Bacteria-Killing Type IV Secretion Systems

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Bacteria have been constantly competing for nutrients and space for billions of years. During this time, they have evolved many different molecular mechanisms by which to secrete proteinaceous effectors in order to manipulate and often kill rival bacterial and eukaryotic cells. These processes often employ large multimeric transmembrane nanomachines that have been classified as types I–IX secretion systems. One of the most evolutionarily versatile are the Type IV secretion systems (T4SSs), which have been shown to be able to secrete macromolecules directly into both eukaryotic and prokaryotic cells. Until recently, examples of T4SS-mediated macromolecule transfer from one bacterium to another was restricted to protein-DNA complexes during bacterial conjugation. This view changed when it was shown by our group that many Xanthomonas species carry a T4SS that is specialized to transfer toxic bacterial effectors into rival bacterial cells, resulting in cell death. This review will focus on this special subtype of T4SS by describing its distinguishing features, similar systems in other proteobacterial genomes, and the nature of the effectors secreted by these systems and their cognate inhibitors.

Keywords: bacterial competition, Xanthomonadales, type IV immunity protein, type IV secretion effector, type IV secretion system, X-Tfe, X-Tfi, X-T4SS

INTRODUCTION

Type IV secretion systems (T4SSs) have been studied since the birth of modern molecular biology, starting with the description of bacterial conjugation over 70 years ago (Lederberg and Tatum, 1946). It quickly became evident that the T4SS-mediated horizontal transfer of genetic material is a major contributor to bacterial evolution, making it necessary to consider lateral connections between lineages for a complete description of the bacterial tree of life (de la Cruz and Davies, 2000). Horizontal gene transfer is also one of the principal mechanisms for the spread of genes conferring resistance to antibiotics (Cabezon et al., 2015). Moreover, many pathogenic bacteria use T4SSs to facilitate their proliferation and survival inside eukaryotic hosts, typically by the secretion of protein effectors or protein-DNA complexes (Gonzalez-Rivera et al., 2016). T4SSs are thus important virulence factors in a variety of human diseases, including whooping cough (Bordetella pertussis; Locht et al., 2011; Carbonetti, 2015), cat-scratch fever (Bartonella henselae; Siamer and Dehio, 2015), brucellosis (Brucella spp.; Ke et al., 2015), Legionnaire’s pneumonia (Legionella pneumophila;
Finsel and Hilbi, 2015), Q fever (Coxiella burnetii; Moffatt et al., 2015) and peptic ulcer and gastric cancer (Helicobacter pylori; Naumann et al., 2017). One of the most well-characterized T4SSs is that of Agrobacterium tumefaciens which injects nucleoprotein complexes and protein factors into plant cells (Alvarez-Martinez and Christie, 2009; Li and Christie, 2018). Furthermore, specialized T4SSs from Neisseria gonorrhoeae or H. pylori secrete DNA to the extracellular milieu or uptake DNA from the environment to the bacterial cytoplasm, respectively (Hofreuter et al., 2001; Hamilton et al., 2005; Callaghan et al., 2017). Finally, the plant pathogen Xanthomonas citri (Oliveira et al., 2016; Sgro et al., 2018; Souza et al., 2015) and, more recently, the opportunistic human pathogen Stenotrophomonas maltophilia (preprint: Bayer-Santos et al., 2019), have been shown to use a T4SS to inject toxic effectors into target bacteria, thus inducing the death of rival cells (Figure 1).

T4SSs are structurally very diverse. For example, the related pKM101 and R388 plasmid-encoded conjugation systems (Chandran et al., 2009; Fronzes et al., 2009; Rivera-Calzada et al., 2013) and the pathogenic Legionella Dot/Icm (Ghosal et al., 2017; Chetrit et al., 2018) and H. pylori Cag (Frick-Cheng et al., 2016; Chang et al., 2018) effector-secreting systems, while all exhibiting an outer membrane-associated complex with 14-fold or 13-fold symmetry, present significantly different features in terms of their overall size. These systems also display a varied set of both functional and structural subunits, and even the homologous subunits have very low sequence similarity and frequently present modified domain architectures (Alvarez-Martinez and Christie, 2009; Christie et al., 2014; Guglielmini et al., 2014; Christie, 2016; Grohmann et al., 2017). For these reasons, the T4SSs from Gram-negative bacteria have been divided into two major classes, denoted A and B (Christie and Vogel, 2000), and classification systems based on detailed phylogenetic analysis have divided Gram-negative and Gram-positive T4SSs into up to 8 classes (Guglielmini et al., 2014).

The canonical class A, best represented by the A. tumefaciens vir system and those coded by conjugative plasmids pKM101, R388, and RP4, have the basic set of 12 conserved subunits, named VirB1 to VirB11 plus VirD4 (Tzfira and Citovsky, 2006). The overall organization of the canonical class A T4SSs has been revealed in electron microscopy studies (Low et al., 2014; Redzej et al., 2017) and can be divided into two general (sub)complexes (Figure 1). The inner membrane complex is made up of subunits embedded in, or associated with, the inner membrane: VirB3, VirB4, VirB6, VirB8, VirB11, and VirD4. The outer membrane or “core” complex is comprised of the subunits VirB7, VirB9, and VirB10. These two complexes are connected by a flexible “stalk” of unknown composition, though it has been proposed to be made up, at least in part, by the disordered N-terminal domain of VirB10 (which also has an N-terminal transmembrane helix embedded in the inner membrane) and/or the C-terminal domain of VirB8 (Christie, 2016; Waksman, 2019). In addition to this transmembrane structure, there are extracellular pili made of subunits VirB2 and VirB5, that are presumably involved in making contact with the membrane of the target cell or organelle (Alvarez-Martinez and Christie, 2009). Even within the class A T4SSs, a large degree of sequence and size diversity has been observed for many of the subunits in different species. This is perhaps most starkly exemplified when considering the H. pylori Cag T4SS which, in addition to orthologs of the basic set of canonical class A subunits, possesses another five subunits that are required for proper function (Backert et al., 2015; Frick-Cheng et al., 2016).

The even more distantly related class B includes T4SSs found in the pathogens L. pneumophila, C. burnetii, and Rickettsiella grylli as well as in the IncI conjugative plasmids R64 and ColIb-P9 (Sexton and Vogel, 2002). L. pneumophila causes Legionnaire’s disease in humans by infecting alveolar macrophages where it replicates within a specialized vacuole (Backert and Meyer, 2006; Ensminger and Isberg, 2009). Its Dot/Icm T4SS is made up of 27 components and secretes more than 30 effector proteins that manipulate signal transduction pathways in the host cell, primarily affecting organelle trafficking (Hilbi et al., 2017; Qiu and Luo, 2017). The bacteria-killing T4SSs, which is the topic of this review, belong to the canonical class A T4SSs, although they do have some structurally distinguishing features as described below.

The Xanthomonadales order of Gammaproteobacteria (Saddler and Bradbury, 2007), recently divided into two orders, Xanthomonadales (families Xanthomonadaceae and Rhodanobacteraceae) and Nevskiales (Naushad et al., 2015), include several hundred phytopathogenic species of the genera Xanthomonas and Xylella as well as important and ubiquitous soil, water and plant-associated bacteria of the genera Stenotrophomonas, Lysobacter, Luteimonas, Pseudoxanthomonas, Rhodanobacter, Luteibacter, Dyella, Fraterea, Aquimonas, and others (Van Sluys et al., 2002; Saddler and Bradbury, 2007; Looney et al., 2009; Mansfield et al., 2012). Some Stenotrophomonas strains are opportunistic pathogens of immunosuppressed human patients (Chang et al., 2015) and some Stenotrophomonas and Lysobacter strains have been recognized as potential biological control agents in combating plant diseases caused by fungi or other bacteria (Hayward et al., 2010; Mukherjee and Roy, 2016; Panthee et al., 2016). Other species from the genera Lysobacter and Luteimonas have been isolated from extreme environments (Brito et al., 2013; Zhang et al., 2015). Although the role of types II and III secretion systems in the virulence of species of the genus Xanthomonas is already well established (Buttner and Bonas, 2010), until a few years ago there was little information available on the functions of other secretion systems in these bacterial species. An accompanying article in this series deals with the recently discovered role of the X. citri Type VI secretion system (T6SS) in protection against predation by phagocytic amoebas (Bayer-Santos et al., 2018). In this review, we will focus on the special characteristics of the Xanthomonadales Type IV secretion systems, first described in X. citri, and their role in the contact-dependent killing of rival Gram-negative species. The review will focus on describing the distinguishing structural features of the T4SS components encoded by the chromosomal virB locus of X. citri, their conservation in homologous systems in the order Xanthomonadales and other proteobacterial genomes, the nature of the effectors secreted by these systems and the cognate inhibitors of these effectors.
FIGURE 1 | Schematic model of the structure and function of the bacteria-killing Xanthomonadales-like Type IV secretion systems (X-T4SSs). The model shows the interface between two bacterial cells. The killer cell (below) is armed with an X-T4SS whose general architecture is based on the negative-stained electron microscope map of the R388 T4SS shown in the background (Low et al., 2014; Redziej et al., 2017) and the cryo-EM structure of the X. citri core complex (VirB7, VirB9, and VirB10; Sgro et al., 2018) associated with the outer membrane (OM). The disordered N-terminal domains of the VirB10 subunits extend down from the core complex and pass through the inner membrane. The inner membrane (IM) complex is made up of VirB3, VirB6, VirB8, the three ATPases VirB4, VirB11, and VirD4 as well as the aforementioned N-terminal segments of VirB10. Pili, made up of VirB2 and VirB5, mediate intercellular contacts. X-T4SS effectors (X-Tfes) interact, via their XVIPCD domains, with VirD4 and are subsequently transferred to the T4SS for translocation into the target cell where they will degrade target structures such as membrane phospholipids or carbohydrate and peptidoglycan (PG) layer. Prior to secretion, X-Tfes whose activities could target cytosolic substrates can be inhibited by cytosolic variants of their cognate immunity proteins (X-Tfis). If X-Tfes make their way into the periplasm, either by leakage from the secretion channel or by injection by neighboring cells of the same species, they will be inhibited by the periplasmic lipoprotein forms of the cognate X-Tfi. Portions of the Figure were adapted from Low et al. (2014) and Sgro et al. (2018) with permission from the publishers.
THE CHROMOSOMALLY CODED T4SS OF Xanthomonas citri

The T4SS encoded by the chromosomal vir locus of X. citri contains the canonical set of 12 structural components found in other class A T4SSs (Figure 2; Alegria et al., 2005; Souza et al., 2011). The presence of chromosomally encoded homologs in several other Xanthomonadas species (see below) suggested an important function in Xanthomonas biology. A role in bacterial conjugation or nucleic acid transfer was deemed unlikely since the chromosomal virB locus does not contain genes coding for homologs of the DNA processing components of the relaxosome or characteristic palindromic oriT sites (Alegria et al., 2005). Furthermore, a knockout of the virB7 gene in X. citri did not affect the development of canker symptoms in citrus plants (Souza et al., 2011) and the deletion of a large part of the homologous operon in Xanthomonas campestris pv. campestris 8004 did not modify the phenotype of infection in several plants of the Brassicaceae family (He et al., 2007), ruling out a direct involvement of the T4SS in Xanthomonas virulence (at least in these two species). Our group subsequently demonstrated that this secretion system confers to X. citri the capacity to kill other Gram-negative cells in a contact-dependent manner (Souza et al., 2015). The first bacterial killing experiments were performed by confronting X. citri with common laboratory strains of Escherichia coli as well as the Betaproteobacterium Chromobacterium violaceum (Souza et al., 2015) and subsequent experiments demonstrated similar T4SS-dependent killing of several other Gram-negative bacteria but not Gram-positive bacteria (DPS, GUA, WC, and CSF; unpublished). Recently, a CPRG-based colorimetric assay has been employed to monitor the real-time kinetics of T4SS-dependent bacterial killing by both X. citri (Sgro et al., 2018) and S. maltophilia (Preprint: Bayer-Santos et al., 2019). Time-lapse microscopy clearly showed that bacterial killing by X. citri and S. maltophilia requires cell-cell contact and that the death of target cells is evidenced by the loss of cell turgor and contents over a very short period of time (Souza et al., 2015; Preprint: Bayer-Santos et al., 2019). These T4SSs share with some T6SSs the ability to transfer their toxic effectors directly into rival bacterial species of different orders and phyla and so are important factors for interspecies competition. In this sense, they differ from contact-dependent growth inhibition (CDI) systems (Hayes et al., 2014) and the Staphylococcus aureus Type VII secretion system (T7SS; Cao et al., 2016) that seem to be important for competition between cells of the same or closely related species (intrasppecies competition).

IDENTIFICATION OF HOMOLOGOUS SYSTEMS IN THE ORDER Xanthomonadales AND OTHER PROTEOBACTERIAL GENOMES

Several of the X. citri T4SS components have some interesting features that distinguish them from their homologs in other more distantly related class A T4SSs involved in horizontal transfer of genetic material. For example, the VirB7 and VirB8 subunits have C-terminal extensions absent in most of their more distantly related homologs (see below). Genes coding for T4SSs with similar characteristics to that of X. citri can be identified in the chromosomes of many other Xanthomonadas species (Figure 2 and Table 1), for example Xanthomonas campestris pv. campestris B100 (Vorholter et al., 2008), Xanthomonas albilineans GPEC73 (Pieretti et al., 2009), X. campestris pv. vasculorum NCPPB702 (Studholme et al., 2010) and X. campestris pv. musacearum NCPPB2005 (Wasukira et al., 2012). The corresponding locus is fragmented in X. campestris pv. campestris strains ATCC33913 (Da Silva et al., 2002) and 8004 (Qian et al., 2005), with the virB5 and virB6 genes found in other regions of the genomes. X. campestris pv. vesicatoria 85-10 (Thieme et al., 2005) lacks a significant part of the vir locus (all that remains is the 5′ region coding for VirD4, VirB7, VirB8, and VirB9). The system is also absent in Xanthomonas oryzae strains KACC10331 (Lee et al., 2005), MAFF311018 (Ochiai et al., 2005), PX099A (Salzberg et al., 2008), and BLS256 (Salzberg et al., 2007), in Xanthomonas fuscans subsp. aurantifoliia strains 10535 and 11122 (Moreira et al., 2010) and in all Xylella species sequenced to date. Homologous loci can be found in some Xanthomonadales species of the genera Stenotrophomonas, Pseudoxanthomonas, Luteimonas, Lysobacter, Thermomonas, Rhodanobacter, Dyella, Fratetia, and Luteibacter (Table 1). Interestingly, homologous systems are also found in some species of the Betaproteobacteria orders Burkholderiales (genera Hydrogenophaga, Variorvax) and Neisseriales (genera Neisseria and Morococcus) (Table 1). This is consistent with the observation that in some phylogenetic analyses, Xanthomonadales species are observed to branch anomalously with Betaproteobacteria, most probably due to horizontal gene transfer events (Martins-Pinheiro et al., 2004; Comas et al., 2006; Naushad and Gupta, 2013). We will therefore employ the term X-T4SS to designate all Xanthomonadales-like Type IV secretion systems.

Figure 2 presents the organization of genetic loci, homologous to the X. citri chromosomal vir locus, that are found in the genomes of a few bacterial species selected from genera of the Xanthomonadaceae family (Xanthomonas, Stenotrophomonas, and Lysobacter), and Rhodanobacteraceae family (Dyella and Luteibacter) within the order Xanthomonadales. Also shown are examples of genetic loci that code for X-T4SSs found in the more distant Betaproteobacteria genera of the Comamonadaceae family (Hydrogenophaga) and Neisseriaceae family (Neisseria). One interesting observation is that the loci in species from the Xanthomonadales order seem to have one operon coding for all 11 VirB components, beginning with the virB7 gene. On the other hand, in Hydrogenophaga crassostreae, the operon has been divided into two segments (virB7-11 and virB1-6) and in Neisseria mucosa and N. flavescens it has been divided into three or more segments (Figure 2). The positions of the virD4 genes also vary: in most Xanthomonas and Stenotrophomonas species it is found immediately upstream of the virB7 gene while in the more distantly related species it appears upstream, downstream or inserted between segments coding for the virB genes (Figure 2).
**FIGURE 2** | Xanthomonas citri chromosomal vir locus and its homologs in other species. The top line presents the T4SS encoded by the chromosomal vir locus of *X. citri* 306 (Da Silva et al., 2002; Alegria et al., 2005). It contains the canonical set of 12 components found in other class A T4SSs. Genes coding for T4SSs with similar characteristics to that of *X. citri* can be identified in the chromosomes of many other species (see Table 1 for an extensive list). Shown here are representative examples from *Serratia marcescens* K279s (Crossman et al., 2008), *Xanthomonas citri* DSM16549 (unpublished; GenBank accession CP014841), *Hydrogenophaga crassostreae* LPB0072 (unpublished; GenBank accession LW00000013), *Neisseria mucosa* C102 (unpublished, GenBank accession GCA_000186165) and *Neisseria flavescens* SK114 (unpublished; GenBank accession ACQV01000009). VirB and VirD4 genes are shown in yellow and orange, respectively. Xanthomonadaceae-like T4SS effectors (X-Tfes) and immunity proteins (X-Tfis) are colored red and green, respectively. Other open reading frames coding for proteins of unknown function are shown in gray.

Xanthomonas citri chromosomal vir locus and its homologs in other species. The top line presents the T4SS encoded by the chromosomal vir locus of *X. citri* 306 (Da Silva et al., 2002; Alegria et al., 2005). It contains the canonical set of 12 components found in other class A T4SSs. Genes coding for T4SSs with similar characteristics to that of *X. citri* can be identified in the chromosomes of many other species (see Table 1 for an extensive list). Shown here are representative examples from *Serratia marcescens* K279s (Crossman et al., 2008), *Xanthomonas citri* DSM16549 (unpublished; GenBank accession CP014841), *Hydrogenophaga crassostreae* LPB0072 (unpublished; GenBank accession LW00000013), *Neisseria mucosa* C102 (unpublished, GenBank accession GCA_000186165) and *Neisseria flavescens* SK114 (unpublished; GenBank accession ACQV01000009). VirB and VirD4 genes are shown in yellow and orange, respectively. Xanthomonadaceae-like T4SS effectors (X-Tfes) and immunity proteins (X-Tfis) are colored red and green, respectively. Other open reading frames coding for proteins of unknown function are shown in gray.
DISTINGUISHING STRUCTURAL FEATURES OF THE BACTERIA-KILLING XANTHOMONADALES-LIKE T4SSs (X-T4SSs)

Supplementary Figures S1–S12 present multiple amino acid sequence alignments of VirB1–VirB11 and VirD4 (respectively) X-T4SS components coded by the homologous loci presented in Figure 2. What follows in this section is a brief description of some interesting structural features that can be identified from these alignments and, in some cases, their correlations with known structures and site-directed mutagenesis studies. The observations gleaned from these comparisons are likely to apply to most of the X-T4SSs listed in Table 1.

VirB7, VirB9, and VirB10: Components of the Core Complex

The 2.9 Å resolution crystal structure of the outer membrane layer of the pKM101 core complex (Chandran et al., 2009) and the recently published 3.3 Å resolution cryo-electron microscopy (cryo-EM) structure of the complete X. citri core complex (Sgro et al., 2018), along with the lower resolution EM maps of the complete pKM101 (Fronzes et al., 2009; Rivera-Calzada et al., 2013), R388 (Low et al., 2014) and A. tumefaciens (Gordon et al., 2017) core complexes have provided us with the greatest detail as yet of the periplasmic channel that connects the inner and outer membranes of class A T4SSs. These structures, are all made of 14 copies of VirB7–VirB9–VirB10 heterotrimers (named TraN-TraO-TraF in pKM101 and TrwH-TrwF-TrwE in R388) and can be divided into two layers: the O-layer associated with the outer membrane, consisting of VirB7 and the C-terminal domains of VirB9 and VirB10, and the I-layer made up of the N-terminal domains of VirB9 and VirB10 (Figures 3, 4; Fronzes et al., 2009).

The VirB7 lipoprotein component of X-T4SSs is much larger (ranging from 130 to 185 amino acids; Supplementary Figure S7) than that found in other class A T4SSs (normally ~40 amino acids). This large size is due to an extra globular C-terminal domain called N0 (Souza et al., 2011). Interestingly, similar N0 domains are also found in a myriad of transport systems located in Gram-negative bacterial outer membranes, ranging from secretins of T2SSs, Type IV pilus biogenesis machineries (Korotkov et al., 2009), T3SSs (Spreter et al., 2009), filamentous phages (Spagnuolo et al., 2010), long-tailed bacteriophages (Kanamaru et al., 2002; Kondou et al., 2005), signal-transduction domains in TonB-dependent receptors (Garcia-Herrero and Vogel, 2005; Ferguson et al., 2007) and membrane-penetrating devices in T6SSs (Leiman et al., 2009). The N0 domain is also the C-terminal domain of the outer membrane lipoprotein DotD of the class B T4SSs found in the human pathogens L. pneumophila and C. burnetii (Nakano et al., 2010). The presence of this domain in many outer membrane transport systems could reflect an unexplored evolutionary relationship between them (Souza et al., 2011). The function of the VirB7 N0 domain is possibly related to the observation that it mediates VirB7 oligomerization and, as the VirB7 subunits are highly concentrated in the context of the core complex, it was predicted that the VirB7 domain could assemble an extra peripheral ring in the O-layer of the X-T4SS (Souza et al., 2011), subsequently confirmed by the resolution of the X. citri core complex structure (Sgro et al., 2018). This external ring of N0 domains give the X. citri core complex its characteristic profile that resembles a flying saucer (Figure 3; Sgro et al., 2018). The motifs that mediate VirB7 oligomerization (specific residues in the N0 domain and a [T/S]EIPL motif) that immediately precedes it, contribute to T4SS assembly in the X. citri periplasm and are essential for its antibacterial activity (Souza et al., 2011; Oliveira et al., 2016; Sgro et al., 2018). The multiple sequence alignment in Supplementary Figure S7 shows that these motifs, as well as the region involved in interaction with VirB9, are conserved among X-T4SS VirB7 proteins.

Like their homologs in other class A T4SSs, the X-T4SS VirB9 proteins have two domains connected by a central linker. They all possess an N-terminal signal peptide with a cleavage site immediately after a highly conserved alanine residue (Supplementary Figure S9). The cryo-EM structure of the X. citri core complex (Sgro et al., 2018) and NMR solution structure of the X. citri VirB7–VirB9 binary complex (Oliveira et al., 2016) showed that the X. citri VirB9 C-terminal domain interacts with VirB7 and with the VirB10 C-terminal domain in the O-layer of the core complex in a manner similar to that observed for pKM101 (Figure 4). The X. citri core complex structure provided us with the first high resolution structure of the I-layer, composed of 14 VirB9 N-terminal β-sandwich domains that pack against each other side-by-side, forming a ring with an internal diameter of 80 Å (Figure 3; Sgro et al., 2018). At the base of the I-layer, small 12-residue helices from the N-terminal domains of VirB10 fit into grooves at the interfaces between VirB9 subunits (Sgro et al., 2018). The multiple sequence alignment of X-T4SS VirB9 proteins shows that the N- and C-terminal domains are quite well conserved but the central linker is variable in both sequence and length (Supplementary Figure S9). This is consistent with the observation that deletion of six residues in the linker region did not affect the stability or function of the T4SS in X. citri (Sgro et al., 2018).

VirB10 subunits in class A T4SSs can be divided into three substructures: the N-terminal cytosolic portion with its contiguous transmembrane helix that spans the bacterial inner membrane, the periplasmic portion of the N-terminal domain that is largely unstructured in the NMR analysis of the isolated domain and the cryo-EM structure of the X. citri core complex (Sgro et al., 2018) and the C-terminal domain localized in the O-layer of the core complex. The alignments shown in Supplementary Figure S10 reveal that the C-terminal domains of X-T4SS VirB10 subunits are very well conserved. The sequences are also very similar to their counterparts in pKM101, R388 and A. tumefaciens (Sgro et al., 2018). One region in this domain that presents a relatively high degree of variability is the “antenna” (Chandran et al., 2009), made up of two alpha helices (α1 and α2) that form the actual pore through the outer membrane. Figure 4 presents a comparison of the relative orientations of the VirB10 antennae when the X. citri core complex and pKM101 O-layer structures are superposed. In the X. citri core complex
### TABLE 1 | Bacterial strains that code for a putative X-T4SS and X-Tes substrates.

| Organism                          | Accession‡       | Organism                      | Accession‡       |
|-----------------------------------|------------------|-------------------------------|------------------|
| **Xanthomonadaceae**              |                  |                               |                  |
| Luteimonas sp. 83-4               |                  |                               |                  |
| Lysobacter antibioticus 76        |                  |                               |                  |
| Lysobacter capsici A778           |                  |                               |                  |
| Lysobacter enzymogenes C3         |                  |                               |                  |
| Lysobacter gummosus 3.2.11        |                  |                               |                  |
| Lysobacter maris H29B              |                  |                               |                  |
| Lysobacter silvestris AM20-91      |                  |                               |                  |
| Lysobacter sp. 4284/11             |                  |                               |                  |
| Lysobacter sp. cf.310              |                  |                               |                  |
| Lysobacter sp. Root494             |                  |                               |                  |
| Lysobacter sp. Root604             |                  |                               |                  |
| Lysobacter sp. TY2-98              |                  |                               |                  |
| Lysobacter sp. yr284               |                  |                               |                  |
| Lysobacter sp. zong215             |                  |                               |                  |
| [Pseudoxanthomonas] geniculata N1  |                  |                               |                  |
| Pseudoxanthomonas sp. CF125        |                  |                               |                  |
| Pseudoxanthomonas sp. GM95         |                  |                               |                  |
| Pseudoxanthomonas sp. KAs_5_3      |                  |                               |                  |
| Pseudoxanthomonas spadix DSM18855  |                  |                               |                  |
| Pseudoxanthomonas suwonensis S2    |                  |                               |                  |
| Pseudoxanthomonas wuyuanensis CGMCC1.10978 |                  |                               |                  |
| Stenotrophomonas chelatiphasa DSM21508 |                  |                               |                  |
| Stenotrophomonas daejeonensis JCM16244 |                  |                               |                  |
| Stenotrophomonas geniculata CFBP7113 |                  |                               |                  |
| Stenotrophomonas indicativa WS40    |                  |                               |                  |
| Stenotrophomonas koreensis DSM17806 |                  |                               |                  |
| Stenotrophomonas lacttubii M15     |                  |                               |                  |
| Stenotrophomonas maltophilia Ab5555 |                  |                               |                  |
| Stenotrophomonas maltophilia K279a |                  |                               |                  |
| Stenotrophomonas maltophilia MF89  |                  |                               |                  |
| Stenotrophomonas maltophilia R551-3 |                  |                               |                  |
| Stenotrophomonas maltophilia RA8    |                  |                               |                  |
| Stenotrophomonas maltophilia WJ66   |                  |                               |                  |
| Stenotrophomonas pavani DSM25135    |                  |                               |                  |
| Stenotrophomonas rizhophila OG2     |                  |                               |                  |
| Stenotrophomonas sp. 92mfc06.1      |                  |                               |                  |
| Stenotrophomonas sp. AG209         |                  |                               |                  |
| Stenotrophomonas sp. CC120222-04    |                  |                               |                  |
| Thermomonas fusca DSM15424          |                  |                               |                  |
| Xanthomonas albilineans GPEPC73     |                  |                               |                  |
| Xanthomonas alfalfa GEV-Rose-07     |                  |                               |                  |
| Xanthomonas arboricola pv. araucaria CFBP7407 |                  |                               |                  |
| Xanthomonas arboricola pv. coriina CFBP2565 |                  |                               |                  |
| Xanthomonas arboricola pv. fragariae CFBP7773 |                  |                               |                  |
| Xanthomonas arboricola pv. guizhous CFBP7408 |                  |                               |                  |
| Xanthomonas arboricola pv. juglandis Xj417 |                  |                               |                  |
| Xanthomonas arboricola pv. populi CFBP3122 |                  |                               |                  |
| Xanthomonas arboricola pv. pruni MAFF301420 |                  |                               |                  |
| Xanthomonas arboricola pv. pruni RSMAFF311562 |                  |                               |                  |
| Xanthomonas axonopodis pv. begoniae CFBP2524 |                  |                               |                  |

(Continued)
structure, the antenna helices are twisted clockwise (~50°) and tilted vertically (~20°) with respect to the corresponding helices in pKM101, producing a more open outer membrane pore in the *X. citri* structure (45 Å versus 35 Å; Figure 4; Sgro et al., 2018). We also observe variability in sequence and length of the linker between the alpha helices that is expected to form a loop on the extracellular face of the outer membrane. This loop is particularly rich in threonine, serine and glycine residues and is longer in the Xanthomonadaceae VirB10 proteins than in their counterparts in *A. tumefaciens* (Garza and Christie, 2013) and *B. subtilis* (Jakubowski et al., 2009). Whether similar interactions occur in the *X. citri* VirB10 subunits (Supplementary Figure S10) remains to be investigated.

The periplasmic portions of the *X. citri* VirB10 subunits interact with VirD4 of the *X. citri* T4SSs from *A. tumefaciens* (Garza and Christie, 2013) and plasmids R388 (Llosa et al., 2003; Segura et al., 2013) and R27 (Gilmour et al., 2003). Whether similar interactions occur in the *X. citri* T4SSs remains to be investigated.

In contrast to the VirB10 C-terminal domain, its N-terminal domain is known to be highly variable in size and sequence. Xanthomonadaceae VirB10 proteins have relatively long N-terminal cytosolic segments that precede the transmembrane helix that passes through the inner membrane. While this cytosolic peptide is only 38 or 22 residues long and lacks proline residues in pKM101 and *A. tumefaciens*, it is 57–68 residues long and is rich in proline residues (around 15%) in most T4SSs. Species names are organized in alphabetical order within each bacterial family. Species discussed in more detail in the main text are shown in bold.

### Table 1

| Organism | Accession | Organism | Accession |
|----------|-----------|----------|-----------|
| **Rhodanobacteraceae** | | | |
| *Dyella jiangningensis* SBZ3-12 | CP007444.1 | Luteibacter sp. 329MFSha | NZ_FOJ01000007.1 |
| *Dyella marensis* UNC179MFts3.1 | FONH01000012.1 | Luteibacter sp. OK325 | QAOX01000004.1 |
| *Dyella sp.* 333MFSha | FNBR01000009.1 | Luteibacter sp. UNCMF331Sha3.1 | FOBU01000002.1 |
| *Dyella sp.* 4MSK11 | NZ_QRB01000004.1 | Luteibacter sp. UNCMF366Sha5.1 | FIPS01000002.1 |
| *Dyella sp.* AD56 | NRDP01000015.1 | Luteibacter yeojunensis SU11 | JZPB01000062.1 |
| *Dyella sp.* AtDHG13 | NZ_QICJ01000003.1 | Rhodanobacter fulvus Jip2 | NZ_AJXU01000014.1 |
| *Dyella sp.* OK004 | FOZIO1000001.1 | Rhodanobacter sp. 67-28 | MKU01000080.1 |
| *Dyella thioxydans* ATB10 | CP014841.1 | Rhodanobacter sp. C01 | NZ_MUN01000011.1 |
| Fratetria terreae CGMCC1.7053 | FOXL01000004.1 | Rhodanobacter sp. C06 | NZ_MUN01000039.1 |
| *Luteibacter rhizovicinus* DSM16549 | CP017480.1 | Rhodanobacter sp. OK091 | FRCH01000001.1 |
| *Luteibacter sp.* 22Crub2.1 | FUYTI01000025.1 | Rhodanobacter sp. Root627 | NZ_LMGR01000002.1 |
| Neisseriaceae | | | |
| *Morococcus cerebrus* CIP81.93 | NZ_JUFZ01000115.1 | Neisseria sp. HMSC069H12 | NZ_KV810249.1 |
| *Neisseria dentiae* NCTC13012 | NZ_U0QR01000001.1 | Neisseria sp. HMSC06F02 | KQ000148.1 |
| *Neisseria flavescens* SK114 | ACQV01000009.1 | Neisseria sp. HMSC070A01 | LTI00100031.1 |
| *Neisseria mucosa* C102 | GL635793.1 | Neisseria sp. HMSC071C03 | KV838738.1 |
| *Neisseria sp.* HMSC066A03 | KV821597.1 | Neisseria sp. oral taxon 014 str. F0314 | GIL349413.1 |
| Neisseria sp. HMSC064D07 | KV831649.1 | Neisseria subflava C2012011976 | NZ_FOOG0100001.1 |
| Comamonadaceae | | | |
| *Hydrogenophaga crassostreae* LPB0072 | LVWD01000013.1 | Hydrogenophaga sp. H7 | MCIO01000014.1 |
| *Hydrogenophaga sp.* A37 | MUNZ01000181.1 | Variorax sp. Root318D1 | NZ_LMGR01000008.1 |
| Others | | | |
| *Acinetobacter baumannii* 43000STDY704581 | UFGS01000006.1 | Burkholderiales bacterium GWF1_66_17 | MERT0100006.1 |
| *Bacillus sp.* SRB_336 | NZ_NADW01000001.1 | Xanthomonadaceae bacterium Rif00X1AFULL_69_10 | MID000100097.1 |
| *Bacterium Am6* | MUYX01000125.1 | Xanthomonadaceae bacterium 14-68-21 | NCKH01000003.1 |
| *Betaproteobacteria bacterium HQW* | PHCO01000002.1 | | |

X-T4SSs were identified according to the following criteria: (i) located in chromosomal DNA, (ii) VirB7 subunits have C-terminal N0 domains, (iii) genes for all twelve class A T4SS subunits are present, and (iv) the genomes carry genes for putative effectors (X-Tfes) with C-terminal XVxPCD required for recognition by X-T4SS VirD4. Species names are organized in alphabetical order within each bacterial family. Species discussed in more detail in the main text are shown in bold. *NCBI Reference Sequences or GenBank accession number.*

**TABLE 1 | Continued**

| Organism | Accession | Organism | Accession |
|----------|-----------|----------|-----------|
| *Neisseria* sp. SRB_336 | NZ_NADW01000001.1 | Luteibacter sp. 67-28 | MKU01000080.1 |
| *Neisseria* sp. Root318D1 | NZ_LMCQ01000008.1 | Rhodanobacter sp. Luteibacteryeojunensis SU11 | JZPB01000062.1 |
| *Neisseria* sp. H7 | MCIO01000014.1 | Rhodanobacter sp. C06 | NZ_MUN01000039.1 |
| *Neisseria* sp. HMSC066A03 | KV831649.1 | Neisseria subflava C2012011976 | NZ_FOOG0100001.1 |
| **Comamonadaceae** | | | |
| *Hydrogenophaga crassostreae* LPB0072 | LVWD01000013.1 | Hydrogenophaga sp. H7 | MCIO01000014.1 |
| *Hydrogenophaga sp.* A37 | MUNZ01000181.1 | Variorax sp. Root318D1 | NZ_LMGR01000008.1 |
| Others | | | |
| *Acinetobacter baumannii* 4300STDY704581 | UFGS01000006.1 | Burkholderiales bacterium GWF1_66_17 | MERT0100006.1 |
| *Bacillus sp.* SRB_336 | NZ_NADW01000001.1 | Xanthomonadaceae bacterium Rif00X1AFULL_69_10 | MID000100097.1 |
| *Bacterium Am6* | MUYX01000125.1 | Xanthomonadaceae bacterium 14-68-21 | NCKH01000003.1 |
| *Betaproteobacteria bacterium HQW* | PHCO01000002.1 | | |

X-T4SSs were identified according to the following criteria: (i) located in chromosomal DNA, (ii) VirB7 subunits have C-terminal N0 domains, (iii) genes for all twelve class A T4SS subunits are present, and (iv) the genomes carry genes for putative effectors (X-Tfes) with C-terminal XVxPCD required for recognition by X-T4SS VirD4. Species names are organized in alphabetical order within each bacterial family. Species discussed in more detail in the main text are shown in bold. *NCBI Reference Sequences or GenBank accession number.*
**FIGURE 3** | Comparison of core complex structures. **(A)** Comparison of the electron microscopy maps of the full-length core complexes from pKM101 (12.4 Å resolution; top row; Rivera-Calzada et al., 2013) and X. citri T4SSs (3.3 Å resolution; middle row; Sgro et al., 2018). Also shown is the electron density map of the O-layer of the pKM101 core complex obtained by X-ray crystallography (2.9 Å resolution; lower row; Chandran et al., 2009). General features and dimensions are shown for side and top views, and for a central section. **(B)** Side-by-side comparison of the atomic models of the X. citri core complex (gray) and pKM101 (orange) O-layers. General features and dimensions are shown for side and top views, and for a central section. **(C)** Side-by-side comparison of the atomic models of the VirB7–VirB9–VirB10 trimer and TraN-TraO-TraF trimer in the X. citri core complex and pKM101 O-layer, respectively. Colors: VirB10 and TraF (blue), VirB9 and TraO (green), VirB7 and TraN (red). Side (left) and top (right) views are shown of diametrically opposed trimers taken from the side-by-side comparisons shown in B. NTD, N-terminal domain; CTD, C-terminal domain. Portions of the Figure were adapted from Rivera-Calzada et al. (2013) and Sgro et al. (2018) with permission from the publishers.
A

Antennae

VirB9/TRAoCTD
and VirB7/TRA

VirB10/NTD

VirB9/NTD

Lever arms

FIGURE 4 | Relative orientations of the antennae that form the outer membrane pore in the X. citri and pKM101 core complexes. (A) Superposition of the atomic models of diametrically opposed VirB7–VirB9–VirB10 trimers of the X. citri core complex (blue) and TraN-TraO-TraF trimers of the pKM101 O-layer (yellow). (B) Details of the relative orientations of the VirB10 and TraF C-terminal domains. The structures shown correspond to the red rectangle in (A). The blue and yellow circles represent the planes that contain the central axes of the two antenna helices (a1 and a2). The blue and yellow rods represent the average vector between the two helices in each protein. The angles between the planes (~50°) and between the rods (~20°) are shown. Figure derived from Sgro et al. (2018) with modifications. NTD, N-terminal domain; CTD, C-terminal domain. This Figure was adapted from and Sgro et al. (2018) with permission from the publishers.

X. citri core complex structure (Sgro et al., 2018). The VirB9NTD-VirB10NTD interaction, in addition to the VirB7–VirB7 interactions mentioned above (Souza et al., 2011) are two examples of interactions that are relatively weak when measured in isolation but reveal themselves to be physiologically relevant in the context of a large multi-subunit complex whose assembly is expected to be highly cooperative. The helical region is in fact the only well-conserved sequence in the N-terminal domains of the Xanthomonadales and H. crassostreae X-T4SS VirB10 subunits and can be described as a P[S/T]Lh[E/D/Q]RRh motif where h is a hydrophobic residue (Supplementary Figure S10). The VirB10 subunits from the two Neisseria species shown in Supplementary Figure S10 do not seem to carry this motif.

One interesting observation is the very small number of sterospecific contacts between the I- and O-layers in the X. citri core complex. This immediately brings up the question as to what maintains the relative orientations between the two layers. The answer may be that individually weak interactions, multiplied fourteen times in the mature complex, could together be strong enough to favor specific conformational states between the two layers. Another interesting observation is that the long flexible N-terminal linkers that emerge from the VirB9 and VirB10 C-terminal domains in the direction of the I-layer point in opposite directions and pass by each other with their main chain atoms coming within 7 Å of each other (Figure 3C; Sgro et al., 2018). Therefore, the covalent linkages between the VirB9 and VirB10 N- and C-domains can be looked upon as forming an intricate cross-weave pattern at the interface of the I- and O-layers. This detail could have a role in maintaining the two layers in a preferential orientation by restricting the excessive relative rotations in both clockwise and counter-clockwise directions. Enigmatically, however, small (6 or 8 residue) deletions in these linkers had only moderate effects on T4SS-dependent bacterial killing by X. citri (Sgro et al., 2018).

VirB1

In A. tumefaciens, VirB1 undergoes cleavage of its N-terminal signal peptide upon transport to the periplasm and a second cleavage reaction that produces two fragments: (i) an N-terminal SLT (soluble lytic transglycosylase) domain predicted to be involved in peptidoglycan remodeling during T4SS biogenesis and (ii) a 76 residue C-terminal fragment (named VirB1∗) that is subsequently transported to the extracellular milieu (Baron et al., 1997; Llosa et al., 2000). The VirB1 proteins in the X-T4SSs all have a well-conserved 150 residue N-terminal domain with predicted SLT activity (Supplementary Figure S1). These proteins lack an N-terminal signal sequence, however, and so their mechanism of transport into the periplasm is not yet known. The X-T4SSs VirB1 C-terminal domains vary in length from 130 to over 200 residues and are highly variable in sequence (Supplementary Figure S1). Whether X-T4SS VirB1 proteins undergo C-terminal processing in a manner analogous to that observed in A. tumefaciens remains to be investigated.

VirB2 and VirB5: The Components of the T4SS Pilus

The VirB2 pilin subunits in the Xanthomonadales species and more distant H. crassostreae, N. flavescens, and N. mucosa all have similar sizes and a very well conserved central hydrophobic region as well as a predicted cleavable 31–39 residue long N-terminal signal peptide (Supplementary Figure S2). After removal of the signal peptide, these pilins are predicted to have lengths between 80 and 104 residues. This is significantly greater than the 70 and 64 residue long mature F-pilin and its close homolog from plasmid pED208 (respectively) whose cryo-EM...
structures have been determined in the context of the assembled sex pilus (Costa et al., 2016). Sequence-based secondary structure predictions for the X-T4SS VirB2 subunits correspond well with the two pilin structures and this allowed us to predict the positions of the two major helices (α2 and α3) in X-T4SS VirB2 as well as the intervening positively charged loop which interacts with the head groups of bound phospholipids in the pilus lumen (Supplementary Figure S2; Costa et al., 2016). The larger size of the X-T4SS VirB2 subunits is due to highly variable C-terminal extensions (Supplementary Figure S2). It is not clear whether these C-terminal extensions decorate the external pilus surface in X-T4SSs, perhaps providing binding sites for species-specific targets, or whether they are processed as has been observed for some P-pili (Eisenbrandt et al., 1999).

VirB5 is thought to be associated with the T4SS pilus, perhaps as a minor pilin at the pilus tip (Schmidt-Eisenlohr et al., 1999; Aly and Baron, 2007; Alvarez-Martinez and Christie, 2009). Due to the very high sequence variability in VirB5 proteins, its annotation as a bona-fide T4SS component in deposited genomic sequences is often ambiguous. In the bacteria-killing T4SSs under consideration here, these proteins have between 200 and 280 residues and are highly variable in sequence. They all have a predicted N-terminal cleavable signal peptide as well as a pair of cysteine residues found in the central portion of the amino acid sequence that are separated by 11 to 33 residues (Supplementary Figure S5). Interestingly, the genetic loci coding for X-T4SSs in the two Lysobacter species shown in Figure 2 each carry two virB5 genes in tandem. These protein pairs are 73% and 43% identical in Lysobacter antibioticus and Lysobacter enzymogenes, respectively. The predicted involvement of VirB5 in mediating the binding of the pilus to specific structures on the target cell (Alvarez-Martinez and Christie, 2009), could be a causative factor in this subunit’s accelerated evolution.

**VirB8, VirB6, and VirB3: Integral Membrane Proteins of the Inner Membrane Complex**

VirB8 is an integral membrane protein with an N-terminal cytosolic peptide, a transmembrane helix and a globular C-terminal domain localized in the periplasm, the latter of which has been shown to interact with several other T4SS components, including VirB6, VirB9, and VirB10 (Alvarez-Martinez and Christie, 2009; Sivanesan et al., 2010; Villamil Giraldo et al., 2012). High resolution structures of the soluble C-terminal region of VirB8 proteins and homologs from diverse T4SSs have been determined: A. tumefaciens (Bailey et al., 2006), pKM101 (Casu et al., 2016), H. pylori (ComB10) and Brucella suis (Terradot et al., 2005), Clostridium perfringens, Rickettsia typhi and several Bartonella species (Gillespie et al., 2015), L. pneumophila and plasmid R64 (Kuroda et al., 2015). All these structures present the same fold, a β-sheet juxtaposed against a group of α-helices, and in most cases have been shown to oligomerize to different degrees (see references above). Since there are an estimated 12 copies of VirB8 in each class A T4SS (Low et al., 2014), these domains could associate to form an as-yet unknown structure in the bacterial periplasm. Interestingly, the bacteria-killing X-T4SSs have highly distinctive VirB8 components that are significantly longer (between 290 and 370 residues in length) than the canonical VirB8 components observed in the species listed above (all less than 250 residues). This greater size is, in large part, due to a C-terminal extension enriched in Ala, Gln, Gly, and Pro (AQGP) residues (Table 2 and Supplementary Figure S8).

The length of this extension and its enrichment in these residues are particularly evident in the Xanthomonadales order (53–73%) and H. crassostreae (45%) and less so in the X-T4SS VirB8 proteins of Neisseria species (Table 2). The role, if any, of the AQGP-rich extensions in these proteins is not yet known.

VirB6 is predicted to be a polytopic integral protein. In both A. tumefaciens and B. suis, the VirB6 N-terminus is located in the periplasm and the C-terminus is located in the cytosol, implying an odd number of transmembrane helices, estimated to be five in A. tumefaciens (Jakubowski et al., 2004) and seven in B. suis (Villamil Giraldo et al., 2015). Supplementary Figure S6 presents the multiple sequence alignment of X-T4SS VirB6 proteins that also align well with their homologs in A. tumefaciens and B. suis (data not shown). The precise number and positions of the transmembrane helices is again ambiguous since transmembrane helix pairs 3/4 and 5/6 could alternatively be longer single helices (Supplementary Figure S6). Another common feature of these proteins is a large loop between transmembrane helices 2 and 3, predicted to be localized in the periplasm for both A. tumefaciens

**Table 2** | Characteristics of C-terminal extensions in VirB8 proteins from X-T4SSs.

| Organism               | Extension size (aa) | % Ala | % Gln | % Gly | % Pro | % AQGP |
|------------------------|--------------------|-------|-------|-------|-------|--------|
| Xanthomonas citri 306  | 98                 | 21    | 25    | 11    | 15    | 72     |
| Sternotrophomonas maltophilia K279a | 104           | 19    | 19    | 12    | 15    | 65     |
| Lysobacter antibioticus 76 | 97             | 26    | 4     | 10    | 32    | 72     |
| Lysobacter enzymogenes C3 | 100             | 25    | 7     | 10    | 31    | 73     |
| Luteibacter rhizovicinus DSM16549 | 129            | 26    | 11    | 12    | 22    | 71     |
| Dyella japonigenis SB23-12 | 70              | 16    | 9     | 11    | 17    | 53     |
| Dyella thiooxidans ATSB10 | 48              | 33    | 2     | 8     | 27    | 70     |
| Hydrogenophaga crassostreae LPB0072 | 53            | 13    | 6     | 11    | 15    | 45     |
| Neisseria mucosa C102  | 55                 | 6     | 16    | 4     | 13    | 39     |
| Neisseria flavescens SK114 | 52              | 6     | 14    | 6     | 6     | 32     |

Analysis is based on the alignment shown in Supplementary Figure S8.

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of one or more predicted N-terminal transmembrane helices in X-T4SS VirB4 proteins (Supplementary Figure S4B boxes and motifs C, D, and E) and these motifs are present C-terminal domain carries all the conserved motifs required proteins can be divided into N- and C-terminal domains. The a VirB4 hexamer (Low et al., 2014; Redzej et al., 2017). VirB4 other periplasmic components of the inner membrane complex VirB6 per T4SS (Low et al., 2014; Alvarez-Martinez and Christie, 2009; Ding et al., 2003; Alvarez-Martinez and Christie, 2009; Paschos et al., 2006; Ripoll-Rozada et al., 2013). These proteins use the hydrolysis of ATP function (Alvarez-Martinez and Christie, 2009; Christie et al., 2016). These proteins use the hydrolysis of ATP to carry out mechanical work, expected to manifest itself in substrate unfolding, transfer and/or extrusion through the T4SS channel (Atmakuri et al., 2004).

VirB4 is the most well conserved T4SS subunit and has been used in phylogenetic analyses to trace evolutionary relationships and propose models for the emergence of T4SS subclasses (Guglielmini et al., 2014). Studies on different T4SSs have reported evidence that VirB4 interacts, at least transiently, with all of the other inner membrane complex components (Jones et al., 1994; Atmakuri et al., 2004; Cascales and Christie, 2004; Jakubowski et al., 2004; Paschos et al., 2006; Ripoll-Rozada et al., 2013; Low et al., 2014; Christie, 2016).

VirB4 has been localized to two 3-tiered barrel-like pedestals at the base of the inner membrane complex in EM reconstructions of the complete R388 T4SS, with each barrel corresponding to a VirB4 hexamer (Low et al., 2014; Redzej et al., 2017). VirB4 proteins can be divided into N- and C-terminal domains. The C-terminal domain carries all the conserved motifs required for nucleotide binding and hydrolysis (Walker A and Walker B boxes and motifs C, D, and E) and these motifs are present in X-T4SS VirB4 proteins (Supplementary Figure S4). The N-terminal domain, known to mediate interactions with the inner membrane, is expected to correspond to the upper tier in the R388 T4SS structure (Low et al., 2014; Redzej et al., 2017). One variable in the family of VirB4 proteins is the presence or absence of one or more predicted N-terminal transmembrane helices and the question as to their requirement for VirB4 function has proven to be controversial (Rabel et al., 2003), with the additional caveat that VirB4/TraB from pKM101 can be purified in soluble and membrane-bound forms (Durand et al., 2010). Therefore, it is not clear whether VirB4 should be considered an integral or peripheral membrane protein, or both (Arechaga et al., 2008; Christie, 2016; Waksman, 2019). The X-T4SS VirB4 proteins do not have predicted transmembrane helices using the TMHMM v2.0 (Krogh et al., 2001) and PSort (Nakai and Horton, 1999) prediction algorithms.

VirB11 is a soluble membrane-associated AAA+ ATPase that has been shown to interact with both VirD4 and VirB4 (Ripoll-Rozada et al., 2013). The crystal structures of VirB11 homologs from VirB11 is located at the interface between the two domains of the same monomer while in B. suis the nucleotide binds at the interface between the N-terminal domain of one monomer and the C-terminal domain of the neighboring monomer (Hare et al., 2006). VirB11 proteins from X-T4SSs show a high degree of sequence similarity and all have the long version of the linker, which aligns well with the B. suis linker B and α2C sequences (Supplementary Figure S11). We can therefore expect that the X-T4SS VirB11 proteins exhibit a domain-swapped structure similar to that of B. suis (Hare et al., 2006).

The VirD4 ATPase and its homologs are often called coupling proteins due to their role in selecting substrates for export by the T4SS. Analysis of the X-T4SS VirD4 proteins suggests that they have a canonical VirD4-like architecture (Ilosa and Alkorta, 2017) with two N-terminal transmembrane helices with a predicted intervening ∼30 amino acid periplasmic loop and a cytosolic C-terminal domain. The cytosolic domain can be separated into a nucleotide binding domain (NBD), with conserved Walker A and Walker B motifs, and a so-called all alpha domain (AAD) (Supplementary Figure S12; Gomis-Ruth et al., 2002). The VirD4 N-terminal transmembrane domain helices have been implicated in interacting with the VirB10 N-terminal region that includes its transmembrane helix (Segura et al., 2013) and the VirD4 all alpha domain is involved in substrate recognition (Gomis-Ruth et al., 2002; Whitaker et al., 2015). TrwB, the VirD4 homolog of the conjugal plasmid R388, has been crystallized as a hexameric ring (Gomis-Ruth et al., 2002). It has been proposed that VirB4 and VirD4 could form transient heterohexameric complexes during substrate transport (Peña et al., 2012; Waksman, 2019) and low resolution electron microscopy studies of the intact VirB3-B10/D4 T4SS from R388 observed VirD4 dimers sandwiched between the two hexameric VirB4 barrels (Redzej et al., 2017). Therefore, the oligomeric structure of VirD4 in a fully assembled and functioning T4SS is still not clear (Redzej et al., 2017; Chetrit et al., 2018; Waksman, 2019).
Putative Xanthomonadales-like T4SS effectors (X-Tfes) found in selected species that carry an X-T4SS. Shown here are representative examples from *Xanthomonas citri* 306 (Da Silva et al., 2002), *Stenotrophomonas maltophilia* K279a (Crossman et al., 2008), *Lysobacter antibioticus* 76 (de Bruijn et al., 2015), *Lysobacter enzymogenes* C3 (unpublished; GenBank accession CP013140), *Luteibacter rhizovicinus* DSM16549 (unpublished; GenBank accession CP017480), *Dyella thiooxydans* ATSB10 (ATSB10_02540), *Hydrogenophaga crassostreae* LPB0072 (LPB072_11930), *Neisseria mucosa* C102 (unpublished; GenBank accession CP017480), (Continued)
FIGURE 5 | Continued

Dyella jaingningensis SBZ3-12 (Bao et al., 2014), Dyella thiooxydans strain ATSB10 (unpublished; GenBank accession CP014841), Hydrogenophaga crassostreae LPB0072 (unpublished; GenBank accession LWDD01000013), Neisseria mucosa C102 (unpublished, GenBank accession GCA_000186165) and Neisseria flavescentis SK1114 (unpublished; GenBank accession ACQ01000009). Protein domains were identified by sequence comparison with the Pfam (El-Gebali et al., 2019) and/or CDD databases (Marchler-Bauer et al., 2015) and are colored according to the scheme presented at the bottom of the Figure. Domain abbreviations: M10 (Pfam accession PF08548), M13 (Pfam accession PF01431), M23 (Pfam accession PF01551), Lipase6 (Pfam accession PF01764), DUF2974 (Pfam accession PF11187), GH-E (Pfam accession PF14410), GH19 (Pfam accession PF00182), Zeta Toxin (Pfam accession PF06641), SLT (CDD accession cd00254), GysPc (CDD accession cd00044), PGB (Pfam accession PF01471), Amidase (Pfam accession PF01510), A-H (Pfam accession PF14412), DUF4344 (Pfam accession PF14247), Lys (CDD accession cl00222), Phage lyso (Pfam accession PF00959), QA (Pfam accession PF01832), Syru (CDD accession cl03193), DUF2365 (Pfam accession PF10157), NLPC_P60 (Pfam accession PF00877), RibH (Pfam accession PF02267), DUF2235 (Pfam accession PF08994), HExoH (HExoH motif in putative metalloprotease domain; Firczuk and Bochtler, 2007).

XANTHOMONADIES-LIKE T4SS EFFECTORS (X-Tfes) AND THEIR COGNATE INHIBITORS (X-Tfis)

X-Tfes

The first clues regarding the physiological role of the X. citri T4SS came from the identification of T4SS substrates using the VirD4 coupling protein as a bait in yeast two-hybrid assays against a Xanthomonas genomic library (Alegria et al., 2005). The strategy was based on the reasoning that in other well-characterized T4SSs, the VirD4 component is known to interact with the macromolecular substrates prior to transport (Llosa and Alkorta, 2017). This screening originally identified 12 so-called “Xanthomonas VirD4 interacting proteins,” or XVIPs (Alegria et al., 2005), later called Xanthomonadaeae T4SS effectors (Souza et al., 2015) and from here on Xanthomonadales-like T4SS effectors (X-Tfes, Figure 5). In X. citri, the gene for one X-Tfe (XAC2609) is found in the vir locus while the remaining X-Tfe genes are dispersed throughout the genome. Interestingly, all of these proteins have a common C-terminal domain entitled “XVIP conserved domain” or XVIPCD (Figures 1, 5), typically around 120 residues long, required for interaction with VirD4 (Alegria et al., 2005) and for secretion in a T4SS-dependent manner (Souza et al., 2015). The XVIPCD is characterized by a few conserved motifs in its N-terminal region and a glutamine-rich C-terminal region (Alegria et al., 2005).

The discovery of the XVIPCD as the secretion signal for the X. citri X-Tfes allowed for a large-scale bioinformatics identification of X-Tfe genes present in other bacterial genomes (Souza et al., 2015). Figure 5 shows the domain architectures of the X-Tfes identified by bioinformatics analysis of bacterial genomes whose X-T4SSs are described in Figure 2. The N-terminal portions of the X. citri X-Tfes are highly variable in size and architecture and most are predicted to function within the periplasm as peptidoglycan (PG) glycohydrolases, lytic transglycosylases, PG peptidases or lipases (Figure 5). Therefore, these bacterial species probably use their X-T4SSs to inject not one, but a diverse cocktail of X-Tfes that will simultaneously attack multiple structures in the target cell (Figure 1). Two purified X. citri X-Tfes (XAC2609 and XAC0466) with predicted PG hydrolase activities have been shown to lyse PG and induce the lysis of Gram-positive cells, which have exposed bacterial cell walls (Souza et al., 2015). It is interesting that a considerable fraction of X-Tfes have N-terminal sequences with no identifiable domains, opening the possibility that new domain families with antibacterial activities could be characterized in the future. One such X-Tfe, Smlt3024 from S. maltophilia K279a (Figure 5), has been shown to inhibit E. coli growth when heterologously expressed and directed to the periplasm (preprint: Bayer-Santos et al., 2019).

It is worth noting that we often encounter several open reading frames that code for small proteins, sometimes possessing little more than an intact XVIPCD; for example XAC0323, XAC1165, and XAC3404 in X. citri (respectively 136, 127, and 132 residues in length; Figure 5; Souza et al., 2015). In some cases, these open reading frames appear to be fragments of ancestral X-Tfes genes that suffered frameshift mutations. One example of this phenomenon is provided by the XAC1165 gene whose first 37 nucleotides overlap with the 3′ end of the upstream XAC1164 gene which codes for a 437 protein of unknown function. The amino acid sequences of XAC1164 and XAC1165 align very well with the N-terminal and XVIPCD regions, respectively, of the Smlt0113 X-Tfe protein from S. maltophilia (Figure 5). Thus X. citri XAC1164 and XAC1165 proteins are homologous, and probably the non-functional, fragments of a functional X-Tfe (Smlt0113) in S. maltophilia.

X-Tfis

To protect against the toxicity of endogenous or exogenous X-Tfes, X. citri and S. maltophilia produce specific immunity proteins that bind to their cognate toxins (Alegria et al., 2005; Souza et al., 2015; preprint: Bayer-Santos et al., 2019). These inhibitors have been termed Xanthomonadaeae T4SS immunity proteins (Souza et al., 2015) and from here on Xanthomonadales-like T4SS immunity proteins (X-Tfis). The genes coding for X-Tfis are usually found upstream and are probably co-transcribed with their cognate X-Tfe (Souza et al., 2015; preprint: Bayer-Santos et al., 2019). All bacterial species identified so far that carry an X-T4SS also code for multiple X-Tfe/X-Tfi pairs and, in most cases, the genes for at least one pair is found within, or in close proximity to, the locus that codes for the structural components of the X-T4SS (see Figure 2). Furthermore, in almost all cases where the X-Tfe is predicted to act upon periplasmic structures (glycosidic and peptide bonds in peptidoglycan or ester linkages in phospholipids), the cognate X-Tfi carries an N-terminal signal peptide and lipobox for periplasmic localization and anchoring in the outer membrane (Souza et al., 2015; preprint: Bayer-Santos et al., 2019). On the other hand, some X-Tfes with N-terminal
FIGURE 6 | Possible alternative translation start codons that could lead to the production of soluble cytosolic X-Tfis in Xanthomonas citri. The first two columns list the names of X. citri X-Tfe/X-Tfi pairs in which the X-Tfi is predicted to be a lipoprotein (Souza et al., 2015). The third column presents the N-terminal amino acid sequence of the X-Tfi in which the signal sequence and Lipobox are shown in bold. The basic nucleotides at the N-terminus of the signal sequence are shown in blue. The four Lipobox residues are shown in red. Underlined residues are those from the absolutely conserved Cys residue at the site of cleavage in the Lipobox to the next Met residue (green) in the protein sequence. The last column presents the nucleotide sequence (lowercase letters) immediately upstream of the putative alternative start codon (green). The putative Shine–Dalgarno sequence (ribosome binding site) for this alternative start codon is shown in red.

TABLE 3 | List of proteins in the KEGG database with greatest similarity\(^a\) to the N-terminal domain (residues 1–240) of Smit0332 from S. maltophilia K279a.

| Organism | Accession\(^b\) | E-value | Description |
|----------|----------------|---------|-------------|
| Xanthomonas campestris pv. campestris B100 | xcc-b100_0624 | 3 E-88 | X-T4SS X-Tfe |
| Xanthomonas campestris pv. campestris 8004 | XC_9909 | 2 E-83 | X-T4SS X-Tfe |
| Xanthomonas campestris pv. campestris ATCC33913 | XCC3567 | 2 E-83 | X-T4SS X-Tfe |
| Xanthomonas vasicola pv. vasculorum SAM119 | CTV42_13240 | 2 E-79 | X-T4SS X-Tfe |
| Xanthomonas vesicatoria ATCC35937 | BJU12_15640 | 2 E-76 | X-T4SS X-Tfe |
| Metakosakonia sp. MRY16-398 | MRY16398_39390 | 2 E-26 | Type VI secretion system secreted protein VgrG |
| Pantoea ananatis LMG20103 | PANA_2352 | 9 E-26 | Type VI secretion system secreted protein VgrG |
| Pantoea vagans FDAARGOS_160 | AL522_12495 | 4 E-25 | Type VI secretion system secreted protein VgrG |
| Kosakonia radicic坦ans GXGL-4A | A3780_13240 | 4 E-22 | Type VI secretion system secreted protein VgrG/Rhs |
| Enterobacter hormaechei subsp. xiangfangensis LMG27195 | BFV63_12735 | 4 E-21 | Type VI secretion system secreted protein VgrG/Rhs |
| Enterobacter hormaechei subsp. xiangfangensis 34399 | L666_12735 | 9 E-21 | Type VI secretion system secreted protein VgrG/Rhs |
| Enterobacter hormaechei subsp. hoffmannii ECR091 | ECR091_12370 | 9 E-21 | Type VI secretion system secreted protein VgrG |
| Enterobacter hormaechei subsp. hoffmannii ECNIH5 | ECNIH3_12435 | 2 E-20 | Type VI secretion system secreted protein VgrG/Rhs |
| Enterobacter cloacae ECNIH5 | ECNIH5_12380 | 3 E-20 | Type VI secretion system secreted protein VgrG/Rhs |
| Burkholderia stabilis ATCCBAA-67 | BB4_1_25130 | 2 E-20 | Type VI secretion system secreted protein VgrG |
| Enterobacter hormaechei subsp. hormaechei 34983 | L64_12500 | 2 E-20 | Type VI secretion system secreted protein VgrG/Rhs |
| Methylocaldum marinuxi S8 | sSl_3556 | 6 E-20 | Type VI secretion system secreted protein VgrG |
| Ralstonia solanacearum FQY_4 | F504_2863 | 2 E-19 | Type VI secretion system secreted protein VgrG |
| Ralstonia solanacearum Po28 | RSPO_00015 | 2 E-19 | Type VI secretion system secreted protein VgrG |
| Burkholderia territii RR8-non-BP5 | WS51_08400 | 4 E-19 | Type VI secretion system secreted protein VgrG |
| Cronobacter tunicinis 23032 | CTU_00910 | 3 E-19 | Type VI secretion system secreted protein VgrG |
| Ralstonia pseudosolanacearum RS 476 | CDC45_17535 | 5 E-19 | Type VI secretion system secreted protein VgrG |
| Ralstonia solanacearum GM1000 | RS23430 | 5 E-19 | Type VI secretion system secreted protein VgrG |
| Ralstonia solanacearum PS307 | RPS07_0016 | 1 E-17 | Type VI secretion system secreted protein VgrG |
| Burkholderia stagnalis MSMB735WGS | WTP7_22275 | 3 E-17 | Type VI secretion system secreted protein VgrG |
| Xanthomonas fragariae Fap21 | BER92_04245 | 2 E-17 | Type VI secretion system secreted protein VgrG |
| Ralstonia solanacearum FQY_4 | F504_3476 | 4 E-17 | Type VI secretion system secreted protein VgrG |
| Xanthomonas campestris pv. raphani 756C | XCR_2915 | 4 E-17 | Hypothetical protein |
| Mycolicibacterium hastiacum DSM41999 | MHA5_03665 | 1 E-16 | Hypothetical protein |
| Burkholderia ubonensis MSMB22 | BW23_4367 | 3 E-16 | Type VI secretion system secreted protein VgrG/Rhs |
| Enterobacter sp. R4-386 | H560_00935 | 2 E-12 | Type VI secretion system secreted protein VgrG |
| Kosakonia cowanii 888-76 | BW195_18245 | 7 E-09 | Type VI secretion system secreted protein VgrG/Rhs |

\(^a\)only proteins with significant coverage in the alignment are shown.  
\(^b\)KEGG (Kyoto Encyclopedia of Genes and Genomes) accession number.
domains predicted to act in the cytosol of the target cell (for example the X. citri X-Tfe XAC3266 with an N-terminal AHH domain with predicted nuclease activity; Figure 5) have a cognate X-Tfi (for example XAC3267) lacking a lipoprotein signal (Souza et al., 2015). Finally, some X-Tfes are expected to be active in both the cytosol and periplasm, as are the cases of the X-Tfes with predicted lipase domains with phospholipase activities (NFB, DPS, BM, and CSF; manuscript in preparation; Figure 5). This brings up the question regarding the cellular localization of the X-Tfis. An analysis of the X. citri X-Tfis with putative N-terminal signal peptide and Lipobox sites indicates that their coding genes have potential alternative downstream start (ATG) codons with associated ribosome binding sites (Figure 6). This raises the possibility that many X-Tfes can be produced in two versions: (i) a membrane-associated periplasmic lipoprotein and (ii) a soluble cytosolic protein (Figure 1). Thus, if X-Tfes make their way into the periplasm, either by leakage from the secretion channel or by injection by neighboring cells of the same species, they will be inhibited by the periplasmic lipoprotein forms of the cognate X-Tfi. On the other hand, X-Tfes whose activities could target cytosolic substrates can be inhibited by cytosolic variants of their cognate X-Tfis. If this is in fact the case, for at least this latter subset of X-Tfes, transport will necessarily involve previous dissociation of the X-Tfe/X-Tfi cytosolic pair.

Parallels Between X-T4SS X-Tfe/X-Tfi, T6SS Effector/Immunity Protein and Plasmid-Encoded Toxin/ Antitoxin (TA) Pairs

X-Tfe/X-Tfi pairs share many of the characteristics observed for T6SS effectors and their inhibitors (Russell et al., 2011, 2013). For example, one immunity protein from X. citri (X-TfiXAC2610) inhibits the GH19 family PG hydrolase X-TfeXAC2609 and has a very similar topology, though very little sequence similarity, to the PG hydrolase inhibitors Piil and Ts1 (Van Herreweghe et al., 2010; Russell et al., 2011) the latter of which is an inhibitor of the T6SS effector Tse1 from Pseudomonas aeruginosa (Souza et al., 2015). Another example is provided by the X-Tfe Smlt0332 from S. maltophilia K279a, which has many X-Tfe homologs in other Xanthomonadales species (data not shown) but whose N-terminal region has no similarity with annotated domains in the Pfam or CCD databases. Interestingly, Blast searches against the curated KEGG database (Kanehisa et al., 2017) using this domain identified a large number of homologous sequences fused to VgrG domains in effectors predicted to be secreted by T6SSs of Metakosakonia, Pantoaea, Kosakonia, Enterobacter, Burkholderia, Methyllocaldum, Ralstonia, Cronobacter, and Xanthomonas species (Table 3). Searches against the non-redundant protein sequence database (Altschul et al., 1990) identified many more such homologs in other bacterial species (data not shown). These findings raise interesting questions regarding the evolution, distribution and exchange of T4SS and T6SS effectors in the biosphere. In fact, we can make a general observation that anti-bacterial Type IV and Type VI secretion systems share many enzymatic effector and cognate inhibitory modules and differ only in the specific sequences recognized for transport. It also raises the possibility that the acquisition of immunity proteins could be advantageous even in the absence of the cognate effector by offering a defense against the toxic activity of substrates launched by both T4SS and T6SSs during encounters with rival bacteria. We have in fact recently observed the reciprocal T4SS-dependent dualing between S. maltophilia and X. citri cells which could mimic similar encounters between soil- and/or plant-associated bacteria in the environment (preprint: Bayer-Santos et al., 2019). The differences in X-Tfe and X-Tfi repertoires between rival pairs will contribute to the outcome of these encounters.

Effector/immunity protein pairs associated with T4SS and T6SSs show intriguing parallels with toxin/anti-toxin modules that function to guarantee vertical transmission of mobile elements (Jensen and Gerdes, 1995). For example, Harms et al. (2017) have shown that the pVbh plasmid of Bartonella schoenbuchensis, codes for a toxin/antitoxin module in which the toxin component, VbhT, acquired a C-terminal BID (Bep Intracellular Delivery) domain that confers its transfer to recipient cells during conjugation. They propose that its function may be to support intercellular DNA transfer by pre-emptively addicting the recipient cell to the plasmid, which also carries the gene for the antitoxin antidote. It is not difficult to imagine a scenario in which the T4SS coded by such a plasmid could lose its capacity to transfer DNA but retain its capacity to transfer the toxin, and in this way evolve into a single-purpose bacteria-killing T4SS. Therefore, we can expect bactericidal T4SSs, perhaps with very different recognition signals, to have arisen on multiple occasions in distantly related bacterial species.

CLOSING REMARKS

These are early days in the characterization of antibacterial T4SSs. The structure of the X. citri core complex has provided a good reference for comparison with other T4SSs, and has illustrated the structural variability that we can expect to encounter even within the class A T4SSs. While X-T4SS activities have only been experimentally verified for X. citri and S. maltophilia, bioinformatics analysis allowed us to confidently expand the list of bacterial families both within the Gammaproteobacteria class as well as to other families within the Betaproteobacteria (Table 1) that carry proteins with many of the characteristic X-T4SSs features, including VirB7 proteins with N0 domains, VirB8 proteins with AQGP-rich extensions as well as recognizable X-Tfe/X-Tfi pairs in which the effector carries a C-terminal XVIPCD. The list of X-T4SSs will most surely expand significantly in the future. However, it is unlikely that bactericidal T4SSs are restricted to the X-T4SSs described here. It is more probable that many other bacterial species carry as yet uncharacterized and perhaps unrecognized T4SSs that recruit effectors with recognition signals significantly different from the XVIPCDs associated with X-T4SSs as predicted (Souza et al., 2015) and as illustrated by the results obtained for the T4SS encoded by the B. schoenbuchensis pVbh plasmid (Harms et al., 2017).
AUTHOR CONTRIBUTIONS

GS, GO, EB-S, and CF produced the figures and tables. CF wrote the manuscript. All authors contributed with critical discussions and revisions that led to the final version of the manuscript.

FUNDING

This work was supported by grants from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) to CF (Grant # 2017/17303-7), CA-M (Grant # 2018/01852-4), and EB-S (Grant # 2017/02178-2).

ACKNOWLEDGMENTS

GS, GO, DS, WC, BM, and EB-S acknowledge scholarships from FAPESP. DS, TDs, and NB acknowledge fellowships from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01078/full#supplementary-material
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Sgro et al. Bacteria-Killing Type IV Secretion Systems

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Conflict of Interest Statement: The authors declare that the research was conducted in accordance with any commercial or financial relationships that could be construed as a potential conflict of interest.

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