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Bacterial Type IV Secretion Systems in Human Disease

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Summary

Type IV secretion (T4S) systems are versatile machines involved in many processes relevant to bacterial virulence, such as horizontal DNA transfer and effector translocation into human cells. A recent Workshop organized by the International University of Andalousia (UNIA) in Baeza, Spain, covered most aspects of bacterial T4S relevant to human disease, ranging from the structural and mechanistic analysis of the T4S systems to the physiological roles of the translocated effector proteins in subverting cellular functions in infected humans. This review reports the highlights from this workshop, which include the first visualization of a T4S system core complex spanning both membranes of Gram-negative bacteria, the identification of the first host receptors for T4S systems, the identification and characterization of novel T4S effector proteins, the analysis of the molecular function of effector proteins in subverting human cellular functions, and an analysis of the role of T4S systems in the evolution of pathogenic bacteria. Our increasing knowledge of the biology of T4S improves our ability to exploit them as biotechnological tools or to use them as novel targets for a new generation of antimicrobials.

Introduction

Type IV secretion (T4S) systems are the most versatile bacterial secretion machines. These trans-membrane multi-protein complexes can secrete protein and DNA molecules to the milieu or directly into virtually any prokaryotic or eukaryotic target cell type. Accordingly, bacteria have recruited T4S systems for a variety of biological processes, such as horizontal DNA transfer or infection of mammalian target cells. Both processes have a significant impact in bacterial virulence. The elucidation of the molecular architecture of T4S systems, the mechanism of secretion, the nature of the secreted molecules and their roles in human target cells, are crucial to understanding pathogenicity and to designing strategies to combat bacterial virulence. These issues were addressed at the workshop “Bacterial Type IV Secretion Systems in Human Disease”, which was organized by the International University of Andalousia (UNIA) in the series of workshops “Current Trends in Biomedicine”. The event took place in Baeza (Spain) on October 14–16, 2008, and gathered from all over the world around 50 scientists working on the biology of T4S systems. The scientific organizers (the authors of this report) structured the workshop in five thematic sessions that addressed all aspects of T4S biology, with special emphasis in their involvement in human disease. The excellent scientific presentations and informal discussions contributed significantly to our understanding of the biology of T4S systems. The order of the thematic sessions followed the path of the secreted substrate: from the T4S system organization in the

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bacterial envelope to the role of the effectors in the infected human cell, going through the recruitment of substrates of different nature, the secretion mechanism, interaction with the host cell surface, effector translocation, and subversion of host cell functions. The following sections highlight most of the new and interesting discoveries presented at the workshop.

T4S system structure and secretion mechanism

The first part of the workshop focused on T4S system architecture and structure-function analysis. A panoply of different T4S systems were addressed, including the prototypical VirB T4S system of *Agrobacterium tumefaciens*, the T4S systems of conjugative plasmids (both from Gram-negative and Gram-positive bacteria), and T4S systems from the human pathogens *Brucella spp.* and *Legionella pneumophila*, the latter belonging to the Type IVB family of T4S systems that shows important differences in genetic organization and protein components from the major Type IVA family of T4S systems (Sexton & Vogel, 2002).

The workshop started with an impressive talk by Gabriel Waksman from the University College London and Birkbeck, London, who showed the first high-resolution EM structure of a T4S system "core complex" (Fronzes *et al.*, 2009) (Figure 1A). The Waksman lab had previously reported the 3D structures of the periplasmic domains of the components believed to form the central channel spanning both membranes, from different T4S systems: *Brucella* VirB8, the VirB10 homologue ComB from *H. pylori* homologue (Terradot et al., 2005), and the TraN-TraF complex (homologues of VirB7-VirB9) of pKM101 (Bayliss *et al.*, 2007). Homologues of these four elements from the T4S system of the conjugative plasmid pKM101 were produced together in the same cell in order to purify T4S system sub-assemblies. An image of the purified complex was obtained by cryo-electron microscopy at 15 Å resolution. Surprisingly, the VirB8 homologue was shown not to form part of this central complex, which was formed by 14 monomers of each TraN, TraO and TraF proteins (homologues of VirB7, VirB9 and VirB10, respectively). The cup shaped structure, of about 1 MDa, spans both membranes, and resembles a secretin-like ring of 185 Å in diameter, with a central channel 55 Å wide at one pole and 10 Å at the other. A constriction in the middle of the ring, which would be located in the periplasmic space, separates the I- and O-layers. At each layer, a double wall surrounds an internal chamber. By limited proteolysis, protein tagging, and docking of the pre-existing 3D structures, the authors conclude that the wide part of the channel faces the cytoplasmic membrane; that the internal wall is composed mainly by VirB9, and that VirB10 forms the external wall. VirB7 and the C-terminal domains of both VirB9 and B10 form the O-layer, and the N-terminal domains of both VirB9 and VirB10 form the I-layer.

The resolution of this structure is crucial to understand the secretion mechanism. It is exciting that the image fits so well with the predicted topology of each protein, and with the molecular interactions that were revealed by the TrIP assay (Cascales & Christie, 2004b), which predicted that the substrate DNA would be in contact with VirB7-B9 (forming the inner cavity) but not with VirB10 (which mostly forms the external wall, and thus would not be in direct contact with the substrate). The presence of a 10 Å constriction in the outer membrane suggests that the channel expands during substrate translocation. This is in agreement with previous data showing that VirB10 undergoes a conformational change upon energization of the cytoplasmic ATPases VirD4 and VirB11, which strengthens an interaction with the VirB7/B9 complex (Cascales & Christie, 2004a). Figure 1B shows how this core structure can reflect a secretion mechanism based on the VirB10-mediated opening of the outer-membrane constriction delimited by VirB7/B9. It should be noted that the I and O layers are linked by thin stretches of density. This periplasmic constriction is the weakest point of the core structure, perhaps allowing a periplasmic entrance for certain substrates. This would help explain how pertussis toxin, for example, can be secreted from the

periplasm by the Ptl T4S system after the five different toxin subunits are exported to the periplasm by the Sec system and assembled correctly (Covacci & Rappuoli, 1993, Weiss *et al.*, 1993).

Peter J. Christie (University of Texas-Houston Medical School, Texas, USA) and Joseph P. Vogel (Washington University, St. Louis, USA) presented their significant progress on the elucidation of the structure-function relationship of the prototypical Type IVA and IVB secretion systems (*A. tumefaciens* VirB and *L. pneumophila* Dot/Icm, respectively). The Christie lab has performed a detailed analysis of the *A. tumefaciens* VirB10 functional domains by analyzing a collection of mutants *in vivo* and *in vitro* (Jakubowski *et al.*, 2009). Their results reinforce a central role for VirB10 in connecting the inner and outer membranes. Mutants were obtained in each subdomain of the protein and analyzed for pilus formation, T-DNA transfer, and interactions with other VirB components. They obtained "uncoupled" mutants abolishing T pilus formation but not T-DNA transfer, supporting the intriguing possibility that the T4S system can assemble either as a pilin secretor or as a DNA transporter. J.P. Vogel centred his presentation on localization studies of the Dot/Icm T4S system. He showed that polar secretion of *Legionella* T4S substrates is due to polar localization of the T4S system and that this polarity is important for bacterial alteration of the host endocytic pathway. In addition, he showed that biogenesis of this T4S system first occurs in exponential phase by deposition of several Dot/Icm components at the mid-cell. Patricia Zambryski (University of California, Berkely, USA) used EM and deconvolution fluorescence light microscopy images to show helical localization of the *A. tumefaciens* T4S system along the bacterial long axis, arguing against the previously-reported polar localization of *A. tumefaciens* T4S system (Atmakuri *et al.*, 2003). Recent kinetic studies suggest that the helical localization pattern is established early and maintained through late times post *virB* induction (J. Aguilar and P. Zambryski, unpublished results). Previous results suggesting predominantly polar localization likely reflect the limited resolution of wide-field epifluorescence microscopy. It remains to be determined what are the cellular mechanisms that regulate the assembly and distribution of the T4S system in the bacterial cell.

The structure-function analysis of T4S systems also opens new avenues for the design of inhibitors with potential therapeutic applications. Substances that inhibit the T4S could potentially disarm an important mechanism contributing to virulence without killing bacteria, which makes T4S inhibitors attractive candidates for a novel class of antimicrobials (Baron & Coombes, 2007). Within this line of research, Christian Baron (Universite de Montreal, Canada) presented a two-hybrid approach to screen for inhibitors of T4S system protein interactions. Several small molecules from a chemical compound library affected VirB8 interactions, and the structure of VirB8 bound to one of these compounds was presented. Inhibition of T4S was also addressed in a talk by Fernando de la Cruz (Universidad de Cantabria, Santandar, Spain), who searched for natural inhibitors of bacterial conjugation by using *E. coli* mutant collections as recipient cells. Only certain mutants involved in lipopolysaccharide biosynthesis showed a modest decrease in conjugation; the take-home message is that bacteria have no way to avoid receiving DNA by conjugation. Curiously, the same set of mutants that inhibited conjugation modestly on agar surfaces inhibited conjugation in liquid to a much larger extend (Perez-Mendoza & de la Cruz, 2009).

A different session at the meeting covered newly identified T4S systems that are being characterized at the genetic and biochemical level. Elisabeth Grohmann (Technical University, Berlin, Germany) showed work in progress on the molecular characterization of the T4S system from the broad host-range conjugative plasmid pIP501 from Gram-positive bacteria. T4S systems from Gram-positives contain proteins with homology to VirB1, VirB4

and VirD4, but not to the rest of Vir elements, in accordance with the lack of a two-membrane system in these bacteria. However, other Tra gene products required for conjugation were shown to be membrane-localized and to interact with the Vir homologues. Protein complexes from this system are being analyzed, which should yield the first interactome of a T4S system that is functional in Gram-positive bacteria (Abajy *et al.*, 2007). Yasuko Rikihisa (Ohio State University, Columbus, USA) presented an analysis of the *vir* genes involved in the assembly of a T4S system in the Gram-negative bacterium *Ehrlichia*, in which it has an unusual genetic organization. *Ehrlichia chaffeensis* has five *virB/D4* genetic loci that are co-ordinately regulated by EcxR to allow developmental stage-specific expression of the T4S system (Cheng *et al.*, 2008). The *vir* genes include four encoding VirB6 paralogues that form a molecular subassembly with VirB9 (Bao *et al.*, 2009).

Role of T4S system pilus-like structures in host cell contact

Whether or not T4S systems need to interact with specific cellular receptors in order to translocate their effectors into target cells has been an important question that has stimulated debate for many years. Steffen Backert (University College Dublin, Ireland) reported at this workshop that the Cag T4S system of *Helicobacter pylori* targets β1 integrins to translocate the effector protein CagA. The CagL protein was identified as an adhesin located at the T4S system pilus surface, where it bound to and activated integrin α5β1 receptor of gastric epithelial cells through an arginine-glycine-aspartate (RGD) motif (Kwok *et al.*, 2007). Based on sequence analysis and homology modelling, CagL was proposed to represent a homologue of the minor pilus associated component VirB5 of the canonical VirB/VirD4 T4S system of *A. tumefaciens* (Backert *et al.*, 2008). Rainer Haas (Ludwig Maximilians University, Munich, Germany) also presented data supporting a crucial role of β1 integrin receptors in CagA translocation, and provided evidence for the involvement of several Cag components other than CagL in direct binding to these important cellular receptors. Thus, the evidence supporting a role for β1 integrins being a crucial receptor required for delivery of CagA into host cells by the *H. pylori* T4S system is very strong, although the mechanism that mediates T4S system interaction with host integrin receptors appears to be complex and might involve specific interactions mediated independently by several components of the Cag apparatus. In addition, Haas presented a comprehensive interaction analysis of the components of the Cag T4S system, which is more distantly related to the canonical VirB T4S system of *A. tumefaciens* than other Type IVA secretion systems. Data from this study suggests that functional homologues of most of the 12 canonical VirB/VirD4 components are found among the 14 essential components of the Cag system (Kutter *et al.*, 2008).

Based on yeast two-hybrid interactions and other functional data, David O'Callaghan (INSERM Espri26, Universite de Montpellier 1, Nimes, France) presented evidence that indicated binding mediated by the VirB T4S system of *Brucella* to specific receptors on human host cells. In particular, the major pilus subunit VirB2 was found to interact directly with CD98, a membrane protein involved in the activation of integrins. CD98 was shown to be critical for the initial steps of infection. Muriel Vayssier-Taussat (INRA, Maisons-Alfort, France) reported data indicating a role of the Trw T4S system of *Bartonella spp.* in mediating specific adhesion to erythrocytes. This T4S system is essential for establishing intraerythrocytic infections in animal models (Saenz *et al.*, 2007, Seubert *et al.*, 2003). Moreover, the *Bartonella* Trw system is closely related to the canonical Trw conjugation system of the broad-host-range antibiotic resistance plasmid R388, but lacks a coupling protein (T4CP) and recognizable substrates (Seubert *et al.*, 2003) and, thus, is not considered to translocate effector proteins into erythrocytes (Dehio, 2008). Based on the analysis of genetically modified *Bartonella* strains in *in vitro* assays of erythrocyte binding and invasion, M. Vayssier-Taussat proposed that the Trw T4S system of *Bartonella* spp.

mediates direct binding to a yet unidentified class of host-specific erythrocyte receptors. The likely candidate T4S components for this interaction are the TrwL and TrwJ homologues of the major and minor pilus subunits VirB2 and VirB5, respectively, which are encoded by fast evolving genes that are repeated in tandem arrays (Dehio, 2008, Nystedt *et al.*, 2008, Seubert et al., 2003). Taken together, novel data presented at this workshop for three distinct T4S systems of mammalian pathogens provided compelling evidence for a direct role of specific cellular receptors of T4S systems in establishing host cell interaction and, for at least two systems, the subsequent step of effector translocation.

Pathogen-induced responses mediated by T4S systems

Several presentations at this workshop discussed the role of T4S systems in bacterial pathogenesis and the marked physiological responses triggered by these devices important for pathogenesis. Christoph Dehio from the University of Basel, Switzerland, has used an integrated genomics approach to infer the role of T4S systems in the evolution of the genus *Bartonella*. The bartonellae are host-restricted pathogens that colonize endothelial cells and erythrocytes of their respective mammalian reservoir host(s), thereby causing long-lasting intraerythrocytic infections. As a representative of an ancestral lineage that does not contain any T4S system, the deadly human pathogen *Bartonella bacilliformis* holds a unique position in the *Bartonella* phylogeny. The other species evolved in a separate “modern” lineage by adaptive radiation, all representing highly host-adapted pathogens with rather low virulence potential. Unlike *B. bacilliformis*, genomes of the species of the modern lineage encode at least one of the closely related T4S systems, called VirB/VirD4 or Vbh. Moreover, Trw is present as an additional T4S system in a sub-branch of the modern lineage. A comprehensive comparative and functional genomic analysis indicated that VirB-like T4S systems represent major host adaptability factors that contribute to the remarkable evolutionary success of the modern lineage (Saenz *et al.*, 2007). At the molecular level, the VirB/VirD4 T4S system is known to translocate a cocktail of effector proteins into endothelial cells to subvert cellular functions critical for establishing chronic infection (Schulein *et al.*, 2005). Fast evolution of these effectors is proposed to account for the remarkable capacity of the VirB T4S system to mediate host adaptability (Dehio, 2008).

Host cell apoptosis is one of the responses modulated by T4S system-dependent processes. Cell death represents an important mechanism by which infected host cells limit the replication of intracellular pathogens. T4S systems in *Bartonella henselae* (Schmid *et al.*, 2006), *Coxiella burnetii* (Luhrmann & Roy, 2007, Voth *et al.*, 2007) and *Legionella pneumophila* (Zink *et al.*, 2002) have been implicated previously in interference with host cell apoptosis as a mechanism to ensure host cell survival. Y. Rikihisa showed that the AnkA protein from *Anaplasma phagocytophilum* is a T4S effector protein that is subject to host tyrosine phosphorylation after translocation, and is critical for bacterial infection (Lin *et al.*, 2007). At the meeting, she presented new data on a second T4S substrate, Ats-1, which is found in the cytosol of *A. phagocytophilum*-infected neutrophils and is able to interfere with apoptosis. Ats-1 was found to co-localize with mitochondria in neutrophils, suggesting that it might interfere with mitochondrial-associated factors important in the release of pro-apoptotic signalling molecules. In addition to Ats-1, other T4S effectors might activate anti-apoptotic signalling pathways, including the *H. pylori* protein CagA (S. Backert).

Host colonization by pathogens with T4S systems often involves the subversion of membrane transport pathways to create a vacuole for intracellular survival, and evasion of the immune system. Thus, it is not surprising that many of the pathogen-associated T4S systems are involved in modulating host immune responses and membrane trafficking. This was illustrated in several presentations focusing on the host response to *Brucella* spp. Rodolfo A. Ugalde (Universidad Nacional de General San Martin, Buenos Aires, Argentina)

described in detail how the *Brucella* VirB system is essential for bacterial replication inside mammalian cells. His work indicated that intracellular expression of the genes encoding the VirB system is tied to a regulatory network that controls histidine catabolism, suggesting that intracellular bacteria might activate the VirB system in response to metabolic stress. Intracellular expression of the VirB system enables *Brucella* to subvert membrane transport pathways within the host cell and results in the creation of a vacuole derived from host endoplasmic reticulum (ER) membrane, as was further demonstrated in a talk by Suzana P. Salcedo from the group of Jean-Pierre Gorvel (Centre d'Immunology Marseille Luminy, Marseille, France). Salcedo also showed that a *Brucella* protein called Btp1 inhibits signalling through the mammalian Toll-like receptor 2 (TLR2) protein (Salcedo *et al.*, 2008). Although the mechanism remains to be determined, the *Brucella* Btp1 protein has a predicted TIR domain that could compete with a host TIR domain-containing protein involved in TLR signalling. This protein is predicted to dampen the inflammatory response following *Brucella* infection and data presented indicate Btp1 can also inhibit maturation of dendritic cells, which would interfere with the priming of adaptive immune responses. Whether Btp1 is delivered into host cells by the VirB system has not been determined; however, this protein remains an enticing effector candidate.

In addition to dampening host immune responses, T4S can also trigger innate immune responses and be targeted by adaptive responses. This was highlighted in a talk by Wendy Brown (Washington State University, USA), who found that strong CD4+ T cell responses were elicited upon immunization of cattle with bacterial membrane vaccinates from *Anaplasma marginale* (Lopez *et al.*, 2007). Antigenic T4S components include VirB9, VirB10, virB11, VirD4 and VirB2, but not VirB4 and VirB6. Renée Tsolis from the University of California, Davis, USA, showed secretion of the pro-inflammatory cytokine IL-1βis triggered in response to *Brucella* by a VirB-dependent mechanism (Rolan & Tsolis, 2008). IL-1βproduction induced by *Brucella* required inflammasome-mediated activation of caspase-1. VirB-mediated activation of caspase-1 by *Brucella* did not require the host protein Ipaf (NLRC4) and was not stimulated in response to bacterial flagellin, but was found to require the caspase-1 adapter protein ASC. The signal generated by the VirB system remains unknown, as does the host sensor protein that triggers ASC-dependent caspase-1 activation.

In summary, these talks illustrated that immune evasion mechanisms mediated by T4S systems may be counteracted by specific immune responses elicited by T4S system components and effectors. This balanced T4S-dependent interplay between the host and pathogen could prove important for maintaining a persistent state of bacterial infection.

Spatial and temporal regulation of T4S effectors in the host cell

Pathogen-associated T4S systems usually deliver effector proteins with diverse biochemical functions into the host cell. Over 100 different effector proteins have been determined for the *L. pneumophila* Dot/Icm system, and several Bep proteins have been detected as substrates of the VirB/D T4S system of *Bartonella*. In contrast, the *H. pylori* Cag system seems to translocate only a single protein, CagA, and substrates have not yet been identified for some of the T4S systems such as for the Trw T4S system of *Bartonella* discussed above (Cambronne & Roy, 2006, Dehio, 2008). The identity of the molecules secreted by the VirB T4S system of *Brucella* has remained elusive. Searching among *virB* co-regulated genes and using a beta-lactamase reporter assay for protein translocation into macrophages, R. Tsolis presented solid evidence that *Brucella* proteins VceA and VceC are translocated through the VirB T4S system (de Jong *et al.*, 2008). These two proteins are conserved among all *Brucella* species, but their function is unknown. VceC could also be translocated by the *L. pneumophila* Dot/Icm system, although the levels of translocation appeared lower than for

endogenous substrates. Robert A. Heinzen (NIH, Hamilton, USA) presented data indicating that a large number of effector proteins are also delivered by a related Dot/Icm secretion system encoded by the intracellular pathogen *Coxiella burnetii*. For both *L. pneumophila* and *C. burnetii* there is evidence that a subset of the effectors are proteins containing eukaryotic-like motifs, such as ankyrin repeat homology domains (Pan *et al.*, 2008, Voth *et al.*, 2009). Other effectors do not share any homology to proteins in either prokaryotes or eukaryotes, making determination of function more challenging.

Interestingly, despite the fact that *L. pneumophila* and *C. burnetii* have similar T4S systems, there is no clear evidence that they share effectors. Perhaps this is not too surprising given that these pathogens have evolved distinct strategies on intracellular infection. *L. pneumophila* is found in fresh-water environmental ecosystems where it lives inside of free-living protozoan hosts, and the vacuole that supports *L. pneumophila* replication is a neutral pH organelle derived by T4S mediated subversion of the host secretory pathway. In contrast, *C. burnetii* resides primarily in mammalian hosts and replicates in an acidified vacuole derived from fusion with lysosomes.

The vacuole containing *L. pneumophila* undergoes distinct changes during maturation, changes that are reflected in the composition of effectors associated with the vacuole at different stages of development. Several effectors translocated early after *L. pneumophila* contact with the host cell participate in remodelling of the initial plasma membrane-derived vacuole into a vacuole with markers found on early secretory vesicles (Ensminger & Isberg, 2009). In a talk by Craig Roy (Yale University School of Medicine, USA), it was shown that a C-terminal region in the effector protein DrrA mediates targeting of this effector to the plasma membrane, and that the DrrA plasma membrane-targeting motif is found in several other *L. pneumophila* effectors of unknown function. Hubert Hilbi (ETH Zurich, Switzerland) showed that as the *L. pneumophila*-containing vacuole begins to acquire early secretory markers, phosphatidylinositol(4) phosphate (PI4P) levels on the vacuole membrane increase. Several effectors, including SidC, have PI4P binding domains that mediate the localization of protein to vacuoles during this intermediate stage of maturation (Ragaz *et al.*, 2008). It was also shown that *Legionella* replication occurred more efficiently in the protozoan host *Dictyostelium discoideum* with an inactivated inositol polyphosphate 5-phosphatase Dd5P4, which might interfere with the ability of host cells to combat infection by reducing the kinetics of cargo transport by the endocytic pathway (Weber *et al.*, 2009). These results provide a nice demonstration of how T4S systems and their effectors target the specificity of PI metabolism to coordinate events that permit intracellular replication.

Hiroki Nagai (Osaka University, Japan) presented data indicating that the effector protein LubX has ubiquitin ligase activity that can target cytosolic proteins for degradation by the host proteosome. The LubX protein is produced, and thus translocated, only several hours after *L. pneumophila* has entered a eukaryotic host (Kubori *et al.*, 2008). The timing of LubX translocation suggests it might play a temporal role in the inactivation of proteins that are no longer necessary on the vacuole. Indeed, it was shown that an effector called SidH is targeted for degradation shortly after LubX is produced by *L. pneumophila*, providing a beautiful example of how temporal production of an effector can modulate the spatial localization and stability of another effector. Similarly, C. Roy presented data indicating that the effector protein PieA is specifically recruited to the mature vacuole in which *L. pneumophila* replicates by a process that requires modifications to the vacuole directed by other effectors (Ninio *et al.*, 2009). Thus, these studies are providing new and exciting examples of how effectors play important roles in controlling the activities and localization of other effectors during infection.

DNA as an effector: secretion and conjugation

T4S systems are well known secretors of both protein and DNA substrates. DNA secretion and conjugation are involved in horizontal DNA transfer and, thus, they have a relevant role in evolution of pathogens, and in the dissemination of antibiotic resistance and other virulence traits. The current model presents T4S systems as protein secretion machines and, so, when a DNA molecule is to be secreted, it is piloted by a protein that is considered to be the direct T4S substrate (Llosa et al., 2002). The secretion of a DNA molecule poses the problem of processivity. It is believed that coupling proteins (T4CP), in addition to having a role in substrate recruitment, act as DNA-dependent ATPases to facilitate translocation of the DNA strand initially recruited to the T4S system by a pilot protein attached covalently to the 5′ end of the molecule (Llosa et al., 2002). Thus, in conjugative T4S systems, a complex has to form between the T4S system, the T4CP, and the substrate complex, called the relaxosome. The relaxosome is formed by the pilot protein/relaxase linked to the T-DNA strand, and often other so-called “accessory proteins”, both from the T4S system and from the host, which affect the formation of the relaxase-DNA covalent complex. Ellen Zechner (University of Graz, Austria) has addressed relaxosome formation and substrate recruitment in the F-like R1 conjugative plasmid. Evidence was presented that the T4CP interacts extensively with the relaxosome, enhancing the DNA cleavage and helicase reactions, probably by protein-protein interactions with the helicase domain of the relaxase TraI. In addition, the CRAFT assay (Vergunst *et al.*, 2000) was used to map two independent T4S signals on the TraI protein. Like the T4CP, the accessory proteins not only affect topology of the DNA, but also enhance helicase activity at *oriT*. Matxalen Llosa (Universidad de Cantabria, Santander, Spain) presented a comparative analysis of two closely related T4S systems displaying a high level of amino acid identity, yet with very different biological roles: the Trw systems of the conjugative plasmid R388 (devoted to conjugative DNA transfer to a bacterial cell) and the human pathogen *Bartonella* spp. (involved in establishment of the bacteria in the erythrocytes). Several molecular components of both T4S systems were shown to be functionally or structurally interchangeable (de Paz *et al.*, 2005). Substrates of the *Brucella* Trw T4S system have been identified, so it is believed that it may play a role in interaction with the erythrocyte (see previous sections). In an attempt to test recruitment of R388 substrates by the *Bartonella* T4S system, *B. henselae* cells harbouring R388 derivatives devoid of their own T4S system and carrying an eGFP cassette were used to infect human cells. 1–2% of the cells expressed the DNA coming from the bacteria. This DNA transfer depended on the activity of the R388 T4CP. Since the R388 pilot protein TrwC can catalyze site-specific integration of the incoming DNA in recipient cells (Draper *et al.*, 2005), introduction of these nucleoprotein complexes into specific human cell types could have biomedical applications.

The only known T4S system involved in DNA secretion into the milieu is that of *Neisseria gonorrhoeae*. Two labs are characterizing this intriguing system. Previous work by Joseph P. Dillard (University of Wisconsin, Madison, USA) showed that a relaxase-like protein is required for DNA export, and that the secreted substrate is a single-stranded DNA molecule with a protected 5′ end (Salgado-Pabon *et al.*, 2007), suggesting the recognized substrate is similar to that in bacterial conjugation. Now they have found an *oriT*-like sequence in the genomic island that encodes the T4S system; its deletion affects DNA secretion, although it does not abolish it. Addition of the *oriT* sequence to a replicating plasmid facilitated transfer of the plasmid by DNA secretion and natural transformation. Further work will be required to assess the similarities and differences with the conjugative systems. Another intriguing feature of this system is the presence of a pilin, TraA, which is not required for DNA secretion. Chris van der Does (Max Planck Institute for Terrestrial Microbiology, Marburg) showed that the TraA pilin from the laboratory strain MS11A is a C-terminal truncated version, while most wild-type strains carry a full-length TraA protein. Interestingly, the

presence of this full-length TraA abolishes DNA secretion, thereby decreasing the transfer of DNA between cells. The function of the presence of the two forms of the pilin subunit in the population is currently being investigated.

The most recently described T4S family members are T4S systems involved in the dissemination of genomic islands (GI) in a broad spectrum of bacteria (Juhas *et al.*, 2007). Mario Juhas (University of Oxford, UK) presented the results of the functional characterization of the T4S system involved in mobilization of the GI of *Haemophilus influenzae*, which has a relevant role in the spread of antibiotic resistance in this pathogenic species. This family of T4S systems is novel and evolutionarily distant from the previously described systems, leading to the proposal that they constitute a third family, different from the Type IVA and B previously defined (Juhas *et al.*, 2008).

Conclusions and prospects

The last international meeting specialized on T4S systems was a EURESCO Conference that took place in September 2003 in Giens, France. The report of this meeting (Llosa & O'Callaghan, 2004) presented as new challenges for this field the visualization of the secretion apparatus, the understanding of the different T4S secretion signals, as well as the identification of the cellular targets for the newly identified T4S effectors, and their effects on the biology of the recipient organisms. It is noteworthy that some of the highlights presented at the Workshop address these issues satisfactorily.

Structural biology studies from the Waksman lab, which in recent years has provided the scientific community with a view of individual T4S components, release now the 3D structure of the core complex, a major step forward in the understanding of T4S architecture. Other Type IV components will undoubtedly add to this structure in the near future, until a full T4S system can be detailed structurally. Complementary functional studies are essential to end up with a view of the secretion mechanism. In spite of recent advances, some key questions, such as if the substrates are folded, or if there is a periplasmic step in secretion, remain unanswered.

The role of the pilus in T4S has been long debated. One of the highlights of this workshop was the increasing evidence coming from several labs that the pilus mediates contact with specific host receptors. It remains to be determined if, in addition to a role as a contact structure, the pilus can function as a channel itself. Recent evidence suggests that the conjugative pilus might serve as the DNA secretion channel (Babic *et al.*, 2008, Wang *et al.*, 2009).

The number of identified T4S substrates increased dramatically in recent years. An update of the T4S systems addressed in this workshop, together with their known substrates, hosts and functions, is shown in Table 1. In this workshop, we have learned that the T4S system of *Coxiella* functions similarly to the homologous system in *Legionella*, although the nature of the translocated effectors is specific for each system. Also, after many fruitless attempts, several translocated effectors have finally been identified for the T4S of *Brucella*. As the number of identified T4S effectors is increasing, the determination of their differences and communalities in their secretion signals will ultimately lead to a better understanding of the basis for substrate recruitment by different T4S systems, allowing the manipulation of the recognition signals to obtain heterologous secretion.

Significant progress has been made recently in unravelling the molecular function of T4S effectors and their cellular targets in subverting host functions, most prominently presented at this workshop for several effector proteins of *Legionella*, for which over 100 effector proteins have now been identified. Interestingly, knock-out mutants in individual effector

genes often fail to result in gross quantitative differences in intracellular replication of *Legionella*. This has led to speculation that there might be extensive redundancy in the function of effectors. Arguing against this view, however, are several examples that show elimination of an effector with a well-defined activity usually results in a complete loss of that biochemical function in the mutant. Additionally, it has been shown for effectors that are part of a large gene family consisting of multiple paralogues, such as the SidH family (Laguna *et al.*, 2006), clear infection phenotypes are revealed when one effector is eliminated, and this same phenotype is not revealed by elimination of any other family member. Thus, there is no clear indication that other bacterial proteins with redundant activities exist for many effectors of known function. A better understanding of the complex interactions of the transferred cocktail of effector proteins in subverting host functions will require an integrated approach involving systems-based analysis of effectors and continued elucidation of the biochemical activities associated with each effector.

This meeting illustrated also novel aspects on the evolution of T4S systems. On the one hand side, several talks highlighted the adaptation of the ancestral bacterial conjugation systems to diverse functions such as DNA uptake, inter-kingdom DNA or protein transfer, or adherence to target cells without transfer of a dedicated substrate, the latter suggested for the Trw system of *Bartonella*. One the other hand, T4S systems were addressed as facilitators of enhanced adaptive evolution to host organisms, using the VirB T4S of *Bartonella* as an example. Further studies on the evolution of T4S systems, including the numerous newly identified T4S systems in various bacteria, should increase our appreciation of the role these versatile transporters play in bacterial pathogenesis as well as in commensal or even mutualistic interactions with humans and other higher eukaryotes.

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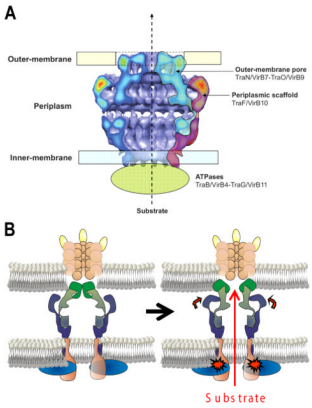

Figure 1. Structure of a T4S system core complex

(A) Cryo-EM 3D structure of the core complex at 15 Å resolution (Fronzes et al., 2009). **(B)** A structure-based functional model showing channel opening upon energization of VirB10 by the cytoplasmic ATPases. The concomitant conformational change of VirB10 would cause the opening of the VirB9/B7 constriction allowing passage of the substrate.

Table 1

T4S systems discussed at the workshop

Organism/plasmid	Name	Function	Known substrates	Main new results presented[1]
a) T4S systems involved in animal cell infection (chromosomally encoded)				
Anaplasma spp	VirB/D4	Infection of macrophages	AnkA, Ats-1	New substrate locating in mitochondria
				Immunogenicity of T4S proteins
Bartonella henselae	Trw	Adhesion to erythrocytes	None detected	Role in host specificity
	VirB/D4	Infection of endothelial cells	BepA-G	Role in host adaptation
Brucella spp.	VirB	Infection of macrophages	VceA, VceC	First T4S substrates detected
				VirB2 interaction with host cell surface
				Metabolic regulation of *virB* expression
Coxiella burnetii	Dot/Icm	Infection of phagocytes	Multiple effectors	Novel substrates identified
Ehrlichia chaffensis	VirB/D4	Infection of macrophages	None	Regulation of expression, Vir subassemblies
Helicobacter pylori	Cag	Infection of gastric cells	CagA, peptidoglycan	T4S contact with β1 integrins
Legionella pneumophila	Dot/Icm	Infection of macrophages	DNA, multiple effectors	Polar secretion of substrates
				Temporal/spatial distribution of effectors
b) T4S systems involved in DNA transfer (present in plasmids or genomic islands)				
Agrobacterium tumefaciens pTi	VirB/D4	T-DNA transfer to plants	T-DNA, VirD2, VirD5, VirE2, VirE3, VirF	VirB10 structure/function analysis
				T4S helical localization pattern
Escherichia coli pKM101	Tra	Conjugative plasmid transfer	DNA, TraI[2]	3D structure of core complex
Escherichia coli R1	Tra	Conjugative plasmid transfer	DNA, TraI[2]	Helicase loading at relaxosome
Escherichia coli R388	Trw	Conjugative plasmid transfer	DNA, TrwC	Transfer through *Bartonella* T4SS
Haemophilus influenzae GI	Tfc	GI horizontal transfer	DNA	New family of T4S systems
Neisseria gonorrhoeae GI	Tra	DNA secretion and transfer	DNA, TraI[2]	Search for *oriT*; role of pilin
Streptococcus pIP501	Tra	Conjugative plasmid transfer	DNA, TraA[2]	Protein-protein interactions

[1] See text for details and labs reporting the results.

[2] Transport of the relaxase protein is assumed based on homology with other DNA transfer systems

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