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Phytobacterial Type VI Secretion System – Gene Distribution, Phylogeny, Structure and Biological Functions

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1. Introduction

Microbes, and their distant relatives, plants, are thought to have co-evolved during the last 2 billion years. Most of the plant-associated prokaryotes are commensals, found primarily on leaf surfaces or roots, and have no discernible or known effects on plant growth or physiology; others evolved more or less intimate relationships with plants such as N-fixing symbioses, endophytic existence or plant growth-promoting (rhizobacterial) associations; yet others, the minority, wage outright hostility with plants, inciting various diseases.

Although some phytopathogenic bacteria internalize themselves in the plant vascular system, most of them colonize plant tissues extracellularly and target plant cell wall and membrane or internal cellular structures, signaling systems and metabolic machinery from the outside. For targeting they deploy phytotoxic metabolites, hormones, polysaccharides, enzymes for the hydrolysis of cell walls and other catalytic macromolecular effectors (and, exceptionally, DNA) as "ballistic missiles". To accomplish efficient transport of macromolecules across the bacterial and/or the plant cell envelop (plant cell wall and membrane), Gram-negative bacteria possess a suite of specialized transport systems, dedicated to the transport of selected sets of proteins from the bacterial cytoplasm to the external environment or into other living cells. Type I to type VI secretion systems (abbreviated T1SS to T6SS) form channels by assembling oligomeric macromolecular complexes of varying composition and sophistication. These assemblies function as molecular machines, are broadly conserved across Gram-negative bacteria and

play important roles in the virulence of pathogens. In general, most of these systems require a component providing energy to the secretion process (usually an ATPase), an outer-membrane protein, and various components involved either in scaffolding the macromolecular complex into the cell envelope or in the specific recognition of secreted substrates. It is noteworthy that certain components of these secretion machines are thought to be derived from other membrane-bound multiprotein structures serving a different purpose (see below).

2. Historical highlights

The T6SS is a relatively recent discovery, first identified as a protein secretion apparatus involved in virulence of *Vibrio cholerae* in the *Dictyostelium* (Mougous et al., 2006) and *Pseudomonas aeruginosa* in mouse models (Pukatzki et al., 2006). In several cases it has been shown to be important for bacterial virulence (host-pathogen interaction) and has attracted strong interest because it has been found via *in silico* analysis in the genomes of a large number of Gram-negative bacteria associated with human and animal diseases. While initially considered an atypical type IV secretion system (T4SS), various lines of evidence have established its identity as a distinct protein transport system (Bladergroen et al., 2003; Roest et al., 1997; Pukatski et al. 2006, Boyer et al., 2009).

An interesting twist to the T6SS story is the discovery of multiple copies of gene clusters coding for T6SS homologs in a large number of sequenced eubacterial genomes, including those of several plant-associated species (KEGG gene database; http://www.genome.jp/kegg-bin/get_htext?ko02044.keg). These species are mostly within the class of *Proteobacteria*, but also within the *Planctomycetes* and *Acidobacteria* (Tseng et al., 2009; Boyer et al., 2009). Several T6SS gene clusters are within "pathogenicity islands", for example, *P. aeruginosa*-HSI (Hcp-secretion island), enteroaggregative *Escherichia coli* (EAEC-*pheU*), *Salmonella typhimurium*-SCI (*Salmonella* Centrisome Island), *Francisella tularensis*-FPI (*Francisella* Pathogenicity Island), *Agrobacterium tumefaciens* (Wu et al., 2008), *Pectobacterium atrosepticum* (Liu et al., 2008) and *Xanthomonas oryzae* (Tseng et al., 2009), which indicates relationship to virulence or survival in the host. Bioinformatic analysis revealed that most of the "avirulent" bacterial species studied (i.e. bacteria that have no known host) lack T6SS orthologs but active protein secretion or the ability to invade hosts await experimental testing (Shrivastava & Mande 2008; Bingle et al., 2008). Many interesting highlights are best expressed by the imaginative titles of many publications cited in this chapter.

3. T6SS genes, proteins, injectisomes

3.1 Gene content and proteins of T6SS clusters

T6SSs are typically encoded by clusters of 12 to over 20 genes, with 13 genes thought to constitute the minimal number needed to produce a functional apparatus (Boyer et al., 2009). They are found mostly in α-, β-, and γ-proteobacteria (about 25% of the sequenced genomes; Bingle et al., 2008). Recently, Chow and Mazmanian (2010) characterized a T6SS in *Helicobacter hepaticus*, which belongs to the ε-(epsilon) subgroup of proteobacteria. These clusters (frequently referred to as T6SS loci in the literature) often occur in multiple, non-orthologous copies/genome (i.e. are not the result of simple duplication), indicating that they have probably been acquired by horizontal gene transfer (Sarris at al., 2011). Detailed

studies in a few bacteria further suggest that each T6SSs assumes a different role in the interactions of the harbouring organism with others. However, it is not known if there are T6SSs that can target both prokaryotes and eukaryotes. Unlike the Type III secretion systems (T3SSs), only few T6SS substrates have been identified and experimentally verified to date, but others may merely await identification.

It is now becoming increasingly clear that T6SS probably represents an evolutionary adaptation of a transmembrane protein translocation mechanism and at least some of its core components may share a common ancestor with bacteriophages. Common evolutionary ancestry and similar design are features ostensibly shared between other macromolecular transport systems and other bacterial devices that have evolved to serve entirely different biological functions (e.g. between T2SS and type IV pili, T3SS and flagella, or T4SS and conjugative pili). Indeed, the study of the macromolecular assembly process in these systems cross-feeds our understanding about structure, function and molecular mechanisms of these bacterial nanomachines.

The T6SS appears to be an injectisome, with some of its core component proteins structurally related to the cell-puncturing devices of tailed bacteriophages, and at least in some well-studied cases, have been shown capable of translocating effector proteins into the host cell cytoplasm (Bingle et al., 2008; Cascales, 2008; Filloux et al., 2008, 2011a; 2011b; Shrivastava & Mande 2008; Russell et al., 2011; Zheng et al., 2011), as is the case with the T3SS and T4SS. In the human pathogenic species *V. cholerae* and *P. aeruginosa* T6SS exports haemolysin-coregulated proteins (Hcp) and Valine-glycine repeat (Vgr) proteins; for these proteins the role of effectors associated with cytotoxicity in some *in vitro* models has been proposed (Pukatzki et al., 2006; Mougous et al., 2006). However, VgrG and Hcp display mutual dependence for secretion in *V. cholerae*, *Edwardsiella tarda* and enteroaggregative *E. coli* (Pukatzki et al., 2007; Zheng & Leung, 2007; Dudley et al., 2006), suggesting that these proteins might be not only passengers but also components of the secretion machine, a fact also supported by recent structural studies (see section 3.3). Such "dual function" could be related to distinct protein domains. In particular, the N-terminal domains of Vgr proteins show strong homology with the T4 bacteriophage base plate components gp27 and gp5 (Pukatzki et al., 2007) and a conserved core followed by a highly polymorphic C-terminal domain. The *V. cholerae* VgrG1 protein has a C-terminal domain homologous to the actin cross-linking domain (ACD) of the RtxA toxin, while VgrG1 from *Aeromonas hydrophila* possesses actin ADP-ribosylating activity (Pukatzki et al., 2007; Sheahan et al., 2004; Suarez et al., 2010). Some VgrGs ("evolved" VgrGs) from various bacterial species possess various effector-like C-terminal domains: a) a tropomyosin-like domain, which is thought to manipulate actin filaments during *Yersinia* infections, b) a pertactin-like, YadA-like, mannose-binding-like, or fibronectin-like domains or share similarities with peptidoglycan- or fibronectin-binding sequences and c) homologs of the eukaryotic lysosomal cathepsin D protein (Cascales, 2008). On the other hand, it was suggested that some VgrG orthologs may not be injected but may remain attached to the bacterial cell surface (Pukatzki et al., 2007).

Similarly to T3S systems, Hcp-secreted proteins lack N-terminal hydrophobic signal sequences, indicating secretion in a Sec- or Tat-independent manner, and a probable crossing of the bacterial cell envelope in a single step (Bingle et al., 2008; Pallen et al., 2003). Furthermore, Hcp-secreted proteins seem to have intracellular targets in eukaryotic hosts.

Thus, the Hcp protein of *A. hydrophila* was found in culture supernatants, as well as in the cytosol and the membrane of human epithelial cells after infection. Hcp secretion was independent of the T3SS and the flagellar system and the secreted protein was capable of binding to the murine macrophages from the outside, in addition to being translocated into mammalian model host cells; heterologous expression of this protein in HeLa cells increased the rate of apoptosis mediated by caspase 3 activation (Suarez et al., 2008). These findings are consistent with Hcp being secreted/translocated by T6SS, along with other yet unidentified effectors. The Hcp1 protein from pathogenic *P. aeruginosa* was also shown to be actively secreted in cystic fibrosis patients resulting in Hcp specific antibody production. Likewise, a novel T6SS protein, VasX, which is required for pathogenicity against the amoeboid host model *Dictyostelium discoideum* has recently been described. VasX is unique because it contains a putative pleckstrin homology domain which is typically only found in eukaryotic and not in bacterial proteins. VasX can bind to mammalian membrane lipids, an interaction mediated by the putative pleckstrin homology domain. It has been proposed that this domain may direct VasX to specific targets within the host cell resulting in disruption of host cell signaling (Miyata et al., 2011).

Another hallmark of T6SSs clusters is a gene coding for an AAA+ Clp-like ATPase , named 17 ClpV, belonging to a sub class of ClpB ATPases which comprise hexameric enzymes involved in protein quality control. A possible role of T6SS Clp-ATPase members might be the unfolding of substrates to be secreted, as demonstrated for the T4SS and the T3SS ATPases (Cascales, 2008). However, the *Salmonella enterica* T6SS Clp protein forms oligomeric complexes with ATP hydrolytic activity but fails to unfold aggregated proteins (Schlieker et al., 2005). A study by Bonemann et al. (2009) revealed the involvement of the ClpV protein of *V. cholerae* in remodelling supramolecular assemblies formed by two core components VipA/VipB (synonyms: ImpB/ImpC) which are crucial for T6SS secretion and virulence (see section 3.3). However, some bacterial species (e.g. *Rhizobium leguminosarum* and *Francisella tularensis*) may not contain functional Clp homologs within their T6SS clusters (Filloux et al., 2008).

Another T6SS-linked gene, *icmF* (intracellular multiplication in macrophages), has been previously studied in the context of T4SS secretion and shown to be necessary for efficient secretion. This protein carries three transmembrane domains (Sexton et al., 2004) and is partially required for *Legionella pneumophila* replication in macrophages (Purcell & Shuman 1998). Furthermore, the lack of IcmF resulted in a reduced level of another core protein, DotU, suggesting that the two proteins interact or are co-regulated. It was also shown that the lack of DotU and/or IcmF affected the stability of other core components, which suggests that DotU and IcmF assist in assembly and stability of a functional T6SS (Filloux et al., 2008). Furthermore, IcmF has been proposed to function as a further energizing component (Bonemann et al., 2009). Amino acid sequence analysis predicts that IcmF is located in the inner membrane and consists of a cytosolic and a periplasmic domain. The cytosolic domain has a conserved Walker-A motif, indicating a function as an ATPase during secretion, consistent with the finding that IcmF mutations prevent Hcp secretion (Pukatzki et al., 2006; Zheng & Leung 2007). An *icmF* mutant of avian pathogenic *E. coli* had decreased adherence to and invasion of epithelial cells, as well as decreased intra-macrophage survival and was also defective for biofilm formation on abiotic surfaces (Pace et al., 2011).

3.2 Regulation

Although T6SS gene expression inside macrophages has been demonstrated for several animal and human pathogens (e. g. *Burkholderia pseudomallei, S. enterica, V. cholerae* and *Francisella* (Shalom et al., 2007; Parsons & Heffron 2005; de Bruin et al., 2007), and to a small extent in plant pathogens, the signals triggering T6SS expression are largely unknown. For example, in *V. cholerae,* upon phagocytosis, expression of the T6SS induces cytoskeleton rearrangements through the secretion of the actin cross-linking domain of VgrG (Ma et al., 2009). Likewise, the Hcp1 of *P. aeruginosa* is induced during the infection of cystic fibrosis patients (Mougous et al., 2006), while Hcp3 is expressed upon addition of epithelial cell extracts (Chugani & Greenberg, 2007). No information concerning the expression of Hcp2 is available.

The T6SS regulation involves various transcriptional activators such as AraC, TetR-, and MarR-like proteins, σ^{54}-like factors and heat-stable nucleoid-structural (H-NS) proteins (Bernard et al., 2010). Likewise, two-component systems, like the ferric uptake regulator Fur and the Quorum sensing (QS) related regulators like LuxI, LuxR and acyl homoserine lactones (AHL) are reported to be involved in regulation of T6SS expression (Bernard et al., 2010). It has been also reported (Mougous et al., 2007) that the regulation of the HSI-1 in *P. aeruginosa* PAO1 is influenced by the sensor kinases RedS and the LadS, resulting in opposite patterns of regulation for the type III and type VI secretion systems in this bacterium, as in *S. enterica* (Parsons & Heffron 2005). T6SS gene expression in *P. syringae pv. syringae* B278a is also regulated by the two sensor kinases RetS (negatively) and LadS (positively). Two more proteins, PpkA and PppA, seem to play an important role in T6SS gene regulation (Mougous et al., 2007). PpkA is a kinase, which becomes activated by auto-phosphorylation under certain environmental conditions whereas PppA is a phosphatase, which counteracts with the action of PpkA. Both proteins act on a common protein substrate, Fha1 (*f*ork *h*ead-*a*ssociated domain protein; Mougous et al., 2007). Gene expression in HSI-2 and HSI-3 is proposed to be regulated by two σ^{54} factors which are encoded in the respective T6SS clusters of *P. aeruginosa*, as well as in their *V. cholerae* and *A. hydrophila* homologs.

Recent work reported the identification of Fur as the main regulator of the enteroaggregative *E. coli sci1* T6SS gene cluster. A detailed analysis of the promoter region showed the presence of conserved motifs, which are target of the DNA adenine methylase Dam (Brunet et al., 2011). The authors showed that the *sci1* gene cluster expression is under the control of an epigenetic methylation-dependent switch: Fur binding prevents methylation of a conserved motif, whereas methylation at this specific site decreases the affinity of Fur for its binding box. In other work (Bernard et al., 2011), several clusters were identified (including those of *V. cholerae*, *A. hydrophila*, *P. atrosepticum*, *P. aeruginosa*, *Pseudomonas syringae* pv. *tomato*, and a *Marinomonas* sp.) as having typical –24/–12 sequences, enhancer binding motifs recognized by the alternate sigma factor σ^{54} which directs the RNA polymerase to cognate promoters and requires the action of a bacterial enhancer binding protein (bEBP), which binds to *cis*-acting upstream activating sequences. The authors further showed that putative bEBPs are encoded within the T6SS gene clusters possessing σ^{54} boxes and, through *in vitro* binding and *in vivo* reporter fusion assays, they demonstrated that the expression of these clusters is dependent on both σ^{54} and bEBPs (Bernard et al., 2011).

A study by Zheng et al. (2011) provides new insights into the functional requirements of secretion as well as killing of bacterial and eukaryotic phagocytic cells by *V. cholerae* by analyzing non-polar mutations (in-frame deletions) in each gene predicted to code for *V. cholera* T6SS components. They grouped 17 proteins into four categories: twelve proteins (VipA, VipB, VCA0109–VCA0115, ClpV, VCA0119, and VasK) are essential for Hcp secretion and bacterial virulence, and thus likely function as structural components of the apparatus; two proteins (VasH and VCA0122) were thought to be regulators that are required for T6SS gene expression and virulence; another two (VCA0121 and VgrG-3) were not essential for Hcp expression, secretion or bacterial virulence, and their functions are unknown; one protein (VCA0118) was not required for Hcp expression or secretion but still played a role in both amoebae and bacterial killing and may therefore be an effector protein. ClpV was required for *Dictyostelium* virulence but was less important for killing *E. coli*. In addition, VgrG-2 which is encoded outside of the T6SS cluster was required for bacterial killing but VgrG-1 was not and several genes in the same putative operon as *vgrG*-1 and *vgrG*-2 also contributed to *Dictyostelium* virulence but had a smaller effect on *E. coli* killing.

3.3 Structure and functions of T6SS proteins and injectisomes

In contrast to other bacterial secretion systems (e.g. T3SS) there is only a small number of experimentally determined structures of T6SS core proteins or of their macromolecular assemblies. These structural studies have allowed within a relatively short time to understand important aspects of T6SS function to a considerable detail. Additional insights into structure-function relationships of T6SS have been deduced from structural/sequence similarities observed between T6SS proteins and a) components of cell puncturing devices utilized by tailed bacteriophages for DNA delivery or b) proteins from other types of bacterial secretion systems; experimental verification of the relationships derived by analogies to other systems remains largely to be delivered.

Medium to high resolution crystal structures exist for the *P. aeruginosa* Hcp1 protein, the N-terminal fragment of VgrG from the uropathogenic *E. coli* CFT073, and EvpC from *E. tarda*. Electron microscopy has been used in the study of the assemblies of various T6SS proteins including *P. aeruginosa* Hcp1, Hcp2 and Hcp3, the *E. coli* CFT073 Hcp, and *V. cholerae* VipA/VipB complex.

The structures of Hcp and VgrG provide the strongest evidence about the evolutionary relatedness between proteins of T6SS and phage tails with extended homologies existing at the levels of structure, assembly and function. The structure of Hcp1 (Mougous et al., 2006) was determined at a resolution of 1.95 Å (PDB ID 1Y12) and was found to be strikingly similar to that of the gpVN tail tube protein of phage lambda, with only minor deviations between the two structures (Pell et al., 2009). In the Hcp1 crystal, symmetry-related molecules assemble into hexameric ring which can be superimposed onto the trimeric pseudohexamer formed by the tube domains of the T4 bacteriophage gp27 trimer. Sequence analyses suggest that Hcp is evolutionary related to a further viral protein, the T4 tail tube protein gp19. Strikingly, the packing of Hcp1 hexamers in the crystals studied produces tube-like structures which are geometrically nearly identical to the T4 tail tube which is composed of stacked gp19 hexamer. The Hcp hexameric rings can also be induced to polymerize *in vitro* into stable nanotubes through the introduction of cysteine mutations capable of engaging in disulfide bridges formation across the hexamers (Ballister et al., 2008).

The crystal structure of the *E. tarda* EvpC protein, an Hcp1 homolog from the virulent protein gene cluster EVP which contains a conserved T6SS, has been recently determined at 2.8 Å resolution (PDB accession code 3EAA) and revealed a high structural similarity with Hcp1 (Jobichen et al., 2010). In solution, EvpC exists as a dimer at low concentrations and as a hexamer at high concentrations. In the crystals symmetry-related EvpC molecules form hexameric rings which stack to form tubes similar to Hcp1. Structure-based mutagenesis has revealed a critical role for EvpC secretion for three negatively charged N-terminal residues, and three positively charged C-terminal ones (Jobichen et al., 2010). This secretion impairment of EvpC decreases the virulence of the T6SS-containing pathogenic bacteria.

The structure of the N-terminal fragment (residues 1-483 out of 824) of the VgrG protein encoded by the *E. coli* CFT073 gene c3393 was determined at a resolution of 2.6 Å (PDB ID 2P5Z). The protein shows striking structural similarities (Leiman et al., 2009) to the structure of the complex $(gp5)_3$-$(gp27)_3$ of the T4 bacteriophage cell-puncturing device (PDB ID 1K28). VgrG can be described as a fusion of T4 gp27 and gp5; at the level of equivalent domains VgrG shares the highest structural homology with gp27 and exhibits only minor modifications relative to gp5. The VgrG structure comprises a domain (residues 380-470) of unknown function which is conserved in all VgrGs. This domain (DUF586) is the equivalent of the oligosaccharide/oligonucleotide-binding (OB)-fold domain of T4 gp5. The secondary structure prediction of the C-terminal part of VgrG (residues 490-820) which follows the OB-fold domain, shows repetitive β-strands (5-10 residues each) flanked by glycines. It is likely, that these strands form a β-helix that is equivalent to the triple stranded β-helix in trimeric gp5 which is involved in membrane penetration. The trimeric structure of the N-terminal VgrG fragment in the crystal, probably indicates that the complete VgrG protein may also adopt a trimeric structure that is equivalent to the $(gp5)_3$-$(gp27)_3$ complex. Sequence analyses suggest that effector domains are fused to the C-termini of many VgrG proteins ('evolved' VgrGs). The gp5 C-terminal β-helix has a 23 KDa extension of unknown function corresponding to a VgrG effector domain. Since many VgrG proteins do not contain an additional C-terminal domain, it may be concluded that different T6SS injectisomes service separate sets of effector proteins.

The structural similarities of Hcp and VgrG to components of the injection apparatus of tailed bacteriophages are highly suggestive that the two proteins might be structural components of T6SS, rather than effector proteins. The absence of 'evolved' VgrGs in many T6SSs suggests also that Hcp/VgrG might act as a conduit for T6SS effectors. The inner diameter of the Hcp tubule (40 Å) would allow the passage of proteins in an unfolded form; delivery of effectors to target cells might involve the cell-puncturing activity of VgrG.

The energy for the translocation of secreted proteins to the extracellular environment is provided by two components of the T6SS that have been introduced in section 3.1: IcmF and ClpV (Bonemann et al., 2009). IcmF is a membrane-embedded component that forms a structure spanning the inner and outer membranes. ClpV does not interact with the exoproteins Hcp and VgrG, but binds specifically with its N-domain to two cytosolic proteins, VipA (COG3516) and VipB (COG3517), that are conserved and essential components of T6SS (Bonemann et al., 2009, 2010). VipA and VipB interact with each other forming a tubular, cogweel-like structure larger than 200 kDa, with a diameter of approximately 300Å and a central channel of 100Å in diameter, and a length ranging from 25 to 500nm. Electron microscopy studies of VipA/VipB suggest that there is an overall

resemblance between the VirA/VirB tubules and the T4 tail sheath structure which accommodates the viral tail tube proteins (Aksyuk et al., 2009); the diameter of the inner channel of VirA/VirB tubules is sufficient to encase Hcp tubes. In-frame deletion mutants of *vipA* and *vipB* genes could no longer secrete Hcp and VgrG proteins, although the total levels of the proteins were not affected (Bonemann et al., 2009), thus suggesting a crucial role of VirA and VirB for T6SS function. Importantly, there is no evidence for an interaction of VipA and VipB with the cytosolic proteins Hcp and VgrG. VipA/VipB cogwheel-like tubules are disassembled by ClpV; this ClpV-mediated remodelling of VipA/VipB tubules into smaller complexes (100kDa), has been suggested as an essential step in T6SS secretion, revealing an unexpected role for this ATPase component in a bacterial protein secretion system. The recent characterization of the *P. syringae* pv. *syringae* T6SS proteins ImpB (177aa) and ImpC (500aa) (homologs of VipA and VipB respectively) suggest that the two proteins form supramolecular structures of comparable size to the assemblies of VipA/VipB (M. Kokkinidis, unpublished results). These ongoing studies represent the first structural analysis of the T6SS of a plant pathogen.

4. T6SS role in host colonization and interbacterial interactions

The T6SS was initially thought to play a role primarily in bacterial pathogenicity and host colonization. Roest et al. (1997) and Bladergroen et al. (2003) characterized *Rhizobium* loci (*imp*) that hinder effective nodulation on certain plants. Imp mutants (impaired in nodulation) were deficient in the secretion of an effector protein (RbsB-like) and were mapped in a cluster of 16 genes (from pRL120462 [impN-like] to pRL120480 [vgrG-like], Fig. 1). The later authors predicted that these genes encoded a new protein secretion system, which was later named T6SS by Pukatzki et al. (2006). However, it is also found in environmental isolates and recent findings suggest that they may also function in a broader biological context: to mediate cooperative or competitive interactions between bacteria, including bacterial biofilm formation, or to promote the establishment of commensal or mutualistic relationships between bacteria and eukaryotes (Aschtgen et al., 2008; Hood et al., 2010; Jani & Cotter, 2010; Schwarz et al., 2010a; Russell et al., 2011; Zheng et al., 2011). For example, *P. aeruginosa* is capable of secreting the antibacterial factors Tse (*T*ype VI secretion *e*xported) (Hood et al., 2010). Tse2 is a toxic protein from *P. aeruginosa* (PA2702) and arrests growth of both prokaryotic and eukaryotic cells when expressed intracelularly. It is proposed to be an export substrate of the *P. aeruginosa* HSI-I (Hood et al., 2010). Tse2 expressing bacterial cells produce also an immunity protein, Tsi2 (PA2703), preventing cell death when co-expressed with Tse2. It is noteworthy that *tse2/tsi2* are not found in other, phylogenetically close *Pseudomonas* species (*P. entomophila* and *P. mendocina*), suggesting that it is a species-restricted regulon that responds to specific needs (Sarris & Scoulica, 2011). That the T6SS can target bacteria has also been demonstrated experimentally for *Burkholderia thailandensis* (Hood et al., 2010; Schwarz et al., 2010b) and *V. cholerae* (MacIntyre et al., 2010; Zheng et al., 2011). In *B. thailandensis* two of the five T6SSs assume specialized functions: either in the survival of the organism in a murine host (T6SS-5), or against other bacteria (T6SS-1), since strains lacking the bacterial-targeting T6SS-1 could not persist in a mixed biofilm with competing bacteria (Schwarz et al., 2010b). Miyata et al. (2010) speculate that *V. cholerae* uses its T6SS to outcompete bacterial neighbours as well as eukaryotic predators

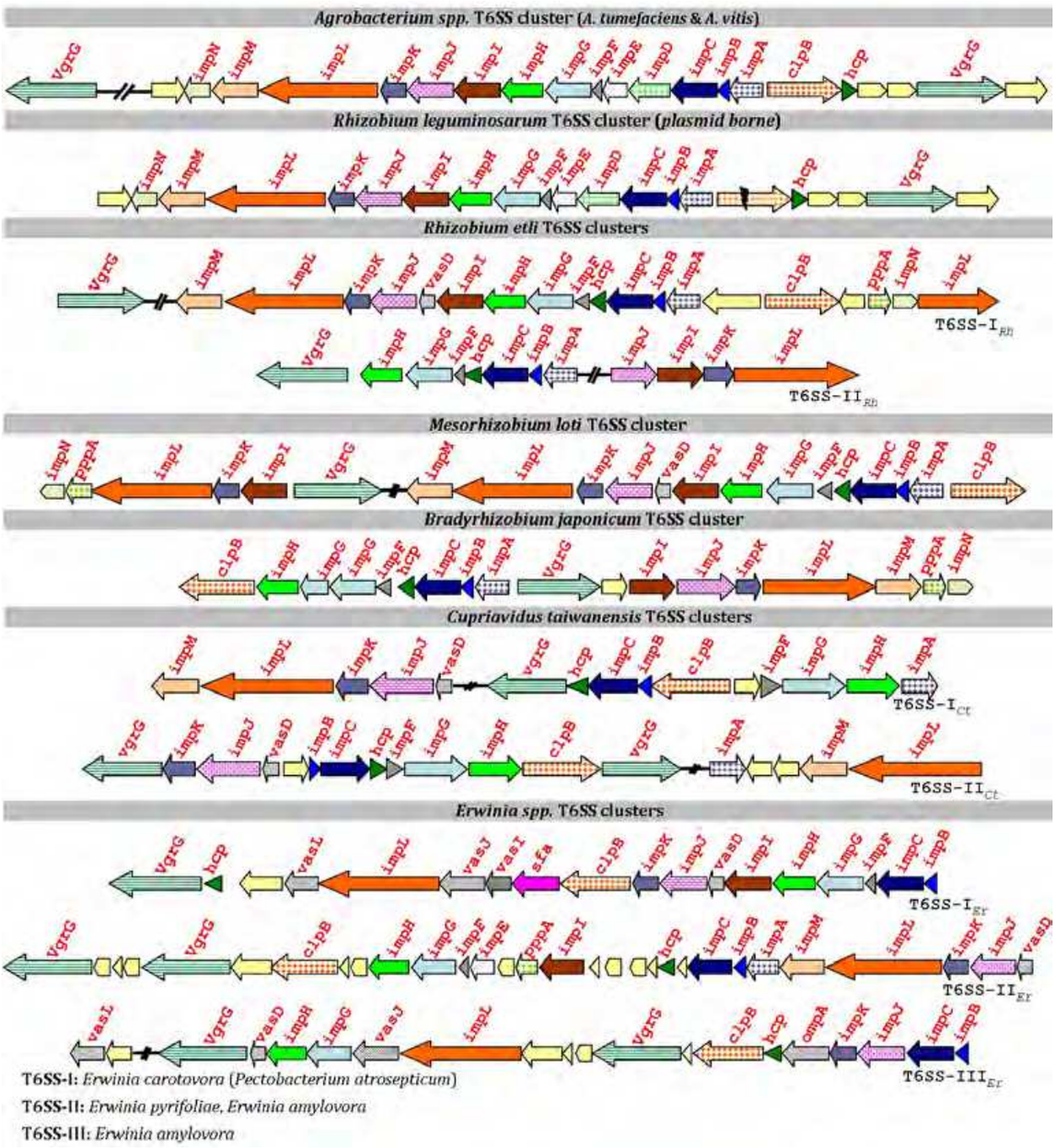
Agrobacterium spp. T6SS cluster (A. tumefaciens & A. vitis)
Rhizobium leguminosarum T6SS cluster (plasmid borne)
Rhizobium etli T6SS clusters
T6SS-I$_{Rh}$
T6SS-II$_{Rh}$
Mesorhizobium loti T6SS cluster
Bradyrhizobium japonicum T6SS cluster
Cupriavidus taiwanensis T6SS clusters
T6SS-I$_{Ct}$
T6SS-II$_{Ct}$
Erwinia spp. T6SS clusters
T6SS-I$_{Er}$
T6SS-II$_{Er}$
T6SS-III$_{Er}$
T6SS-I: Erwinia carotovora (Pectobacterium atrosepticum)
T6SS-II: Erwinia pyrifoliae, Erwinia amylovora
T6SS-III: Erwinia amylovora

Fig. 1. *Part 1*

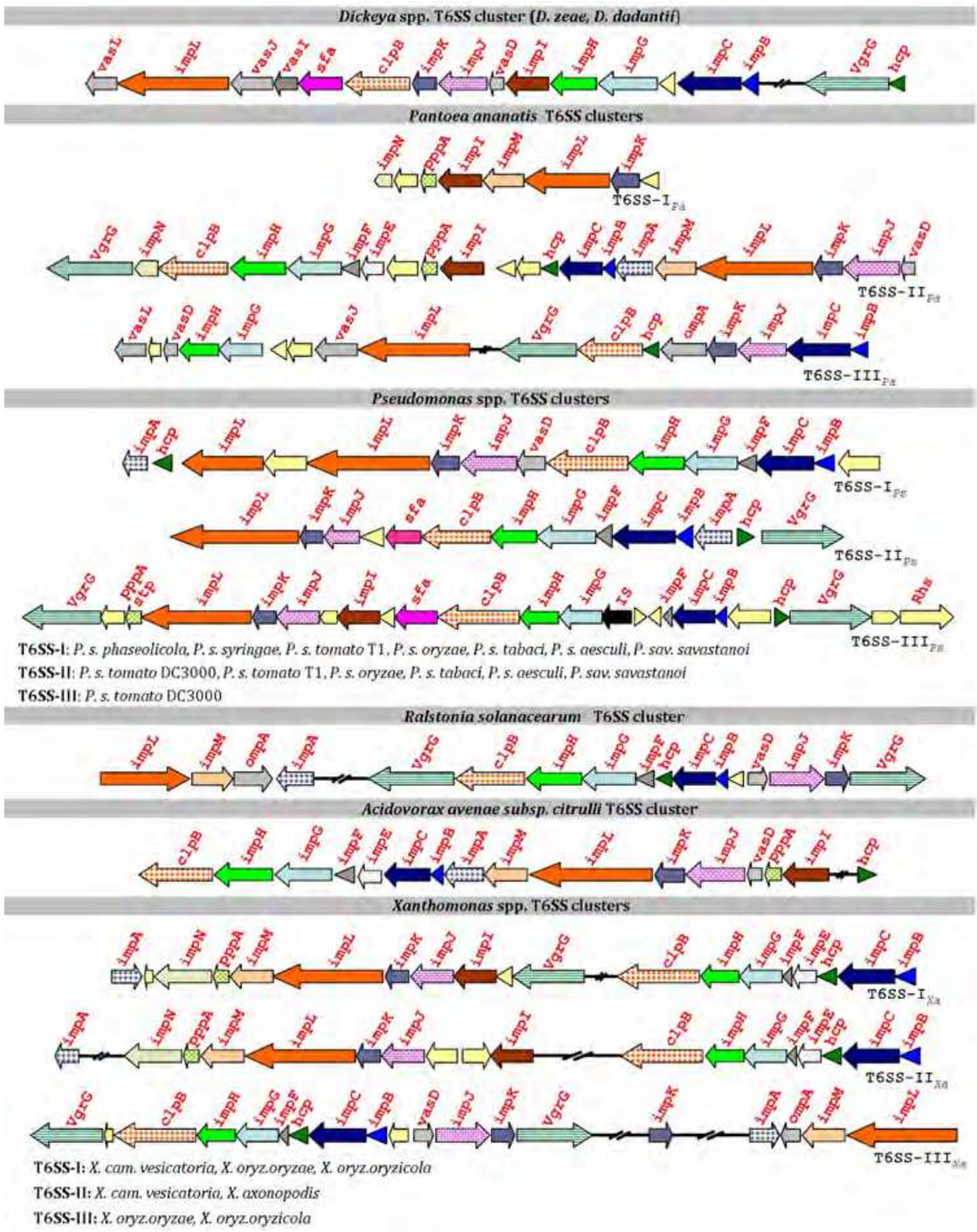

Fig. 1. *Part 2*

Fig. 1. Maps of T6SS clusters of plant-associated bacteria. Orthologs are indicated by the same color. The genes adjacent to or encoded by the T6SS gene clusters but not recognized as orthologs are indicated by light beige arrows. Arrows indicate the transcriptional direction. The gene locus numbers are referred in the text and the published or annotated gene designations are indicated above the genes of each cluster.

like amoebae and mammalian immune cells. Chow & Mazmanian (2010) further propose that pathobionts of the human gastrointestinal tract, such as *H. hepaticus,* may have evolved a T6SS as a mechanism to actively maintain a non-pathogenic, symbiotic relationship in the GI tract by regulating bacterial colonization and host inflammation; they hypothesize that alteration in the composition of the microbiota, known as dysbiosis, may be a critical factor in various human inflammatory disorders such as inflammatory bowel disease and colon cancer.

Knowledge on the functionalities and biological roles of T6SS in plant-associated bacteria is poor and limited to relatively few systems (reviewed in Records, 2011). With regard to phytopathogens, functionality has been demonstrated for *A. tumefaciens, P. atrosepticum*, and two pathovars of *Pseudomonas syringae*. A study with *P. s.* pv. *tomato* DC3000 (Wang et al., 2008) has shed some light on the possible role of the T6SSs in pathogenicity. Deletions of the entire T6SS clusters (T6SS-II or T6SS-III; grouping is based on our phylogenetic analysis, see sections 5, and 6) or both copies of *icmF* (*icmF1* and *icmF2*) caused reduction of bacterial population in *Nicotiana benthamiana* and milder symptoms on tomato leaves. When either the T6SS-II or T6SS-III cluster was deleted, both symptoms severity and bacterial populations were reduced. However, *vgrG1* or *vgrG2* deletions had no effect on disease development on tomato or on *N. benthamiana*. However, an insertional mutant of the *clpV/B* gene of the T6SS maintained the ability for *in planta* multiplication and produced disease symptoms similar to those caused by wild-type strain (Records & Gross 2010). RNA transcripts of the *icmF* homologs of the *P. syringae* pv. *tomato* DC3001 and *P. syringae* pv. *phaseolicola* 1448a were detected by RT-PCR in both rich and minimal media, indicating that the gene is probably expressed in both strains (Sarris et al., 2010). Microarray analysis showed that the *A. tumefaciens* T6SS is induced by mildly acidic conditions, such as encountered in plant tissues and in the rhizosphere (Yuan et al., 2008) and deletion of *hcp* resulted in reduced tumorigenesis on potato tuber slices (Wu et al., 2008). The *P. atrosepticum* T6SS is induced by potato tuber extracts (Mattinen et al., 2007). Transcriptome profiling (Liu et al., 2008) also indicated regulation of the T6SS of *P. atrosepticum* by quorum sensing, as deletion of *expI*, a gene responsible for N-(3-oxohexanoyl)-L-homoserine lactone synthesis. Furthermore, deletions in either ECA3438 (*impJ*) or ECA3444 (*vipB*) resulted in slightly reduced virulence in potato stems and tubers. However, mutation of the ECA3432 (*icmF*) gene resulted in increased potato tuber maceration, indicating that the T6SS may be involved in antipathogenesis activity (Yuan et al., 2008). Whether T6SS mechanisms engage other aspects of *P. syringae*-host/vector biology, antagonism and predation in the plant or other micro-environments remain open questions. Among the symbiotic N-fixing bacteria, extended symbiosis phenotypes of certain rhizobia have been linked to a T6SS. The presence of T6SS homologs in the sequenced genomes of many rhizobia presents opportunities to further investigate it's role in bacteria-plant symbiosis (Fauvart & Michiels, 2008).

5. Mining phytobacterial for T6SS homologs

Further to our recently published study (Sarris et al., 2010), a genome-wide *in silico* analysis was carried out for 13 phytobacterial species and more than 30 strains from different genera, to identify conserved gene clusters encoding for T6SSs by BLASTP and reverse BLAST analysis of sequences deposited in various genome databases (e.g. KEGG, NCBI, RizoBase), both complete annotated and draft sequenced phytobacterial genomes. The baits consisted of protein sequences encoded by the *P. syringae* Hcp secretion islands I, II and III (HSI-I, II,

III, here referred to interchangeably as T6SS-I, T6SS-II and T6SS-III, respectively) (Sarris et al., 2010), as well as their homologs from other known T6SS clusters (Boyer et al., 2009). Clusters containing at least five genes encoding proteins with similarity to known T6SS core proteins were considered as part of a putative T6SS locus. The genomic regions thus identified were then extended by examining four kilo-bases up- and down-stream for putative conserved genes associated with T6SS by "orthologue" and "paralogue finder" analysis against all the KEGG deposited genomes. Maps of the genomic islands were constructed manually in PowerPoint Microsoft office software. For sequence alignment and phylogenetic tree construction, the conserved proteins ImpL, ImpG, IpmC and ImpH from all phytobacterial species deposited at the NCBI and KEGG databases were edited with the DNAman computer package (Lynnon Co) and were included for sequence alignment and tree construction. Phylogenetic relations were inferred using the neighbour-joining method (Saitou & Nei 1987) offered in MEGA4 software (Tamura et al., 2007). In Table 1 and Fig. 1 the clusters are identified by the organsim's initials, and in the phylogenetic trees the organism's name/strain number and the T6SS groupings used in the text and figure legends are given.

6. Phylogenetic analysis of phytobacterial T6SS

6.1 Phylogenies based on overall gene content

Table 1 shows the presence/absence of a T6SS protein homolog (indicated with the plus sign [+] and minus [-] sign, respectively). Homologs of each T6SS-related bait protein exist in members of all phytobacterial species/strains, but with substantial differences among strains in gene content and copy number. Nine core genes, *impK*, *impB*, *impC*, *impG impH*, *impJ*, *hcp*, *impL* and *clpB*, are found in 100% of the species examined, with *clpB* seemingly present as a pseudo-gene in *Rhizobium leguminosarum)*. On the other hand, homologs of genes such as *ompA* and *impD* are present only in 14% of the species/strains examined, a finding of unclear significance. Several instances of multiple T6SS clusters were identified in the same strain, mostly in distant locations with respect to each other. These clusters are depicted in Fig. 1, based on the phylogenetic analysis described in the sections below.

To analyse the evolutionary history of phytobacterial T6SS, a distance tree was initially constructed, depicting the phylogenetic relationships and gene composition among the T6SSs of all species examined based on the data in Table 1. Initially, the evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei 1987) by scoring each locus as present (+) or absent (-), including all the data (Fig. 2). The constructed tree essentially gives a graphic representation of the data in Table 1 and reveals two distinct T6SS clusters, in terms of the presence or absence of the genetic elements. The tree shows extensive intermixing among the T6SSs of various bacterial species/groups, with several deep and shallow branches, many with low bootstrap values. Noteworthy in this tree is the close relationship between: *a*. the *Xanthomonas* T6SS-I and II and rhizobial T6SSs (except *Rhizobium leguminosarum*), *b*. the two phytopathogenic enterobacteria T6SS-II (*Erwinia amylovora*, and *Erwinia pyrifoliae*) and the *Acidovorax* T6SS, *c*. the sole T6SS of the *Agrobacteria* spp., *Rh. leguminosarum* and *Xanthomonas* spp. T6SS-II *d*. the *Ralstonia solanacearum* and *Xanthomonas* spp. T6SS-III and *e*. between other phypathogenic enterobacterial T6SSs (*E. amylovora* and *P. ananatis* T6SS-III, the *P. atrosepticum* T6SS-I and the T6SS of the two *Dickeya* species).

Species*	Imp K COG 3455	Imp L COG3 523	Imp B COG 3516	Imp C COG 3517	Imp G COG 3519	Imp H COG 3520	Imp J COG 3522	Hcp COG 3157	Clp B COG 0542	Vgr G COG 3501	Imp A COG 3515	Imp F COG 3518	Imp M COG 3913	Vas D COG 3521	Imp I COG 3456	Ppp A COG 0631	Imp E COG 4455	Vas L COG 3515	Vas J COG 3515	Sfa COG 3604	Omp A	Imp D COG 3604	Imp N COG 0515
	100**	100	100	100	100	100	100	100	96	86	77	86	64	59	50	41	36	18	18	23	14	14	37
AAsC	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	-	-	-	-	-
AT	+	+	+	+	+	+	+	+	+	++	+	+	+	-	+	-	+	-	-	-	-	+	+
AV	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	-	-	-	-	+	+
BJ	+	+	+	+	++	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	-	+
*PA_I***	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	-	-	-	+	-	-	-
EA_II	+	+	+	+	+	+	+	+	+	++	+	+	+	+	+	+	+	-	+	-	-	-	-
EA_III	+	+	+	+	+	+	+	+	+	+	-	-	-	+	-	-	+	+	+	-	+	-	-
EP_II	+	+	+	+	+	+	+	+	+	++	+	+	+	+	+	+	-	-	-	-	-	-	-
ML	++	+	+	+	+	+	+	+	+	+	+	+	-	+	++	+	-	-	-	-	-	-	+
PS_I	+	+	+	+	+	+	+	+	+	-	+	+	-	+	-	-	-	-	-	-	-	-	-
PS_-II	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-
PS_III	+	+	+	+	+	+	+	+	+	++	-	+	-	-	-	+	-	-	-	+	+	-	-
RS	+	+	+	+	+	+	+	+	+	++	+	+	+	+	-	-	-	-	-	-	+	-	-
RE	+	++	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	+
RL	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+
Xsp_I	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	+
Xsp_II	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	-	-	-	-	-	+
Xsp_III	++	+	+	+	+	+	+	+	+	++	+	+	+	+	-	-	-	-	-	-	+	-	-
CT_I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-
CT_II	+	+	+	+	+	+	+	+	+	++	+	+	+	+	-	-	-	-	-	-	-	-	-
DZ	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	+	-	-	-
DD	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	+	-	-	-
PA_I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+
PA_III	+	+	+	+	+	+	+	+	+	+	-	-	-	+	-	-	-	+	+	-	-	-	-

Table 1. Gene content of the putative T6SS genes of phytobacterial species, indicating locus names and COG numbers (not available for OmpA). (+): present, (++): present in a second copy, (-): missing. *Species abbreviations: AAsC: *Acidovorax avenae* subsp. *citrulli*; AT: *Agrobacterium tumefaciens*; AV: *Agrobacterium vitis*; BJ: *Bradyrhizobium japonicum*; PA: *Pectobacterium atrocepticum*; EA: *Erwinia amylovora*; EP: *Erwinia pyrifoliae*; ML: *Mesorhizobium loti*; PS: *Pseudomonas syringae*; RS: *Ralstonia solanacearum*; RE: *Rhizobium etli*; RL: *Rhizobium leguminosarum*; Xsp: *Xanthomonas* spp.; CT: *Cupriavidus taiwanensis*; DZ: *Dickeya zeae*; DD: *Dickeya dadantii*; PA: *Pantoea ananatis*. **The numbers in the second row of the table denote the percentage of cases where the gene/protein is present among the species/strains examined. ***Latin numerals I, II, III denote T6SS-I, T6SS-II and T6SS-III, respectively. A table of the locus numbers from the KEGG database is available upon request from P.F. Sarris.

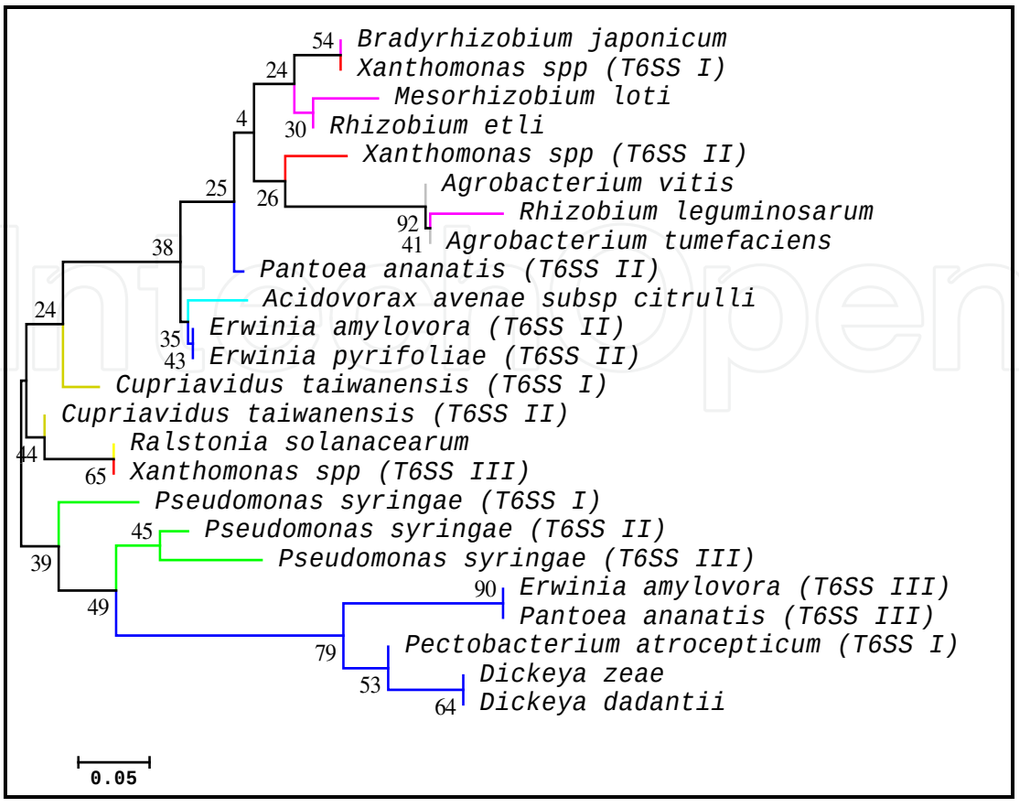

Fig. 2. Distance tree of T6SS of various plant pathogenic bacteria; constructed with data of Table 1, through a matrix where each gene locus was scored as (+) when present or as (-) when not present. The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The bootstrap consensus tree inferred from 5000 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the species analysed (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (5000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl & Pauling 1965) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 29 positions in the final dataset. Phylogenetic analyses were conducted with MEGA4 (Tamura et al., 2007).

6.2 Phylogenetic analysis of four core proteins

Subsequently, we carried out a phylogenetic cluster analysis of four highly conserved T6SS core proteins (ImpC, ImpG, IpmH and ImpL-like) by combining the protein sequences and forcing the software to reckon phylogenetic analysis of all four proteins. Data clustering was accomplished by the Neighbor-Joining method (Saitou and Nei, 1987) and are presented as a tree for the evaluation of similarity/distances among each T6SS in each species (Fig, 3) and is used as a basis to infer the T6SS phylogenies discussed below.

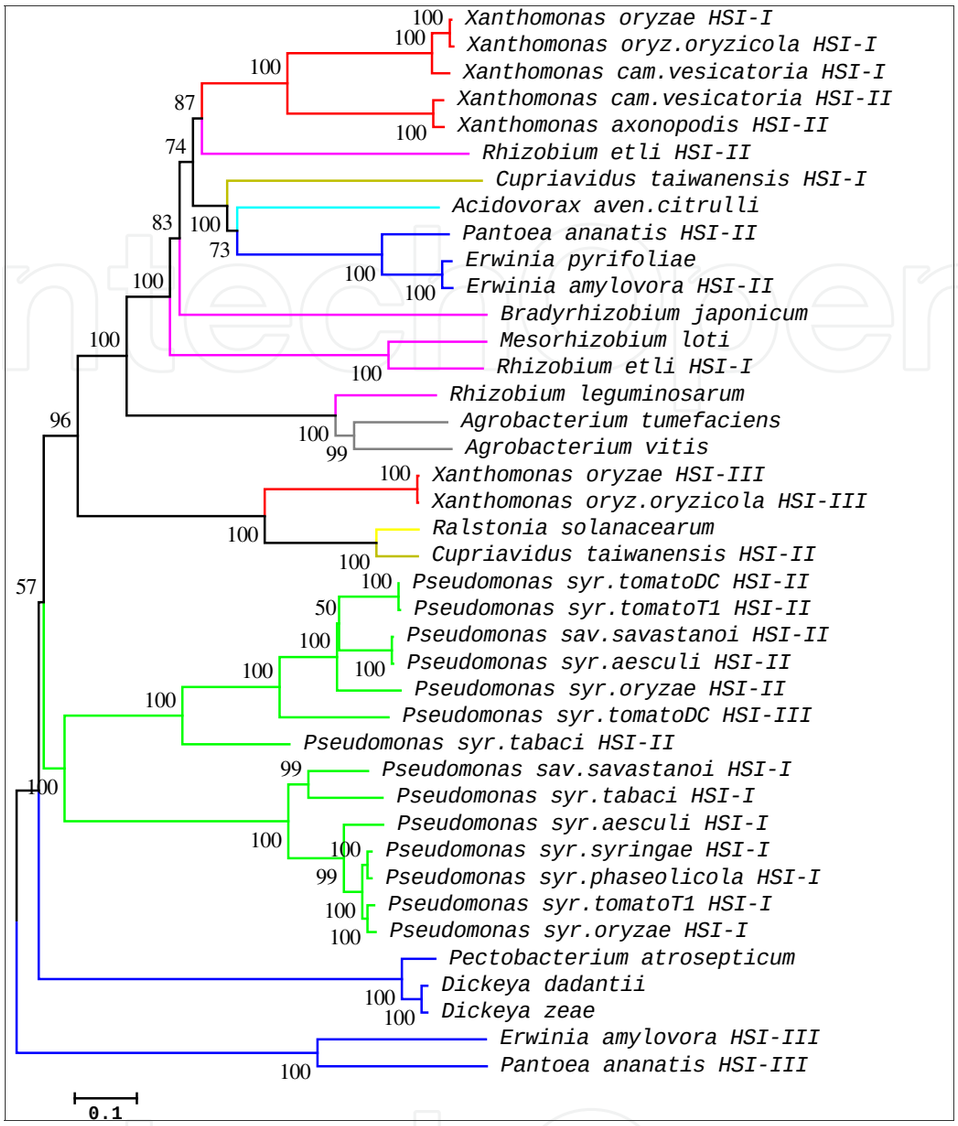

Fig. 3. Phylogenetic clustering of the plant-associated bacterial species based on the sequence of four T6SS core proteins (ImpC ImpG, ImpH and ImpL). The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei 1987). The bootstrap consensus tree inferred from 5000 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the proteins analysed replicates (Felsenstein 1985). For other details see Fig. 2 legend. There were a total of 1729 positions in the final dataset. Phylogenetic analyses were conducted with MEGA4 (Tamura et al., 2007). Difference in tree branch colors indicates the different bacterial species.

The consensus phylogenetic tree obtained shows four deep branches. One branch hosts the majority of the phytobacterial T6SSs, except for those of *P. syringae* pathovars (T6SSs-I, II and III), the *Dickeya* spp. T6SS and *P. carotovorum* T6SS. In this branch several T6SS groups are evident. One group includes the *Xanthomonas* spp. T6SS-I and T6SS-II together with the T6SS of *Rhizobium etli* T6SS-II. A second group includes the *C. taiwanensis* T6SS-I, *A. avenae* subsp. *citrulli*, *P. ananatis* T6SS-II and the *Erwinia* spp. T6SS-II. The *B. japonicum* T6SS

appears distinct (with bootstrap value 83) but very close to a subgroup formed by the *M. loti* and *R. etli* T6SS-II. Another group in this phylogenetic branch includes the *R. leguminosarum* and *Agrobacterium* spp. T6SSs. Finally, in the same branch are the *Xanthomonas* spp. T6SS-III, grouped together with the *R. solanacearum* and *C. taiwanensis* T6SS-II. In the second phylogenetic branch are grouped only the *P. syringae* pathovars. As previously reported (Sarris et al., 2010), in this group there are two distinct sub-groups. The first one includes the *P. syringae* T6SS-II and T6SS-III, while the second carries only the *P. syringae* T6SS-I. Finally, two more branches were formed but without bootstrap value. The first one includes the T6SSs of *Dickeya* spp. and *P. atrosepticum*, while the second consists of the *P. ananatis* T6SS-II and *Erwinia* spp. T6SS-III.The phylogenetic relationships of the *P. syringae* T6SSs were further examined by constructing an additional phylogenetic tree of the four core proteins (ImpC, ImpG, IpmH and ImpL-like) by including representatives of fully sequenced non-phytopathogenic fluorescent *Pseudomonas* and the *R. solanacearum T6SS, C. taiwanensis* T6SS-II, *P. ananatis* T6SS-II and *Erwinia* spp. T6SS-III, as these appear as reference species based on their distant relationships in the tree of Fig. 3. The *Pseudomonas* tree shows three deep branches (not including out-group species), each including species with high boostrap values (Fig. 4). The first branch includes a group which is formed by the *P. syringae* T6SS-II

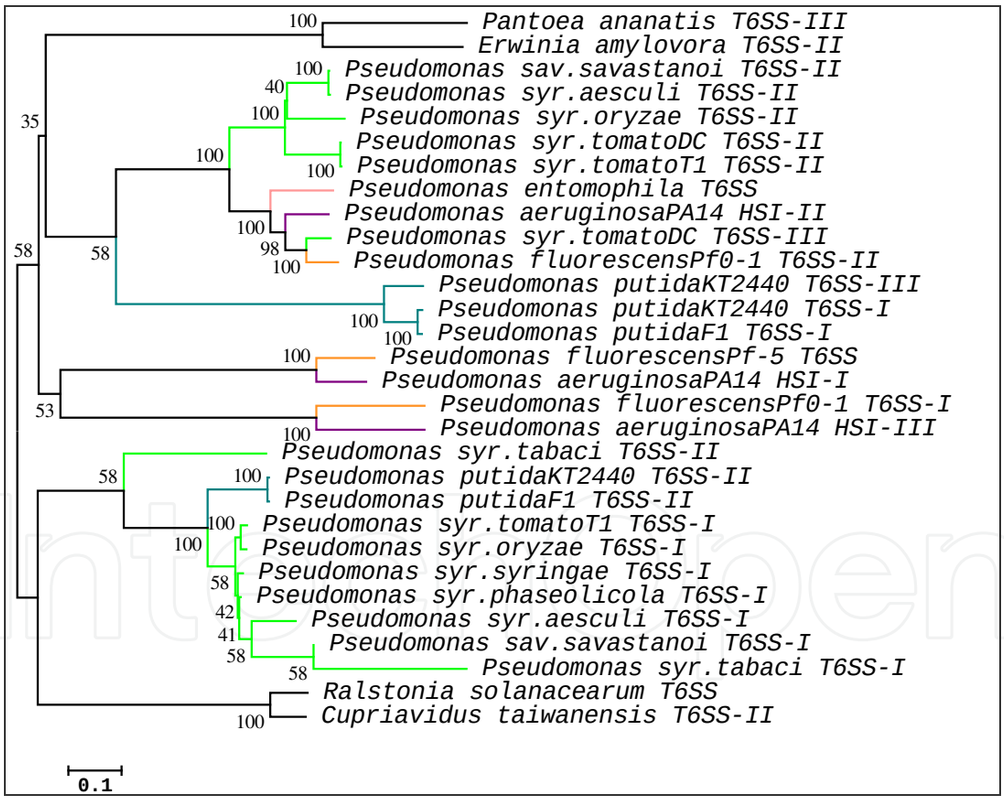

Fig. 4. T6SS evolutionary relationships of 30 fluorescent *Pseudomonas* T6SSs. The evolutionary history was inferred using four (4) T6SS core proteins (ImpC, ImpG, ImpH, ImpL), by the Neighbor-Joining method (Saitou & Nei, 1987). The optimal tree with the sum of branch length = 6.49662029 is shown. For other details see Fig. 2 and Fig. 3 legends). Difference in tree branch colors indicates the different *Pseudomonas* species, while the out-group species are presented with black.

and T6SS-III, the *P. aeruginosa* HSI-II, *P. fluorescens* T6SS-II and the *P. entomophila* four T6SS core proteins. In this group, the *P. syringae* T6SS-III is phylogenetically very close to the *P. aeruginosa* HSI-II and *P. fluorescens* T6SS-II. The *P. syringe* T6SS-III is present only in *P. syringae* pv. *tomato* strain DC3000, and has the same gene order and high protein homology with the T6SS-II of the *P. aeruginosa* PA14 HS-II (data not shown), which reinforces the view of Yan and colleagues (Yan et al., 2008) that the model plant pathogen *P. s.* pv. *tomato* DC3000 is a very atypical tomato strain. Thus, it appears that the *P. syringae* T6SS-III may have been horizontally acquired and maintained through vertical transfer and is remarkably well conserved in *P. s.* pv. *tomato* DC3000, *P. aeruginosa* and *P. fluorescens* species with distant genetic relatedness and very distinct, opportunistic relationships with plants. A second distinct group in the same branch is formed by the T6SSs –I and –III of *P. putida*. The second branch in the four-protein tree is formed by the *P. fluorescens* T6SS-I and the *P. aeruginosa* HSI-I and –III. Finally, the third branch of the four protein tree includes the *P. syringae* T6SS-I grouped with the *P. putida* T6SS-II. Remarkably, in the two *P. fluorescens* strains analysed the sole T6SS in Pf-5 and the T6SS-I and T6SS-II in Pf0-1 are quite distant. Two of the three T6SSs found in the *P. putida* strains studied (T6SS-I in Pf-5 and PKT2440, and T6SS-III in the later strain) are very close relatives while the third one (T6SS-II) is very distant from the others (Fig. 4).

7. T6SS in phytopathogenic bacterial species

7.1 *Pseudomonas* spp. (*P. syringae* and *P. savastanoi*)

The species *P. syringae* (*P.s.; γ-proteobacteria*) comprises phytopathogenic members that are placed in infra-sub-specific groupings (pathovars); collectively, they infect a wide range of plant species and can also live as epiphytes in the plant phyllosphere, until conditions are favourable for disease development (Hirano & Upper, 2000). Some non-pathogenic strains of *P. syringae* have been used as biocontrol agents against post-harvest rots (Janisiewicz & Marchi, 1992).

Bioinformatics analysis (Sarris et al., 2010) revealed the presence of multiple T6SS clusters and putative effectors in six fully or partially sequenced genomes of *P.s.* pathovars. In a subsequent study, two more draft genomes of the bleeding cankers pathogen of the horse chestnut trees (*Aesculus hippocastanum*), *P. syringae* pv. *aesculi* (*Pae*), became available in the NCBI gene database (Green et al., 2010). The two strains show genomic differences implicated in host association and fitness: one strain was isolated recently from bleeding stem cankers on European horse chestnut in Britain (E-*Pae*), and the second (I-*Pae*) from leaf spots on Indian horse chestnut in India in 1969 (Green et al., 2010).

Genome comparisons between the sequence assemblies of E-*Pae* and I-*Pae* revealed differences in a number of genomic regions including two T6SS clusters and a large number of putative effectors that are present in I-*Pae* but absent from E-*Pae*. In the phylogenetic trees of Figs 3 and 4, the two *Pae* T6SS clusters group together with the *P. syringae* T6SS-I & II respectively. Analysis of the draft genome sequence of a close relative, *P. savastanoi* pv. *savastanoi* strain NCPPB 3335 (Rodriguez-Palenzuela et al., 2010) indicated also the presence of two putative T6SS gene clusters:, one (AER-0002618 to AER-0002633) highly similar to the *P. syringae* T6SS-I and another (AER-0002971 to AER-0002983) that is more similar in gene order and protein sequence similarity to the T6SS-II (Figs. 3 and 4).

7.2 *Ralstonia solanacearum*

R. solanacearum (*R.s.*, previously known as *Pseudomonas solanacearum; β-proteobacteria*), the causative agent of bacterial wilt of solanaceous plants, is a soil-resident bacterium with wide geographical distribution in all warm and tropical areas and the causal agent of vascular wilt disease in over 200 annual and perennial plant species, representing over 50 botanical families, both monocots and dicots. It also causes disease on the model plant *Arabidopsis thaliana*, and along with *P. syringe* pv. *tomato* DC3000, is one of the leading models in the study of plant-microbe interactions.

The genome of *R.s.* strain GMI1000, as was annotated in KEGG gene data base has one T6SS cluster comprising 16 genes, lying between and *impL*-like (RS01945; also designated as RSp0763) and a *vgrG*-like RS01970, also designated as RSp0738). This cluster seems to be interrupted by some apparently T6SS-unrelated putative open reading frames (11 ORFs) indicating possible gene rearrangements. The gene clusters RS01945-46-47 (also designated as RSp0763-62-61) and RS01967-68-69-70 (also designated as RSp0741-40-39-38) have been annotated as putative *impL-impM-ompA* and *vasD-impJ-impK-vgr* respectively, and are found in reverse orientation compared to the middle section of the cluster, which include genes annotated as *vgr-clpB-impH-impG-impF-hcp-impC-impB* [from RS01949 to RS01966 (also designated as RSp0743 to RSp0749)]. Thus far, there is no experimental evidence for a role of the T6SS in the *R.s.* pathogenicity.

The consensus phylogenetic analysis tree of four *R.s.* T6SS core proteins revealed the closest phylogenetic proximity of the *R.s.* T6SS core components to those of the T6SS-II of *Cupriavidus taiwanensis* which belongs to the phylogenetically distant β-Rhizobium group (Fig. 3), with the same gene order, and the next closest relatives being those of the *Xanthomonas* T6SS-III and the *Ralstonia* T6SS studied (also β-proteobacteria), indicating very recent common ancestry.

7.3 *Xanthomonas* spp.

The genus *Xanthomonas* (γ-*Proteobacteria*) consists of 27 plant-associated species, each with many pathovars, which collectively, cause disease on at least 124 monocot species and 268 dicot species, including fruit and nut trees, solanaceous and brassicaceous plants, and cereals (Hayward, 1993; Leyns et al., 1984). *X. campestris* pv. *vesicatoria* (Doidge; syn. *Xanthomonas vesicatoria*) has tomato and pepper as principal hosts. However, various other *Solanaceae*, mainly weeds, have been recorded as incidental hosts. *X. axonopodis* pv. *citri* (*X.a.c.*) is the causal agent of "citrus canker", which affects most commercial citrus cultivars, resulting in significant losses worldwide. (Stall & Seymour, 1983). *X. oryzae* includes the two non-European rice pathogens pvs. *oryzae* and *oryzicola* (*X.o.* & *X.o.o.*). The principal host of both pathovars is rice and high-yielding cultivars are often highly susceptible. *Oryza sativa* subsp. *japonica* is usually more resistant than subsp. *indica* to pv. *oryzicola*. *X.o.* invades the vascular tissue, while *X.o.o.* proliferates in the parenchyma (Nino-Liu et al., 2006).

Experimental evidence for a role of T6SS in *Xanthomonas* pathogenicity or other aspects of its life cycle is limited. Our data base search revealed a variable situation vis-à-vis T6SS homologs among several sequenced strains of *Xanthomonas*; All three *X. campestris* pv. *campestris* (*X.c.c.*) strains (ATCC33913, Xcc8004 and B100) and the avirulent *X. populi* and *X. codiaii* seem to lack T6SS-related genes, while two distinct T6SS loci are found in *X.*

campesrtis pv. *vesicatoria* XCV2120 (*X.c.v.*), one in *X. axonopodis* 4122 (*X.a.c.*) and three in each, *X.o* and *X.o.o.* One T6SS cluster in XCV2120 extends from XCV2120 (*impB*-like) to XCV2143 (*impA*-like) and is referred to here as T6SS-I, while a second cluster is located from XCV4202 (*impA*-like) to XCV4243 (*impB*-like) and referred as T6SS-II (Fig. 1). This cluster contains two interruptions by some apparently T6SS-unrelated putative ORFs, one, between XCV4214 (*impI*-like) and XCV4236 (*clpB*-like), and another between XCV4202 (*impA*-like) and XCV4208 (*impM*-like). Two T6SS clusters are found in *X.c.v.*: one of them is phylogenetically closer to the T6SS-I found in *X.o.* and *X.o.o* while the second seems to more closely related to the sole T6SS locus of *X.a.c* (referred to as T6SS-II in Fig. 3) which spans from XAC4112 (*impA*-like) to XAC4147 (*impB*-like) (Fig. 1). The *X.a.c.* T6SS locus is almost collinear for the T6SS related genes and for the two T6SS-unrelated putative ORF insertions with the T6SS of *X.c.v*. Whole genome comparisons (Potnis et al., 2011) recently enabled an extensive analysis of the presence and distribution of T6SS among *Xanthomonas* strains representing 15 pathovars. *X.o.* and *X.o.o.* carry the T6SS-I, located from XOO3034 (*impA*-like) to XOO3052 (*impB*-like) and Xoryp_12330 (*impA*-like) to Xoryp_12445 (*impB*-like), and a second T6SS which is referred to as T6SS-III in Fig. 1 and is located between XOO3517 (*impL*-like) and XOO3474 (*vgrG*-like) in *X.o.* and Xoryp_06365 (*impL*-like) and Xoryp_06645 (*vgrG*-like) in *X.o.o.* This locus contains a large number of T6SS unrelated ORF insertions, and is phylogenetically distant from the *Xanthomonas* T6SS-I and T6SS-II, while is phylogenetically close to the *R. solanacearum* and the *C. taiwanensis* T6SS-II.

7.4 *Erwinia* spp. (*E. pyrifoliae* and *E. amylovora*)

Bacteria of the genus *Erwinia* (γ-proteobacteria) are plant-associated as pathogens, saprophytes and epiphytes and exhibit considerable heterogeneity, forming four phylogenetic clades that are intermixed with members of other genera, such as *E. coli*, *Klebsiella pneumoniae*, and *Serratia marcescens* (Kwon et al., 1997). *E. amylovora* (*E.a*) originated in North America and has spread to other continents, threatening the native apple germplasm in Central Asia. *E. pyrifoliae* (*E.p.*) is a newly described necrotrophic pathogen initially isolated in the middle 1990's from Japanese pear (*Pyrus pyrifolia*), but could not be found in later years in the previously affected orchards (Kim et al., 2001). *E.p.* causes fire blight symptoms essentially indistinguishable from those of *E.a.* infection (Kim et al., 2001) but has more limited host range. Both pathogens share many common virulence factors, including two distinct type III secretion systems (T3SS) and genes for desferrioxamine biosynthesis. However, *E.p* lacks a third T3SS cluster found in *E. amylovora.*

The genome sequence of the highly virulent strain Ea273 (ATCC 49946) isolated from diseased apple (*Malus pumila* cv. Rhode Island Greening) in New York State, is over 99.99% identical to that of the European isolate CFBP 1430 (ERWAC), indicating minimal divergence since the global dispersion of *E.a.* However, a large-scale rearrangement of the genome resulting in repositioning of two large portions of the chromosome has taken place. Additionally, ERWAC has only the smaller plasmid (pEA29) found in *E.a.* and its two T3SSs on genomic islands PAI-2 and PAI-3 have high homology to the insect endosymbiont *Sodalis glossidinius* str. *morsitans* (SODGM) and to the mammalian pathogens *Salmonella* and *Yersinia* spp., indirectly suggesting a closer insect association than mere passive dispersal (Smits et al., 2010).

E.p. strain Ep1/96 harbors one T6SS cluster which spans the region from EpC_06150 (*vasD*-like) to EpC_06440 (*vgrG2*-like) and shares sequence and gene order homology with the one of the *E.a* T6SS clusters [designated T6SS-II in Fig. 1, 2 and 3), starting from EAMY_3027 (*vasD*-like) to EAMY_3000 (*vgrG*-like)]. These clusters are very close phylogenetic relatives and have as next closest relative the *Pantoea ananatis* T6SS-II (Fig. 3). Furthermore, a small T6SS cluster of four ORFs (not presented in Fig. 1) is present in both *E.p.* and *E.a.* (*E.p.*:EpC_19520-EpC_19550 and *E.a.*: EAMY_1620-EAMY_1623). *E.a.* also harbors a second T6SS cluster (designated T6SS-III in Figs. 1, 2 and 3) which spans from EAMY_3228 (*impB*-like) to EAMY_3201 (*vasL*-like). This cluster exhibits gene order and sequence relatedness to *P. ananatis* T6SS-III (Fig. 1). Similar results were reported for *E.p.* DSM 12163T and *E.a* CFBP 1430 based on whole genome sequence analysis (Smits et al., 2010). There are no experimental data concerning the biological role of the T6SS in *Erwinia* spp. and most genes within the T6SS clusters are uncharacterized.

7.5 *Pantoea ananatis*

The genus *Pantoea* (γ-proteobacteria) consists of both important plant pathogens and clinically relevant species. Clinical isolates have been reported to cause bacteraemia in humans. *P. ananatis* (*P.a.*) is considered an unconventional plant associated species, being associated with plants as an epiphyte, endophyte, pathogen, or symbiont, but can also occupy unusual ecological niches (e.g. contaminating aviation jet fuel tanks). It's ice nucleation activity has been exploited in the food industry and in the biological control of insects (Coutinho & Venter, 2009).

The exact role of T6SS in the adaptive capacity of *P.a.* is not known, but such a role might be considered likely as there has been no evidence of T2SS or T3SS in any of the strains sequenced so far (De Maayer et al., 2010). Our database search in the sequenced *P.a.* strain LMG 20103 revealed one truncated and two entire T6SS clusters named T6SS-I, II and III respectively in Figs 1 and 3. The truncated T6SS-I is located from PANA_1650 (a possible T6SS related protein kinase A; PknA=ImpN) to PANA_1656 (*impK*-like). The obvious absence of some basic T6SS core genes raises questions about the functionality of this T6SS locus. The T6SS-II locus is embedded between PANA_2352 (*vgrG*-like) to PANA_2372 (*vasD*-like) while the T6SS-III locus is divided in two sub-loci the first one is located from PANA_4130 (*vasL*-like) to PANA_4138 (*impL*-like), the second one, being located a few apparently T6SS-unrelated putative ORFs away, from PANA_4144 (*vgrG*-like) to PANA_4151 (*impB*-like). Phylogenetically, the *P.a.* T6SS-I seems to be related to some random distributed T6SS related ORFs in the genome of *E. amylovora* (data not shown). The *P.a.* T6SS-II is phylogenetically closer in protein sequence and the gene order to the *Erwinia* spp. T6SS-II (Figs 1 and 3). Interestingly, the *P.a.* T6SS-III forms a distant phylogenetic branch with the *E.a.* T6SS-III and appears as an out-group in our analysis (Fig. 3).

7.6 *Pectobacterium atrosepticum*

P. atrosepticum (*P.a.*; formerly *Erwinia carotovora* subsp. *atroseptica;* γ-proteobacteria) is a member of the pectolytic *Erwinia* responsible for the soft rot and blackleg of potato stems and tubers. A T6SS locus is found in *P.a.* strain SCRI1043 extending from ECA3427 (*impB*-like) to ECA3445 (*vgrG*-like) and is here referred as *Erwinia* spp. T6SS-I (Fig. 1). One additional solitary locus, designated as *vgrG*-like gene (ECA2104), as well as four other loci, designated as *hcp*-like

genes (ECA4275, ECA2866, ECA0456 and ECA3672), are also present. Interestingly, virulence assays, performed with mutants in ECA3438 and ECA3444, in potato stems and tubers, showed significantly reduced virulence compared with the wild type strain in both cases (Liu et al., 2008). In our phylogenetic analysis the *P.a.* T6SS is presented as a member of a distinct phylogenetic branch comprising the *P.a.* and *Dickeya* spp. T6SSs (Fig. 3).

7.7 *Dickeya* spp. (*D. dadantii* and *D. zeae*)

Dickeya dadantii (*D.d.*; formerly *Erwinia chrysanthemi;* γ-proteobacteria) is an opportunistic plant pathogen causing soft-rot, wilt, and blights on a wide range of plant species, such as maize, pineapple, banana, rice, tobacco, tomato, *Brachiaria ruziziensis* and *Chrysanthemum morifolium*. It possesses two O-serogroups, O: 1 and O: 6. *D.d.* is also highly virulent on the pea aphid *Acyrthosiphon pisum*, and possesses four genes encoding homologs of the Cyt family of insecticidal toxins from *Bacillus thuringiensis* (Grenier et al., 2006). *Dickeya zeae* (*D.z.*; formerly *Erwinia chrysanthemi*) was isolated from soft rot and wilt of a various range of plants, such as *Zea mays*, *Ananas comosus*, *Brachiaria ruziziensis*, *Chrysanthemum morifolium*, *Musa* spp., *Nicotiana tabacum*, *Oryza sativa* and *Solanum tuberosum*, as well as from water samples. *D.z.* in contrast to *D.d.* possesses more than nine O-serogroups.

Two strains, *D.d.* Ech586 and *D.z.* Ech1591 (Lucas et al., 2009) that have been examined contain identical T6SS loci consisting of 17 genes lying from Dd586_1304 (*vasL*-like) to Dd586_1272 (*hcp*-like) for *D.d.*, with a disruption of several apparently T6SS-unrelated putative ORF insertions between Dd586_1290 (*impB*-like) and Dd586_1273 (*vgrG*-like) genes (Fig. 1). The *D.z.* T6SS locus spans from Dd1591_2793 (*vasL*-like) to Dd1591_2826 (*hcp*-like) with a disruption of several apparently T6SS-unrelated ORF insertions between Dd1591_2807 (*impB*-like) and Dd1591_2825 (*vgrG*-like) genes (Fig. 1). The two clusters are almost identical and the four core T6SS proteins examined form a distinct phylogenetic branch which includes the *P. atrosepticum* T6SS (Fig. 3).

7.8 *Acidovorax avenae* subsp. *citrulli*

A. avenae subsp. *citrulli* (*A.c.;* β-proteobacteria) is formerly known as *Pseudomonas pseudoalcaligenes* subsp. *citrulli* and is the causal agent of bacterial fruit blotch. It spreads by infested seeds, infected transplants, and occurs naturally in wild hosts. It can be asymptomatic on older plants, which can lead to high numbers of infected young plants early in the planting season. A T6SS cluster is found in *A.c.* strain AAC00-1 consisting of 16 genes between Aave_1482 (*clp*-like ATPase) and Aave_1465 (*hcp*-like) as annotated in Fig. 1. The T6SS locus is contiguous, except of two putative ORF insertions between the Aave_1468 (*fha*-like) and Aave_1465 genes that are apparently T6SS-unrelated. The *A.c.* T6SS cluster lacks a *vgrG* homolog, which potentially raises questions about the system's functionality. To date, there is no experimental evidence concerning a role of this system in *A.c.*-host interactions. Phylogenetically the *A.c.* T6SS cluster forms a sub-group with the *C. taiwanensis* T6SS-I, *P. ananatis* T6SS-II, *Erwinia* spp. T6SS-II (Fig. 3).

7.9 *Agrobacterium* spp. (*A. tumefaciens* and *A. vitis*)

Agrobacterium strains (α-proteobacteria) invade the crown, roots and stems of a great variety of plants via wounds causing overgrowths (crown gall, hairy root, and cane gall). *A.*

tumefaciens (A.t.) have a wide host range among dicotyledonous plants, whereas other possess a very limited host range [*A. rubi* (*A.r.*) and *A. vitis* (*A.v.*) form galls on raspberries and grapes, respectively]. *A. rhizogenes* causes hairy roots on many plants. The ability to cause disease is associated with transmissible plasmids which may move from one strain to another. *A.t.* strain C58 is a representative of biovar I, which has also been extensively modified for biotechnological uses. *A.v.* strain S4 is a virulent biovar III isolated from *Vitis vinifera* (grape) cv. Izsaki Sarfeher crown galls in Kecskemet, Hungary in 1981 (Szegedi et al., 1988; 1996). *A.v.* strains not only cause galls on grapevines but also necrosis on grapevine roots and a hypersensitive response on non-host plants.

The two sequenced species, *A.t.* strain C58 and *A.v.* strain S4 seem to harbor almost identical T6SS gene cluster. According to our database search, those loci are lying between Atu4330 (*impN*-like) and Atu4348 (*vgrG*-like) in *A.t.* C58 and between Avi_6039 (*impN*-like) and Avi_6054 (*hcp*-like) in *A.v.* S4 (Fig. 1). *A.v.* seems to lack the *vgrG*-like gene upstream of the *clpV*-like gene (Fig. 1), while *A.t.* carries a *vgrG*-like gene in the solitary locus Atu_3642 outside the T6SS cluster. However, the *A.v.* has five additional *vgrG*-like genes at the solitary loci Avi_1646, Avi_2758, Avi_3254, Avi_7056, Avi_7557, which also are located outside the T6SS cluster. Phylogenetically the *Agrobacterium* spp. T6SSs branch more closely to the *Rhizobium leguminosarum* T6SS phylogenetic sub-group (Fig. 3).

8. T6SS in plant symbiotic bacteria

8.1 *Rhizobium leguminosarum*

R. leguminosarum bv. *viciae* 3841 (*R.l.*; α-proteobacteria) has a genomic portfolio consisting of seven circular DNA modules (totalling about 7,8 Mb): one circular chromosome of about 5 Mb and six plasmids varying in size from 147 kb to 870 kb. A T6SS is one of the many protein secretion systems identified in *R.l.*, (Krehenbrink & Downie, 2008). The T6SS gene cluster was initially reported to contain 14 genes (*impA*-*impN*); later an *hcp* and a *vgrG* gene homolog (pRL120477 and pRL120480), coding for secreted proteins, were added. Our data mining in the genome of *R.l.,* 3841 leave open the presence of a functional Clp-like ATPase because of multiple sequence insertions and point mutations in the *clpV/B* gene (locus No: pRL120476) (Fig. 1), which is annotated as pseudogene in the KEGG, RhizoBase and NCBI databases. Phylogenetically, the *R.l.* T6SS clusters with the *Agrobacterium* spp. T6SSs and form a distinct phylogenetic branch (Fig. 3).

8.2 *Rhizobium etli*

R. etli (*R.e.* a-proteobacteria) contributes to a significant proportion of nitrogen coming to the earth through microorganisms and is the prime species found associated with cultivated beans in the Americas. Although there is no experimental evidence for a functional T6SS in *R.e.*, bioinformatic analysis revealed two putative T6SSs, one of which (T6SS-I) is similar in organization to that of *R.l.* (Bladergroen et al., 2003) (Fig. 1), and in our phylogenetic distance tree (Fig. 3) it branches with the *Mesorhizobium loti* T6SS.

Our data mining revealed two interesting features. First, of the two strains of *R.e.* sequenced to date, *R.e.* CFN 42 and *R.e.* CIAT 652, only the latter seems to have T6SS core genes. Second, in this strain there are two T6SS gene clusters (T6SS-I and –II) located in distinct

genomic islands (Fig. 1). The T6SS-I spans from RHECIAT_PC0000958 (*vgrG*-like) to RHECIAT_PC0000933 (*impL*-like). While the T6SS-II seems to be divided in two segments with opposite gene orientations, located from RHECIAT_PB0000227 (*impL*-like) to RHECIAT_PB0000224 (*impJ*-like) and from RHECIAT_PB0000217 (*impA*-like) to RHECIAT_PB0000210 (*vgrG*-like). Furthermore, the *Clp*-like ATPase is absent in T6SS-II, which raises questions about the functionality of the cluster (Fig. 1). Phylogenetically, the four core proteins of the *R.e.* CIAT 652 T6SS-I are more closely related to those of the *Mesorhizobium loti* T6SS, forming a distinct sub-group, while those of the T6SS-II branch with the *Xanthomonas* spp. T6SS-I and T6SS-II, forming a separate sub-group (Fig. 3).

8.3 *Bradyrhizobium japonicum*

B. japonicum (*B.j.* α-proteobacteria; *Rhizobium japonicum* in earlier references) utilizes similar mechanisms, as the other symbiotic bacteria, to establish a symbiotic relationship. The *B.j.* strain USDA110 genome consists of a single circular chromosome of about 9 Mbp and has no plasmids. Our i*n silico* genome mining results were in agreement to Boyer et al. (2009) and Records et al. (2011) revealing the presence of one T6SS gene cluster consisting of 17 ORFs located between blr3604 (*ImpN*-like) and bll3587 (*clpB*-like) (Fig. 1). Phylogenetically, the *B.j.* T6SS forms a distinct branch from other *Rhizobium* T6SS and clusters closest to the *Xanthomonas* T6SS-I and III (Fig. 3).

8.4 *Mesorhizobium loti* (*Rhizobium loti*)

M. loti (*M.l.* α-proteobacteria), a symbiont of *Lotus japonicus* contains a chromosomal symbiosis island, similar to what is observed with other rhizobacteria. *M.l.* strain MAFF303099 contains one T6SS which is located between mlr2363 (*impN*-like) and mll2335 (*clpB*-like) gene loci (Fig. 1). In contrast to *R.l.*, *M.l.* possesses *vasD* while it apparently lacks *impE* (Figs. 1 and 3).

8.5 *Cupriavidus taiwanensis*

C. taiwanensis (*C.t.* β-proteobacteria; originally called *Ralstonia taiwanensis* or *Wautersia taiwanensis*), belongs to the β-rhizobia group. It was first isolated from a nodule from the legume *Mimosa pudica* in Taiwan. The type strain LMG19424 has a three-replicon genome made up of two chromosomes of 3.5 Mb, and 2.4 Mb, and a 0.5 kb symbiotic plasmid which carries the genes essential for nodulation and nitrogen fixation (Amadou et al., 2008). In our *in silico* genome mining, two T6SS gene clusters were found in the LMG19424, one (T6SS-I) consisting of 15 genes located between RALTA_A0602 (*impM*-like) and RALTA_A0622 (*impA*-like), with six apparently T6SS-unrelated putative ORF insertions between RALTA_A0611 (*vgrG*-like) and RALTA_A0618 (*vasD*-like) genes (Fig. 1). Phylogenetically, the *C.t.* T6SS-I groups together with the T6SSs of *A. avenae* pv. *citrulli*, *P. ananatis* T6SS-II, *E. pyrifoliae* and *E. amylovora* T6SS-II (Fig. 3). The second cluster (T6SS-II) consists of 18 genes located between RALTA_B1008 (*vgrG*-like) and RALTA_B1029 (impL-like) and containing four putative ORF insertions apparently unrelated to T6SS, between RALTA_B1019 (*clpB*-like ATPase) and RALTA_B1025 (*impA*-like) genes (Fig. 1). Based on gene order and sequence similarities, the C. t. T6SS-II and *R. solanacearum* T6SS appear phylogenetically close (Fig. 3). There are no studies concerning the functionality and/or the contribution of these T6SS clusters in host colonization.

9. Prospects

Protein secretion is fundamental to bacterial virulence and several systems mediate pathogenesis and other types of bacteria-host interaction. Beyond the other recognized secretion systems of Gram-negative bacteria with established role in host-pathogen interactions, the T6SS is of particular interest in this respect and has been shown to be important for bacterial virulence and for interaction with the host in various ways, often leading to "anti-pathogenesis" (Jani & Cotter, 2010). Nevertheless, its role and function in most bacteria is not clearly established and formal evidence for protein secretion/translocation into plant cells is scant. At present, we do not fully understand how the T6SS works and are only beginning to understand the biological role/s of the T6SS in plant-associated bacterial life. Multiple copies of T6SS in a single bacterial strain appear to be a frequent phenomenon, and this holds true for many plant associated species. Recent studies (Boyer et al., 2009; Filloux et al., 2008) have established the presence of multiple copies of apparently complete and/or degenerate T6SS loci in about one quarter of the proteobacterial genomes examined, that they generally display different phylogenetic origins and are not a result of recent duplication events, suggesting sustained and constrained mechanisms that favour this trend. Based on our own analysis (Sarris & Scoulica, 2011; Sarris et al., 2011), most strains of *Pseudomonas syringae,* the insect pathogenic *Pseudomonas entomophila* strain L48, the human opportunistic pathogen *Pseudomonas mendocina* strain ymp, and most of the *Pseudomonas* strains studied by Barret et al. (2011) typically carry T6SS from more than one phylogenetic clade and/or additional *vgrG* and *hcp* genes.

Although the majority of the recent studies concern the contribution of T6SS in bacterial pathogenicity (positively), many bacteria with genomes encoding putative T6SS are not known to be pathogens or symbionts, and T6SS may also function in non-pathogenic bacteria-host interactions and/or in interactions not involving eukaryotic partners. In *R. leguminosarum* the T6SS limits host-range and in *S. typhimurium* and *H. hepaticus* the evidence suggests a possible role of T6SS in limiting of bacterial virulence and, therefore, contribution to host colonization (Bladergroen et al., 2003; Parsons & Heffron, 2005; Jani & Cotter, 2010; Chow & Mazmanian, 2010). A relatively new twist in the system's repertoire of biological functions in a broader context is the finding that bacteria engage each other in a T6SS-dependent manner and can provide protection for a bacterium against cell contact-induced growth inhibition caused by other species of bacteria (Hood et al., 2010; Schwarz et al., 2010a). This leads to speculation that this pathway is of general significance to interbacterial interactions in polymicrobial diseases and the environment.

T6SS clusters occur with high frequency, have divergent phylogenies and individual strains or species often possess non-orthologous clusters with distinct or overlapping functions in bacterial interactions with multiple hosts, antagonists or predators unsuspected at present. In a recent study of the ocean metagenome (Persson et al., 2009) the T6SS was more abundant among γ-proteobacteria than other protein transport systems. The weight of present evidence suggests, at least indirectly, an apparently rampant lateral transfer of T6SS clusters/genes in the microbial world, which could be a significant driver for newly emerging pathogens, as proposed for the gastroenteritis agent *E. tarda* (Leung et al., 2011).

Future studies are needed and expected to further advance the T6SS field. It is important to remember that formal evidence of the translocation of effector proteins into plant cells through T6SS is presently lacking, as is also the case for the molecular targets of these effectors. Paraphrasing Schwarz et al. (2010a), outstanding questions for future research on T6SS include the following: What are the physiological role(s) and adaptive significance of T6S-mediated plant cell targeting in disease, symbiosis, and interbacterial interactions in the environment? What is the significance of additional *vgrG* and *hcp* genes Are host- and bacterial cell-targeting T6SSs discernible by sequence or gene content? Are there T6SSs that can target both eukaryotic and prokaryotic cells? Are there other T6S substrates that await identification? Given the resemblance T6SS components to bacteriophage proteins it is also tempting to ask if T6SS transports only protein substrates and/or other macromolecules as well.

It is instructive to point out that the genes coding for several secretion systems, including T6SS, were first identified in phenotypic screens of mutants altered in their interaction with higher eukaryotes. It is conceivable that new secretion systems may be identified in other appropriately designed screens in multi-organism settings. Bioinformatic sourcing of genome, transcriptome and proteome data may point to new potential candidates, as occurred historically with the T6SS. Finally, the striking similarities between secretion systems and type IV pili, flagella, bacteriophage tail, or efflux pumps invite speculation that new systems may even be predicted, "the way Mendeleïev had anticipated characteristics of yet unknown elements" (Filloux, 2011b).

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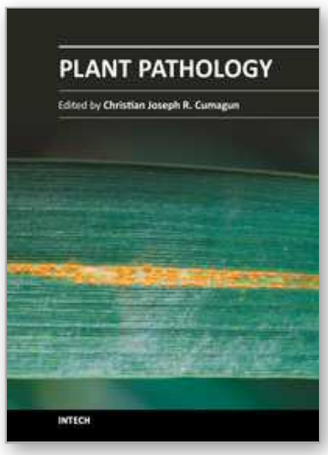

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