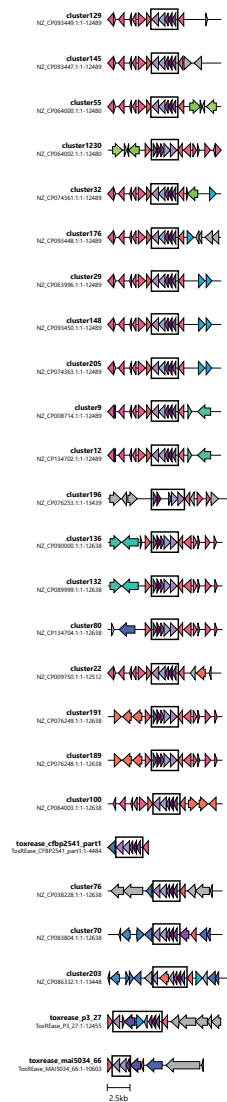
- Group 1
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- Group 3
- Group 4
- Group 5
- Group 6
- Group 7
- Group 8
- Group 9
- Group 10
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- Group 12
- Group 13
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- Group 100



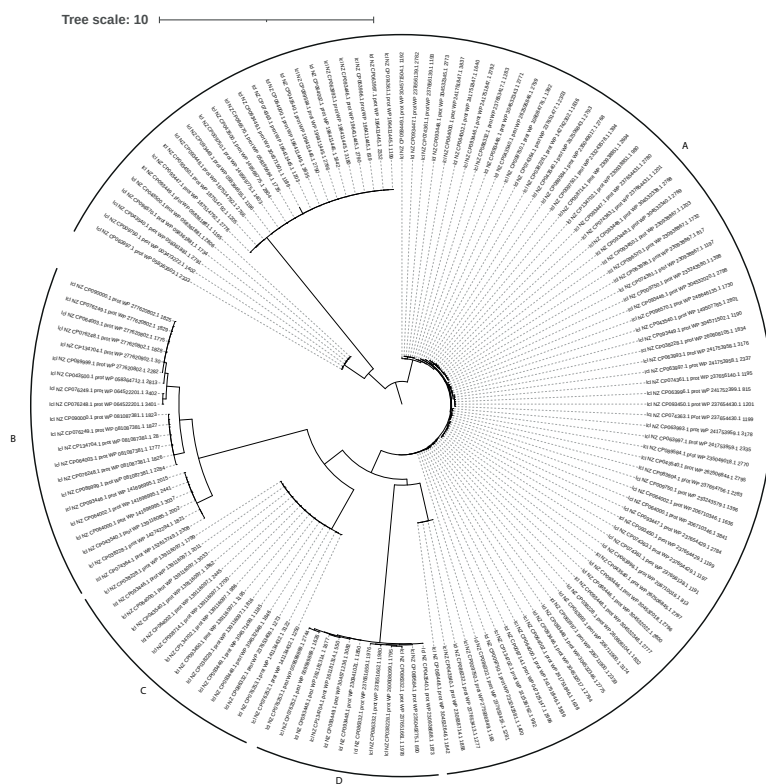


Supplementary Fig.5 Phylogenetic tree of ToxREase domain containing proteins in *Xanthomonas translucens*

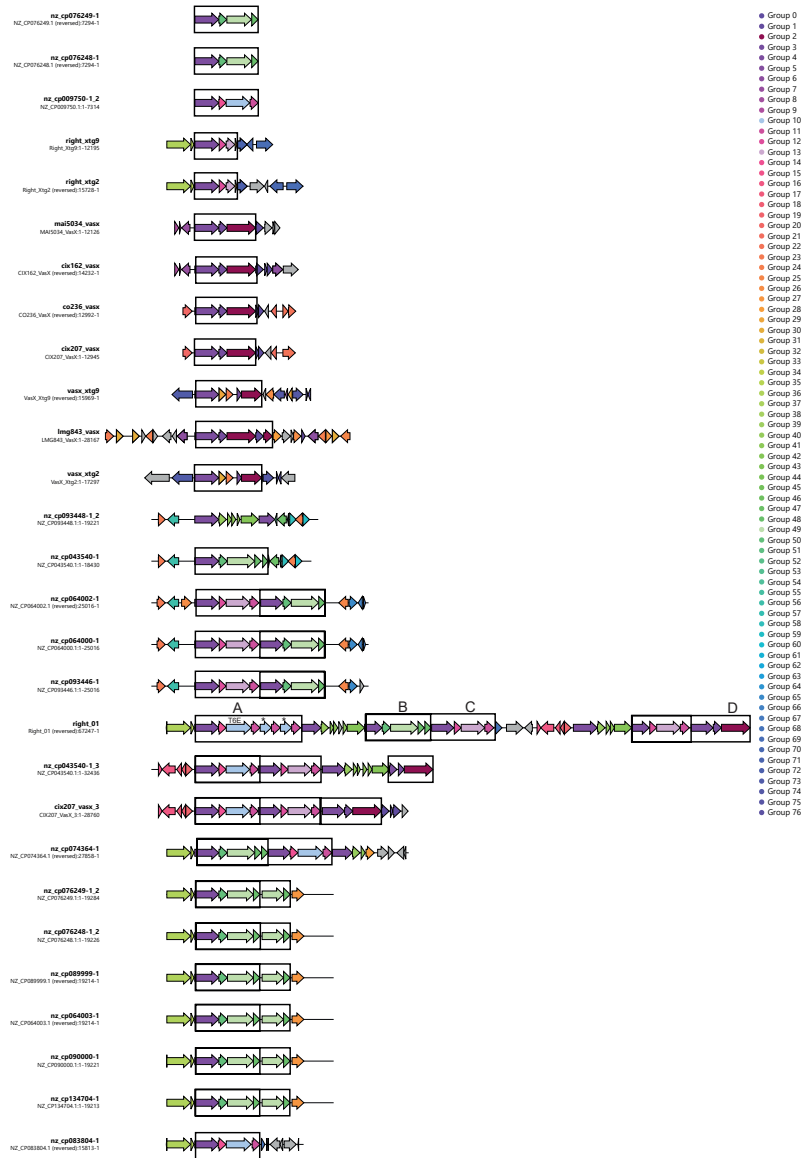


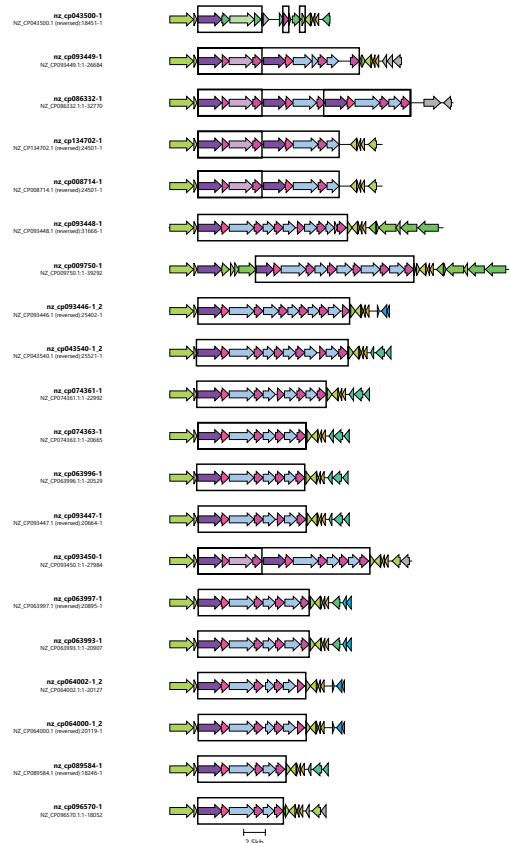


Supplementary Fig.6 Clinker comparison of T6SS Effector-Immunity modules including ToxREase domain-containing protein variants in *Xanthomonas translucens*

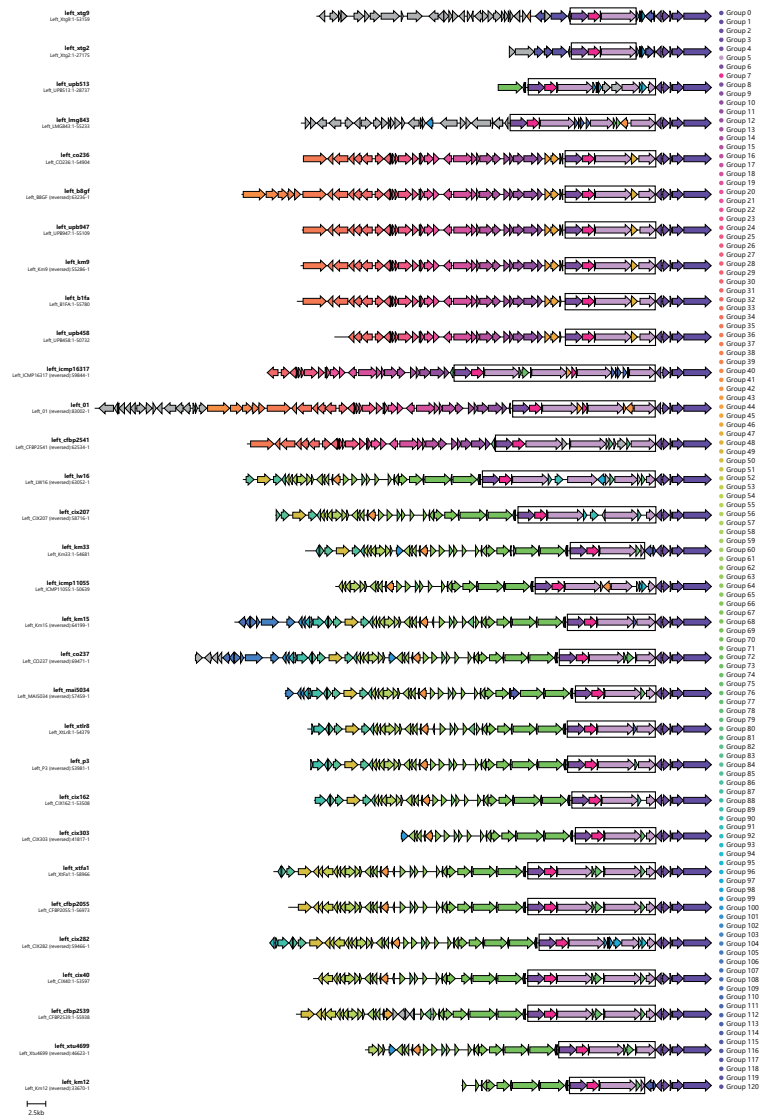


Supplementary Fig.7 Phylogenetic tree of VasX domain containing proteins in *Xanthomonas translucens*





Supplementary Fig.8 Clinker comparison of T6SS Effector-Immunity modules including VasX domain-containing protein variants in *Xanthomonas translucens*



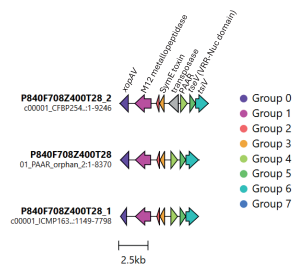




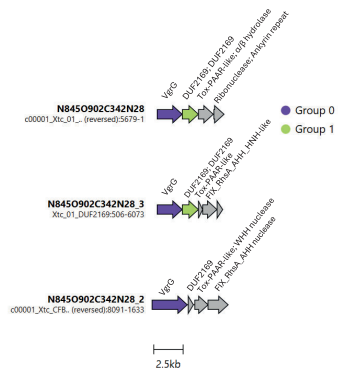
Supplementary Fig.10 Clinker comparison of T6SS Effector-Immunity modules including PGAP1 domain-containing protein variants in *Xanthomonas translucens*



Supplementary Fig.11 Clinker comparison of T6SS Effector-Immunity modules including M23 peptidase domain-containing protein variants in *Xanthomonas translucens*



Supplementary Fig.12 Schematic representation of PAAR and TseV with VRR Nuc domain module

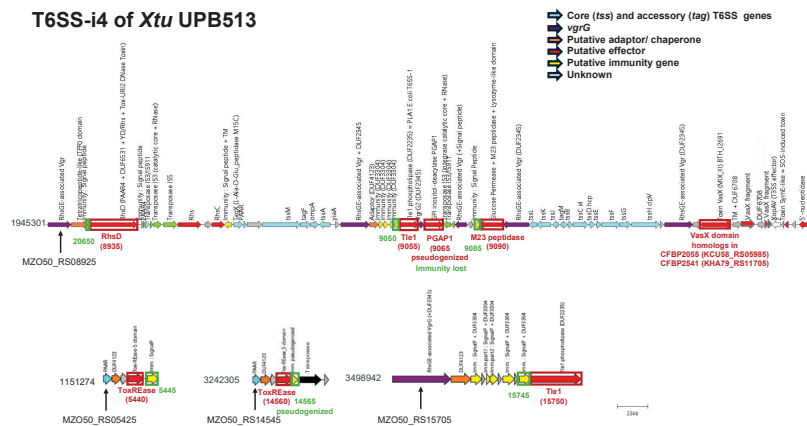


Supplementary Fig.13 Schematic representation of PAAR-fused nuclease module with DUF2169 adaptor.

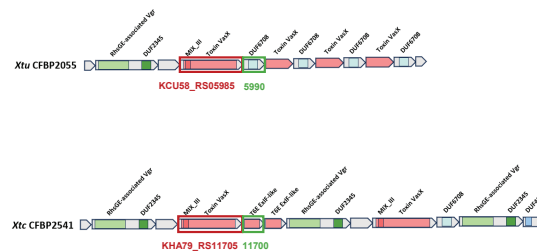
Supplementary Fig.15 Comparison of T6SS variable regions highlight T6E-T6I modules. Clin-ker comparison of T6SS-i4 variable regions of the 31 Xt T6SS-i4+ complete genome sequences, comprised between tssA and tssL core genes. Module homologs to Xt UPB513 T6E-T6I candidate modules are framed in black. Other T6SS effector-immunity modules are framed with dotted lines. Genes are color-coded according to homology.

Supplementary information

T6SS-i4 of *Xtu* UPB513



VasX in *Xt* CFBP2055 and *Xt* CFBP2541



Supplementary Fig.16 Schematic representation of T6SS-4 effector candidates in the reference strain Xt UPB513 and VasX effector candidates in Xt CFBP2055 and Xt CFBP2541 functionally tested for bacteriolytic activities, framed in red. Cognate immunity proteins used in neutralization assays are framed in green.

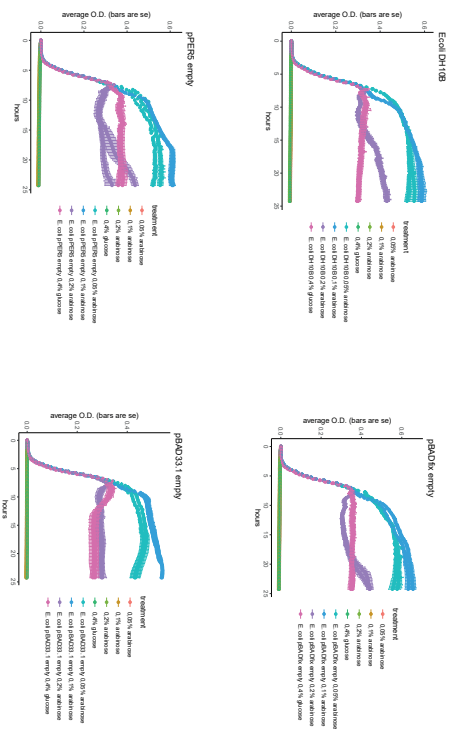
Supplementary Table 1. Bacterial strains and plasmids used in the present study

Bacterial strains and plasmids used in the present study		
Strains	Genotype or relevant features	Reference or source
Δ ⁺ UFB513	Wild-type Δ ⁺ UFB513 (isolation: 1987, <i>Triticum</i> , Mexico)	UFB collection
Δ ⁺ CFBP2541	Wild-type Δ ⁺ CFBP2541 (isolation: 1941, <i>Bromus inermis</i> , USA)	CFBP collection
Δ ⁺ CFBP2055	Wild-type Δ ⁺ CFBP2055 (isolation date: 1966, <i>Triticum durum</i> , Canada)	CFBP collection
<i>E. coli</i> DH10B	Wild-type <i>E. coli</i> DH10B	Lab collection
<i>E. coli</i> DH10B	pBADfix empty, Kan ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pPER6 empty, Kan ^R , araBAD promoter, pelB signal	This study
<i>E. coli</i> DH10B	pBAD33.1 empty, Chlor ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pBADfix empty, Kan ^R , araBAD promoter and pBAD33.1 empty, Chlor ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pPER6 empty, Kan ^R , araBAD promoter and pBAD33.1 empty, Chlor ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pBADfix:RS09055, Kan ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pPER6:RS09055, Kan ^R , araBAD promoter, pelB signal	This study
<i>E. coli</i> DH10B	pBAD33.1:RS09050, Chlor ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pBADfix:RS09055, Kan ^R , araBAD promoter and pBAD33.1:RS09050, Chlor ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pPER6:RS09055, Kan ^R , araBAD promoter, pelB signal and pBAD33.1:RS09050, Chlor ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pBADfix:RS08935, Kan ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pPER6:RS08935, Kan ^R , araBAD promoter, pelB signal	This study
<i>E. coli</i> DH10B	pBAD33.1:RS20650, Chlor ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pBADfix:RS08935, Kan ^R , araBAD promoter and pBAD33.1:RS20650, Chlor ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pPER6:RS08935, Kan ^R , araBAD promoter, pelB signal and pBAD33.1:RS20650, Chlor ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pBADfix:RS09065, Kan ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pPER6:RS09065, Kan ^R , araBAD promoter, pelB signal	This study
<i>E. coli</i> DH10B	pBADfix:RS05440, Kan ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pPER6:RS05440, Kan ^R , araBAD promoter, pelB signal	This study
<i>E. coli</i> DH10B	pBAD33.1:RS05445, Chlor ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pBADfix:RS05440, Kan ^R , araBAD promoter and pBAD33.1:RS05445, Chlor ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pPER6:RS05440, Kan ^R , araBAD promoter, pelB signal and pBAD33.1:RS05445, Chlor ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pBADfix:RS14560, Kan ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pPER6:RS14560, Kan ^R , araBAD promoter, pelB signal	This study
<i>E. coli</i> DH10B	pBADfix:RS15750, Kan ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pPER6:RS15750, Kan ^R , araBAD promoter, pelB signal	This study
<i>E. coli</i> DH10B	pBAD33.1:RS15745, Chlor ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pBADfix:RS15750, Kan ^R , araBAD promoter and pBAD33.1:RS15745, Chlor ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pPER6:RS15750, Kan ^R , araBAD promoter, pelB signal and pBAD33.1:RS15745, Chlor ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pBADfix:RS09090, Kan ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pPER6:RS09090, Kan ^R , araBAD promoter, pelB signal	This study
<i>E. coli</i> DH10B	pBAD33.1:RS09085, Chlor ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pBADfix:RS09090, Kan ^R , araBAD promoter and pBAD33.1:RS09085, Chlor ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pPER6:RS09090, Kan ^R , araBAD promoter, pelB signal and pBAD33.1:RS09085, Chlor ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pBADfix:RS11705, Kan ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pPER6:RS11705, Kan ^R , araBAD promoter, pelB signal	This study
<i>E. coli</i> DH10B	pBAD33.1:RS11700, Chlor ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pBADfix:RS11705, Kan ^R , araBAD promoter and pBAD33.1:RS11700, Chlor ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pPER6:RS11705, Kan ^R , araBAD promoter, pelB signal and pBAD33.1:RS11700, Chlor ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pBADfix:RS05985, Kan ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pPER6:RS05985, Kan ^R , araBAD promoter, pelB signal	This study
<i>E. coli</i> DH10B	pBAD33.1:RS05990, Chlor ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pBADfix:RS05985, Kan ^R , araBAD promoter and pBAD33.1:RS05990, Chlor ^R , araBAD promoter	This study
<i>E. coli</i> DH10B	pPER6:RS05985, Kan ^R , araBAD promoter, pelB signal and pBAD33.1:RS05990, Chlor ^R , araBAD promoter	This study
Plasmids		
pBADfix	Kan ^R , araBAD promoter	[1]
pPER6	Kan ^R , araBAD promoter	[1]
pBAD33.1	Chlor ^R , araBAD promoter	[1]

[1] Fridman, C. M., Kappel, K., Rudenko, V., Altuna-Avarez, J., Albasa-bwé, D., Bosis, E., & Salomon, D. (2025). A new class of type VI secretion system effectors can carry two toxic domains and are recognized through the WHIX motif for export. *PLoS biology*, 23(3), e3003053.

Supplementary Table 2. Primers used in the present study

[illegible]



Supplementary Fig. 17 Growth of *E. coli* wild-type or carrying empty plasmids monitored for 24h:30 at 37°C in LB medium supplemented with kanamycin or chloramphenicol when appropriate and either varying concentrations of L-arabinose (0.05%, 0.1%, or 0.2% w/vol) for expression, or D-glucose (0.4% w/vol) for repression as a control.

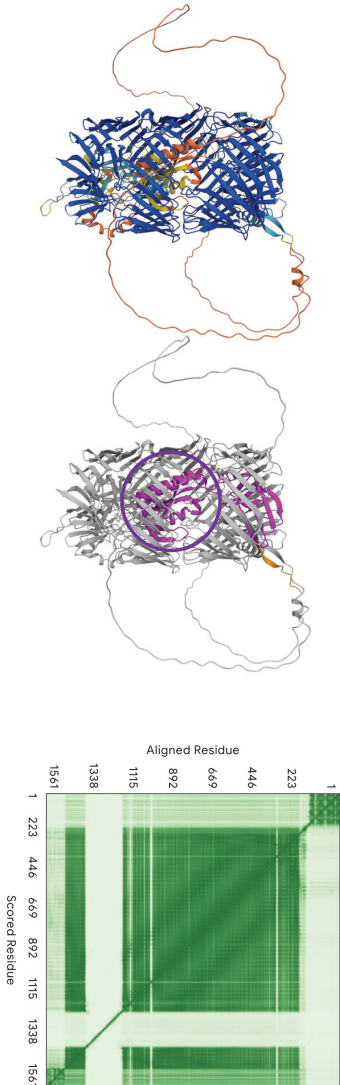
Supplementary Table 3. List of tested AlphaFold predictions of T6SS effector-immunity pairs, chaperones and YgrG/ PAAR interactions and resulting pTM and ipTM.

MZO50_	XTU_UP8513	ipTM	pTM
RS20650-RS08935	RnsD Imm-upstream	0.26	0.69
RS08940-RS08935	RnsD Imm-downstream	0.54	0.68
RS09025-RS09030-RS09055	Tle1(RS09055) YgrG-adaptor-effector complex	0.71	0.56
RS09035-RS09055	Tle1(RS09055) Imm_RS09035	0.83	0.85
RS09040-RS09055	Tle1(RS09055) Imm_RS09040	0.79	0.87
RS09045-RS09055	Tle1(RS09055) Imm_RS09045	0.77	0.86
RS09050-RS09055	Tle1(RS09055) Imm_RS09050	0.77	0.87
RS09060-RS09065	PGAP1	0.76	0.78
RS09085-RS09090	M23 peptidase	0.85	0.91
RS09165-RS09170	VasX-Imm upstream	0.64	0.70
RS09175-RS09170	VasX Imm downstream	0.72	0.54
RS09220-ig_ORF-RS09215	Nucleotidase tripartite complex	0.12	0.55
RS05425-RS05430-RS05435-RS05440	ToxREase PAAR-adaptors-effector complex	0.78	0.69
RS05430-RS05435-RS05440	ToxREase-tripartite complex (1)	0.60	0.61
RS05425-RS05435-RS05440	ToxREase-tripartite complex (2)	0.36	0.40
RS05425-RS05430-RS05440	ToxREase-tripartite complex (3)	0.42	0.39
RS05425-RS05430-RS05435	ToxREase-tripartite complex (4)	0.90	0.70
RS05440-RS05445	ToxREase	0.92	0.84
RS15710-RS15715-RS15750	Tle1(RS15750) YgrG-adaptor-effector complex	0.57	0.66
RS15745-RS15750	Tle1(RS15750)	0.14	0.62

RS08935	RhSD	/	0.74
RS20650-RS08935	RhSD-Immunity (upstream)	0.26	0.69
RS08940-RS08935	RhSD-Immunity (downstream)	0.54	0.68
RS08935₁₄₇₆₋₁₅₆₃	RhSD	/	0.80
RS08935 ₁₄₇₆₋₁₅₆₃ -RS20650	RhSD-Immunity (upstream)	0.14	0.34
RS08935 ₁₄₇₆₋₁₅₆₃ -RS08940	RhSD-Immunity (downstream)	0.17	0.62

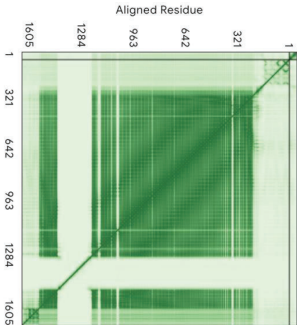
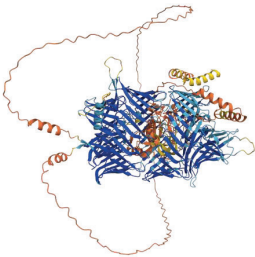
RhSD alone

Pink domains inside the barrel cage (Toxin encircled)

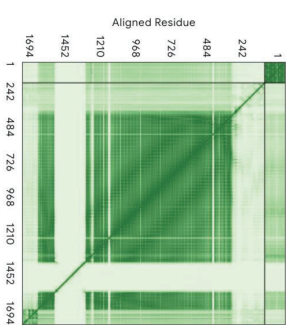
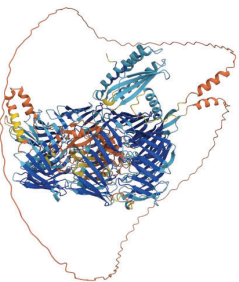


RS08935	RhSD	/	0.74
RS20650-RS08935	RhSD-Immunity (upstream)	0.26	0.69
RS08940-RS08935	RhSD-Immunity (downstream)	0.54	0.68

T6E & upstream candidate



T6E & downstream candidate



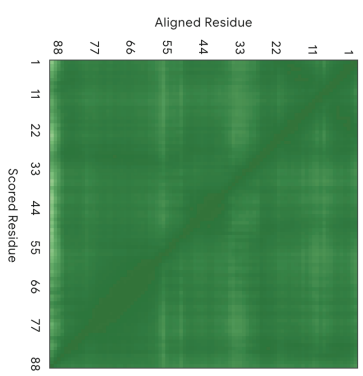
RS08935₁₄₇₆₋₁₅₆₃

RhSD

/

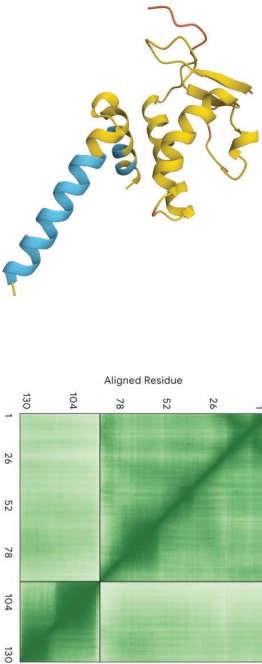
0.80

Toxin domain

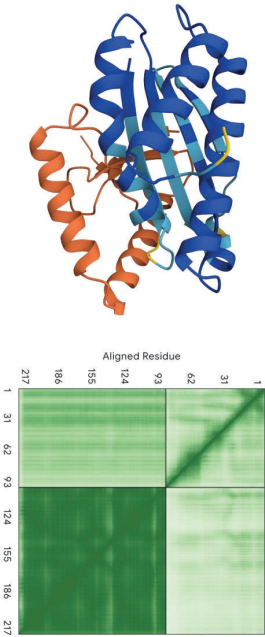


RS08935 ₁₄₇₆₋₁₅₆₃	RhSD	/	0.80
RS08935 ₁₄₇₆₋₁₅₆₃ -RS20650	RhSD-Immunity (upstream)	0.14	0.34
RS08935 ₁₄₇₆₋₁₅₆₃ -RS08940	RhSD-Immunity (downstream)	0.17	0.62

Toxin & upstream candidate

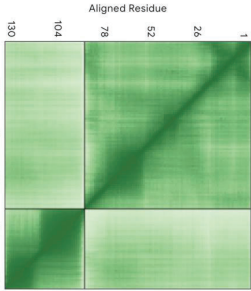
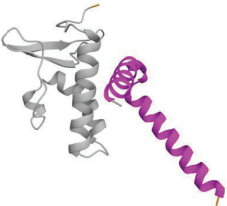


Toxin & downstream candidate



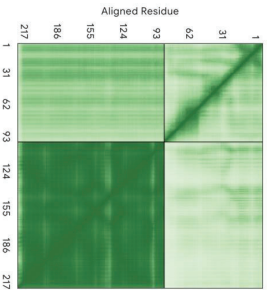
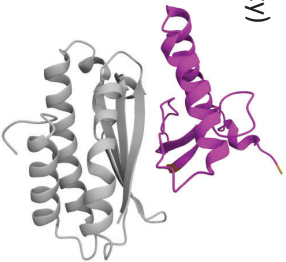
RS08935 ₁₄₇₆₋₁₅₆₃	RhSD		/	0.80
RS08935 ₁₄₇₆₋₁₅₆₃ ~RS20650	RhSD-Immunity (upstream)		0.14	0.34
RS08935 ₁₄₇₆₋₁₅₆₃ ~RS08940	RhSD-Immunity (downstream)		0.17	0.62

Toxin & upstream candidate

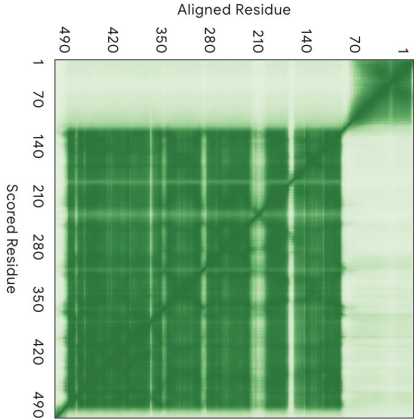
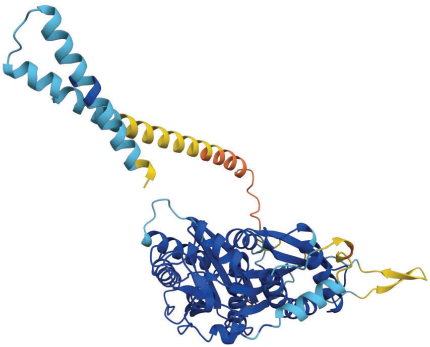


(turned, pink: candidate immunity)

Toxin & downstream candidate

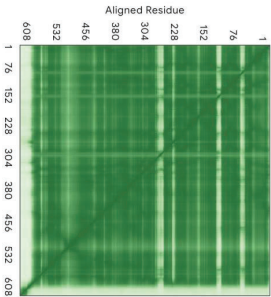
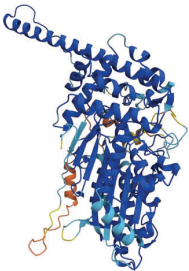


RS08995	Hypothetical protein	/	0.76
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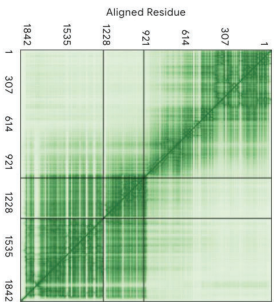


RS09055	Tle1(RS09055)	/	0.90
RS09025-RS09030-RS09055	Tle1(RS09055)-VgrG-adaptor-effector complex	0.71	0.56

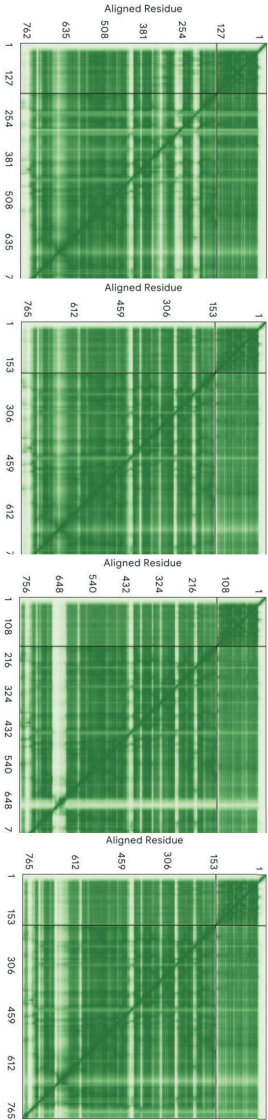
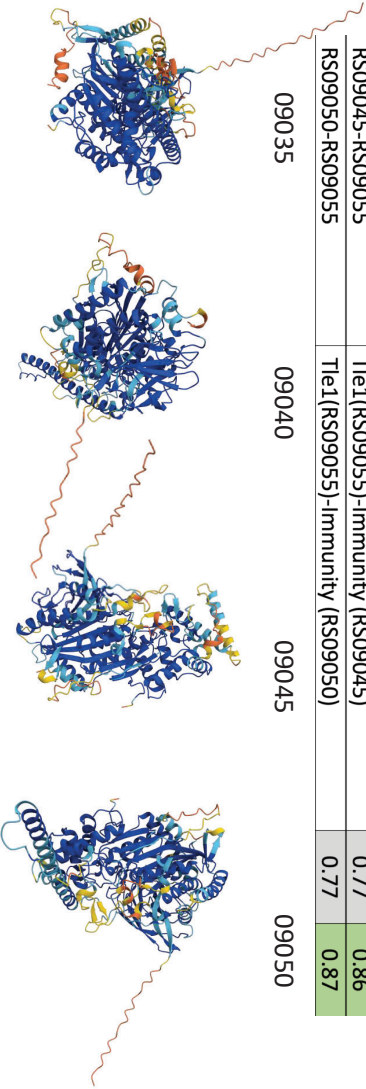
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Complex

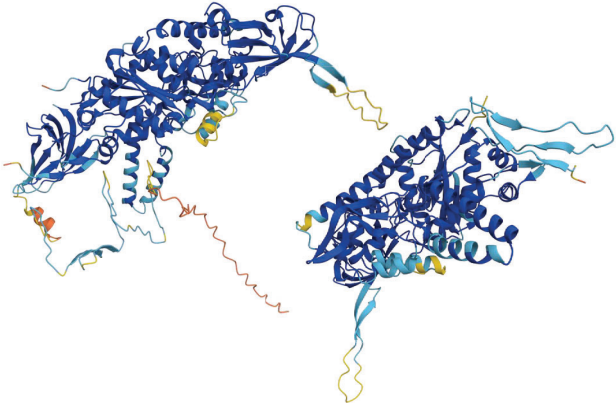


RS09035-RS09055	Tle1(RS09055)-Immunity (RS09035)	0.83	0.85
RS09040-RS09055	Tle1(RS09055)-Immunity (RS09040)	0.79	0.87
RS09045-RS09055	Tle1(RS09055)-Immunity (RS09045)	0.77	0.86
RS09050-RS09055	Tle1(RS09055)-Immunity (RS09050)	0.77	0.87

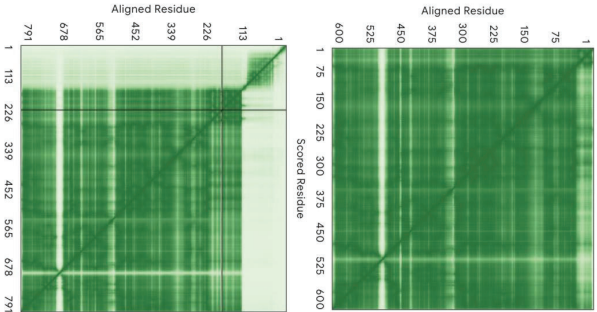


RS09065	PGAP1		
RS09060-RS09065	PGAP1-Immunity	/	0.92
		0.76	0.78

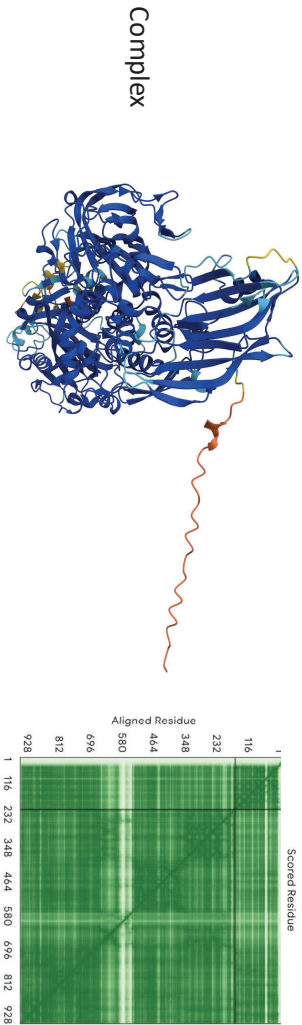
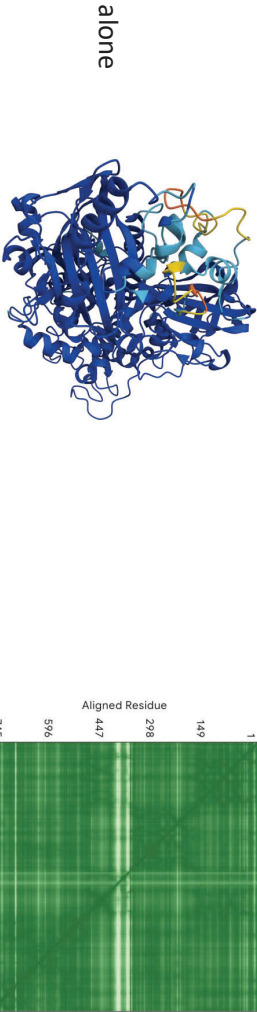
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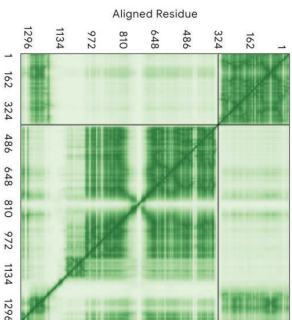
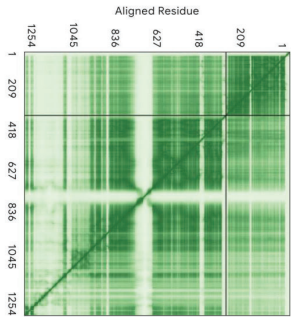
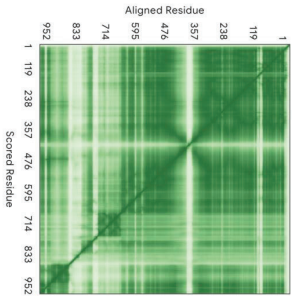
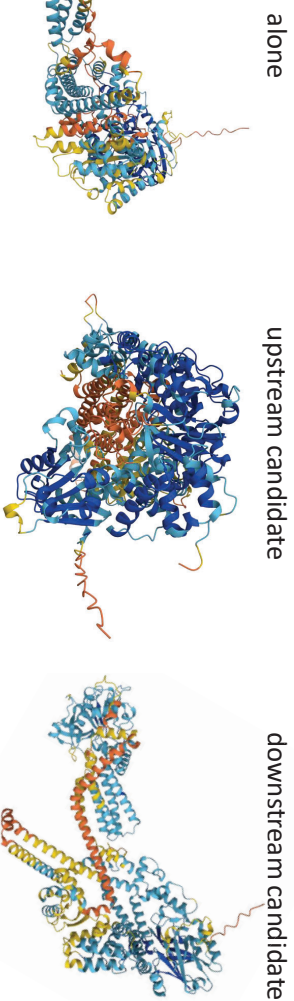
Complex



RS09090	M23 peptidase	/	0.95
RS09085-RS09090	M23 peptidase-Immunity	0.85	0.91

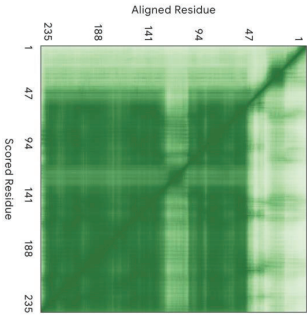
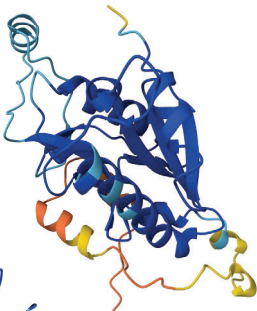


RS09170	VasX		
RS09165-RS09170	VasX-Immunity (upstream)	0.64	0.70
RS09175-RS09170	VasX-Immunity (downstream)	0.72	0.54

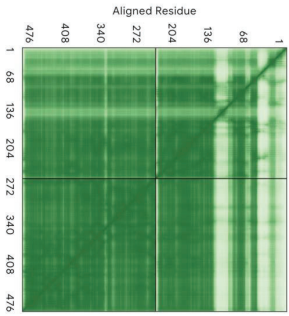
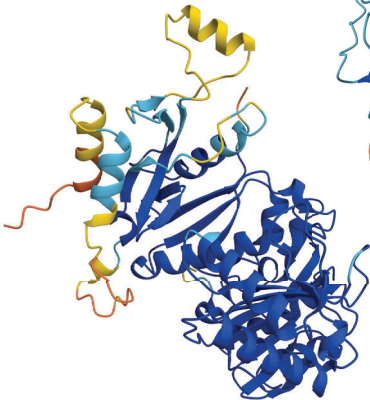


RS05440	ToxREase		
RS05440-RS05445	ToxREase-Immunity	0.92	0.84

alone

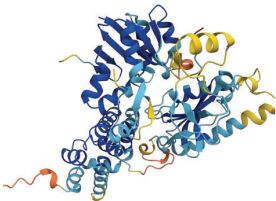


Complex

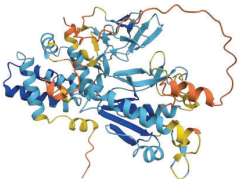


RS05430-RS05435-RS05440	ToxREase-tripartite complex (1)	0.60	0.61
RS05425-RS05435-RS05440	ToxREase-tripartite complex (2)	0.36	0.40
RS05425-RS05430-RS05440	ToxREase-tripartite complex (3)	0.42	0.39
RS05425-RS05430-RS05435	ToxREase-tripartite complex (4)	0.90	0.70

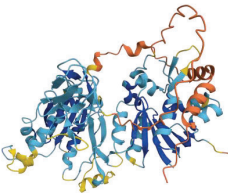
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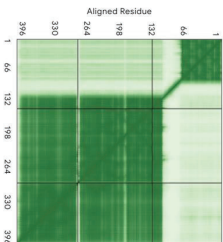
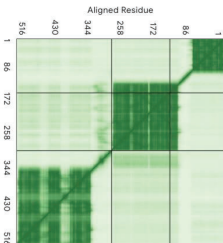
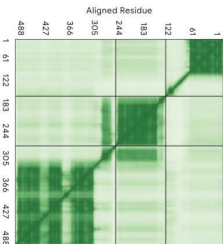
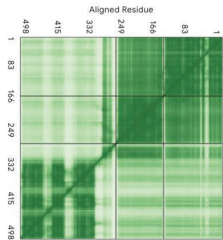
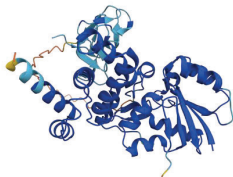
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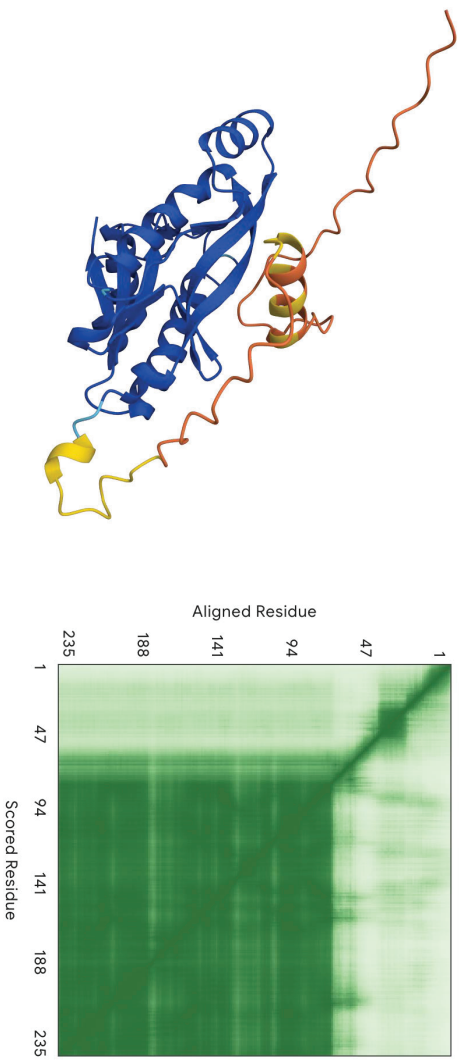
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4

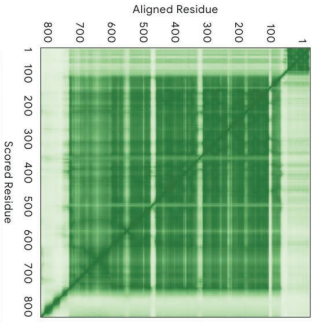
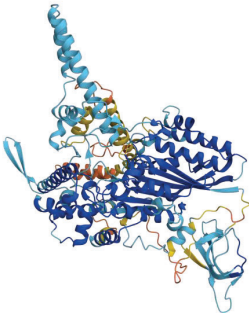


RS14560	ToxREase	/	0.71
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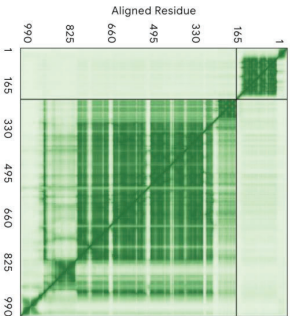


RS15750	Tle1(RS15750)	/	0.78
RS15745-RS15750	Tle1(RS15750)-Immunity	0.14	0.62

alone

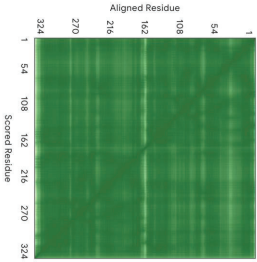
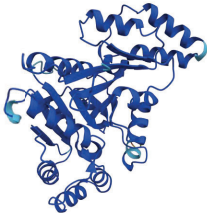


Complex

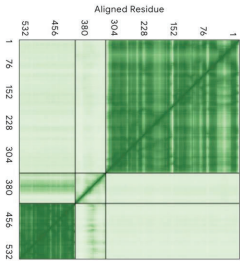
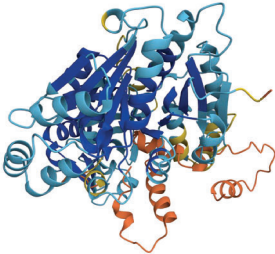


RS09220	Nucleotidase		
RS09220-ig_ORF-RS09215	Nucleotidase tripartite complex	0.12	0.55

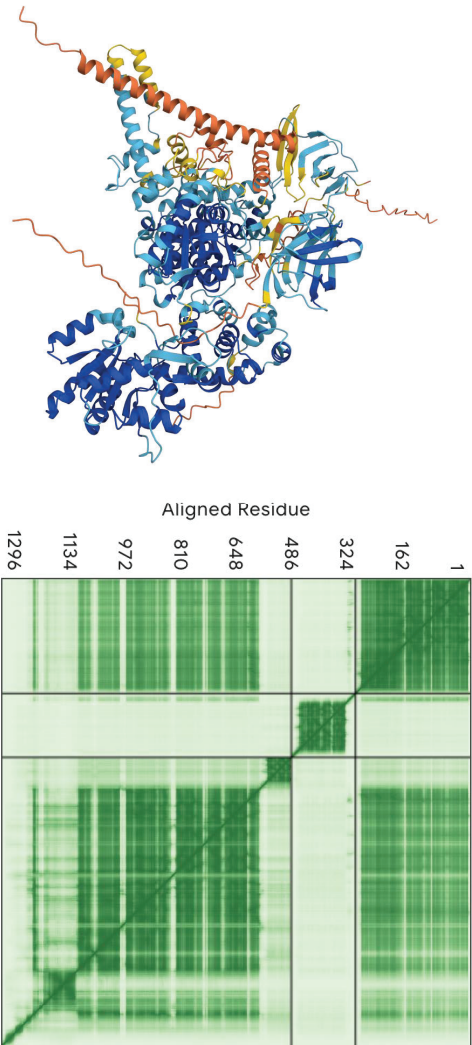
alone



Complex



RS15710-RS15715-RS15750	Tle1(RS15750)-VgrG-adaptor-effector complex	0.57	0.66
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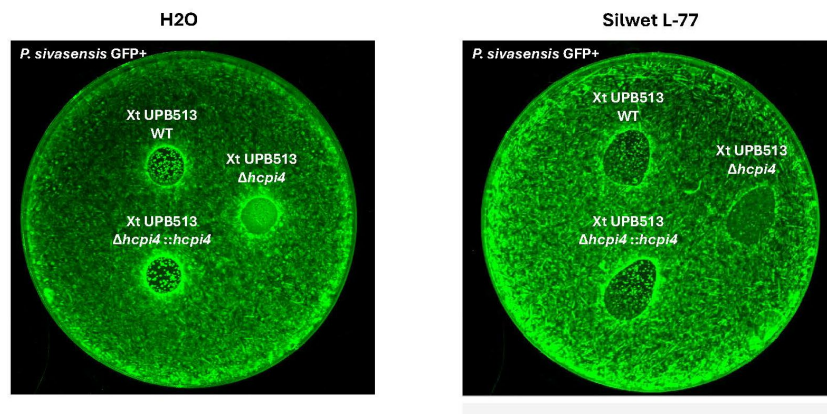
Supplementary Fig. 18 AlphaFold predictions for each candidate TSS effectors along 384 or 486 complex with surrounding 1033 residues and resulting pTM and ipTM. Bottom left : predicted structure for protein or protein complex. Bottom right : PAE plot for the predicted structure.

Appendix 4

Supplementary material pertaining to Chapter 4 can be found here in Appendix 4.

Silwet L-77 does not impact T6SS-dependent killing

Silwet L-77 is a wetting agent added to the formulation of some phytosanitary products. It limits droplet drift and ensures uniform coverage of the leaf surface. This additive is also frequently used in laboratory pathogenicity tests to introduce and spread xanthomonads epiphytically at the leaf surface and to reproduce natural entry through stomata and/or hydathodes. We therefore tested the effect of 0.05% (v/v) Silwet L-77 on T6SS-i4 killing efficiency on solid media *in vitro*. We selected *P. sivasensis* as a susceptible strain to Xt UPB513 T6SS-i4, which was further labeled with GFP for survival monitoring (Delitte et al., 2025). We did not observe any difference in the bacterial killing of Xt UPB513 in the presence of Silwet L-77, compared to the control plates without Silwet L-77 (Supplementary Figure 1).



Supplementary Figure 1: Silwet L-77 did not affect Xt T6SS-i4 killing against *P. sivasensis* on solid NA.