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Review

# Secrete or perish: The role of secretion systems in *Xanthomonas* biology

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ABSTRACT

Bacteria of the *Xanthomonas* genus are mainly phytopathogens of a large variety of crops of economic importance worldwide. *Xanthomonas* spp. rely on an arsenal of protein effectors, toxins and adhesins to adapt to the environment, compete with other microorganisms and colonize plant hosts, often causing disease. These protein effectors are mainly delivered to their targets by the action of bacterial secretion systems, dedicated multiprotein complexes that translocate proteins to the extracellular environment or directly into eukaryotic and prokaryotic cells. Type I to type VI secretion systems have been identified in *Xanthomonas* genomes. Recent studies have unravelled the diverse roles played by the distinct types of secretion systems in adaptation and virulence in xanthomonads, unveiling new aspects of their biology. In addition, genome sequence information from a wide range of *Xanthomonas* species and pathovars have become available recently, uncovering a heterogeneous distribution of the distinct families of secretion systems within the genus. In this review, we describe the architecture and mode of action of bacterial type I to type VI secretion systems and the distribution and functions associated with these important nanoweapons within the *Xanthomonas* genus.

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**Contents**



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## 1. Introduction

Bacterial pathogens cope with diverse hostile conditions and usually can successfully adapt to and colonise both host and non-host environments. In this context, secretion systems play essential roles in adaptation and colonisation processes by, among other functions, facilitating the acquisition of nutrients, contributing to biofilm formation, killing other bacterial or protozoan species and modifying host cell behaviour in a manner that promotes bacterial survival and growth [1]. The genus *Xanthomonas* comprises more than 30 species of plant pathogens with the distinguishing feature of causing severe diseases in many economically important mono-and-dicot plants throughout the world, including pepper and tomato (Bacterial leaf spot), citrus (Citrus canker), rice (Bacterial blight), soy (Bacterial pustules), sugarcane (Leaf scald), banana (Enset wilt) and many others [2,3]. *Xanthomonas* spp. belong to the order Xanthomonadales (syn. Lysobacterales), an early-branching group of the Gammaproteobacteria, and form part of the *Xanthomonadaceae* family (syn. *Lysobacteriaceae*), which also includes important phytopathogenic bacteria of the genus *Xylella*, the human opportunistic pathogen *Stenotrophomonas maltophilia* and environmental predatory bacteria of the genus *Lysobacter*, among other genera [4].

Based on host range and tissue specificity during colonisation, *Xanthomonas* species can be further distinguished into pathovars. The life cycles of most *Xanthomonas* species studied to date include at least three niches: outside of the host (e.g. on dead organic material, in the soil or water droplets), epiphytic (on the surface of host tissue), and endophytic (within the host tissue). Outside of the host, *Xanthomonas* has to deal with hostile environments, bacterial competitors, and amoebal predators that often feed on bacteria. The epiphytic stage initiates after *Xanthomonas* cells are transported by wind, rain, or insects to the aerial tissues of a new host. At this stage, *Xanthomonas* species may compete with other microorganisms and encounter new stressors such as nutrient limitation, drought, UV radiation, and larger oscillations in temperature, against which they may find some protection by forming biofilms. After passively or actively gaining entrance into

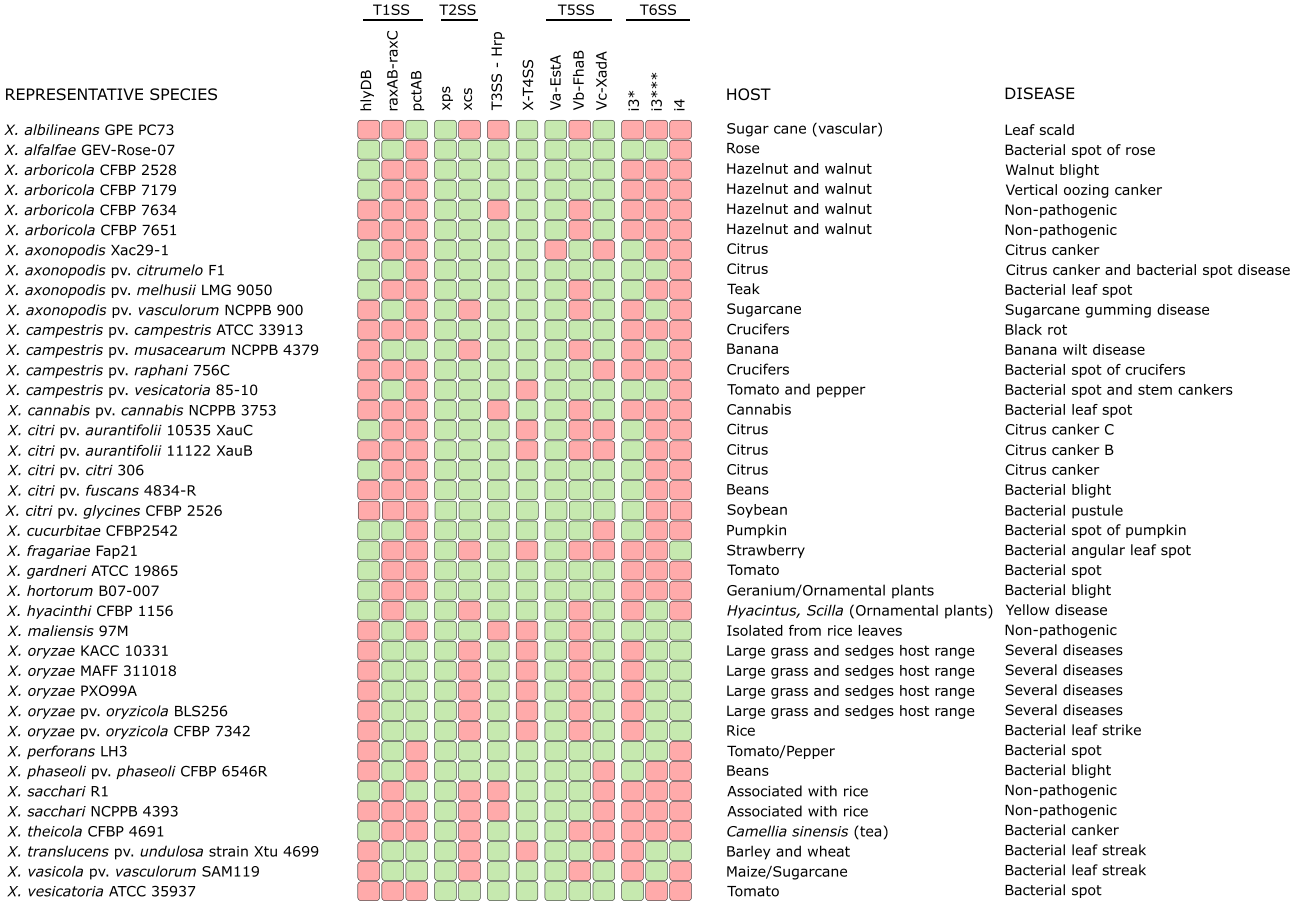
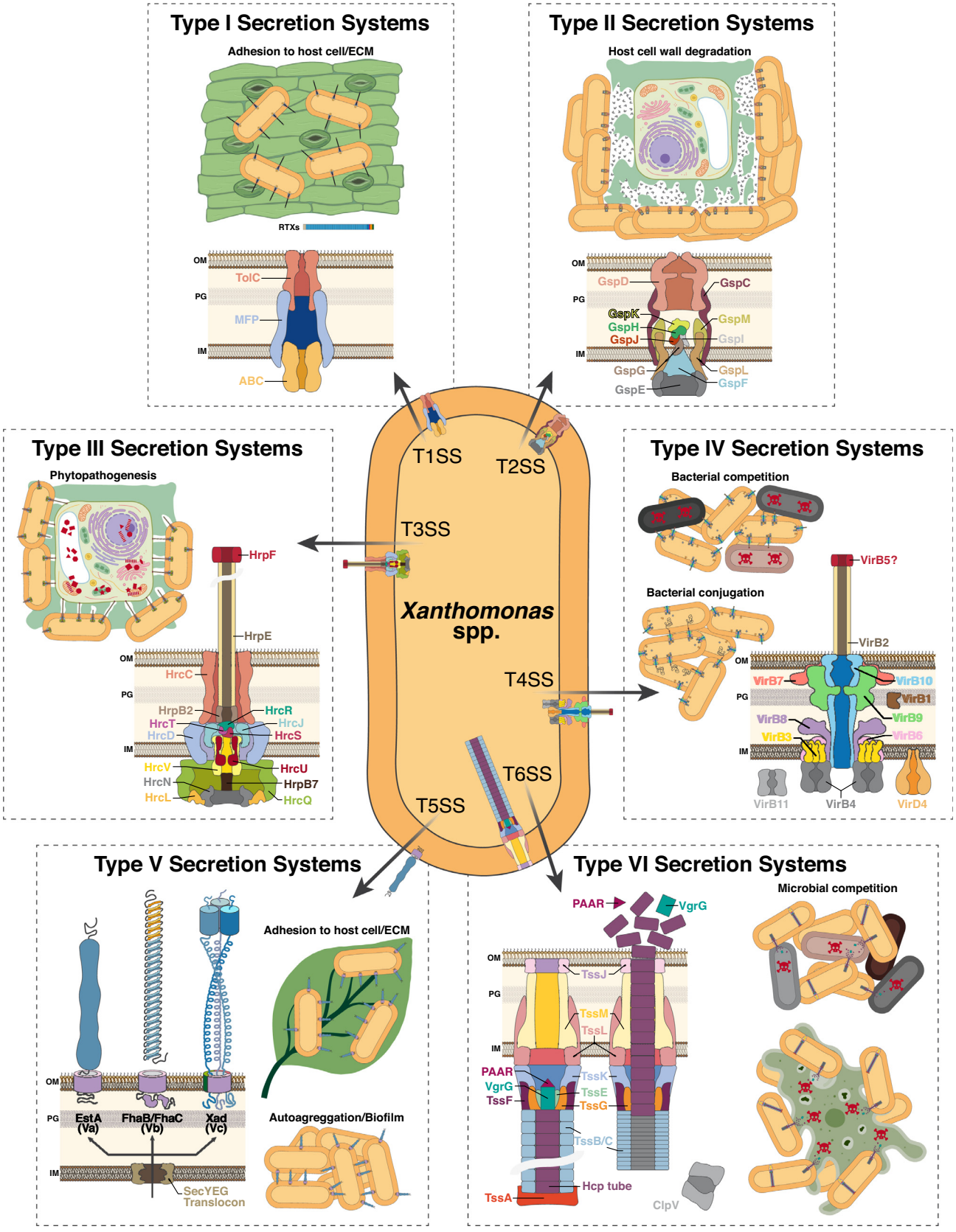



**Fig. 1.** Distribution of Secretion Systems in representative species of the *Xanthomonas* genus. Secretion systems in each species/strain were identified using the annotated genomes found in the KEGG (https://www.genome.jp/kegg/), NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and IMG (https://img.jgi.doe.gov/) databases [298,365,366]. Presence or absence of the particular secretion system is indicated by green and red squares, respectively. Plant host species and associated diseases (where appropriate) are indicated. See Table S1 for more detailed information. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the host tissue via natural openings (hydathodes or stomata) or wounds, bacterial cells need to adjust their physiology to the specific conditions they encounter when colonising the host mesophyll (non-vascular pathogen) or xylem vessel tissues (vascular pathogen). Comparative analyses of sequenced genomes from many *Xanthomonas* species have identified a large number of potential



mechanisms that contribute to bacterial fitness in these diverse environments. One class of such mechanisms is composed of a set of secretion systems that are able to transport proteins or protein-DNA conjugates across the bacterial envelope directly into target cells or to the extracellular medium. The purpose of this review is to bring together the most recent advances covering these macromolecular secretion systems, termed type I to type VI (T1SS to T6SS) in *Xanthomonas* species. Secretion by outer membrane vesicles (OMVs) is also briefly discussed. For each secretion system, we will describe its structural and genetic organization, mechanism of action, the effectors they secrete, factors involved in their regulation, and the general importance of these systems for *Xanthomonas* physiology and fitness. We also performed a survey of the distribution of these secretion systems among representative genomes within the *Xanthomonas* genus, which we hope will provide the reader with an overview of their distinct representation according to species and host range (Fig. 1, Table S1). A number of excellent recent reviews that have covered some aspects of specific secretion systems will be mentioned and cited where appropriate.

## 2. Type I secretion system

Type I secretion systems (T1SSs) are responsible for the secretion of unfolded cognate substrates from the cytoplasm directly to the extracellular medium either in a single step or via two steps that involves an intermediary periplasmic state [5–8]. The T1SS machinery consists of a heterotrimer, composed of an inner membrane transporter of the ATP-binding cassette (ABC) superfamily, a periplasmic membrane fusion protein (MFP) and a TolC-like outer membrane protein (OMP) (Fig. 2) [6,9–11]. The MFP is anchored in the inner membrane, extending a large periplasmic domain that connects to the OMP [8,12,13]. The ABC transporter is responsible for recognizing the leader sequence of the effector and using the energy of ATP hydrolysis to translocate it through the inner membrane channel [5,14–18]. Effector binding promotes a conformational change of the MFP-ABC transporter complex, that is transmitted to the OMP, causing a structural change that allows the effector to be translocated from the cytoplasm to the extracellular environment [8]. Upon substrate release, the MFP-ABC transporter complex separates from the OMP, making it available to participate in subsequent transport events [5,19,20].

T1SS substrates can be subdivided into three groups: i) bacteriocins, ii) proteins containing a nonapeptide repeat in toxins (RTX) motif, GGxGxD which can be followed or preceded by xUx, where × corresponds to any amino acid and U is a large hydrophobic residue, and iii) large adhesins that also contain RTX repeats in the C-terminal region [5,21,22]. Antimicrobial bacteriocins secreted by T1SSs possess an N-terminal double glycine (GG) leader peptide that is processed by a C39-like peptidase domain of its cognate ABC transporter, prior to its secretion through the channel [5,23]. RTX proteins, which constitute the majority of T1SS effectors in other bacterial species, are proteases, lipases, adhesins, or toxins possessing a variable number of RTX motifs, followed by an uncleavable T1SS-specific C-terminal secretion signal [5,21,24,25]. These RTX proteins are translocated in their unfolded state and upon secretion, extracellular $Ca^{2+}$ binds to the RTX motif promoting folding and facilitating the process [6,26–28]. The third family of T1SS substrates are large adhesins which are secreted in two steps. Genes encoding for bacterial transglutaminase-like cysteine proteinases (BTLCPs) and proteins containing RTX motifs are often found in the neighboring region of T1SS structural genes in the bacterial genome, which suggests a link between their activities [6,29]. Recently, Smith et al., showed that BTLCPs are T1SS accessory proteins, with an essential role in the secretion of large RTX adhesins. These adhesins possess an N-terminal dialanine (AA) cleavage site, multiple C-terminal RTX motifs, and are secreted in a two-step process [5]. After substrate translocation by the ABC transporter-MFP complex through the inner membrane, the unfolded C-terminal portion is translocated to the extracellular medium, but the N-terminal domain folds into a large structure in the periplasm, which clogs the pore formed by the OMP, preventing adhesin secretion. BTLCPs are required to process the effector by cleaving the AA motif allowing its release [5].

The RaxABC from *Xanthomonas oryzae* pv. *oryzae* is a well-studied T1SS in *Xanthomonas* spp. RaxA (MFP), RaxB (ABC transporter), and RaxC (OMP) are components of a predicted T1SS that recognizes effectors with a double glycine leader peptide. RaxA and RaxB are encoded in the same operon along with RaxST, a sulfotransferase like protein [30]. The genes for a two-component regulatory system, composed by RaxH and RaxR, responsible for the regulation of the RaxABC T1SS are found downstream of the *raxSTAB* operon [30,31]. The *X. oryzae* pv. *oryzae* RaxX virulence factor mimics the plant peptide hormone PSY (plant peptide containing sulfated tyrosine), which promotes root growth [32,33]. Mutation of *raxX* impaired virulence in rice, demonstrating that it promotes *X. oryzae* pv. *oryzae* infection by an unknown mechanism that pos-

**Fig. 2.** Schematic representation of type I to type VI secretion systems identified and characterized *Xanthomonas*. The T1SS is made up of a TolC-like outer membrane protein, a membrane fusion protein (MFP) and an ABC transporter, which is responsible for recognizing the leader sequence of the substrates and providing the energy to translocate them to the extracellular milieu. T1SS substrates can be subdivided in bacteriocins, proteins containing a nonapeptide repeat in toxins motif (RTX) and large RTX adhesins, having antimicrobial activities and roles in host cell invasion and adhesion to the host cell and extracellular matrix (ECM). The T2SS is made up of multiple copies of at least eleven different subunits and spans the bacterial envelope. It exports folded periplasmic proteins that have been first transported across the inner membrane by the Sec or Tat pathways. Two distinct T2SS gene clusters can be found in the *Xanthomonas* genus, *xps* (conserved throughout the genus), and *xcs* (present in strains of species such as *X. citri* and *X. campestris*). In *Xanthomonas* spp., the *xps* T2SS is involved in the secretion of extracellular enzymes targeted to the degradation of the plant cell wall, which is believed to aid the assembly of the extracellular appendages of other secretion systems, such as the Hrp pilus of T3SS. The T3SS is the main virulence determinant of most *Xanthomonas* spp. with a pathogenic lifestyle, and is encoded by the conserved *hrp*/*hrc* locus. More than 20 proteins, many of them present in multiple copies, constitute the major T3SS subassemblies: the sorting platform (HrcQ, HrcL, HrcN, HrpB7), the export apparatus (HrcV, HrcR, HrcS, HrcT, HrcU), the needle complex (HrcD, HrcJ, HrcC, HrpE), and the translocon (HrpF). Many T3S effector proteins are directed to suppress immune responses in host plants, allowing the colonisation of host milieu and consequent disease development. The chromosomally-encoded bacteria-killing X-T4SS has 12 subunits, each in multiple copies, that form a greater than 3-MDa apparatus that crosses both bacterial membranes. This system translocates a cocktail of effectors with antibacterial enzymatic activities into the cells of other bacterial species [203]. Many *Xanthomonas* species also carry mobilizable plasmids that code for separate T4SSs predicted to mediate conjugation. The T5SS consists of just one or two proteins that localize in the outer membrane. Translocation across the inner membrane is mediated by the general Sec pathway. *Xanthomonas* spp. T5SSs belong to the Va, Vb and Vc subgroups, and are involved in several processes such as attachment to plant surfaces, epiphytic fitness and colonization of host leaves, and in cell-to-cell attachment and biofilm formation. The T6SS nanomachine is organized into three main structures that assemble to form a functional contractile system: the membrane-spanning complex (TssJ, TssL and TssM), a baseplate (TssK, TssF, TssG, TssE, VgrG and PAAR), and an extended inner tube (Hcp) surrounded by a contractile sheath (TssB and TssC). The T6SS directly translocates proteins to prokaryotic and eukaryotic target cells and, in *Xanthomonas* spp., it has been associated with contact-dependent interbacterial antagonism and with promoting resistance against predation by soil amoeba. IM, inner membrane; PG, peptidoglycan; OM, outer membrane.

sibly involves stimulation of host tissue growth [32,33]. Pruitt et al. speculated that the plant *Oryza longistaminata* evolved the receptor XA21 to counteract RaxX, by specifically recognizing it and inducing immune responses [33]. Accordingly, rice lines lacking the receptor XA21 are more susceptible to *X. oryzae* pv. *oryzae* infection and mutation of *raxX* improves infection in rice cultivars containing XA21 [33]. Recognition of RaxX by XA21 requires the sulfation of its single tyrosine at position 41 by RaxST [32,34]. After RaxX sulfation, RaxB cleaves the double glycine leader peptide from RaxX and directs it for secretion through the RaxA and RaxC [34]. Curiously, although RaxC is essential for RaxX secretion, RaxA and RaxB are partially compensated by PctA and PctB, which may form an alternate T1SS [34]. Further studies are needed to confirm the role of PctAB as a new T1SS, such as identification of its cognate substrates. Homologous *raxX-raxSTAB* systems have been identified in the following *Xanthomonas* lineages: *X. oryzae*, *X. vasicola*, *X. campestris* pv. *musacearum*, *X. campestris* pv. *vesicatoria*, *X. axonopodis* pv. *manihotis* and *X. maliensis* [4,35–48] (Fig. 1, Table S1). Other species, such as *Xanthomonas albilineans*, contain the *pctAB* but not the *raxSTAB system*, and *Xanthomonas citri* pv. *citri* 306 possesses *hlyDB,* a T1SS that presumably secretes the two RTX-related effectors XAC2197 and XAC2198 [34,49]. The *X. citri* pv. *aurantifolii* pathotype B genome lacks the *hlyDB* T1SS, an observation that may explain why it is less aggressive than *X. citri* pv. *citri* [49].

## 3. The knowns and unknowns of the Xps and Xcs type II secretion systems

Type II secretion systems (T2SSs) are large protein complexes with components spanning the envelope of Gram-negative bacterial cells (Fig. 2) that are evolutionarily related to the molecular machineries responsible for type IV pili, competence pili in Gram-positive bacteria, and archaeal flagella [50–52]. T2SSs export folded periplasmic proteins that are first translocated across the inner membrane by the Sec or Tat pathways [50,53]. Effectors are generally released through an outer membrane channel to the extracellular medium, but some are known to anchor or otherwise bind to the cell surface after secretion [54]. In human and plant pathogens, T2SSs are known to secrete a numerous and diverse set of virulence factors, toxins and degradative enzymes [55].

The core components of a T2SS can be named using the letters that commonly describe each protein of the system in different bacteria (except in the *Pseudomonas* genus) with the suggested standard prefix "Gsp" (referring to the general secretory pathway), though other nomenclatures persist, including in the *Xanthomonas* genus as discussed below. The T2SS machinery is mainly composed of 12 proteins that include a cytoplasmic ATPase (GspE), an inner membrane platform complex (GspC, GspF, GspL, and GspM), an outer membrane channel (GspD), a prepilin peptidase (GspO), and a pseudopilus (GspG, GspH, GspI, GspJ, and GspK) to eject the substrate. GspE is a hexameric ATPase that powers the system from the cytoplasm and is associated with copies of GspL [56]. Integral membrane proteins GspM and GspF also compose the inner membrane platform, which is responsible for assembling the periplasmic pseudopilus [57]. The pseudopilus is composed of the major pseudopilin GspG [58,59], as well as minor pseudopilins GspI, GspJ, and GspK, which form a ternary complex that sits at the tip of the filament [60,61]. Minor pseudopilin GspH is known to interact with the major pseudopilin, but is possibly non-essential to the system, as observed for PulH from *Klebsiella oxytoca* [53,59,62]. Similar to the pilins that compose type IV pili, pseudopilins are the products of prepseudopilins processed by a prepilin peptidase (called GspO in some systems), being cleaved and methylated at their N-terminus [53,63]. GspC is an inner membrane protein that connects the assembly platform to the outer membrane channel of the system through its periplasmic "homology region" (HR) domain [64]. GspD is a secretin family protein that multimerizes to form the outer membrane channel, which in some bacteria requires an additional pilotin lipoprotein for its correct localization and assembly [53,65]. The precise mechanism of secretion by the T2SS machinery has not been fully elucidated. Generally, it is proposed that ATP hydrolysis by GspE leads to the assembly and extension of the pseudopilus from the inner membrane platform, promoting the displacement of periplasmic substrates through the secretin and into the extracellular space [50].

Two distinct T2SS gene clusters can be found in the *Xanthomonas* genus: *xps*, conserved throughout the genus; and *xcs*, present in addition to *xps* in some species such as *X. citri* and *X. campestris*, but absent in others, such as *X. oryzae* strains [66,67] (Fig. 1, Table S1). Following the nomenclature of the T2SS machinery components detailed above, but with their own prefix, the 11 genes in the *xps* cluster code for proteins E, F, G, H, I, J, K, L, M, N (C), and D, in this respective order. The gene originally annotated as coding for XpsN, despite its name, actually corresponds to GspC and was therefore renamed "XpsC" [68,69]. The missing GspO prepilin peptidase function is supplanted by PilD, coded in another cluster with genes related to the type IV pilus (for which it is also an essential component), similarly to what has been observed in *Pseudomonas aeruginosa* and other bacteria [53,68]. Additionally, the XpsD secretin was experimentally verified to be lipidated at its N-terminus [70], thus probably dispensing the necessity of a lipidated pilotin for correct assembly and localization in the outer membrane as has been described for other liposecretins [65]. The *xcs* cluster found in some strains contains genes for proteins C, D, E, F, G, H, I, J, K, L, M, and N, in this order and again with their own prefix. In this case, XcsN, a homolog of GspN proteins in other species, is of unknown function and possibly non-essential for secretion [53,62].

Notably, the *xps* T2SS was found to be responsible for the secretion of a range of effectors and to influence virulence in different *Xanthomonas* species, while no function has so far been attributed to the *xcs* T2SS [71–74]. In fact, there have been no mutations in the *xcs* cluster reported to affect pathogenicity in any *Xanthomonas* strain. The *xcs* T2SS was seemingly acquired by horizontal transfer from a member of the Alphaproteobacteria, in which homologous systems have been encountered in environmental organisms such as *Caulobacter crescentus* [66,67,75]. Nevertheless, in *X. campestris* pv. *vesicatoria*, *xcs* genes were capable of at least partially complementing mutants of homologous *xps* genes (coding for equivalents of GspE and GspD), suggesting redundancy for some components of these systems [71].

Effectors of the *xps* T2SS identified in *Xanthomonas* species include cellulases, lipases, xylanases, endoglucanases, polygalacturonases, pectate lyases, and proteases, some of which have been shown to directly influence virulence [71–74,76]. By promoting degradation of the plant cell wall with the secretion of degradative enzymes, the *xps* T2SS of *Xanthomonas* spp. has been proposed to aid the assembly of the extracellular appendages of other secretion systems, such as the Hrp pilus from the T3SS [66,72,76]. Co-regulation of components of both systems by HrpG and HrpX seems to support this hypothesis (as can be seen below in the section describing secretion system regulation). A further functional interplay between these secretion systems was observed in *X. oryzae* pv. *oryzae*. Secreted T2SS effectors generate host cell wall degradation products that elicit plant defenses (such as callose deposition in rice leaves) which are, in turn, circumvented by bacterial T3SS effectors [77].

The functions of the *xcs* cluster remain unknown, but a possible clue comes from an RNA-seq study with mutants for stringent response regulators in *X. citri* pv. *citri*. Mutants for genes essential

for this response, i.e. the transcription factor DskA and the (p)ppGpp synthases SpoT and RelA, presented significantly reduced expression of most *xcs* genes, while no difference was observed for the *xps* cluster [78]. This is interesting since the stringent response is induced by stress factors such as nutrient deprivation, which may point to a possible, yet unexplored role for this enigmatic T2SS in cell survival under these adverse environmental conditions. This hypothesis gains support from studies on the *C. crescentus* T2SS cluster, that is most closely related to the *Xanthomonas xcs* T2SS. In this bacterium, this system was found to be essential for the secretion of the extracellular ElpS lipoprotein, triggered by phosphate starvation [75]. ElpS is thought to be involved in the mobilization of inorganic phosphate, and the fact that the *xcs* clusters found in *Xanthomonas* species also contain a gene coding for an ElpS homologue (XAC0692 in *X. citri* pv. *citri* 306, for example) is again suggestive of a possible role in environmental adaptation. Interestingly, this specific role in response to stress could be mirrored in *P. aeruginosa*, which also possesses a secondary T2SS involved in phosphate acquisition, coded by the *hxc* cluster [79].

## 4. Type III secretion system: a major virulence determinant

The Type III secretion system (T3SS) is one of the most studied secretion systems due to its correlation with bacterial pathogenesis. The first steps towards determining its role in triggering animal and plant diseases by pathogenic bacteria were taken more than 30 years ago [80]. Subsequent studies also linked T3SSs to symbiotic interactions between bacteria and plant species, broadening its role and relevance in nature [81]. Today, the T3SS (Fig. 2) is a well-characterized multi-protein molecular machine specialized in the translocation of a diversity of effectors into a wide variety of hosts, such as plants and animals including vertebrates, nematodes and insects [80–83].

T3SSs contribute to the pathogenicity of many species of great importance to human health, such as *Yersinia pestis*, *Shigella* spp., *Salmonella enterica*, enteropathogenic and enterohemorrhagic *Escherichia coli* (EPEC and EHEC) and *Vibrio cholerae*. They are important for the pathogenicity of plant-associated species of the genera *Xanthomonas*, *Erwinia*, *Ralstonia* and *Pantoea* and can also be found in species of the genera *Sinorhizobium*, *Mesorhizobium*, *Bradyrhizobium* and *Rhizobium* that establish symbiotic relationships with plants. *P. aeruginosa* represents an interesting case, as its T3SS contributes to pathogenicity in both plant and animal hosts, including humans.

### 4.1. Evolution and genetic organization of T3SSs in Xanthomonas spp

For a long time, the origin of bacterial T3SSs was subjected to intense debate, but it is now understood that they probably evolved by exaptation from a primal flagellar-like apparatus, a surface structure required for swimming motility and with which they share several features [1,84–86]. During the course of evolution, further diversification occurred, thus creating seven distinct families of non-flagellar T3SSs (Hrp1, Hrp2, Rhizo, SPI-1, SPI-2, Ysc and Chlamy) [87], although a recent survey indicates that the number of categories can reach thirteen [88]. Each bacterial genus generally encodes for a T3SS from one specific group, although some bacteria with complex lifestyles may carry more than one T3SS from different groups. From these families, Hrp1, Hrp2 and Rhizo are found in plant-interacting bacteria: the first two are exclusive to phytopathogens and the last one to plant symbionts [87]. Hrp1 (found in species of *Pseudomonas* and *Erwinia*) and Hrp2 (found in species of *Ralstonia*, *Xanthomonas*, *Burkholderia* and *Acidovorax*) groups differ from each other in gene composition, arrangement and transcriptional regulation [80,87].

Structural T3SS-coding genes are grouped in pathogenicity islands (PAIs) of ~20–50 kbp in size, most often located on the chromosome, but in some cases also in plasmids [80,89,90]. In phytopathogenic species, T3SS genes were historically designated as "hypersensitive response and pathogenicity" (*hrp*) due to their importance for the development of plant response and disease in resistant and susceptible plants, respectively. Depending on the degree of conservation among different species and role in T3SS biogenesis, *hrp* genes can be further divided into *hrc* (*hrp*-conserved) and *hpa* (*hrp*-associated) genes. Over the years, these T3SS components received unique names in each bacterial species under study (from both animal and plant pathogens), which generated some degree of confusion when comparing them. In 1998, Hueck [91] proposed a unified nomenclature for the conserved components of T3SSs, the prefix "secretion and cellular translocation" (*sct*), although in plant-related bacteria this nomenclature is not commonly used.

Although the Hrp2-family T3SS is the major virulence determinant for *Xanthomonas* species [92], an exception is found in *X. albilineans* which instead carries a SPI-1-family T3SS [93,94], apparently not involved in pathogenicity [95]. SPI-1-family T3SSs are also present in three strains of *X. axonopodis* pv. *phaseoli* (CFBP 2534, CFBP 6164 and CFBP 6982), which all carry a second, Hrp2-family T3SS [94,96]. *Xanthomonas cannabis* strains NCPPB 3753 and NCPPB 2877 [41], the non-pathogenic *Xanthomonas sacchari* NCPPB 4393 [46] and R1 [97], and *X. maliensis* 97M [44] all lack an Hrp-T3SS and its associated effectors. Additionally, in *Xanthomonas arboricola*, some strains infecting walnut and stone fruit trees are typical Hrp-T3SS-dependent pathogens, while others strains are non-pathogenic (termed commensal) with a reduced effector repertoire or with no T3SS at all [98] (Fig. 1, Table S1). In light of recent findings based on genomic comparison using several newly sequenced *Xanthomonas* species, genome erosion and gains and losses of genes associated with life history traits between different bacteria are the apparent driving forces for speciation and the appearance of commensal and pathogenic *Xanthomonas* strains [98–102].

### 4.2. Structural organization of the T3SS machinery

The T3SS apparatus (Fig. 2) is made up of more than 20 different gene products, each usually in more than one copy, which together form a large supramolecular structure that spans the diderm cell envelope of Gram-negative bacteria [91,103]. This so-called *injectisome* (due to its similarity with a syringe-like structure) is composed of three subassemblies, each of which plays a specific role during the process of selecting the effectors in the bacterial cytoplasm and their subsequent secretion to the external milieu or delivery into the target host cell [104–110]. The sorting platform is a large (~25 nm height by ~35 nm width) complex formed by the scaffold proteins $HrcQ_{SctQ}$ and $HrcL_{SctL}$, and the AAA+-like ATPase $HrcN_{SctN}$ that supplies energy to the system [109,111–113]. This cytoplasmic structure creates a chamber-like space, where the T3SS effectors are recognized and partially unfolded [114] before moving on to the next stages of the transfer process through the system [115]. The ~3.5 MDa needle complex subassembly mediates the passage of effectors out of the cell [110]. It has a multi-ring tubular base that traverses the bacterial envelope plus an external needle-like filament (pilus) that is connected to the former by the inner rod, made of several copies of $HrpB2_{SctI}$ [110]. The base is mainly composed of three different subunits: $HrcD_{SctD}$, $HrcJ_{SctJ}$ and $HrcC_{SctC}$. As recently seen in enterobacteria, the former two proteins create an inner ring with 24-fold symmetry while the third makes up the outer ring and the neck, of mixed

15- and 16-fold symmetry, respectively [110,116]. Enclosed at the base of the needle complex, several protein subunits ($HrcV_{SctV}$, $HrcR_{SctR}$, $HrcS_{SctS}$, $HrcT_{SctT}$ and $HrcU_{SctU}$) together form the export apparatus, which serves as a duct for substrates to pass through the internal bacterial membrane towards the lumen of the pilus [110,117]. The sorting platform is connected to the export apparatus (via $HrcV_{SctV}$) by a stalk made of $HrpB7_{SctO}$ [109]. The T3SS pilus is made of a homopolymer of the self-polymerizing $HrpE_{SctF}$ protein. Phytopathogenic T3SS pili have been found in all major plant pathogens coding for an active T3SS [118–121], exhibiting greater lengths (in the micrometer range) compared to the T3SS needles from animal pathogenic bacteria, presumably due to the need to penetrate the thick plant cell wall [122]. Finally, in order to deliver the substrates into the eukaryotic cell, it is proposed that T3SSs use a pore-forming multimeric ring structure made up of the $HrpF_{SctE}$ protein, called translocon, and assembled at the most distal end of their pili in contact with the target cell membrane. Consistent with this hypothesis, HrpF is required for pathogenicity but is dispensable for the secretion of effector proteins *in vitro* [123].

The localization and role of the components described above is well described in the literature for animal-pathogenic species, such as *S. enterica*, *Shigella flexneri* and *Yersinia enterocolitica*. In many cases this allows for a straightforward extrapolation to *Xanthomonas* spp. T3SS components. However, there are some *hrp*/*hpa* gene products conserved in the genus which are less studied and characterized to date [124–130], and their precise role in the function of the T3SS remains incipient and unclear. Therefore, many questions regarding the mechanisms for regulation, assembly, substrate recognition and action of T3SS in *Xanthomonas* species are still unanswered.

### 4.3. T3SS effectors

Upon entering the plant host tissue, *Xanthomonas* is subjected to immune responses such as microbe- or damage-associated molecular pattern (M/DAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) [76,131–134]. To cope with these host immune responses, *Xanthomonas* uses the T3SS to deliver into host cell cytosol type III effector (T3E) proteins, also known as Avr (avirulence) or Xop (*Xanthomonas* outer proteins) proteins [76]. T3E proteins suppress immune responses in host plants promoting colonisation of the host milieu [76,131,132], and were initially identified through the characterization of resistance (*R*) gene triggered responses in non-host plants [135–137]. Later studies employing reporter fusion assays with well-known Avr proteins and calmodulin-dependent adenylate cyclase (Cya) have provided direct evidence for T3SS-dependent secretion and translocation of effectors to plant cells [138–140]. More recently, machine-learning approaches have been developed for searching for T3E candidates in *Xanthomonas* genomes [100,141], relying on multiple criteria such as GC content, codon usage, homology to known and validated effectors, amino-terminal secretion signals, structural patterns, and regulation by the HrpG/HrpX signaling pathway. Effectors are not only essential to *Xanthomonas* pathogenicity, but also are determinants of host specificity and pathogen fitness [100]. *Xanthomonas* T3Es have evolved to target different components of the PTI pathways in host plants, however some of them may show functional redundancy (Table S2). For example, XopR, AvrBs2, XopK, XopN, XopP, XopL, XopB, AvrAC, and XopS target and suppress components that mediate PTI responses [142–150], while XopQ, XopX, XopZ, and XopN inhibit DAMP-triggered immunity, and XopH and XopQ interfere with both PTI and ETI responses [151–156]. In addition, XopAU, a Ser/Thr kinase, contributes to disease development by manipulating MAPK signaling through phosphorylation and activation of the immunity-associated MKK2 (Mitogen-activated protein kinase kinase 2) [157]. Effectors such as XopD and XopJ interfere with the host ubiquitin proteasomal system, targeting PTI components and/or salicylic acid-mediated defense to suppress immunity [158–162]. Whilst effectors eliciting ETI in non-host plants were initially identified in studies of avirulence (*avr*) genes, molecular mechanisms involved in cell death are unclear for some of them [132,163]. Other effectors such as XopQ and AvrBsT can also function as ETI suppressors [155,164]. A screening for *X. oryzae* pv. *oryzae* effectors led to the identification of another five effectors, XopU, XopV, XopP, XopG, and AvrBs2, which could individually suppress the immune responses elicited by XopQ-XopX in rice [146]. These results suggest a complex interplay of *Xanthomonas* T3Es in suppressing both PTI and ETI to promote virulence on rice [146].

A list of identified T3E proteins can be found at the *Xanthomonas* resource website (http://xanthomonas.org/t3e.html). Table S2 also presents a list of *Xanthomonas* T3Es, describing predicted protein domains, identified roles in bacterial pathogenesis, and links to protein structures deposited in the protein data bank (https://www.rcsb.org/) [165]. It has been suggested that most *Xanthomonas* species carry a common set of nine T3Es (“effectorome”) [131,166] (Table S2). However, as the number and diversity of *Xanthomonas* genomes sequenced grows, so has the list of exceptions to this rule. For example, *X. campestris* pv. *raphani*, formerly classified as *X. campestris* pv. *armoraciae*, carries only XopP and XopR from the *Xanthomonas* effectorome (Table S2) [166]. In addition, *X. campestris* pathovars *incanae*, *raphani* and NP carry a limited core of type III effectors (XopP, XopF1, and XopAL1) of which only XopP is a component of *Xanthomonas* effectorome [167]. Likewise, *X. arboricola* contains strains that possess T3SS and variable numbers of effectors as well as commensal strains lacking T3SS but possessing four effectors (XopF1, XopM, XopR, and AvrBs2) [100]. Because of these recent findings from genome sequence data, the concept of a core effectorome of *Xanthomonas* including nine effectors is currently under debate [100,131]. Indeed, it remains to be determined whether the four effectors found in *X. arboricola* commensal strains allow them to overcome basal immune responses without a T3SS machinery, or if those strains behave as profiteers and survive within the plant host only in association with other *X. arboricola* pathogenic strains [100].

Most species in the genus *Xanthomonas* carry members of a distinct family of T3E proteins (AvrBs3/Pth family), which are known as transcription activator-like (TAL) effectors [168,169]. Interestingly, these effectors were also identified in *Ralstonia solanacearum* and *Mycetohabitans rhizoxinica*, an endofungal bacterium [170,171]. TAL effectors bind to host gene promoters in a sequence-specific manner and add plasticity to adaptation of the bacteria to host plants by direct manipulation of host metabolism and susceptibility [172,173]. Specific effector binding elements (EBEs) in the promoters of target genes are recognized by the rearrangeable central-repeat domain of TAL proteins [174,175]. Therefore, the TAL effector repertoire can target different host genes and affect different host physiological processes. The distribution of genes encoding TAL effectors among *Xanthomonas* spp. is variable, being absent in some strains while others, such as *X. oryzae* pv. *oryzicola* strain BLS256, encode up to 27 paralogues of these effectors [2,131,173]. Various TAL effector-associated susceptibility genes, associated with disease development, have been identified in different hosts [168,173]. For example, AvrHah1, a TAL effector in *Xanthomonas gardneri*, triggers expression of two basic helix-loop-helix (bHLH) transcription factors, which in turn activate the expression of a pectate lyase essential for the induction of water-soaked lesions on tomato fruits and leaves during bacterial spot disease [176]. Interestingly, *Xanthomonas translucens* pv. *undulosa*, causal agent of the bacterial leaf streak of wheat, deploys the TAL effector Tal8 to activate expression of the susceptibility gene TaNCED and thus stimulates the abscisic acid (ABA) pathway,

enhancing disease susceptibility [172,177]. Also, *CsLOB1*, a member of the plant-specific lateral organ boundaries (LOB) domain family of transcription factors, is targeted for expression by PthA4 of *X. citri* pv. *citri* and *X. citri* pv. *aurantifolii*, the causal agents of citrus canker [178,179]. Deletion of *pthA4* in *X. citri* or modification of the EBE in the *CsLOB1* promoter by genome editing leads to loss of the typical canker symptoms [180,181]. However, *CsLOB1* activation in some citrus hosts does not always correlate with canker formation and induction/repression of other genes and/or interaction with other host factors might also contribute for full canker development [182–185].

In this way, TAL effectors have been shown to promote host cell transcriptional reprogramming as a virulence strategy [172,183]. Consistent with this functional specialization, *X. oryzae* pv. *oryzae* TAL effectors target the susceptibility *SWEET* genes, which encode sugar transporters in rice, and are responsible for development of bacterial blight disease. Among susceptibility *SWEET* genes, *OsSWEET14* encodes a well-defined sugar exporter and is the target of PthXo3 (TalBH), TalC, and AvrXa7 [173,186,187]. *X. oryzae* pv. *oryzae* induction of *OsSWEET14* leads to sugar accumulation in the apoplast and xylem, promoting bacterial colonisation [173,187]. Likewise, OsSWEET11 and OsSWEET13 susceptibility genes were shown to be targeted by *X. oryzae* pv. *oryzae* PthXo1 and PthXo2 TAL effectors, respectively [188]. In the absence of SWEET gene expression, bacteria fail to effectively colonize rice leaves [184]. Notably, mutations in the EBE sequences of *OsSWEET11-13–14* promoters by CRISP-Cas9-mediated genome editing endowed rice lines with broad-spectrum resistance to bacterial blight disease, a breeding strategy called resistance by loss of susceptibility [188–190]. Furthermore, TAL effectors can trigger resistance associated with the hypersensitive response (HR) by activating an executor resistance (*R*) gene [191,192]. Executor *R* genes *Bs3* in pepper and *Xa10*, *Xa23* or *Xa27* in rice are activated by AvrBs3, AvrXa10, AvrXa23, and AvrXa27 TAL effectors, respectively [193–196]. *Xa10* in rice contains an EBE in its promoter that is recognized by AvrXa10 specifically inducing *Xa10* expression. After activation, XA10 triggers programmed cell death by a mechanism involving disruption of the endoplasmic reticulum and cellular $Ca^{2+}$ homeostasis [194]. Recently, a short PthA effector carrying only 7.5 repeats in its DNA-binding domain ($PthA4^{AT}$) was shown to trigger a host-specific HR in *Citrus limon* [197]. This suggested that $PthA4^{AT}$ may directly activate an executor target gene implicated in the HR and determines host range in citrus. Interestingly, PthA4 activates the expression of LOB1, a homolog of *CsLOB1*, in Meiwa kumquat (*Fortunella crassifolia*), which in this plant works as an executor gene by triggering immunity and limiting *X. citri* host range [198]. Also, it was demonstrated that TAL effectors can elicit an ETI response by binding and forming a complex with specific R proteins [199]. Rice XA1, a nucleotide-binding leucine-rich repeat protein, can recognize and interact with different TAL effectors [200]. Similarly, four *Xa1* allelic R genes *Xa2*, *Xa31* (t),*CGS-Xo1*$_{11}$, and *Xa14*, which were identified in different rice varieties, trigger an ETI response upon interaction with TAL effectors [201]. However, TAL effector-triggered resistance by *Xa1* allelic R genes is suppressed by sets of truncated TAL effector (or interfering TALEs), known as iTALEs, which suppresses recognition mediated by XA1 [200].

## 5. T4SS: a bactericidal nanoweapon

Type IV secretion systems (T4SSs) are used by bacteria to transfer proteins and/or protein-DNA complexes into target prokaryotic or eukaryotic cells. They are responsible for bacterial conjugation (an indeterminately large number of conjugative plasmids), the transfer of T-DNA into plant cells (*Agrobacterium tumefaciens*), the transfer of proteinaceous effectors into mammalian hosts (i.e. *Legionella pneumophila*, *Helicobacter pylori*) and the transfer of toxic effectors into bacterial cells of competitive species (the X-T4SSs first identified in *Xanthomonas* and related species) [202–206].

Type IV secretion systems have traditionally been divided into two classes: the canonical Type IVA (T4ASS) and the larger and more complex Type IVB (T4BSS). However, as more and more of these systems are characterized, the picture that is emerging is that of a continuous spectrum of complexity [207,208]. The chromosomes of many *Xanthomonas* species carry the genes that code for the core 12 subunits found in the T4ASSs: three subunits (VirB7, VirB9, VirB10) that form the outer membrane pore (commonly referred to as the core complex), seven subunits (VirB3, VirB4, VirB6, VirB8, VirB11, VirD4), plus the N-terminal domain of VirB10, that are associated with and form a pore through the inner membrane and two subunits (VirB2 and VirB5) that are expected to form an extracellular pilus structure (Fig. 2). Finally, VirB1 is predicted to be a periplasmic lytic transglycosylase involved in peptidoglycan remodeling during T4SS biogenesis [202–206].

The first study on a chromosomally-encoded *Xanthomonas* T4SS identified protein-protein interactions for baits of individual T4SS subunits in two-hybrid assays against a prey library derived from the entire *X. citri* genome [209]. One interesting observation was that no interactions were observed between components of the plasmid-encoded system and the chromosome-encoded system. The plasmid-encoded system seems to be associated with plasmid mobilization (see below) while the chromosomally encoded T4SS system was shown not to be required for the development of citrus canker symptoms [210] but rather confers a competitive advantage to *X. citri* during physical encounters with other Gram-negative bacterial species such as *E. coli* and *Chromobacterium violaceum* [211]. The chromosomally-encoded T4SSs in *Xanthomonas* species do have some features that distinguish them from the more thoroughly studied T4ASSs from *A. tumefaciens* and the self-mobilizing plasmids R388 and pKM101; for example larger VirB7 and VirB8 subunits [203,210,212]. The cryo-EM structure of the *X. citri* T4SS core complex showed that the characteristic globular VirB7 C-terminal domain decorates the perimeter of the core complex, resulting in a distinctive profile reminiscent of a flying saucer [212].

The two-hybrid assays also identified a set of a dozen *X. citri* proteins that interact with the coupling protein VirD4, the inner-membrane-associated ATPase that recognizes substrates to be secreted [209]. These *Xanthomonas* VirD4-interacting proteins all contain a common C-terminal domain of approximately 120 residues termed XVIPCDs (*Xanthomonas* VirD4 interacting protein conserved domains) [209]. Subsequent studies in *X. citri* [211] and the related *Xanthomonadaceae* species *S. maltophilia* [213] showed these proteins are translocated into target bacterial cells resulting in death and that the XVIPCD is required for secretion. Therefore, these bactericidal T4SS are now known as X-T4SS and their secreted effectors are called X-Tfes (*Xanthomonadales*-like*ceae* T4SS effectors).

X-Tfes are highly variable in size and architecture due N-terminal regions that carry different domains that in many cases are predicted to degrade peptidoglycan and phospholipids in the target cell periplasm as well as other effectors that may act in the cytoplasm. Bioinformatics analysis has identified X-Tfes in many *Xanthomonas* species as well as others from the *Xanthomonadales* order within the Gammaproteobacteria and a few other families within the Betaproteobacteria [203,211,213]. So far, all species that carry a homologous X-T4SS also code for many proteins with XVIPCD domains, potential X-Tfes. Therefore X-T4SSs are used to inject a potent cocktail of toxic enzymes into the target cell (Fig. 2).

All X-Tfes are neutralized by immunity proteins called X-Tfis whose genes are found upstream of those for their cognate X-Tfe [203,211,213]. The end of the X-Tfi gene is often very close to, or overlaps with, the beginning of the X-Tfe genes, suggesting that they are in most cases co-transcribed as a polycistronic message. These X-Tfis can, in principle, protect the cell against the toxic effects of endogenous X-Tfes as well as X-Tfes injected by neighboring cells. Since many predicted X-Tfe targets are found in the periplasm, many X-Tfis are have N-terminal signal sequences with lipoboxes and are predicted to be periplasmic lipoproteins [211,213].

The mechanism by which these X-T4SS recognize their toxins for secretion seems to be well conserved since X-Tfes from one organism can be deployed by the X-T4SS from another [213]. Therefore, the horizontal transfer of genes encoding X-Tfe/X-Tfi pairs could increase the arsenal of toxins that a bacterium can simultaneously deploy upon encounters with competing species. Furthermore, *X. citri* and the closely related *S. maltophilia* can use their homologous X-T4SSs to kill each other, in part due to the fact that their X-Tfe and X-Tfi repertoires are significantly different [213]. In addition to the advantage gained from killing cells of rival species that would compete for space, lysis of the target cell would be expected to increase the local concentration of sugars, amino acids, nucleotides and other valuable nutrients that could be captured, compensating, at least in part, the metabolic cost of producing and maintaining the X-T4SS [214].

### 5.1. T4SS-mediated conjugation

Historically, T4SSs were first characterized as structures that mediate the transfer of DNA, often in the form of plasmids, from one bacterium to another [215]. A large number of sequenced *Xanthomonas* genomes revealed the presence of extrachromosomal plasmids varying in size from a few to hundreds of kilobases. These plasmids play a particularly important role in *Xanthomonas* biology since they often code for proteins that mediate interactions with the host, for example secretion systems and/or secreted effectors, or provide mechanisms to survive environmental stress, for example exposure to divalent metals (for some selected examples see [39,42,93,216–223]). Many of these plasmids carry T4SSs that are distinctly different from the bacteria-killing X-T4SSs described above (significantly lower amino acid sequence identity for all subunits). Furthermore, the structural genes that code for the secretion channel are accompanied by genes coding for DNA processing components of the relaxosome, a multiprotein complex responsible for recognizing and nicking the oriT (origin of transfer) site on the plasmid, separating the two DNA strands, forming a covalent bond with the central relaxase component and directing the relaxase-ssDNA conjugate to the coupling protein (VirD4 homolog) for subsequent secretion [205,206,224,225]. This strongly suggests that these *Xanthomonas* plasmid-encoded T4SSs play a role in conjugation [209,222,223,226]. Horizontal gene transfer of both plasmid and chromosomal DNA between *Xanthomonas* strains in different species has been observed *in planta* [216,217,226]. El Yacoubi et al. (2007) [222] showed that the ability of pXcB to self-mobilize depends on the presence of an intact plasmidial T4SS and observed conjugation between two different *Xanthomonas* strains *in planta*. Phylogenetic analysis of a large number of genomes have provided evidence of the importance of horizontal transfer, plasmid insertion and recombination during the evolution of *Xanthomonas* species (for example: [227–229]).

### 5.2. Origin of bacteria-killing T4SSs

VirD4 proteins and their homologs play a central role in the recruitment of macromolecules for secretion in all T4SSs, whether they are involved in the secretion of a covalent protein-DNA complex in the case of conjugation or solely protein substrates. In X-T4SSs, the evidence so far points to a direct interaction between the VirD4 protein and the XVIPCD domain of X-Tfes [209]. In conjugation, VirD4 homologs interact with specific sequences or domains in the relaxase proteins as well as with auxiliary proteins such as TraM and IHF [224,225,230,231]. Many self-mobilizable plasmids also carry the genes for toxin/antitoxin pairs that are thought to participate in plasmid maintenance. If recombination events lead to the duplication and insertion into a toxin gene of relaxossome component domains that mediate binding to the coupling protein, then the conjugation system will transfer the actual toxin protein (not only its gene) along with the plasmid [232]. Strains with mutations that result in loss of the ability to transfer the plasmid (for example via a random mutation in the gene of an essential relaxossome component) but that maintain the T4SS apparatus intact could continue secreting the toxin. Since this could provide a competitive advantage to the cell, the system could be expected to be maintained. In this way, through only a few rearrangements in the genetic material, a conjugation system could be converted into a bacterial killing system [203,232]. Subsequent integration of the plasmid into the bacterial chromosome will lead to fixation of the bactericidal T4SS in the bacterial lineage.

## 6. Type V secretion systems: non-fimbrial adhesins for colonization

Type V secretion systems (T5SSs) are the simplest among bacterial secretion systems, from a structural point of view. They consist of one or two proteins that localize in the outer membrane of Gram-negative bacteria and do not require ATP or electrochemical gradients as energy sources for transport (Fig. 2) [233,234]. They have been classified into Va-Ve subclasses, and a Vf subclass restricted to *H. pylori* has been recently described [235]. T5SS members are also known as autotransporters, because the translocated effector, called the passenger domain, is found in the same polypeptide chain as the transporter, which forms a β-barrel in the outer membrane. The exception is the Vb subclass, also known as two-partner secretion systems, which are formed by a protein transporter and a separate cognate passenger protein [236]. Translocation from cytoplasm to the periplasm is mediated by the Sec translocase pathway, after recognition of the amino-terminal signal peptide [237]. The exported effectors are either released to the extracellular milieu or remain associated with the transporter, becoming exposed at the bacterial cell surface. Effectors have a plethora of identified roles in animal and plant pathogens, such as adhesins, exoenzymes, toxins and proteins involved in immune evasion [238,239]. All T5SSs described in *Xanthomonas* spp. fall into the Va, Vb and Vc subgroups, which we briefly describe below (Fig. 1, Table S1).

The type Va/classical autotransporter is a monomeric autotransporter subclass which includes proteases, lipases and adhesins. Their C-terminal transporter domain folds into a 12-stranded beta-barrel in the outer membrane, while the passenger domain usually presents a repetitive β-helix fold, although other structures are also possible, such as the α-helix structure typical of EstA [235]. Type Vb/two-partner secretion system (TPS) is the only T5SS subclass consisting of two proteins, the passenger domain with effector function (TpsA) and its transporter TpsB, which forms a pore in the OM for TpsA translocation. These two polypeptides are generally encoded in one operon. TpsB typically contains a 16-stranded beta-barrel domain that forms the outer membrane pore and two periplasmic POTRA domains, responsible for recognition of the cognate partner via binding to a TPS domain in TpsA. TpsA is usually released into the milieu after secretion,

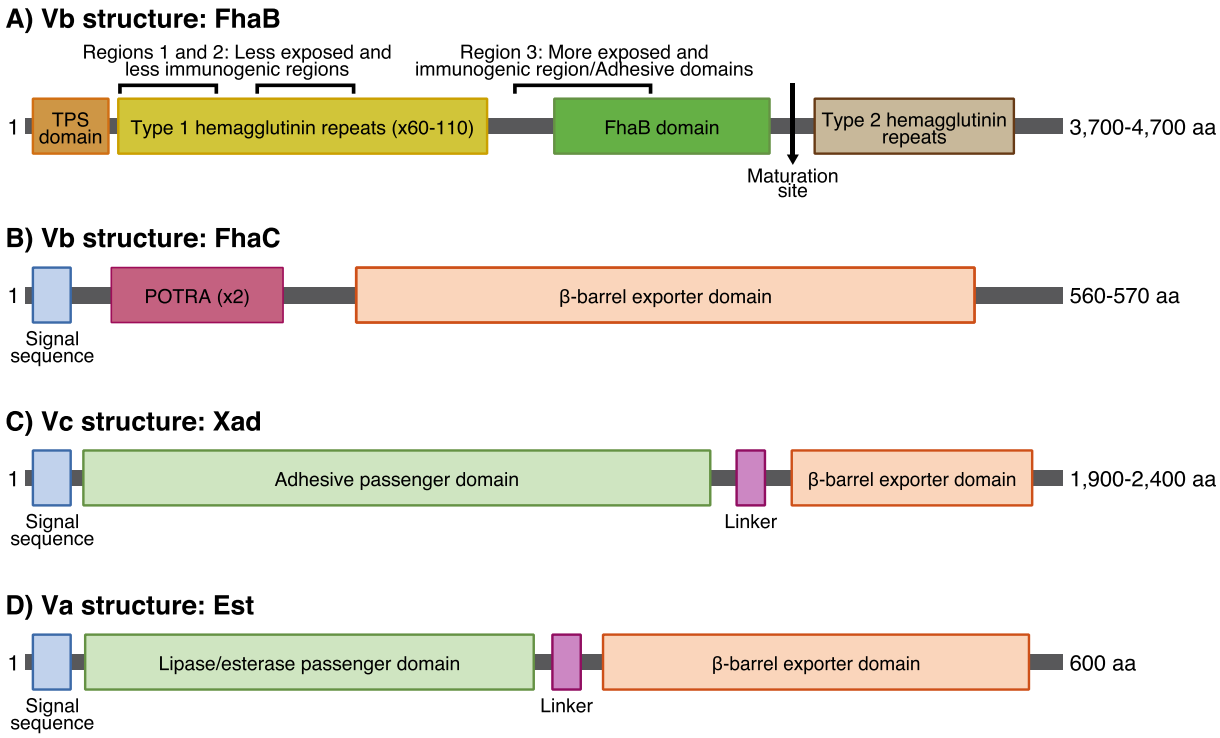



**Fig. 3.** Conserved domains found in prototypical FhaB/C (A/B), Xad (C) and EstA (D) T5SS from *Xanthomonas*. Sec-dependent signal sequences, functional passenger domains and beta-barrel domains that form the outer membrane transporter are indicated by blue, green and red boxes, respectively. (A) Representation of passenger protein FhaB. TPS domain, type 1 and 2 hemagglutinin repeats are depicted as orange, yellow and brown boxes, respectively. Black arrow denotes the maturation site. Regions functionally characterized in XacFhaB are indicated by brackets [252]. (B) Representation of the exporter protein FhaC. (C, D) Purple boxes represent the linker region between passenger and transporter domains. Polypeptide chains are not represented to scale. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

although anchorage to the OM by TpsB association is also possible [236]. The type Vc/trimeric autotransporter adhesins are surface-exposed adhesins that assemble in homotrimeric structures at the outer membrane. The exporter pore is formed by a 12-stranded β-barrel, with each monomer contributing 4 β-strands to the structure [237].

Functional studies of T5SS in *Xanthomonas* spp. have mainly identified roles in adhesion in the context of pathogenic processes. These proteins are classified as non-fimbrial adhesins and play an important role in the first steps of bacteria interaction with host and non-host plants. Four main gene families have been identified, encoding FhaB/C, Xad and YapH adhesins and the lipase/esterase EstA. Genome comparisons of sequenced *Xanthomonas* species and strains from distinct pathovars have shown that the repertoire of T5SS adhesins is highly variable and is possibly an important determinant of host specificity in the genus [166,240,241]. In addition, some genomes present truncated paralogues of some T5SS components encoded in tandem and close to functional ones, which may be a source of variability through recombination. Fig. 3 presents the distribution of domains found in prototypical T5SS components from each group.

It has been suggested that genes encoding adhesins, as well as those involved in environmental sensing – both functions involved in the initial steps of *Xanthomonas* pathogenesis – are under adaptive divergence [241]. Therefore, an intense selection scenario on an ancestor gene set would favor the creation of an optimized combination of adhesins for a specific microenvironment during colonization and infection. Accordingly, in some cases, evolutionary distinct species that infect the same host plant share a similar set of adhesin and sensor proteins, as seen for the sugarcane pathogens *X. axonopodis* pv. *vasculorum and X. vasicola* pv. *vasculorum*, while distinct pathovars of the same species have a more divergent repertoire [241]. Genome comparisons among pathogenic and non-pathogenic strains of *Xanthomonas* indicated a greater prevalence of the T5SS adhesins YapH, XadA and XadB in pathogenic strains [3]. Similarly, the presence of FhaB/C in two pathogenic isolates of *X. arboricola* and absence of this T5VbSS in two non-pathogenic strains of the same species was also reported [98].

The most well characterized VbSS so far is XacFhaB/XacFhaC (TpsA/TpsB) encoded by XAC1815/XAC1814 in *X. citri* pv. *citri*. Null mutations in XacFhaB revealed a highly dysfunctional phenotype in terms of abiotic and leaf surface adhesion, bacterial viability in leaves, bacterial aggregation, biofilm formation and pathogenicity. Mutation of the cognate transporter XacFhaC caused a less severe phenotype, raising the possibility that FhaB could be transported through the putative paralogous Vb transporter XAC4114, an unusual mechanism for Vb/Tps action [242]. A role for XacFhaB in biofilm formation was also suggested by a genome-wide screening for mutants with deficient biofilms [243], and the XacFhaB homologue in *X. citri* pv. *fuscans* is required for biofilm formation in biotic and abiotic surfaces and efficient bacterial transmission from xylem to seeds [244]. XacFhaB contains an adhesive region located N-terminal to a predicted maturation site, which becomes surface-exposed after cleavage, according to the model from FhaB homologues in *Bordetella pertussis* and *Bordetella bronchiseptica* (Fig. 3) [245,246]. Both the maturation site and the C-terminal region are required for *B. pertussis* FhaB function *in vivo,* working as an intramolecular chaperone controlling folding of the adhesion site prior to cleavage [246]. Importantly, genome comparisons in plant-associated bacteria show that the C-terminal effector region of the Vb subtype carries the bulk of the genetic variation [247]. XacFhaB contains around a hundred type 1 hemagglutinin-type repeats (20 residues each), adopting right-handed β-helical folds which act as adhesive domains (Fig. 3) [248]. In addition, a disor-

dered polypeptide sequence with type-2 hemagglutinin repeats is present after the maturation site of XacFhaB. Repetitive domains are a common feature of T5SS adhesins, providing flexibility that facilitate intimate cell-to-cell (bacteria-bacteria and bacteria-host) contact as well as serving as hotspots for recombination that contributes to adaptability [249,250,251].

Syringe infiltration of purified XacFhaB into leaves promotes immune responses and impairs subsequent infections in orange. Furthermore, syringe infiltration of purified XacFhaB regions 2 and 3 (Fig. 3) in orange and tomato leaves promotes callose deposition, ROS production and expression of defense genes such as CsHIR1 (named SlHIR1 in tomato) [252]. These genes are homologs of pepper CaHIR1, a membrane-bound regulator of cell death, salicylic acid accumulation and pathogenesis-related gene expression, which is induced by the PTI pathway [253]. The *X. campestris* pv. *vesicatoria* FhaB homolog, Fha1 (XcvFha1) directly interacts with CaHIR1 when expressed in pepper leaves, triggering plant cell death and suppressing the expression of plant defense genes [254]. Mutation of XcvFha1 resulted in reduced virulence and motility and greater surface attachment [254]. XcvFha1 is a small protein (445 amino acids) devoid of the amino-terminal TPS domain involved in recruitment by the cognate transporter, thus its cellular localization and/or mechanism of secretion remains elusive. This suggests that XcvFha1 enhances bacterial virulence, increasing bacterial spread *in planta*, while suppressing plant defenses and activating pathogen-induced cell death upon interaction with CaHIR1, helping the pathogen to overcome the host basal defense response [254].

Genome analysis of distinct *Xanthomonas* species has shown interesting features regarding distribution of FhaB/FhaC homologues. Genes for a FhaC exporter and three FhaB paralogues (FhaB, FhaB1 and FhaX) are found in a region flanked by ISXo5 transposase insertion sequences in *X. oryzae* pv. *oryzae* PXO99A genome. These genes are absent from strains MAFF311018 and KACC10331 and only one ISXo5 is found in the same region, pointing to a loss through genomic arrangement in these two strains, since *X. oryzae* pv. *oryzicola* BLS256 also contains the same genetic cluster [45]. Interestingly, we have identified two XacFhaB homologues (XACM_1837 from *X. axonopodis* pv. *citrumelo* and EBN15_07745 from *Xanthomonas cucurbitae*) that present an extra C-terminal extension with PT-VENN and Ntox24 toxic domains (data not shown). These domains are found in TpsA from the closely related contact-dependent growth inhibition (CDI) Vb T5SS, which deliver antibacterial toxins and are involved in interbacterial competition. The PT-VENN is required for specific recognition of target cells and toxicity is conferred by the Ntox24, a recently described domain with predicted metal-independent endoRNAse activity [255,256]. Thus, it is tempting to propose that these proteins acquired new toxic functions in these two *Xanthomonas* species.

Functionally characterized YapH and Xad genes in *Xanthomonas* species play important roles in bacterial adhesion. YapH from *X. axonopodis* pv. *glycines* is required for adhesion to plant leaves, whereas it negatively impacts virulence and bacterial egress from leaves. However, when host leaves are incubated under conditions that simulate rainfall, repression or mutation of YapH severely reduces virulence. Altogether, these data indicate that this adhesin is required for attachment to leaves but hampers later stages of infection, after apoplast colonization [257]. Similarly, deletion of YapH in *X. citri* pv. *fuscans* resulted in reduced adhesion and a quasi-inexistent biofilm but greater virulence. XadA2 mutation led to loss of seed infection through the xylem pathway, whilst not affecting virulence or biofilm formation, and XadA1 deletion did not affect any of these aspects [244]. In *X. oryzae* pv. *oryzae,* YapH mutation rendered the strain slightly less able to attach to and enter rice leaves and caused reduced virulence and deficiency for migration *in planta*. Curiously, single mutations in XadA and XadB caused a more pronounced phenotype regarding leaf adhesion and invasion as compared to YapH mutation, with increased severity in the double mutant strain, but did not result in reduced virulence. These observations indicate that multiple adhesins are involved in pathogenicity, at different stages of disease evolution in this species [258]. The *X. citri* pv. *citri* YapH homologue XAC2151 is induced in cells from biofilms and has been proposed to promote surface adhesion [259]. *X. campestris* pv. *campestris* XadA1 has been identified as a cargo component of outer membrane vesicles released by the pathogen upon contact with target cells, suggesting a role in vesicle adhesion to their targets [260]. The xylem-limited species *X. albilineans,* characterized by significant genome reduction and the lack of important virulence factors, carries one copy of XadA (XALC_2666) and FhaB/C homologues as the sole representatives of the T5SS [261], as well as putative and seemingly truncated YapH (XALc_1305) and XadA (XALc_1884) homologs.

The EstA lipase (XOO3370) was identified as a pathogenicity related gene in *X. oryzae* pv. *oryzae* [262]. Xv_EstE from *Xanthomonas vesicatoria* was identified through its similarities to *Xylella fastidiosa*, *Moraxella bovis*, and *P. aeruginosa* EstA homologues and to other lipase/esterase autotransporters such as XOO3370. This EstA was not initially classified as a T5SS because the lipase passenger domain is not translocated to the extracellular environment or cell surface by the transmembrane exporter domain, but remains in the periplasm [263].

Another XOO3370 homolog is the *X. campestris* pv. *campestris* Xcc_Est lipase, identified in extracellular fractions by proteomic analysis [264]. It was successfully used as a target for heterologous protein secretion. Wang et al. (2014) demonstrated efficient secretion of a gama-lactamase from *Sulfolobus solfataricus* when expressed in *E. coli* as a fusion protein to the C-terminal transporter domain of Xcc_Est [265]. The same lipase was also used as the determinant for detection of pathogenic *Xanthomonas* based on the ability to hydrolyze Tween 80 during growth in the differential medium Xan-D (expression of Xcc_Est (and its homologs) results in cleavage of this detergent and the formation of a white halo around the colonies). Twenty-six *Xanthomonas* pathovars were successfully isolated through this method, pointing to the widespread distribution of EstA homologs in the *Xanthomonas* genus [266].

## 7. T6SS: anti-amoeba and anti-bacterial families

This secretion system is the most recently described in diderm bacteria, in two concomitant studies that characterized a cluster of genes required for secretion of virulence proteins in the animal pathogens *P. aeruginosa* and *V. cholerae* [267,268]. Although these studies pioneered the recognition of a conserved new class of secretion system in bacteria, the first functional characterization of members of this group was performed three years earlier in the plant symbiont *Rhizobium leguminosarum* [269]. The *R. leguminosarum* bv. *trifolii* T6SS, originally named *imp* locus (“impaired in nitrogen fixation”), was identified as a conserved cluster of 14 genes that encode a temperature-dependent secretion system involved in inhibition of nodulation and nitrogen fixation in pea, a legume which is not naturally infected by the strain [269]. Comparative genomics studies showed that T6SSs are widespread in pathogenic and non-pathogenic Proteobacteria, commonly found in clusters encoding 13 core protein components, which are phylogenetically separated into 5 clades [270]. Evolutionarily divergent T6SS clusters were later identified and classified as subtypes T6SS$^{ii}$ and T6SS$^{iii}$, each restricted to the *Francisella* genus and the Bacteroidetes phylum, respectively [271,272,273]. More recently, a new distantly related contractile injection system has been identified in the intracellular amoeba symbiont *Amoebophilus asiaticus*

and classified as T6SS$^{iv}$ [274]. Despite its relatively recent history, remarkably rapid progress has been made in the understanding of T6SS function and mechanisms of T6SS assembly, secretion and effector recruitment.

These nanomachines directly translocate proteins to prokaryotic and eukaryotic target cells and are more frequently involved in contact-dependent interbacterial antagonism, by the delivery of toxins that promote bacterial killing. In addition, T6SS have been described as playing roles in killing of fungal cells [275] and delivery of effectors that promote virulence in eukaryotic hosts [276]. T6SSs that are involved in nutrient acquisition by secretion of metal scavenging proteins to the extracellular milieu were identified in *Pseudomonas* species, as well as in *Yersinia pseudotuberculosis* and *Burkholderia thailandensis*, suggesting that distinct modes of protein secretion and a wide variety of functions can be associated with this class of secretion systems [277]. Also, some T6SSs are able to secrete proteins to both prokaryotic and eukaryotic cells, engaging in interbacterial competition and host subversion, as described for T6SSs H2 and H3 of *P. aeruginosa* [278,279].

T6SS$^{i}$ from all 5 clades (i1 to i5) are distributed among plant-associated bacterial species, irrespective of the type of bacteria-plant interaction, pathogenic or non-pathogenic (mutualists and commensals) and are more frequently involved in bacterial competition than virulence, providing fitness advantages against other community members [280]. Importantly, in some cases, effectiveness of bacterial killing was only observed during growth *in planta*, as described for *A. tumefaciens* and in the nodule-forming rhizobacteria *Azorhizobium caulinodans* [281,282]. Despite the initial characterization in *R. leguminosarum* that indicated a role of T6SS in the restriction of nodulation in non-host plants, recent work in *R. etli* has shown a nodule promoting activity of the T6SS during symbiotic interaction with bean plants, suggesting that effects of T6SS in bacteria-legume symbiotic interactions vary according to the pair involved [283]. T6SS mutations affecting plant pathogenesis have been described in bacteria. However, a direct role of T6SS in pathogenicity by secretion of T6SS effector proteins that subvert plant cell functions has not yet been described. Virulence attenuation may result from indirect detrimental effects in ability to colonize the plant tissue, such as impairment in biofilm formation, nutrient acquisition or competition with the beneficial bacterial community.

The T6SS is organized in the bacterial cell envelope in three main structures that assemble to form the functional contractile system: the membrane complex, a baseplate and an extended inner tube surrounded by a contractile sheath (Fig. 2). The membrane-spanning complex is formed by the lipoprotein TssJ, the inner membrane protein TssL and TssM, which contains a cytosolic N-terminal domain that interacts with TssL and a C-terminal domain that traverses the periplasm and interacts with TssJ in the outer membrane [284–286]. TssL and TssM connect the membrane complex with a baseplate formed by TssK, F, G, E, VgrG and PAAR, which is structurally and evolutionarily related to baseplates from contractile phages. The central portion of the baseplate is formed by a VgrG trimer that assembles in a needle-like structure containing a protein with typical PAAR repeats at its tip [287,288]. The VgrG trimer is surrounded by six copies of a complex formed by TssE, F, G and K. Baseplate assembly is required for Hcp polymerization, which then promotes formation of the surrounding sheath formed by subunits of TssB and TssC, generating the extended T6SS phage-like tube in the inactive state [289,290]. Sheath/tube assembly and extension is coordinated by TssA-like proteins [291]. According to the current model, environmental signals that induce T6SS activity are sensed by the transmembrane complex and subsequently transduced to the baseplate, causing it to open and induce sheath contraction, which propels the inner tube decorated with T6SS effectors through the bacterial envelope and into target cells [285]. Cargo effectors are recruited to the system during T6SS assembly, by interaction with VgrG, Hcp or PAAR [277]. Extended versions of VgrG, Hcp and PAAR containing effector domains (specialized effectors) represent another mode of effector delivery identified in several bacteria [277]. Differently from the other secretion systems described above, the T6SS tube disassembles in each firing event, and Hcp is lost to the extracellular milieu, requiring new rounds of synthesis and polymerization. At this stage, another T6SS component comes into play: the ATPase ClpV, which unfolds and recycles the sheath proteins TssB and TssC for new rounds of T6SS assembly [292].

Besides the core components described above, distinct sets of accessory proteins are encoded by T6SS gene clusters from different clades, which are generally referred as Tag (“*tss*-associated genes”) and include transcriptional and post-transcriptional regulators and proteins of unknown function. Post-translational control of T6SS assembly and activation mediated by the sensor Ser/Thr kinase PpkA and the cognate phosphatase PppA has been described in several bacteria [285]. In this model, PpkA activates T6SS assembly/firing upon phosphorylation of the target protein Fha in response to specific signals and PppA turns off T6SS activity by dephosphorylation of Fha [293]. A distinct mechanism whereby the phosphorylation target of PpkA is the membrane core component TssL was described in the plant pathogen *A. tumefaciens* [294]. Additional regulation of T6SS assembly and activity is provided by inhibitory activity of TagF on Fha in these bacteria, although the mechanism involved remains poorly understood [295,296,297]. Nevertheless, distinct mechanisms of regulation of T6SS activity are yet to be identified, since *ppkA/pppA* homologues are restricted to members of clades i3 e i5 [270,280]. Interestingly, *fha* homologues are present in T6SS clusters from clade i1 and several clade i4 gene clusters encode the *tagF* gene despite the absence of *fha* [270,280].

A survey of the distribution of T6SS gene clusters in *Xanthomonas* genomes available in the KEGG database [298] identified representatives from clades i3 and i4, with some species containing two T6SS clusters, consisting of either two copies of clade i3 T6SS or one copy from each clade (Fig. 1, Table S1) [299]. Interestingly, *Xanthomonas* representatives of clade i3 are further subdivided in two distinct subclades, named i3* and i3***, based on phylogenetic analysis using *tssC* gene (XAC4146 in *X. citri*) [299]. T6SS clusters from distinct i3 subclades also present differences in their genetic architectures, i.e. a *paar* homologue is absent in subclades i3*** and genes encoding hypothetical proteins and transcriptional regulators are differentially distributed in each subclade [299]. Importantly, *Xanthomonas* i3 clusters lack the core component TssJ, indicating a distinct mode of assembly in the bacterial outer membrane [270,299]. Absence of *tssJ* has also been described in i5-T6SS clusters from phytopathogenic bacteria, including *A. tumefaciens* and *R. leguminosarum* [270,280]. Distribution of T6SS among *Xanthomonas* species does not correlate with host preference and/or mechanism of plant colonization (vascular versus mesophyll), however, genomes of non-pathogenic *Xanthomonas* generally lack T6SS gene clusters, according to a recent study [3].

The *X. citri* pv. *citri* i3* T6SS was the first functionally characterized in the genus and is required for resistance to predation by the bacterivorous amoeba *Dictyostelium discoideum* [300], which revealed a hitherto unknown aspect of *Xanthomonas* biology that possibly has an important influence on its ability to survive outside the host. In addition, regulation of *X. citri* T6SS i3* gene expression requires a novel signaling cascade, which involves a Ser/Thr kinase and an alternative extracytoplasmic function sigma factor, PknS and EcfK, respectively [300]. These regulators are encoded in association with the T6SS gene cluster and are conserved in other

members of clade i3* [299,300]. T6SSs from clade i3* are found in species from *Xanthomonas* phylogenetic groups 2B and 2C, as classified based on distinct phylogenetic studies (Fig. 1, Table S1) [100,131,301]. No putative effectors with predicted functions against eukaryotic cells have been identified in association with this T6SS gene cluster by *in silico* prediction [299]. Further functional characterization of the *X. citri* i3* T6SS showed that it is not required for virulence, killing of bacterial or fungal cells, or biofilm formation [299,302].

Two recent studies in *X. oryzae* species describe functional characterization of members of clade i3*** and i4 [303,304], which are generally found in genomes from these species (Fig. 1, Table S1) [299]. In *X. oryzae* pv. *oryzicola* $GX_{01}$, a rice non-vascular pathogen that causes bacterial leaf streak, mutations in i4 T6SS impaired bacterial competition with *E. coli* and the rice endophytic bacterium *Ochrobactrum oryzae*, while not affecting virulence-related phenotypes [303]. Indeed, *in silico* prediction of T6SS effectors in *Xanthomonas* genomes identified an enrichment of genes encoding putative antibacterial toxins in association with T6SS clusters from clade i4, suggestive of a role in bacterial killing [299]. Interestingly, i4 T6SS are found in *Xanthomonas* genomes that lack a bacteria-killing X-T4SS, with some strains presenting a probably non-functional version of X-T4SS, such as *Xanthomonas fragariae* whose VirB9 gene contains an internal stop mutation (Fig. 1, Table S1). No role in *X. oryzae* pv. *oryzicola* physiology has been identified for the i3*** T6SS, which was not required for virulence or bacterial competition [303]. Nevertheless, mutations in i3*** significantly reduced virulence and plant colonization in *X. oryzae* pv. *oryzae* PXO99, the causal agent of rice bacterial blight that colonizes the plant xylem [304]. A search for putative effectors encoded in association with i3*** clusters by an *in silico* approach identified a protein with a TIR_2 domain, which cleaves $NAD^+$ in prokaryotic and eukaryotic cells [299,305]. Proteins with TIR_2 domains are found in pathogenic and non-pathogenic bacteria, and some are virulence factors secreted to mammalian cells [306]. The role of i3*** and i4 T6SS in resistance against protist predators have not been addressed in these species.

## 8. Outer membrane vesicles

The release of outer membrane vesicles (OMVs) appears to be universal in Gram-negative bacteria and is perhaps the most basic form of secretion, not requiring specialized protein machineries [307,308]. This has led some authors to name them the "type zero secretion system" [309], but their actual significance for protein export in different species is still being elucidated. For bacterial phytopathogens in particular, relatively few studies have focused on these structures but a number of possible functions have already been proposed, including possible roles in virulence [310,311]. In *Xanthomonas* species, T3SS effectors were found associated with OMVs of *X. campestris* pv. *campestris* when grown in XVM2, a T3SS-derepressing medium [260], while T2SS substrates were identified in OMVs from *X. campestris* pv. *vesicatoria*, including a protease and a xylanase [72]. These findings reveal that OMVs can be carriers of pathogenicity-associated proteins, similar to what has been demonstrated in the closely related plant pathogen *X. fastidiosa* [311,312]. It has also been observed that OMVs from *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* are able to induce plant immune responses in *Arabidopsis thaliana* and this phenomenon was specifically linked to the protein EF-Tu present in these structures triggering pattern recognition receptors [313]. Given the multitude of functions already described for OMVs of different bacterial species [307,308], further studies could reveal yet unexplored roles of this additional mechanism of protein secretion from *Xanthomonas* spp. in interactions with host plants, with other bacteria, and between cells of the same species.

## 9. The interplay of multiple factors controlling secretion system expression

During colonisation of a niche, bacteria face many stressors, including pH, reactive oxygen species, temperature, UV light, drought, humidity, nutrient scarcity, competitors, and predators. Sensor proteins are fundamental for perceiving environmental signals and trigger adaptive responses within bacterial cells. Signal transduction may involve phosphorylation cascades or production of second messenger molecules, which in turn can modulate the expression of specific genes or protein activity at an appropriate time and place [314].

Macromolecular secretion systems are determinants of adaptation strategies and virulence traits in *Xanthomonas* by contributing to survival and colonisation outside or inside the host. Not surprisingly, expression of secretion systems components is fine-tuned to respond to environmental and host cues, which might be important for balancing energy costs and fitness strategy in bacterial cells. Different transcriptional and posttranscriptional regulators that control expression of these secretion systems have been characterized and shown to contribute to the ability of *Xanthomonas* to adapt and survive during epiphytic and/or endophytic stages. *Xanthomonas* expression profiles available at the NCBI GEO (Gene Expression Omnibus) database have been obtained for mutant strains and for bacteria grown under different conditions (for example, rich media or nutrient conditions that mimic the plant environment). Analysis of these datasets allowed us to identify genotypes and conditions associated with the activation or repression of genes associated with specific secretion systems in *Xanthomonas* spp. The results of this analysis are summarized in Fig. 4 and are discussed below.

Due to its important role in virulence and determination of host specificity, regulation of the T3SS has been extensively studied in *Xanthomonas* species [131]. Expression of *Xanthomonas* T3SS and its effectors depends on two master regulators, HrpG and HrpX, which are OmpR- and AraC-type transcriptional regulators, respectively. HrpG retains a low expression level in rich medium, but significant induction is observed during bacterial infection of a host [76]. T3SS expression is triggered after upregulation and phosphorylation of HrpG that activates the expression of HrpX, which binds to a conserved plant-inducible promoter (PIP) box (TTCGC-$N_{15}$-TTCGC) present in the promoter regions of *hrp*/*hrc* and T3E-encoding genes [315,316]. Several T3SS regulators have been characterized in *Xanthomonas* and most of them act indirectly, by controlling *hrpG* and/or *hrpX* expression at transcriptional or post-transcriptional levels (Fig. 5).

GamR, a LysR-transcriptional regulator of galactose metabolism genes, is required for high-levels of expression of *hrpG* and *hrpX* in *X. oryzae* pv. *oryzae*, directly binding to a regulatory region common to both genes, which are transcribed in opposite directions [317]. Induction of *hrpG/X* by GamR is independent of the presence of galactose in the growth medium. In fact, *hrp* genes are induced in response to xylose in this bacterium, through a mechanism that relies on indirect repression of HrpX by the LacI-type transcriptional repressor XylR in the absence of the sugar [317]. Expression of the *X. oryzae* pv. *oryzae* T3SS is also induced in response to low concentration of $Ca^{2+}$ through the PhoPQ two-component system (TCS) and a *phoP* mutant strain is impaired in virulence in rice [318]. Positive regulation of the T3SS gene cluster by PhoP was also demonstrated in *X. citri* pv. *citri* and mutation in *phoP* severely impaired motility, biofilm formation and virulence in this bacterium [319]. Expression of *hrp* genes in *X. oryzae* pv. *oryzae* is also

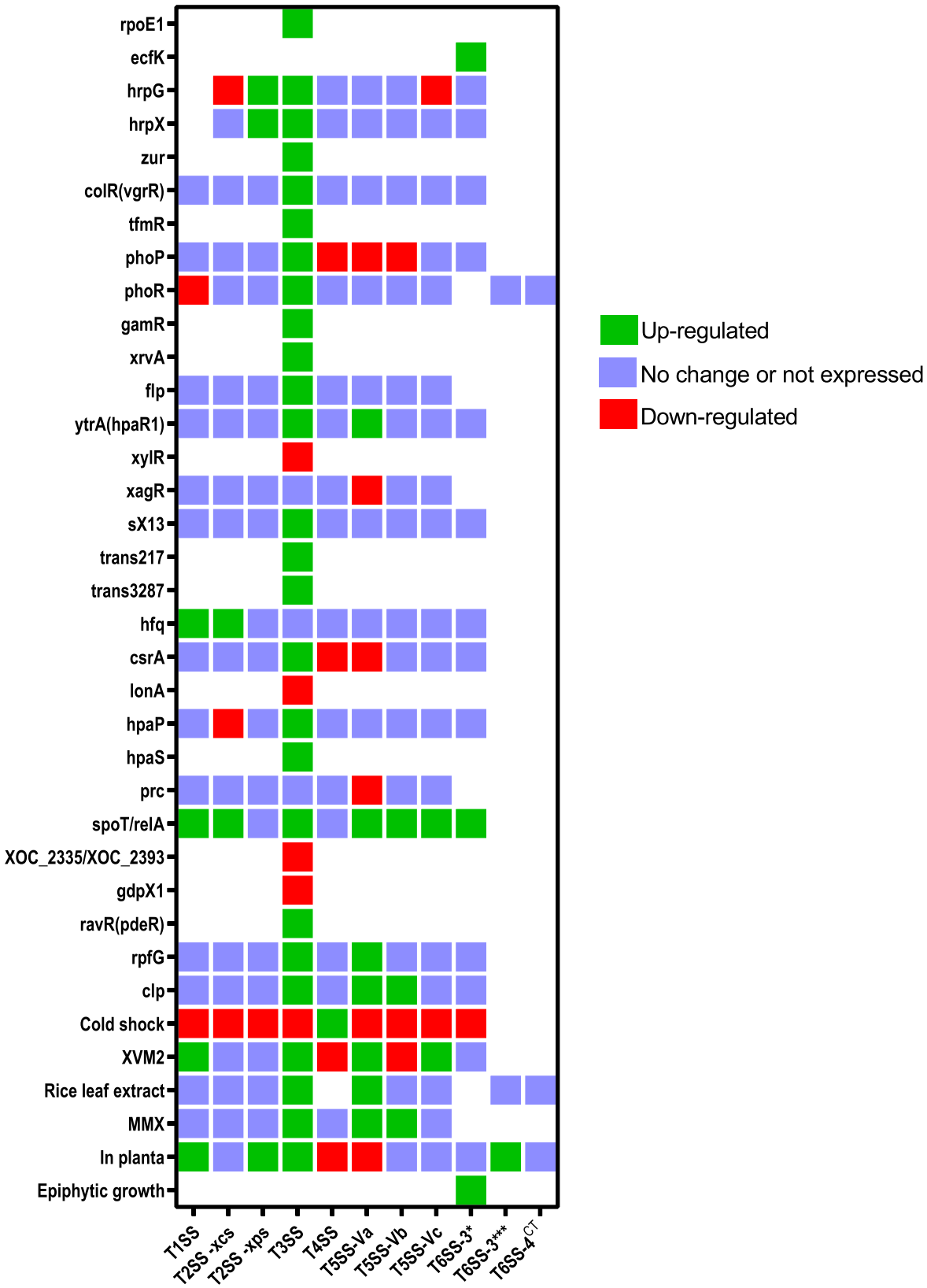



**Fig. 4.** Regulators and specific growth conditions that have been shown to positively or negatively affect expression of the type I to type VI secretion systems in *Xanthomonas*. Data were obtained from gene expression profiles of *Xanthomonas* species that are available in the NCBI GEO (Gene Expression Omnibus) database (www.ncbi.nlm.nih.gov/geo). CT = constitutive expression. All of the relevant references are cited and discussed in the main text.

positively regulated by the phosphate-regulated two component system PhoBR, although the mechanism involved needs to be further investigated [320].

In *X. campestris* pv. *campestris* the Fis-like protein Flp and the alternative sigma factor RpoE1 were shown to directly induce *hrp* gene expression by binding to the *hrpX* promoter [321,322]. Other

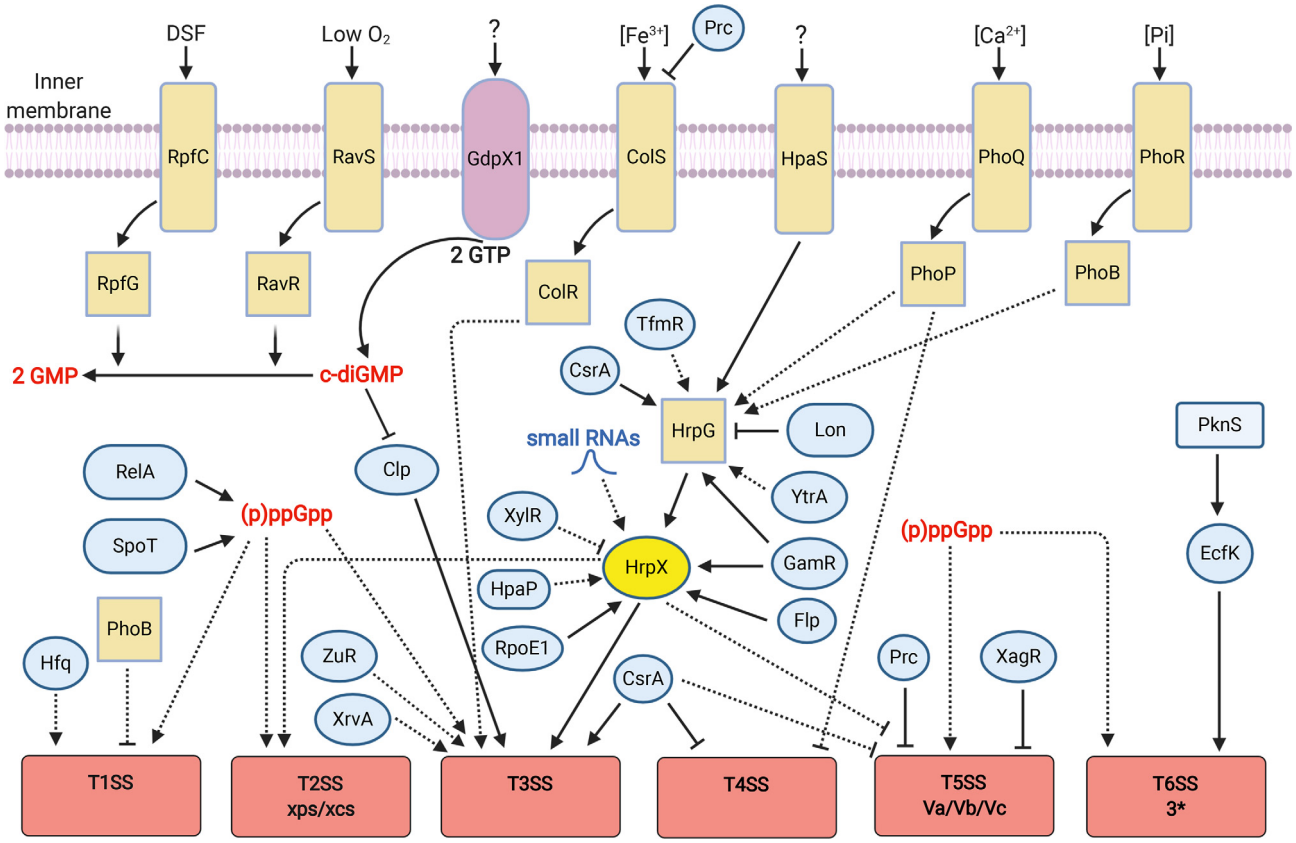



**Fig. 5.** Regulatory signaling pathways controlling the expression of secretion systems in *Xanthomonas*. Overview of observations of regulatory pathways derived, in most cases, from studies on only a small number of *Xanthomonas* species (see main text). It is therefore to be expected that each species will have evolved significant variations in the regulatory pathways for one or more secretion systems. Membrane-associated sensor proteins of two-component systems transduce environmental cues to cytoplasmic transcriptional factors that regulate the expression of different secretion systems. The intercellular communication signal DSF is sensed by the two-component signaling system RpfC/RpfG, which mediates hydrolysis of the second messenger cyclic di-GMP. Likewise, RavS/RavR is activated at low oxygen levels and contributes to hydrolysis of cyclic di-GMP. Environmental signals can trigger GdpX1, a diguanylate cyclase, which increases the pool of cyclic di-GMP in the cell. High levels of cyclic di-GMP repress the binding of the Clp to the promoter of its target genes. HrpG, an OmpR-type transcriptional regulator, induces the expression of *hrpX*, which encodes an AraC-type transcriptional activator. Other transcriptional regulators that directly or indirectly affect HrpG and HrpX expression levels are indicated. Phosphorylation and activation of HrpG by HpaS kinase was observed in *X. campestris* pv. *campestris*. Expression of *hrp/hrc* operons are also controlled by the two-component systems ColS/ColR, PhoQ/PhoP, and PhoR/PhoB and by the post-transcriptional regulators CsrA, small non-coding RNAs (sX13, trans217 and trans3287), and Lon protease. The stringent response leads to synthesis of (p)ppGpp (by RelA and SpoT), which may be a positive regulator of several *Xanthomonas* secretion systems. T1SS was proposed to be regulated by PhoR/PhoB and Hfq, and Xps-T2SS was shown to be positively regulated by HrpX. T4SS expression seems to be constitutive and its homeostasis is controlled by CsrA and perhaps also by PhoQ/PhoP. T5SS components were shown to be repressed by the transcriptional regulator XagR and Prc, a periplasmic protease, which similarly controls the ColS histidine kinase. Expression of T6SS-3* is activated by the Ser/Thr kinase PknS that phosphorylates the sigma factor EcfK. Positive and negative regulation are represented by arrows and lines, respectively. Dashed lines indicate that regulatory mechanisms or direct targets have not yet been determined.

factors have been proposed to indirectly control T3SS expression in plant-mimicking medium and/or in plant hosts by as yet undetermined mechanisms. Among these regulators are Zur, ColR, TfmR, YtrA (or HpaR1) and XrvA (Fig. 5). Zur, a zinc uptake regulator, was shown to activate T3SS-genes by contributing to *hrpX* induction in *X. campestris* 8004, whereas T3SS-genes in *X. campestris* XC1 appears not to be regulated by Zur [323,324]. Thus, regulation by Zur seems to vary according to the *X. campestris* strain. The two-component system ColSR (also known as VgrSR) regulates the adaptation of *X. campestris* pv. *campestris* to both iron-limiting and osmotic stress conditions [325,326]. Mutant strains lacking *colS* or *colR* genes showed pleiotropic effects, including down-regulation of some T3SS encoding genes in different *Xanthomonas* species [327–329]. The TetR-like transcriptional regulator TfmR (T3SS-and-Fatty acid Mechanism Regulator) is required for virulence by indirectly activating T3SS-gene expression in *X. citri* pv. *citri* and overexpression of *hrpG* restores the virulence of a Δ*tfmR* mutant strain [330]. The GntR-type transcriptional repressor YtrA in *X. citri* pv. *citri* and its homolog in *X. campestris* (HpaR1) indirectly repress expression of T3SS-genes in XVM2 medium. Nevertheless, YtrA is required for virulence and essential for induction of T3SS-genes *in planta* [331,332]. Regulation of *hrpG*/*hrpX* genes by the nucleoid-structuring H-NS domain protein XrvA was also described in *X. oryzae* pv. *oryzae* [333].

The posttranscriptional regulator CsrA has been found to act as a positive regulator of T3SS genes by directly binding to 5′ untranslated regions of the *hrpG* gene and *hrpD* operon. Thus, CsrA stabilizes *hrpG* and *hrpD* mRNAs, leading to increased accumulation of HrpG and HrpQ proteins in *X. citri* pv. *citri* [334,335]. The activation of the T3SS-genes by CsrA via HrpG was further supported by the observation that ectopic overexpression of *hrpG* in a *csrA* mutant restored its full ability to cause disease in host plants and trigger hypersensitive response (HR) in non-host plants [334–336]. Homologues of small non-coding RNAs that control CsrA activity in other Gram-negative bacteria were not found in *Xanthomonas* genomes [337]. However, distinct small RNAs have been identified in some *Xanthomonas* species and at least three of them were suggested to regulate T3SS gene expression: sX13, trans217, and trans3287. sX13 of *X. campestris* pv. *vesicatoria* promoted accumulation of *hrpX* mRNA, possibly by indirectly affecting activation of HrpG by targeting a yet unknown regulator [338]. In addition, *X. oryzae* pv. *oryzae* small RNAs trans217 and trans3287 are essential for virulence,

positively regulating T3SS-genes and promoting the secretion of the T3E protein PthXo1 [337,339]. However, the direct targets and mechanisms of these small RNAs that mediate T3SS-gene regulation still need to be determined, and their functions, if any, in regulating virulence in other *Xanthomonas* pathosystems have not yet been studied.

Recent findings have suggested that expression of T3SS genes is also regulated at the post-translational level. Different mechanisms have been described: phosphorylation and activation of HrpG by HpaS; degradation of HrpG in rich medium by Lon protease; and induction of *hrpX* expression by the HpaP phosphatase. The histidine kinase HpaS physically interacts with and phosphorylates HrpG in *X. campestris* strain 8004. A mutant strain lacking *hpaS* markedly reduced the phosphorylation of HrpG *in vivo* and almost completely abolished virulence in the host [337,339,340]. Interestingly, the *hpaS* homolog in *X. campestris* pv. *vesicatoria* does not seem to regulate HrpG, pointing to a possible degree of variation in the signalling pathways regulating T3SS in different *Xanthomonas* species [337]. In *X. citri* pv. *citri* grown in rich medium, the Lon protease represses T3SS by recognizing the N-terminal of HrpG, leading to its degradation. Genetic and biochemical data indicate that phosphorylation at Ser-654 impairs Lon proteolytic activity and attenuates HrpG proteolysis in the plant host [341] The kinases involved in the regulation of Lon are unknown. Finally, the phosphatase HpaP (HR and Pathogenicity Associated Phosphatase) in *X. campestris* was proposed to regulate the expression of *hrc/hrp* genes by controlling the transcription of *hrpX* by an undetermined mechanism [125].

Despite the advances in understanding the mechanisms that trigger T3SS expression, how *hrc*|*hrp* genes are induced by host signals in an HrpG/HrpX-dependent manner and the chemical identities of those host inducers remain unclear [2,131]. It is reasonable to assume that *Xanthomonas* senses and responds to a wide range of environmental and host signals through the dozens of transmembrane chemoreceptors identified in most genomes, including histidine kinases, diguanylate cyclases, phosphodiesterases, and mechano-chemotaxis sensor systems [2]. A regulatory system dependent on cell density and mediated by diffusible signal factor (DSF) has been implicated in virulence factor production, biofilm dispersion and the expression of T3SS-genes in *Xanthomonas* [324,342,343]. The synthesis and recognition of the DSF signal depends on different Rpf proteins [344–346]. RpfF (an enoyl-CoA hydratase) is required for the synthesis of DSF, while the RpfCG two-component system plays a role in DSF perception and signal transduction [347]. In *X. campestris* pv. *campestris*, RpfC phosphorylates RpfG, triggering its phosphodiesterase activity that hydrolyzes the second messenger cyclic diguanylate (c-di-GMP) [344]. The consequent decrease in intracellular levels of c-di-GMP leads to disinhibition of Clp, a transcription factor repressed by binding of c-di-GMP, activating transcription of virulence factors [348,349]. Likewise, other phosphodiesterases such as RavR (or its homolog PdeR) has been found to contribute to activation of T3SS-genes in *X. oryzae* pv. *oryzae* and *X. campestris*, while the diguanylate cyclase protein GdpX1, XOC_2335 and XOC_2393 play a negative role in T3SS expression in *X. oryzae* pv. *oryzae* [350–353].

Also, stress cues in hostile environments can be detected and processed by *Xanthomonas* signal transduction pathways to adjust cell physiology and promote successful adaptation. For example, scarcity of nutrients triggers the production of (p)ppGpp (guanosine pentaphosphate) by RelA and SpoT enzymes in *Xanthomonas* [78]. This molecule reprograms the transcriptome and regulates growth in response to stress signals, allowing a rapid adjustment of bacterial cells to environmental changes [354]. Indeed, a *X. citri* pv. *citri* double mutant lacking both *spoT* and *relA* showed impaired expression of T3SS-genes and virulence [78].

Apart from T3SS, for which regulation has been well studied at many different levels, our knowledge about the regulation of the other *Xanthomonas* secretion systems is still very sketchy. Analysis of *Xanthomonas* expression profiles in the GEO database suggests that T1SS-genes are upregulated *in planta* or in culture media that mimic the plant environment (Fig. 4) [355–358]. Components of T1SS seem to be positively regulated by Hfq, an RNA binding protein involved in posttranscriptional regulation, and by (p)ppGpp in *X. citri* pv. *citri*, though the mechanisms involved in these processes need further investigation [78,359]. On the other hand, some T1SS genes were repressed in *X. oryzae* pv. *oryzae* by the PhoR/PhoB TCS, which regulates adaptation to phosphate limitation and virulence [320]. Genes encoding the pathogenicity-related *xps* T2SS are activated by HrpG and HrpX and are not affected by quorum-sensing in *X. campestris*, *X. citri* pv. *citri*, and *X. oryzae* pv. *oryzae* [53,324,342,360]. These *xps* T2SS genes are positively regulated by *hfq* and *spoT*|*relA*, since deletion of these genes causes a downregulation of Xcs-T2SS expression in *X. citri* pv. *citri* [78,359]. T4SS, T5SS and T6SS play important roles in survival outside the plant host, including epiphytic colonisation and interactions between *Xanthomonas* and other community microbes in the environment. The *X. citri* X-T4SS, which injects a cocktail of antibacterial proteins into neighboring Gram-negative competitor bacteria, seems to be constitutively expressed under most growth conditions tested [203,211,214], though a negative regulation was observed by the transcriptional regulator PhoP, *in planta* or when bacteria were grown in XVM2 medium [319,355,358]. X-T4SS-genes are downregulated by the posttranscriptional regulator CsrA that binds to the 5′ untranslated region of the chromosomal *virB* operon [214,334]. This negative regulation seems to play an important role in T4SS homeostasis, maintaining the X-T4SS density in the cell envelope more or less constant under a wide variety of growth conditions, thus keeping T4SS-related costs in check while maintaining *X. citri*'s aggressiveness against competitor bacterial species [214,334].

The Type Vb and Vc secretion systems FhaB/C, YapH, XadA and XadB are required for the strong attachment of cells to the leaf surface during the epiphytic stage, thereby conferring resistance to removal by rain [242,257,258,355]. Later on, when cells enter the leaf and reach the apoplast, signal molecules produced by the host are perceived by *Xanthomonas* and may activate and/or stabilize regulators. Accordingly, HrpG/HrpX, PhoP, CsrA, Prc, and XagR were verified to repress some of those T5SS-genes in *X. axonopodis* pv. *glycines*, *X. campestris* and *X. citri* pv. *citri* [257,319,334,358,361]. In contrast, the Rpf-signaling system, *ytrA*, and *relA*/*spoT* appear to activate T5SS expression in *X. citri* pv. *citri* and *X. campestris* [78,324,331,342]. Interestingly, subgroups of T5SS (T5SS-Va, Vb and Vc) present distinct patterns of expression in plant-mimicking media compared to *in planta* conditions [355,358,362]. This could be due to the absence of specific signal molecules in artificial media that are present only during infection of the plant host tissues, and perhaps by divergent capacities among *Xanthomonas* species to sense these signals.

Finally, *X. citri* T6SS-3*, which provides protection against predation by *D. discoideum*, is induced at the transcriptional level in response to contact with amoeba cells by a signalling cascade involving a Ser/thr kinase (PknS) and the extracytoplasmic function (ECF) alternative sigma factor EcfK [299,300,302]. EcfK acts downstream from the kinase PknS in the cascade and, accordingly, the direct phosphorylation of an EcfK homologue by a cognate Ser/thr kinase has been recently demonstrated in *Vibrio parahaemolyticus* [300,302,363]. *X. citri* T6SS genes and *ecfK* are also induced during epiphytic growth. Since the phyllosphere is colonised by bacterial predators, induction of the anti-amoeba T6SS during epiphytic growth suggests the presence of an environmental signal that may trigger the T6SS-3* [302]. Also, production of (p)ppGpp

by RelA/SpoT might have a positive effect in T6SS-3* expression [78]. T6SS-4, another T6SS subgroup that is involved in interbacterial competition in *X. oryzae* pv. *oryzicola*, is constitutively expressed during growth in rich and minimal media in a wide range of pH, actively secreting Hcp [303,304]. Constitutive expression of antibacterial secretion systems during growth in culture media seems to be a common strategy in *Xanthomonas*, as it is also observed for the antibacterial X-T4SS. In contrast, the subgroup T6SS-3*** of *X. oryzae* pv. *oryzicola* showed very low expression levels under all conditions analyzed, and transcriptome studies have shown that it is induced within the host, but its function remains to be elucidated, as it was not required for virulence in this strain [356]. Furthermore, cold stress (15 °C) was shown to downregulate the expression of all secretion systems except for the T4SS in *X. citri* pv. *citri* 306 [364]. This result might imply a low adaptability of *X. citri* for cold temperature, since its citrus hosts are restricted to tropical and subtropical areas.

It is evident that the expression of the distinct secretion systems and their substrates should be fine-tuned and coordinated at different stages of the *Xanthomonas* life cycle. However, our understanding of the signaling mechanisms responsible for this integration in *Xanthomonas* is still very limited and remains an important challenge for future research.

## 10. Summary and outlook

*Xanthomonas* species have a diversified set of secretion systems that enable them to colonize plant hosts, attack bacterial competitors and resist environmental predators. Despite the recent progress in the understanding of the roles played by these secretion systems in xanthomonads, few protein effectors secreted by these nanomachines have been functionally characterized, with the exception of T2SS and T3SS substrates. Therefore, efforts towards identification and characterization of substrates secreted by T1SS, T4SS, T5SS and T6SS are promising avenues for identification of novel cellular functions and mechanisms used by *Xanthomonas* to subvert target cells and thrive in the environment. Likewise, studies aimed at determination of high-resolution structures of these multiprotein machines and subcomplexes, currently available solely for the bactericidal X-T4SS, will provide new clues regarding their mode of action and possible targets for inhibition. Moreover, several of these nanomachines were identified by genome analysis but have not yet been functionally characterized and functional studies have been mostly restricted to a few *Xanthomonas* species. Broadening the studies to other members of the genus will surely reveal variations in secretion system regulation, architecture, function and effector repertoire. These efforts would also be greatly complemented by integrative approaches to understand how the distinct secretion systems are coordinated to colonize the various hosts. These future studies promise to provide new insights not only into the role played by these nanomachines in the ecology of *Xanthomonas* spp., as well as point to new targets for the development of alternative strategies for plant disease management, with positive impact on food security and productivity.

## CRediT authorship contribution statement

**Cristina E. Alvarez-Martinez:** Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing, Visualization, Supervision, Funding acquisition. **Germán G. Sgro:** Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing, Visualization. **Gabriel G. Araujo:** Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing, Visualization. **Mateus R.N. Paiva:** Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing, Visualization. **Bruno Y. Matsuyama:** Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing, Visualization. **Cristiane R. Guzzo:** Conceptualization, Writing - original draft, Writing - review & editing, Visualization, Funding acquisition. **Maxuel O. Andrade:** Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing, Visualization. **Chuck S. Farah:** Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing, Visualization, Supervision, Funding acquisition.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2020.12.020.

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