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Anchoring the type VI secretion system to the peptidoglycan

TssL, TagL, TagP,… what else?

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The recently identified bacterial type VI secretion system (T6SS) has rapidly become one of the most interesting areas of research in microbiology. In a relatively short period of time the relationship between the T6SS and the bacteriophage T4 tail and baseplate has been established. However, a number of questions concerning the T6SS remain the focus of a large number of researchers worldwide. Key questions that need to be addressed include how this system assembles in the cell envelope and the mechanism by which it translocates effector proteins across two membranes, the identification of such effectors and their function, how this secretion system contributes to virulence, interbacterial interactions and/or adaptation to the environment, and the evolutionary relationship between T6SS machine and bacteriophage T4. Focused on how the proteins constituting the secretion system interact, we recently identified a sub-complex of the T6SS comprised of four cell envelope proteins: the inner membrane-anchored TssL, TssM and TagL proteins and the outer membrane-associated TssJ lipoprotein. We further demonstrated that the TagL subunit carries a specific domain allowing anchorage of the secretion system to the peptidoglycan (PG) layer. Herein, we discuss these results, examine whether PG-binding motifs are found within other T6SS subunits and express hypotheses regarding the role of PG-binding motifs in type VI secretion.

Type VI secretion systems (T6SSs) are macromolecular machines spanning the cell envelope in a large number of Gram-negative bacteria. T6SSs have been shown to play a role in pathogenesis in several bacterial species, including *Vibrio cholerae*, *Burkholderia pseudomallei* and *Pseudomonas aeruginosa*.[1-5] Although originally described as a virulence factor, the T6SS is now recognized as playing a role in interbacterial communication in some bacteria.[6,7] For example, it participates in bacteria-bacteria interactions by secreting bacterial toxins, by regulating biofilm formation or stress sensing.[8,9] T6SSs are composed of a minimum of 13 proteins, called "core-components" which assemble in the cell envelope to form the apparatus.[8] According to the current nomenclature proposed by Shalom et al. these core subunits were named Tss (for Type six secretion) whereas additional/auxiliary components were called Tag (for Tss-associated genes).[3] These proteins are predicted to localize in the cytoplasm, inner membrane, periplasm or outer membrane. A number of these core subunits are ancestrally related to proteins of tailed-bacteriophage, including proteins of the tail, the baseplate, the spike or the sheath. Among them, two proteins have been structurally characterized, TssD (Hcp) and TssI (VgrG).[5,10] This revealed that their polypeptide chains folded similarly to their bacteriophage counterparts.[11,12] Apart from the phage-like subunits, a number of components, essentially those anchored in the membranes, do not derive from bacteriophage proteins. Two of them, TssM and TssL, are related to proteins associated with type IVb secretion systems, namely IcmF and IcmH/DotU,

Table 1. Type VI secretion-associated subunits with peptidoglycan-binding domains

Case	Subunit	PG-binding domain (pfam family)	Organism/T6SS gene cluster
1	no PG-binding motif		*Vibrio cholerae*
			Edwardsiella tarda
			Aeromonas hydrophila
			P. aeruginosa/HSI-2
			P. aeruginosa/HSI-3
			Enteroaggregative *E. coli* 042/Sci-2
			Burkholderia pseudomallei/Tss5
			Myxococcus xanthus
			Pectobacterium atrosepticum
2	TssL	OmpA (PF00691)	*Salmonella enterica*
			Agrobacterium tumefaciens
			Pseudomonas aeruginosa/HSI-1
			Yersinia pseudotuberculosis/Yps1
			Yersinia pseudotuberculosis/Yps3
			Rhizobium leguminosarum
			Burkholderia pseudomallei/Tss3, Tss4, Tss6
3	TssL	SPOR (PF05036)	Marinomonas sp. MED121
			Vibrio harveyi HY01
4	TagL	OmpA (PF00691)	Enteroaggregative *E. coli* 042/Sci-1*
			Yersinia pseudotuberculosis/Yps2
			Yersinia pseudotuberculosis/Yps4
			Klebsiella pneumoniae
			Enterobacter sakazawii
			Enterohaemorrhagic *E. coli* Sakai
			Uropathogenic *E. coli* CFT073
			Burkholderia pseudomallei/Tss2
5	TagP	OmpA (PF00691)	*Pseudomonas putida* KT2440
			Pseudomonas syringae B728a
6	TagN	OmpA (PF00691)	*Burkholderia pseudomallei*/Tss1
			Ralstonia solanacearum
6	TagW	OmpA (PF00691)	*Vibrio parahaemolyticus*

The EAEC Sci-1 TagL [indicated by *] is the only protein characterized in terms of PG-binding.[15]

respectively.[13] The others, including the TssJ outer membrane lipoprotein, have no homologs in other secretion or macromolecular systems.[14] It is thus believed that the T6SS results from the assembly of two sub-complexes, one derived from the bacteriophage, and the second resulting from the interaction between the membrane proteins.

In the recent study by Aschtgen et al. we identified a complex composed of at least four proteins: TssM, TssL, TagL and TssJ in enteroaggregative *Escherichia coli* (EAEC).[15] The first three proteins are anchored in the inner membrane through one or several trans-membrane helices. TssJ is a periplasmic protein anchored to the outer membrane by lipidation. TagL is particularly interesting since its C-terminal periplasmic region is homologous to a specific domain of the PF00691 family (OmpA/MotB/Pal).[16,17] This domain is well known to specifically interact with the peptidoglycan (PG), and the three-dimensional structures of proteins carrying this domain, including OmpA, Pal, MotB and RmpM are available.[18-20] We have shown that TagL interacts in vivo and in vitro with the peptidoglycan (PG) and is therefore sufficient to anchor the T6SS to the cell wall. Using targeted mutagenesis and phenotypic characterization, we then showed that the anchorage of the T6SS to the cell wall is absolutely required for its function. Interestingly, TagL is not a core component shared by all T6SSs. The observations that TagL is not conserved in T6SSs but that PG-binding is required for T6S were contradictory. We present below in silico analyses aimed at defining whether PG-binding domains are critical modules in T6SS.

As summarized in **Table 1 and Figure 1**, we have shown that the OmpA/MotB/Pal PG-binding domains can be carried by various T6SS-associated subunits, including TssL. We propose to call these chimeric TssL-PG proteins "evolved TssL proteins." Many of the T6SS gene clusters that specify the "ancestral TssL" (i.e., TssL that is devoid of the PG-binding domain), a PG-binding module can be carried by an additional subunit encoded within the same gene cluster: TagL, TagP, TagN or TagW. Interestingly, the TagL protein and the "ancestral TssL" encoded within the EAEC *sci-1* T6SS cluster interact directly. From an evolutionary perspective, it suggests that "evolved TssL proteins" may have derived from a reduction of the gene content, by fusing the *tssL* gene with the 3' fragment of *tagL* (fusion hypothesis). Conversely, "evolved TssL proteins" may have been split to raise two different subunits, TssL and TagL (split hypothesis). However, genetic and in silico analyses support the former hypothesis. First, we recently engineered a TssL-TagL fusion protein, comprising the full-length TssL protein fused to the periplasmic, PG-binding, domain of TagL (Aschtgen MS and Cascales E, unpublished data). This fusion protein was able to complement a double *tssL-tagL* deletion mutant, suggesting that the first ~300 amino-acids of TagL have no essential function in T6S, and thus could have been eliminated during evolution. Second, the presence of TagL or of "evolved TssL" proteins is not restricted to portions of the global phylogeny (as examples, TagL proteins are shared by *Escherichia coli*, Klebsiella and Yersinia species, whereas no TagL is present in Salmonella, which is a closely

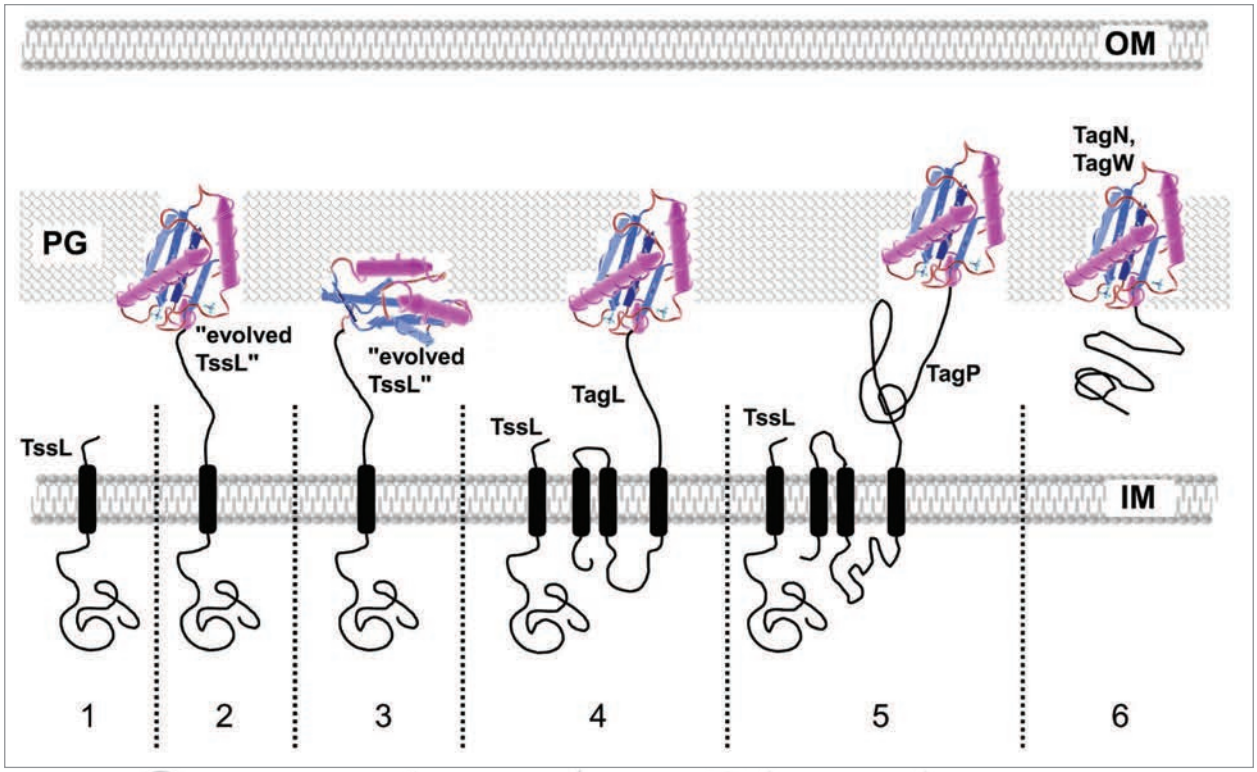

Figure 1. The various cases of PG-binding proteins and combinations found in T6SSs. Case 1: no PG-binding domain on TssL (green in Fig. 2); case 2: TssL proteins carrying a PG-binding domain of the PF00691 family (OmpA/MotB/Pal; yellow in Fig. 2); case 3: TssL proteins carrying a PG-binding domain of the PF05036 family (SPOR); case 4: TssL interacting with TagL (SciZ), TagL carrying the PG-binding domain (pink in Fig. 2); case 5: TssL interacting with TagP (contains the N-terminal cytoplasmic domain and flanking transmembrane helices of TssM fused to the PG-binding domain); case 6: TagN/TagW carrying the PG-binding domain. The localizations and topologies of TssL, TssM and TagL have been determined experimentally.[15,24] The localizations of TagN and TagW are putative. The structures of the PG-binding domains are those of Pal (OmpA/MotB/Pal family [PF00691]; accession 1OAP) and of FtsN (SPOR family [PF05036]; accession 1UTA). OM, outer membrane; IM, inner membrane; PG, peptidoglycan.

related strain to *E. coli*). However, when placed in the T6SS phylogenetic tree,[21,22] it clearly appears that "evolved TssL" and TagL are not randomly distributed, but are rather restricted to distinct families of T6SS gene clusters (see **Fig. 2**). The A and B families contain the "evolved TssL" proteins, whereas TagL is only found in the C and D families. Because A/B and C/D are the two first ramifications in the tree, this means that the fusion or the split is an ancient event in T6SS evolutionary history. However, TagL is distributed among both the C and D families. If it remains difficult to imagine that T6SS gene clusters with different evolutionary histories may have acquired *tagL* genes independently, it is easy to imagine that various T6SS gene clusters may have lost segments with no function. We thus support the fusion hypothesis, in which TagL was originally present, and was then lost following separation of the A/B and C/D families.

The PG-binding domain can be carried by other putative T6SS subunits, TagP, TagN or TagW (**Table 1**). TagP is a TssM derivative in which much of the large C-terminal periplasmic domain has been substituted by the PG domain. Thus, it is a composite protein comprising the N-terminal domain of TssM. TssM proteins have been characterized in *Edwarsiella tarda* and *Agrobacterium tumefaciens*: TssM is a three-transmembrane spanning segment inner membrane protein, directly interacting with TssL by its N-terminal domain.[23,24] Because N-terminal domains are conserved in TagP and TssM, one may hypothesize that TagP would interact with TssL. If this remains to be tested, it highlights the intimate link between TssL and the peptidoglycan. TagN is only found in the T6SSs of a limited number of genera, but it is prevalent in the Burkholderiaceae, including *B. pseudomallei tss1* and the *Ralstonia solanacearum* T6SS.[3] In some members of the genus Vibrio, a large protein (TagW) that possesses a predicted PG-binding domain located in the central region of the polypeptide is encoded adjacent to the T6SS gene cluster. However, it remains to characterize TagN and TagW in terms of localization/topology, PG-binding, interaction with TssL and to check whether TagN and TagW are bona fide T6SS subunits. In almost all cases, the presence of the Tag proteins possessing PG-binding domains reflects the presence of an "ancestral TssL" in the corresponding T6SS. An alignment of PG-binding domains present in evolved TssLs, TagL, TagN, TagP and TagW is shown in **Figure 3**.

"Evolved TssL proteins" may carry another domain, called SPOR (PF05036) (**Table 1 and Fig. 1**). Similar to the OmpA/MotB/Pal PF00691 domain, the SPOR domain is able to interact with the PG layer.[25] However, localization studies recently showed that this domain localizes at the septum, suggesting that it interacts

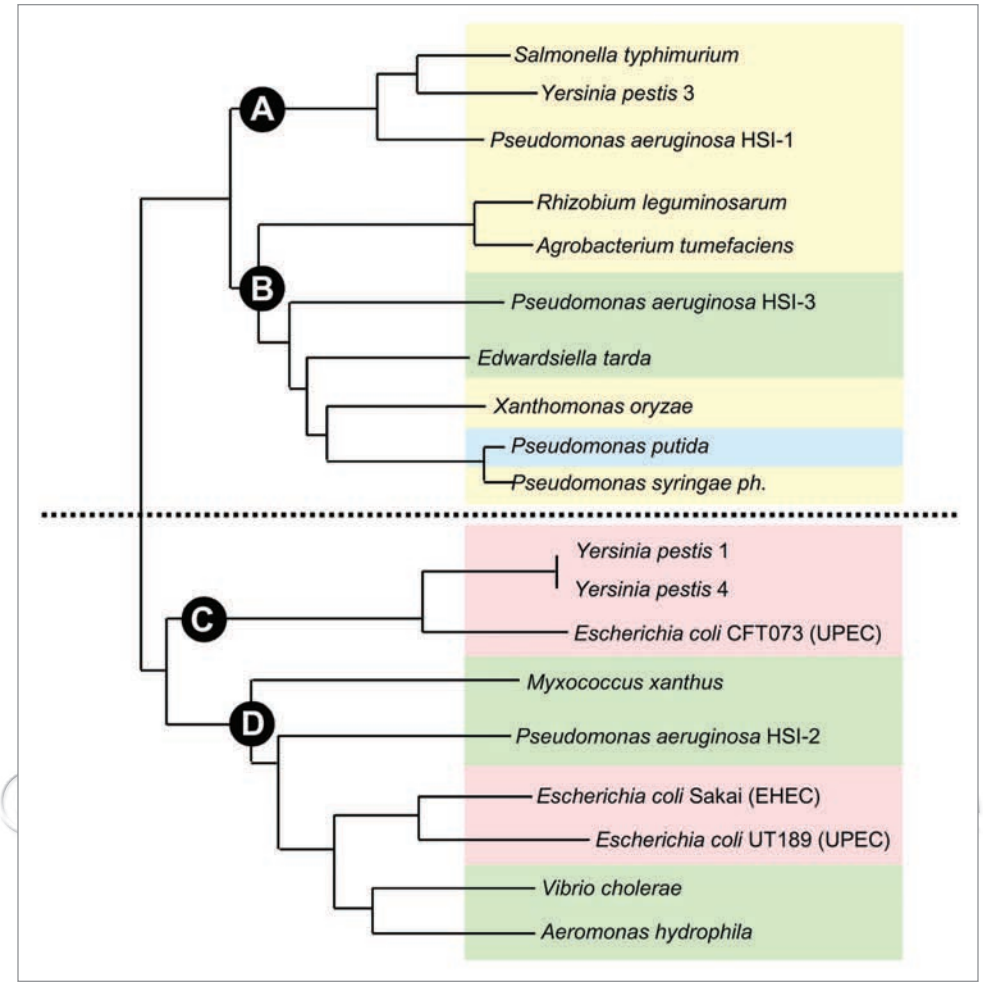

Figure 2. Relationships between PG-associated subunits and T6SS phylogeny. The PG-associated proteins found in the different classes defined by the T6SS phylogeny proposed by Bingle et al.[21] are indicated by a color code (green, no PG-binding domain; yellow, TssL-PG chimera; pink, TssL + TagL; blue, TssM-PG chimera). The figure highlights the observation that TagL proteins are only found in the C and D families, suggesting that the loss of *tagL* in the A and B families (or the acquisition of *tagL* in families C and D) is an ancient event.

with a newly-synthesized PG present at the cell division site.[25-27] Although the mechanistic basis for this recognition is unknown and that it remains to be tested whether these TssL-SPOR fusion proteins localize at the septum, this observation suggests that the TssL proteins may be biogenesis factors, with the PG-binding motif controlling the site of assembly. Several other PG-binding modules have been described, particularly in other secretion systems such as in the T3SS of *Salmonella typhimurium* or the PF01471 domain in the ExeA protein of the *Aeromonas hydrophila* T2SS.[28-30] However, none of these domains are found in T6SS subunits.

What can be the function of PG binding? First, as discussed above, PG-binding of various families can target the T6SS apparatus at specific sites of the cell, and thus may act as localization factors. Second, PG-binding might be important to stabilize the apparatus in the cell envelope. This can help to consolidate the secretion system by providing a strong anchor to the cell wall. Another type of stabilization is to enable wide conformation modifications to occur, without affecting the overall structure of the apparatus. For example, flagella are strongly anchored to the cell wall by PG-binding motifs carried by MotB that allows the rotation of the flagellum to occur.[31] In the Tol-Pal system, TolA undergoes structural modifications which can be stabilized by Pal binding to the PG.[32] However, as noted in **Table 1**, a number of T6SSs apparently do not carry PG-binding modules. This is the case for *Vibrio cholerae* or *Edwardsiella tarda*. Here again, looking back at the T6SS phylogeny (**Fig. 2**), these T6SS gene clusters are distributed in each family, suggesting that upon the separation between *tagL*-containing clusters and evolved-*tssL* clusters, PG-binding domains could have been lost in each ramification. How these T6SS are anchored to the peptidoglycan or how they deal with this loss of function remains to be answered.

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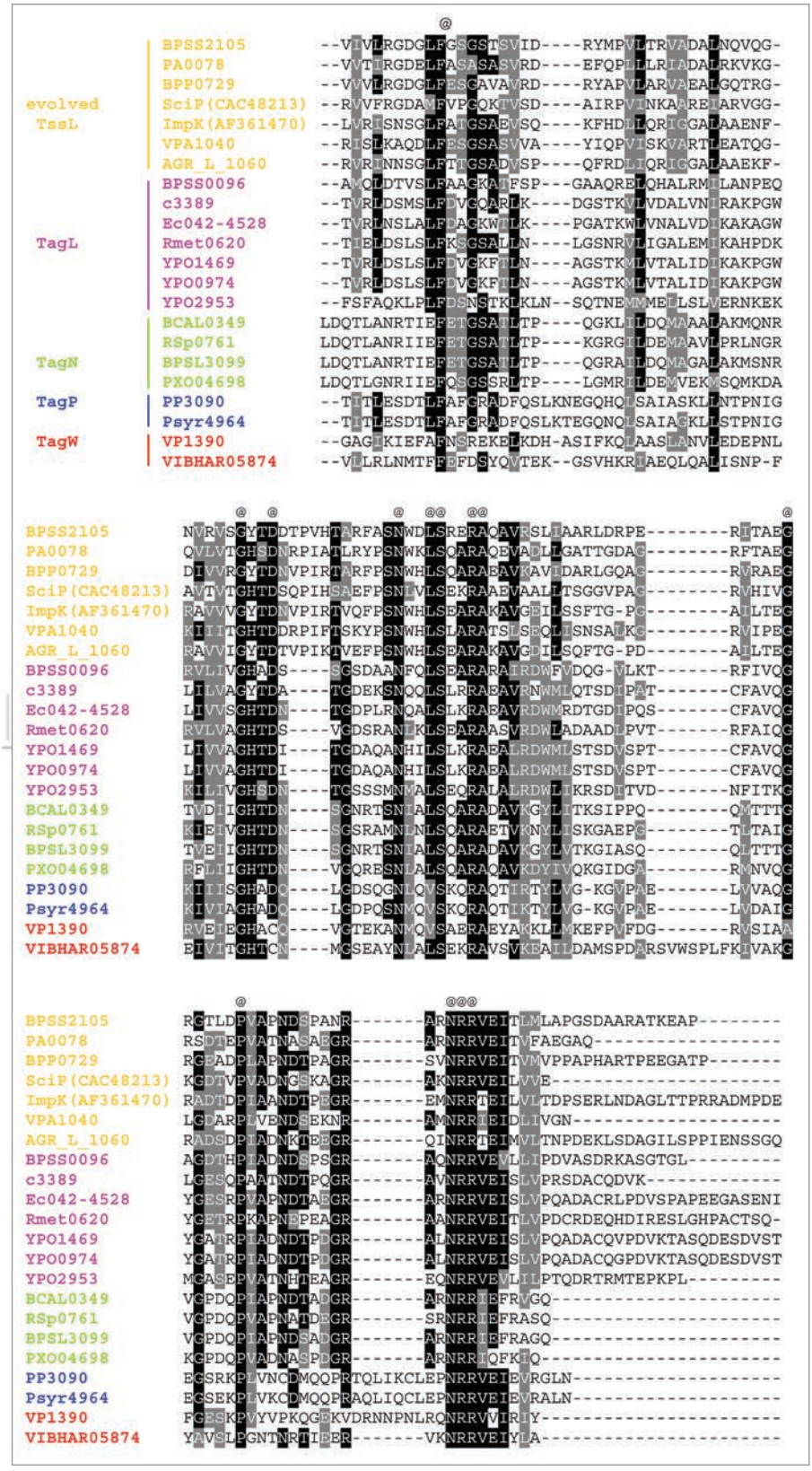

Figure 3. Sequence alignments of PG-binding domains present in evolved TssLs, TagL, TagN, TagP and TagW. Residues of the PG-binding motif consensus are indicated by @.

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