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An ABC-transporter and an outer membrane lipoprotein participate in posttranslational activation of type VI secretion in *Pseudomonas aeruginosa*

Maria G. Casabona^{*,1,2,3,4}, Julie M. Silverman^{*,5}, Khady M. Sall^{1,2,3,4}, Frédéric Boyer⁶, Yohann Couté^{3,4,7}, Jessica Poirel^{1,2,3,4}, Didier Grunwald^{1,2,3,4}, Joseph D. Mougous⁵, Sylvie Elsen^{1,2,3,4}, and Ina Attree^{1,2,3,4,#}

¹INSERM, UMR-S 1036, Biology of Cancer and Infection

²CNRS, ERL 5261, Bacterial Pathogenesis and Cellular Responses

³UJF-Grenoble 1, Grenoble, F-38041, France

⁴CEA, DSV/iRTSV, Grenoble, F-38054, France

⁵Department of Microbiology, University of Washington, Seattle, WA 98195

⁶Laboratoire d'Ecologie Alpine, UMR CNRS 5553, Université Joseph Fourier, F-38041 Grenoble, France

⁷INSERM, U1035, Laboratoire Biologie à Grande Echelle, F-38054 Grenoble, France

Abstract

Pseudomonas aeruginosa is capable of injecting protein toxins into other bacterial cells through one of its three type VI secretion systems (T6SS). The activity of this T6SS is tightly regulated on the posttranslational level by phosphorylation-dependent and -independent pathways. The phosphorylation-dependent pathway consists of a Thr kinase/phosphatase pair (PpkA/PppA) that acts on a forkhead domain-containing protein Fha1, and a periplasmic protein, TagR, that positively regulates PpkA. In the present work, we biochemically and functionally characterize three additional proteins of the phosphorylation-dependent regulatory cascade that controls T6S activation: TagT, TagS and TagQ. We show that similar to TagR, these proteins act upstream of the PpkA/PppA checkpoint and influence phosphorylation of Fha1 and export of Hcp1 and Tse1. Localization studies demonstrate that TagQ is an outer membrane lipoprotein and TagR is associated with the outer membrane. Consistent with their homology to lipoprotein outer membrane localization (Lol) components, TagT and TagS form a stable inner membrane complex with ATPase activity. However, we find that outer membrane association of T6SS lipoproteins TagQ and TssJ1, and TagR, is unaltered in a $\Delta tagTS$ background. Notably, we found that TagQ is indispensable for anchoring of TagR to the outer membrane fraction. As T6S-dependent fitness of *P. aeruginosa* requires TagT, S, R and Q, we conclude that these proteins likely participate in a trans-membrane signaling pathway that promotes H1-T6SS activity under optimal environmental conditions.

[#]Corresponding author: BCI, "Bacterial Pathogenesis and Cellular Responses", iRTSV, CEA-Grenoble, 17 rue des Martyrs, 38054 Grenoble cedex 09, France. Tel: 33 438783483; Fax: 33 438784499; iatreedelic@cea.fr.

^{*}contributed equally to this work

INTRODUCTION

Bacteria cope with their environment through an arsenal of secreted macromolecular products that are transported across the bacterial envelope by protein complexes called secretion systems. Gram-negative bacteria possess six secretion machineries, each of divergent composition and function (Bleves *et al.*, 2010, Desvaux *et al.*, 2009).

Pseudomonas aeruginosa, an opportunistic human pathogen associated with a variety of acute and chronic diseases, possesses five of these, including the types III and VI secretion systems (T3SS and T6SS) (Bleves *et al.*, 2010). A defining feature of these two protein export machines is that they allow for the export of cargo proteins directly into eukaryotic and/or prokaryotic cells (Cornelis, 2010, Schwarz *et al.*, 2010). While the composition and mechanism of the T3SS has been studied in great detail (Galan & Wolf-Watz, 2006, Mattei *et al.*, 2010), the existence of T6SSs has been described only recently and their function and composition are still largely unexplored.

T6SSs are encoded by genes organized in operons within genetic islands. In some instances, multiple T6SSs are present in a given bacterial genome (Boyer *et al.*, 2009, Cascales, 2008, Filloux *et al.*, 2008). Bioinformatics studies revealed that T6SSs are composed of 13 highly conserved core components and a set of additional proteins termed Tags (type six secretion-associated genes) (Boyer *et al.*, 2009, Shalom *et al.*, 2007). Two core components are similar to IcmF and DotU of T4SSs (Fronzes *et al.*, 2009, Ma *et al.*, 2009), and one protein, ClpV, belongs to a family of AAA+ ATPases (Bonemann *et al.*, 2009). A hallmark of all T6SSs is the presence of two conserved proteins, Hcp (haemolysin co-regulated protein) and VgrG (valine glycine rich G). These proteins share sequence and structural homology with tail tube and spike proteins of bacteriophage, respectively (Leiman *et al.*, 2009, Mougous *et al.*, 2006, Pukatzki *et al.*, 2007, Pukatzki *et al.*, 2009), and are proposed to form an injection device. Some specialized VgrG proteins possess C-terminal domains that contain eukaryotic cell effector activities. An example is VgrG-1 of *Vibrio cholerae*, which can cross-link host actin (Pukatzki *et al.*, 2007).

Hcp Secretion Islands I–III (HSI-I to III) of *P. aeruginosa* encode three potential T6SSs. The HSI-I-encoded T6SS (H1-T6SS) has been shown to be active in chronic *P. aeruginosa* infections, as sputum of chronically infected cystic fibrosis patients contains Hcp1 and the serum of these patients contains Hcp1-specific antibodies (Mougous *et al.*, 2006). Furthermore, a mutation in HSI-I operons affected the survival of *P. aeruginosa* in a rat model of chronic respiratory infection (Potvin *et al.*, 2003). The H1-T6SS specifically exports at least three proteins, Tse1, Tse2 and Tse3 (type VI secretion exported 1-3). These proteins are important for fitness in interbacterial competition assays (Hood *et al.*, 2010). While the target of Tse2 is not known, Tse1 and Tse3 are toxins targeting peptidoglycan of adjacent bacteria (Russell *et al.*, 2011).

As with other secretion systems of *P. aeruginosa*, the H1-T6SS is finely regulated at several levels. Goodman *et al.* have shown that HSI-I operons are posttranscriptionally regulated by the Gac/Rsm pathway. Two sensor kinase/response regulator hybrid proteins, RetS and LadS, reciprocally regulate the H1-T6SS through this pathway (Goodman *et al.*, 2004, Moscoso *et al.*, 2011). A second level of regulation is exerted directly on H1-T6SS activity and depends on a set of accessory genes within HSI-I (Figure S1). Among the proteins encoded by these genes are a transmembrane threonine protein kinase, PpkA, a PP2C-type phosphatase, PppA, and a periplasmic protein, TagR, which promotes dimerization-induced activity of the kinase (Hsu *et al.*, 2009, Mougous *et al.*, 2007). The cytoplasmic target of PpkA is a Forkhead Associated domain (FHA)-harboring protein, Fha1. Fha1 is in a complex with the ClpV1 ATPase, and upon phosphorylation of Fha1, effector export is triggered (Hsu *et al.*, 2009, Mougous *et al.*, 2007). This regulatory pathway will be referred

to hereafter as the threonine phosphorylation pathway (TPP; (Silverman *et al.*, 2011)). Reminiscent of T3SS activity, the intoxication of target cells by T6S effectors requires close cell-cell contact (Hayes *et al.*, 2011, Pettersson *et al.*, 1996). Interestingly, the TPP is stimulated when *P. aeruginosa* is grown on a surface, as observed by increased levels of phosphorylated Fha1 and Hcp1 secretion (Silverman *et al.*, 2011). These findings suggest that the TPP responds to specific physiological stimuli. In addition to this pathway, TagF, a protein encoded upstream of *pppA*, has been reported to function as a negative posttranslational regulator of the H1-T6SS that acts independently of the TPP (Silverman *et al.*, 2011).

Three uncharacterized genes, *tagT*, *tagS* and *tagQ*, neighbor genes involved in the TPP. In the current study, we found that the proteins encoded by these genes act upstream of PpkA in the TPP and are required for efficient protein transport through the T6S machinery. We demonstrated that in a heterologous host, TagT and TagS form a membrane-bound complex with ATPase activity, features characteristic of bacterial ABC-transporters. We also investigated the localization of TagQ and showed that its outer membrane localization requires a conserved cysteine within the lipo-box sequence. TagQ, but not TagTS, is required for association of the kinase activator, TagR, with the outer membrane fraction. These findings, together with *in silico* analysis of available genomes, illustrate the complexity and novelty of trans-membrane signaling that lead to tuning of T6SS activity.

RESULTS

TagT, TagS and TagQ participate in posttranslational regulation of the H1-T6SS

TagT, TagS and TagQ are non-conserved T6SS components (Boyer *et al.*, 2009) encoded within the HSI-I operon that contains each of the known posttranslational regulators of the system (*tagR*, *ppkA*, *pppA* and *tagF*) (Mougous *et al.*, 2007, Hsu *et al.*, 2009, Silverman *et al.*, 2011). Furthermore, conserved synteny of *tag* genes with *ppkA* and *pppA* in *Pseudomonas* species, *P. fluorescens*, *P. mendocina* and *P. brassicacearum* (<http://www.pseudomonas.com/>), suggests their functional relationship (Figure S1).

Our previous work on the H1-T6SS has shown that the basal activation of the system in wild-type cells is exceedingly low. Indeed, under planktonic conditions, the quantity of secreted Hcp1 is below standard detection levels. However, using more sensitive detection methods, we found that basal Hcp1 secretion levels can be distinguished from background levels observed in an H1-T6SS-inactive strain ($\Delta ppkA$) (Figure 1A). In this study, we utilize this basal level of Hcp1 secretion as a means to investigate genes involved in H1-T6SS activation. To evaluate the contribution of TagT, TagS and TagQ to T6S activity, Hcp1 secretion levels were assayed in strains with *tagT*, *tagS* or *tagQ* deletions. Secreted Hcp1 levels of these strains were compared to strains that abrogate Hcp1 secretion ($\Delta ppkA$ and $\Delta tagR$). As shown in Figure 1A, individual isogenic PAO1 mutants ($\Delta tagT$, $\Delta tagS$ and $\Delta tagQ$) were impaired in Hcp1 export.

The H1-T6SS exports three low-molecular weight effectors encoded by genes outside of the HSI-1 locus, Tse1, Tse2 and Tse3 (Hood *et al.*, 2010). To determine the effect of *tagT*, *tagS* and *tagQ* genes on Tse export, we analyzed secretion levels of Tse1 in the mutant strains. To detect Tse1, a chromosomal fusion of vesicular stomatitis virus G encoding sequence (VSV-G) to *tse1* (Tse1-V) was used (Hood *et al.*, 2010). As in the case of Hcp1, the quantity of secreted Tse1-V was decreased in strains lacking *tagT*, *tagS* and *tagQ*. To gain information regarding the functional hierarchy of the Tag proteins relative to the TPP, we examined Hcp1 and Tse1 export in mutants prepared in the $\Delta pppA$ background. Strains lacking both the *tag* genes and *pppA* did not display a decrease in Hcp1 secretion levels (Figure 1B), suggesting that these proteins represent regulatory accessory components that act upstream

of the kinase/phosphatase checkpoint. Interestingly, the deletion of *tagS* in strains lacking *pppA* reproducibly resulted in higher levels of exported Hcp1, but not Tse1, relative to *pppA* and the other *tag* genes. Possible explanations for this finding are discussed below.

Phosphorylation of Fha1 requires PpkA and is promoted by growing bacteria on solid medium (Silverman *et al.*, 2011). To determine if TagT, TagS and TagQ affect phosphorylation of Fha1, we assayed phosphorylated Fha1 (*p*-Fha1) levels in *tag* deletion strains. A chromosomal *fha1-VSV-G* fusion was used and *p*-Fha1 levels were detected by electrophoretic mobility shift (Figure 1C). Consistent with previous studies, we observed an increase in *p*-Fha1 levels in the wild-type background when grown on solid versus liquid media. Interestingly, individual *tag* deletion strains abrogated surface growth-dependent *p*-Fha1 levels. As expected, under liquid growth conditions, only low levels of *p*-Fha1 were detected in all strains containing PppA. Taken together with the secretion phenotypes observed, these results suggest that TagT, TagS and TagQ act upstream of PpkA in the TPP.

***tagT*, S and Q are required for T6S-dependent fitness**

Previous studies have shown that the TPP activates the H1-T6SS during surface growth and therefore is required for H1-T6S-dependent fitness against competing bacteria (Silverman *et al.*, 2011). We hypothesized that if TagT, S, and Q act upstream of PpkA in the TPP, these proteins should contribute to H1-T6SS-dependent fitness. Using growth competition assays, we assessed the fitness of donor strains containing in-frame deletion of *tagT*, *S*, or *Q* relative to Tse2-sensitive recipient strain ($\Delta tse2 \Delta tsi2$) (Figure 1D). This experiment confirmed that *tagT*, *S*, or *Q* are required for H1-T6SS-dependent fitness. As an additional control, we included a donor strain lacking TagR, a protein previously demonstrated to act upstream of PpkA in the TPP. This strain also displayed a loss of H1-T6SS-dependent fitness. Together with their involvement in promoting surface-dependent Fha1 phosphorylation, these findings support a critical role for TagT, S, and Q in TPP activation during surface growth.

TagT and TagS form a membrane-bound complex with ATPase activity

TagT and TagS share sequence signatures with bacterial ABC-transporters (Davidson *et al.*, 2008). The *tagT* gene encodes a protein of 26 kDa with Walker domains (Walker A and Walker B) and other conserved features of ATPases associated with ABC transporters as shown in Figure 2A. The *tagS* gene encodes a protein of 42 kDa, predicted to be an integral membrane protein with four hydrophobic transmembrane helices (TMH) and a long periplasmic segment of 233 amino acids between TMH1 and TMH2. BLAST analysis showed high homology of TagS and TagT with membrane components of the lipoprotein outer membrane localization (Lol) complex (Narita & Tokuda, 2006), sharing 56.0%/38.5% and 40.0% similarity with LolE/LolC and LolD of *P. aeruginosa*, respectively (Tanaka *et al.*, 2007) (Figure 2A). Based on sequence homology, the predicted TagT protein belongs to a family of MecA/FtsE/SalX ATPases of bacterial ABC-transporter exporters (see also Discussion).

To investigate the mechanism by which TagT, S and Q regulate the H1-T6SS through the TPP, we purified the proteins and conducted biochemical analyses. Co-production of TagS and TagT resulted in formation of a stable protein complex associated with *E. coli* membranes that could be solubilized by a detergent and purified to homogeneity by affinity chromatography (Figure 2B). As mentioned previously, TagT harbors all conserved signatures of classical ATPases. In order to test whether the TagTS complex is capable of ATP hydrolysis, the complex was incubated in the presence of ATP and magnesium and the formation of inorganic phosphate (Pi) was quantified by a malachite green method (Van Veldhoven & Mannaerts, 1987). Notably, the TagTS complex displayed significant ATPase activity varying, in three independent purifications, between 70 and 100 nmolPi/min/mg.

The specific activities of the TagTS complex was consistent with activities of several bacterial ABC transporters reported to date (Ward *et al.*, 2007, Torres *et al.*, 2009, Ravaut *et al.*, 2006). Moreover, the ATPase activity of the complex was sensitive to orthovanadate (Figure 2C), a small organic molecule that impedes ATP hydrolysis by interfering with binding of ATP to the Walker A motif (Pezza *et al.*, 2002). Finally, to confirm that ATP hydrolysis was due to the TagTS complex, we replaced a conserved amino acid within the Walker A motif of TagT (K/A: Lys 44 to Ala) and purified the complex (Figure 2B). The K/A mutation abolished ATPase activity of the TagTS complex (Figure 2C). In conclusion, these results clearly show that the TagTS complex possesses ATPase activity that is dependent on the conserved Walker A motif within the TagT ATPase.

TagQ encodes an outer membrane lipoprotein

The last gene of the operon, *tagQ*, encodes a 31.7 kDa protein with a stretch of hydrophobic and uncharged amino acids at the N-terminus. This sequence is characteristic of a signal peptide and conserved lipo-box with an invariable Cysteine, a sequence recognized by signal peptidase II (Babu *et al.*, 2006) (Figure 3A). In order to study the localization of TagQ in *P. aeruginosa*, we created a fusion protein between TagQ and the red fluorescent protein mCherry, and examined its localization by confocal microscopy using GFP-expressing *P. aeruginosa* strains (GFP TagQ-mCherry). TagQ-mCherry was readily detected and localized around the periphery of bacterial cells (Figure 3A). To gain information on the precise localization of TagQ, *P. aeruginosa* cells were treated with lysozyme to create spheroplasts, bacterial cells lacking the peptidoglycan layer. *P. aeruginosa* spheroplasts, while maintaining an intact internal membrane, appear to have a crescent-shaped external membrane as it begins to dissociate from the rest of the spheroplast (Lewenza *et al.*, 2008). When *P. aeruginosa* GFP TagQ-mCherry was treated to obtain spheroplasts, the majority of cells retained mCherry fluorescence in the crescent-shaped labeling pattern particularly visible under xyz scan (Figure 3A), strongly suggesting the association of TagQ with the outer bacterial membrane. Lipid modifications occur at a conserved cysteine within a lipo-box sequence of lipoproteins and promote its association with membranes. To test the requirement for Cys30 in TagQ-mCherry localization, we created a mutant fusion protein, TagQΔCys-mCherry, and checked its localization (Figure 3A). *P. aeruginosa* GFP TagQΔCys-mCherry cultures systematically showed a mixed population, with the majority of cells harboring mCherry at the periphery and some cells displaying overlapping cytoplasmic GFP and mCherry. This result suggests that the absence of Cys30 affects the efficient transport of the protein across the inner membrane. The majority of spheroplasts obtained from TagQΔCys-mCherry *P. aeruginosa* lost outer membrane labeling, strongly suggesting that in most cases the protein had lost its lipid anchor and was released by lysozyme treatment (Figure 3A). These observations were immuno-quantified by fractionation of membranes and periplasm of Δ*tagQ* strains ectopically expressing wild-type *tagQ* or *tagQΔCys* (Figure 3B). The deletion of Cys30 resulted in partial processing of the protein, as two anti-TagQ-specific polypeptides were visualized in total bacterial extracts. In addition, while the majority of wild-type TagQ was found to be associated with membrane fractions, the majority of the mutated protein was recovered in periplasmic fractions, in concordance with confocal microscopy observations.

The Lol-like TagTS complex is dispensable for OM localization of TagQ and TssJ1

As the TagTS complex shows significant homology with lipoprotein recycling systems of *E. coli* and *P. aeruginosa*, we first hypothesized that this complex is involved in the transport of specific lipoproteins of the H1-T6SS. To compare membrane distribution of TagQ in different strains, we set up membrane fractionation experiments on discontinuous sucrose gradients coupled to immuno-detection. These experiments were performed using *P. aeruginosa* Δ*retS* background to obtain higher expression of the whole HSI-1 locus. The

quality of separation between inner (IM) and outer membranes (OM) was systematically determined by measuring the activity of NADH oxidase and by Coomassie blue staining of SDS-PAGE gels of each recovered fraction. As shown in Figure 3C, in accordance with microscopy experiments, the majority of TagQ was found in OM fractions. In order to determine whether the Lol-like ABC-transporter TagTS was involved in localization of TagQ, we fractionated membranes of $\Delta retS \Delta tagST$ in an identical manner and detected no significant difference of TagQ distribution between the two strains (Figure 3C and S2 for complete gradients). The second lipoprotein of the H1-T6SS is TssJ1 (PA0080). TssJ1 shares 50% similarity with OM lipoprotein SciN involved in assembly of the *sciI* T6SS of enteroaggregative *E. coli* (Aschtgen *et al.*, 2008). To ascertain whether TssJ1 is targeted to the OM in *P. aeruginosa*, we constructed a fusion between TssJ1 and mCherry, and examined its localization by confocal microscopy (not shown) and by fractionation on sucrose gradients. Similar to our findings with TagQ, TssJ1-mCherry associated with the OM and its localization was not significantly altered in the *tagTS* mutant (Figure S3). Together these results show that the ABC-transporter TagTS, despite its strong homology to the Lol system, does not participate in membrane targeting of two H1-T6SS specific lipoproteins.

TagQ is required for TagR association with the OM

Preliminary nanoLC/LC mass spectrometry data performed on inner and outer membrane fractions (Casabona and Couté, unpublished) indicated the presence of TagR, a positive regulator of the TPP, in OM fractions. This result was intriguing, as TagR is predicted to be a soluble protein and was shown to fractionate with the periplasm, wherefrom it promotes dimerization and activation of PpkA (Hsu *et al.*, 2009). To further address the localization of TagR, we raised anti-TagR antibodies and analyzed fractionated membranes from wild-type PAO1, confirming that at least one portion of TagR associated with outer membranes (Figure 4). This association was significant, as a soluble periplasmic protein, DsbA, was not found in any of the membrane fractions (not shown). Whereas TagR OM localization was not altered in a *tagTS* mutant (Figure S4), the absence of *tagQ* clearly influenced the distribution of TagR between inner and outer membrane fractions (Figure 4). In accordance, a strain expressing *tagQ* Δ *Cys* *in trans*, resulted in TagR mislocalization, demonstrating that the OM-anchored TagQ is essential for OM localization of TagR. Of note, TagR was found dispensable for OM localization of the TagQ lipoprotein (Figure S5).

In silico genome wide analysis of TagTSR-like systems

Participation of the TagTS complex in trans-membrane signaling involving a periplasmic protein TagR and an inner membrane-bound Ser/Thr kinase prompted us to perform *in silico* analysis of all available complete bacterial genomes to search for homologs of these proteins. Both TagT and TagS attributed COGs (COG1136-COG4591) are frequently adjacent to other ABC-transporter-specific COGs involved in peptides and drugs transport, specifically COG0845 and COG0577 which are membrane components of the AcrA and SalY family of multidrug efflux pumps and antimicrobial peptides transport systems, respectively. We found also two strong associations between COG1136-COG4591 tandem and regulatory partners. In 90 *Firmicutes*, ABC-transporters are encoded adjacent to homologs of the OmpR family two-component regulatory system (COG0642 and COG0745). In addition, in 100 *Enterobacteriaceae*, ABC-transporters are encoded upstream of NagC (COG1940), a transcriptional regulator involved in sugar transport. This observation suggests a more general functional relationship between the TagTS family of ABC-transporters with trans-membrane signaling and regulation. Finally, we examined the local organization of chromosomal regions encoding the ATPase component (TagT, COG1136), the predicted permease component (TagS, COG4591) and COG1262 (TagR) in all available microbial genomes. TagQ was excluded from this screen as it lacks an

attributable COG number. As shown by a maximum likelihood tree (Figure 5), *tagT*, *S* and *R* are predominately found within T6SS encoding loci in Pseudomonads (*P. aeruginosa*, *P. fulva*, *P. fluorescens*, *P. brassicacearum*). We found that in *Rhodobacter sphaeroides*, an α -proteobacterium that possess a T6SS locus with similar gene content of HSI-I, *tagT*, *S* and *R* are neighboring genes coding for PpkA and PppA homologs. However, *tagT*, *S* and *R* are not exclusively found in bacterial genomes encoding T6SSs, suggesting that these components may play a role in different cellular processes.

DISCUSSION

Our current study identified TagT, TagS and TagQ as new components of a posttranslational regulatory pathway that modulates the activity of the H1-T6SS of *P. aeruginosa*. We found that these components participate in a phosphorelay system that is stimulated by surface growth conditions. Phosphorelay systems are classical ways for bacteria to regulate adaptive cellular responses induced by external stimuli; however, to our knowledge, this is the first example of an ABC transporter complex, TagTS, participating in trans-membrane signaling that involves a Ser/Thr kinase-dependent phosphorylation pathway.

Some parallels can be made with a large family of “co-sensor” ABC-transporters that interact with membrane-integrated histidine kinases (HK) (Tetsch & Jung, 2009, Tetsch *et al.*, 2008). These proteins clearly play a regulatory role in adaptive responses to certain environmental stimuli (Tetsch *et al.*, 2008). This is well illustrated by the ABC-transporter PstSCAB of *E. coli* that is linked to sensing inorganic phosphate in phosphate-limiting conditions. It has been proposed that the membrane components of this ABC-transporter transmit the signal toward the membrane-integrated HK, PhoR, which signals to a transcriptional regulator (Makino *et al.*, 1989). A role for related systems in resistance against antimicrobial peptides was recently proposed (Coumes-Florens *et al.*, 2011, Dintner *et al.*, 2011). For example, it has been experimentally demonstrated in *Staphylococcus aureus* that the detection of, and response to, antibiotic peptide bacitracin requires the interplay between two ABC-transporters, BraD/BraE and VraD/VraE, and the HK/RR system BraS/BraR (Hiron *et al.*, 2011). Interestingly, our *in silico* analysis showed that genes encoding TagTS like proteins (COG1136-COG4591) frequently co-occur with genes encoding proteins that participate in two component-regulatory systems. In addition, our *in silico* analysis highlights the association of TagTS-like proteins with NagC, a transcriptional regulator involved in the response to N-acetylglucosamine and peptidoglycan in *E. coli* and *P. aeruginosa* (Korgaonkar & Whiteley, 2011, Pennetier *et al.*, 2008), further implying their participation in signal recognition and transmission. It is worth noting that in at least one non-*Pseudomonas* T6SS, *Vibrio anguillarum*, an inner membrane polypeptide belonging to a major facilitator superfamily of transporters is required for regulating Hcp export and two additional periplasmic proteins contribute to this signaling (Weber *et al.*, 2009).

The TagTS complex could be involved in export or import of a small molecule required for activating the H1-T6SS, or it may play a structural role by stabilizing other Tag proteins. Future experiments aim to determine whether TagT ATPase activity and a long periplasmic loop present in TagS are required for its role in signaling. The apparent conflicting effects on Hcp1 export between $\Delta pppA \Delta tagS$ versus $\Delta pppA \Delta tagST$ (Figure 1) suggest a possible second role for the integral inner membrane domain of TagS, independent of its ATPase partner, TagT. Interestingly, a long periplasmic loop present in TagS homologues, LolC and BraB, mediates the detection of cognate substrates, lipoproteins and bacitracin, respectively and it was proposed, for BraB, to interact through the inner membrane with the HK partner.

Outer membrane-localized TagQ is a top candidate for signal detection. The region from amino acids 68 to 114 of TagQ is annotated as PF05433, a family of proteins that include

several *Rickettsia* genus specific 17kDa surface antigens, annotated also as a conserved trans-membrane alpha-helical region containing glycine zipper motifs (<http://pfam.sanger.ac.uk>). There is rising evidence that OM lipoproteins play crucial roles in trans-membrane signaling in bacteria. A well characterized example is the Rcs phosphorelay in *E. coli*, which reflects envelope stress response activated with peptidoglycan stress and antibiotics. In this complex system, RcsF, an OM lipoprotein, is proposed to transmit the signal to the IM-located His-kinase sensor and allow further signal transduction from the cell envelope to the cytoplasm (Farris *et al.*, 2010, Leverrier *et al.*, 2011).

What may be the link between IM TagTS, OM TagQ and the IM PpkA-kinase? The finding that some portion of TagR is also associated with the OM indicates that TagTS, TagR and TagQ may represent a unique complex participating in trans-membrane signaling. Taking into account the sequence predictions for TagR, its role in signal transduction, and our current findings, we hypothesize that TagR associates with membranes through interactions with other OM proteins, such as TagQ. The distribution of TagR between inner and outer membranes was clearly affected by the absence of TagQ, leaving open the possibility that TagQ may correctly position TagR to interact with the kinase.

Finally, what is the nature of the signal(s) that activate the H1-T6SS? Silverman *et al.* discovered that surface growth of *P. aeruginosa* induces the TPP (Silverman *et al.*, 2011) and, here, we show that *tagQ*, *tagR*, *tagS* and *tagT* are important in surface-induced phosphorylation of Fha1. Moreover, using growth competition assays we showed that the fitness of Tag mutants was impaired. All together these results imply that the predicted ABC-transporter, TagTS, and the OM lipoprotein TagQ are essential players in intra-bacterial communication through H1-T6SS activity. The rapid detection of neighboring cells may thus be essential in turning on the injection device.

Although we have no evidence yet demonstrating direct interactions between the four Tag proteins (except for TagT and TagS) and the kinase, PpkA, we propose a model (Figure 6) in which TagTS and TagQ participate in detection and transmission of an environmental signal to PpkA, by modulating either localization or conformation of the kinase activator TagR, leading to rapid response of «attacking» bacteria in highly competitive multi-species niches, such as infecting tissue. Our future work will aim at discovering the signal nature and deciphering the connections between Tag proteins and their link with other components of trans-membrane signaling in T6SS.

EXPERIMENTAL PROCEDURES

Genetic constructions

Genes of interest were PCR amplified using PAO1 genomic DNA as a template, cloned into pCR-Blunt II-TOPO (Invitrogen) and sequenced before cloning into final destination vectors. The list of oligonucleotides with appropriate restriction sites is given in the Supplementary Table 1. For overexpression, *tagT* and *tagTS* were amplified and cloned into pETDuet-1. In the pETDuet-*tagTS* bicistronic vector, only *tagT* is fused to a histidine tag encoding sequence. For Hcp1 overexpression, the gene was cloned into pET52b giving Hcp1-Strep fusion. The *tagQ* gene was amplified so that it lacks the sequence encoding the first 29 amino acids and the predicted lipo-box (TagQ Δ 2-30). It was cloned into pET15b by fusing *tagQ* to a sequence encoding hexa-histidine tag on N-terminus. For TagR overproduction, the *tagR* gene was amplified and cloned into pET52b resulting into TagR-His10 protein. All fusions to *mCherry* were constructed using pJN105-derived plasmids, which contain an arabinose inducible promoter. The gene encoding mCherry was amplified and cloned into *Xba*I and *Sac*I sites of pJN105 (Newman & Fuqua, 1999) giving pJN-*mCherry*. The DNA sequence containing the ribosome binding site (RBS) of *tagQ* and the

whole gene was cloned upstream of *mCherry*-encoding gene using *EcoRI* and *XbaI* sites, giving pJN-*tagQ-mCherry*. *tssJ1*-encoding sequence was amplified and cloned in the same manner to give pJN-*tssJ1-mCherry*. Site-directed mutagenesis (QuikChangeII Site-Directed Mutagenesis kit, Stratagene) was employed to generate TagT^{K/A}S mutant and TagQΔCys, using pETDuet-*tagTS* and pJN-*tagQ-mCherry* as templates, respectively. The TGA stop codon was introduced between TagQ and mCherry encoding sequence by site directed mutagenesis using pJN-*tagQ-mCherry* and pJN-*tagQΔCys-mCherry*. To constitutively express green fluorescent protein (GFP), pX2-*gfp* (Thibault *et al.*, 2009) was transferred in mini-CTX1 (Hoang *et al.*, 2000) and inserted into the chromosome of *P. aeruginosa* strains as described. All replicative plasmids were introduced in *P. aeruginosa* strains by transformation. Constructs used to generate in-frame deletions of *tag* genes or a *tseI-VSV-G* chromosomal fusion were previously reported (Silverman *et al.*, 2011).

P. aeruginosa strains were grown in Luria-Bertani (LB) medium at 37°C supplemented with carbenicillin 100-250 μg mL⁻¹, gentamycin 200 μg mL⁻¹ and tetracycline 200 μg mL⁻¹ when needed. *E. coli* strains were grown in LB medium at 37°C supplemented with ampicillin 100 μg mL⁻¹, gentamycin 50 μg mL⁻¹, kanamycin 25 μg mL⁻¹, tetracycline 10 μg mL⁻¹ as required.

Fha1 phosphorylation assays

Cellular samples of Fha1 from liquid and solid grown cultures were prepared and analyzed as previously described (Hsu *et al.*, 2009, Mougous *et al.*, 2006)). Western blots were developed using chemiluminescent substrate (SuperSignal West Pico Substrate, Thermo Scientific) and imaged with a FluorChemQ (ProteinSimple). Densitometry was performed as previously described (Silverman *et al.*, 2011) using AlphaView®Q software (ProteinSimple). The percentage of phosphorylated Fha1 was determined by measure band intensity of phosphorylated and total Fha1 from three independent experiments. The values were normalized to Δ*ppkA*, which was set at 0% *p*-Fha1.

Interbacterial growth competition assays and quantification

Growth competition assays were performed as previously described (Silverman *et al.*, 2011). Each donor and recipient strain contained constitutively expressing yellow fluorescent protein (YFP) or cyan fluorescent protein (CFP), respectively. pUCP18-mini-Tn7 containing *yfp* or *cfp* (inserted at the neutral phage attachment site, *attB*) was used to construct these strains (Lambertsen *et al.*, 2004). The plasmids were introduced into *P. aeruginosa* via four-parental mating conjugation or electroporation (Choi & Schweizer, 2006). Vector backbones were not removed. To observe a H1-T6SS-dependent fitness advantage, Tse2 and Tsi2 were overexpressed in the donor strain. Donor strains harbored pPSV18::PA2702-PA2703 for constitutive expression of Tse2 and Tsi2, and recipient strains harbored the empty vector, pPSV18 (Rietsch *et al.*, 2004). Overnight cultures were mixed at a 1:1 ratio to a total density of approximately 1.0×10⁸ CFU mL⁻¹ in 1mL LB medium. Competitions were grown on 0.2μM polycarbonate membranes on LB agar for 18h at 37°C, or in 2mL of LB with shaking. Cells were resuspended in LB medium and spotted onto 1.0% agarose PBS pads and imaged as described (Silverman *et al.*, 2011). YFP and CFP filters were used to image the two cell populations. Assays were performed in triplicate. Three fields containing 100 to 200 cells were imaged for each competition. To determine the competitive index (the number of YFP positive cells to CFP positive cells), NIS-Elements computer-assisted morphometry was used to count YFP and CFP cells.

TagTS expression and protein purification

E. coli BL21(DE3)Star cells harbouring pETDuet-*tagTS* were grown in LB medium at 37°C and 200 r.p.m. At OD₆₀₀ of 0.7, the expression of *tagT* and *tagS* was induced with 0.5 mM

IPTG for 4h. Cells were recovered by centrifugation and lysed in buffer containing 25 mM Tris-HCl, 500 mM NaCl, 10 mM imidazole, pH 8.0 and cocktail of protease inhibitors (PIC, Roche). The lysis was achieved by passage of cells through a Microfluidizer (M-100P, Microfluidics, USA) at constant pressure of 10,000 psi. After centrifugation at $200,000 \times g$ for 1h, membranes were recovered in solubilisation buffer containing 25 mM Tris-HCl, 500 mM NaCl, 2% n-dodecyl- β -D-maltopyranoside (DDM), 15% glycerol, pH 8.0 and PIC. The solubilisation was performed in a glass beaker at 4°C for 1h 30 min by agitation. The obtained suspension was further centrifuged at $200,000 \times g$ for 1h. Solubilised material was loaded at 0.5 mL/min on HisTrapHP Ni²⁺-column previously equilibrated in solubilisation buffer. The washes and elution was performed on AktaPurifier FPLC apparatus (GE Healthcare) with buffer A (25 mM Tris-HCl, 500 mM NaCl, 0.05% DDM, 15% glycerol, pH 8.0) and buffer B (25 mM Tris-HCl, 500 mM NaCl, 0.05% DDM, 15% glycerol, 200 mM imidazole, pH 8.0). The washes were performed with 30 and 80 mM imidazole obtained by mixing buffers A and B, and elution was performed using buffer B. Fractions eluted from the column were analyzed on SDS-PAGE and the gel was stained by Coomassie Brilliant Blue R-250.

ATPase activity and orthovanadate assay

ATPase activity was quantified by measuring inorganic phosphate Pi by a malachite green method (Van Veldhoven & Mannaerts, 1987). The reaction mixture (100 μ L) containing 0.5 mM ATP, 1 mM MgCl₂ and 10 mM Tris-HCl, pH 7.5, and 1 μ g of purified TagTS complex was incubated for 30 min at 37°C. When indicated, the orthovanadate was added to protein samples at indicated concentrations before the reaction. The reaction was stopped by adding 800 μ L of malachite green solution. Malachite green solution was prepared 30 min in advance by mixing 3 volumes of 0.045% malachite green with 1 volume of 4.2% ammonium molybdate in 4 M HCl and 1/50 of volume of Triton X-100. Greenish colour resulting from precipitation of Pi was measured at 640 nm.

Secretion assays

Overnight (O/N) cultures were diluted at OD₆₀₀ of 0.02 and incubated at 37°C with shaking up to mid-log phase, in the presence of antibiotics when needed. At this point, 250 μ L bacteria were harvested by centrifugation at $7,000 \times g$ and resuspended in 100 μ L of loading buffer and stored at -20°C until use. Extracellular proteins were precipitated by a TCA-sarkosyl method (0.5% final volume of sarkosyl and 7.5% final volume of TCA) (Chevallet *et al.*, 2007) after a double centrifugation of 1250 μ L of culture and 1h incubation on ice. Pellets were resuspended in a final volume of 25 μ L of loading buffer. Samples were then analyzed by SDS-PAGE and immunoblotting.

Proteomic analyses by mass spectrometry (MS)

Protein bands were manually excised from the gels and washed. Proteins were *in-gel* digested with trypsin (Promega, sequencing grade) and peptides extracted from gel slices (Shevchenko *et al.*, 1996). The dried extracted peptides were resuspended in 5% acetonitrile and 0.1% trifluoroacetic acid and analysed by online nanoLC-MS/MS (Ultimate 3000, Dionex and LTQ-Orbitrap XL, Thermo Fischer Scientific). The nanoLC method consisted in a 15 min gradient ranging from 5% to 40% acetonitrile in 0.1% formic acid at a flow rate of 300 nL min⁻¹. Peptides were sampled on a 300 μ m $\times$ 5mm PepMap C18 precolumn and separated on a 75 μ m $\times$ 150mm RP column (PepMap C18, Dionex). MS and MS/MS data were acquired using Xcalibur (Thermo Fischer Scientific) and processed automatically using Mascot Daemon software (version 2.3, Matrix Science). Searches against the PAO1 database, SwissProt-Trembl_decoy (*Escherichia coli* taxonomy) and contaminants databases (534637 sequences) were performed using an in-house version of Mascot 2.3. ESI-TRAP was chosen as the instrument, trypsin/P as the enzyme and 2 missed cleavages was allowed.

Precursor and fragment mass error tolerances were set respectively at 10 ppm and 0.6 Da. Peptide modifications allowed during the search were: Carbamidomethyl (C, fixed), Deamidated (NQ, variable), Oxidation (M, variable) and Acetyl (Protein N-term, variable). The IRMa soft (Dupierriis *et al.*, 2009) was used to filter the results by query homology threshold $p < 0.01$ and a minimum of 2 peptides per protein.

Spheroplast preparation and confocal microscopy

O/N cultures of *P. aeruginosa*-GFP strains producing TagQ-mCherry, TagQΔC-mCherry or TssJ1-mCherry fusion proteins were diluted at OD₆₀₀ of 0.15 in 3 mL LB. Cultures were incubated until mid-log phase of growth in the presence of antibiotics and then induced for 1.5 h by 0.25% arabinose. At this point, cells were harvested and spheroplasts were created (Imperi *et al.*, 2009). Briefly, 1 mL of culture was centrifuged at $6,000 \times g$ for 5 min. The pellet was resuspended in TSE buffer (0.1 M Tris-acetate, 16% saccharose, 5 mM EDTA, pH 8.2) and lysozyme was added at a final concentration of $50 \mu\text{g } \mu\text{L}^{-1}$. Spheroplasts were incubated on ice and centrifuged at $1,000 \times g$ for 5 min after the addition of MgSO₄ to a final concentration of 0.1 M. Finally, spheroplasts were resuspended in TSM buffer (0.05 M Tris-acetate, 8% sucrose, 10 mM MgSO₄, pH 8.2). 1 mL culture of arabinose-induced bacteria was harvested by centrifugation at $7,000 \times g$ for 5 min and resuspended in 100 μL of LB.

Specimens were analyzed by confocal laser scanning microscopy, using a Leica TCS-SP2 operating system (Manheim, Germany). GFP and mCherry fluorescences were excited and collected sequentially (400 Hz line by line) by using 488 nm for GFP and 543 nm for mCherry excitation. Fluorescence emissions were collected from 500 to 537 nm for GFP and from 557 to 625 nm for mCherry.

Fractionation of *P. aeruginosa*

Cultures of *P. aeruginosa* grown for 16 h were diluted to an OD₆₀₀ of 0.15 in 30 mL LB cultures with antibiotics when needed. Cultures were incubated with agitation until OD₆₀₀ of 0.85 and at this point, 100 μL cells were harvested by centrifugation as a total bacteria fraction. The rest of the cultures was centrifuged at $6,000 \times g$ for 10 min. Pellets were washed with 10 mL TMP buffer (10 mM Tris-HCl, 200 mM MgCl₂, PIC) and re-centrifuged at $6,000 \times g$ for 10 min. Pellets were resuspended in 1 mL TMP and incubated for 30 min at 300 r.p.m. and 23°C in the presence of 0.5 mg mL^{-1} lysozyme. Next, bacteria were centrifuged at $8,000 \times g$ for 15 min at 4°C, obtaining the periplasm fraction (supernatant) and spheroplasts (pellet). At this point, the proteins present in the periplasm fraction were precipitated (as described in Secretion assays). Spheroplasts were then resuspended in 1 mL TMP, recentrifuged and the pellet, recovered in 1 mL TM buffer (30 mM Tris-HCl, 10 mM MgCl₂, pH 8.0), was disrupted by sonication. Unbroken spheroplasts were eliminated by a low speed centrifugation and the supernatant was ultracentrifuged for 30 min at $100,000 \times g$ with a TLA120 rotor at 4°C to obtain the cytosolic fraction (supernatant) and the total membrane fraction (pellet). All fractions were resuspended in loading buffer and heated at 100°C for 10 min before PAGE and immunoblotting analysis.

Inner and outer membrane separation

Inner and outer membranes of *P. aeruginosa* cells were separated by a discontinuous sucrose gradient as described (Viarre *et al.*, 2009). Briefly, 500 mL cultures of *P. aeruginosa* at OD₆₀₀ of 1 were harvested by centrifugation. In the case of PAO1ΔtagQ + tagQΔCys, bacteria were grown with appropriate additives and induced by 0.01% arabinose at OD₆₀₀ 0.5. Pellets were resuspended in 25 mL of 10 mM Tris-HCl, 20% sucrose, 10 mg mL^{-1} DNase, 10 mg mL^{-1} RNase, pH 7.4, and were disrupted by using a Microfluidizer at 15,000 psi. Unbroken cells were removed by 15 min centrifugation at $6,000 \times g$. Total membrane

fraction was obtained by ultracentrifugation at $100,000 \times g$ and resuspended in 500 μL of 20% sucrose containing PIC. The total membrane fraction was then applied at the top of a discontinuous sucrose gradient composed of 1.5 mL layers of 60%, 55%, 50%, 45%, 40%, 35% and 30% of sucrose in 10 mM Tris-HCl, 5 mM EDTA, pH 7.4 (from bottom to top). Sucrose gradients were centrifuged at $90,000 \times g$ at 4°C for 36–72 h, and 500 μL fractions were collected from the top. All fractions were then characterized by SDS-PAGE, Western Blot analysis and NADH oxidase activity. Antibodies against T2SS protein XcpY (Michel *et al.*, 1998), kindly gifted by R. Voulhoux (CNRS, Marseille, France), were used as an inner membrane marker and porines visualized directly on Coomassie blue-stained SDS-PAGE as markers of the outer membrane. NADH oxidase activity was determined as described elsewhere by measuring the NADH consumption at 340 nm of 50 μL of each fraction (Aubert *et al.*, 2010).

Hcp1, TagQ and TagR and Tse1 expression for antibody production

The pET52b-*hcp1*, pET15b-*tagQ* Δ 2-30 and pET15b-*tagR* expression vectors were introduced into *E. coli* BL21(DE3)Star. Expression was induced at OD₆₀₀ of 0.7 by 0.5 mM IPTG and lasted for 4 h. Bacteria were lysed by the Microfluidizer at 10,000 psi and the proteins were purified on appropriate affinity columns using AktaPurifier (GE Healthcare). TagR-His₁₀ was obtained by solubilisation of inclusion bodies using 6 M guanidine. Antibodies were raised in guinea pig for Hcp1 (Eurogentec, Belgium) and in mouse for TagQ and TagR (Agro-Bio, France).

The anti-Tse1 polyclonal rabbit antibody was raised against purified Tse1 (GenScript).

Immunoblotting

For western blotting, antibodies were used at dilutions: anti-Hcp1 1:5000, anti-XcpY 1:1000, anti-TagQ 1:20000, anti-TagR **1:1000 anti-Tse1 1:2000**. Commercial antibodies anti-VSV-G (Sigma Aldrich) and anti-mCherry (Clontech) were used as recommended by the manufacturers. Secondary antibodies were anti-rabbit (Zymed), HRP-coupled anti-guinea pig (Invitrogen) and HRP-coupled anti-mouse (Sigma), all used at 1:5000 dilution. The blots were developed by ECL Detection Kit (Amersham) and Millipore HRP Substrate.

In silico bacterial genome scanning for *tagTSR*

Annotated genomes were downloaded (September 2011) from the Genome Reviews ftp site (ftp://ftp.ebi.ac.uk/pub/databases/genome_reviews/) (Sterk *et al.*, 2006) and <http://www.pseudomonas.com/> for *Pseudomonas* genomes unavailable in Genome Reviews (Winsor *et al.*, 2011). Predicted protein sequences for all genomes were aligned with *rpsblast* (Altschul *et al.*, 1997) against the COG section of the CDD database (September 2011)(Marchler-Bauer *et al.*, 2009). COG hits were considered positive if their alignment covered at least 30% of the COG PSSM and had an E-value 10^{-6} . TagR and *tagR*-like sequences were aligned using *muscle* (Edgar, 2004). Based on this alignment, a maximum likelihood tree with 100 bootstrap replicates was computed using PhyML (Guindon *et al.*, 2010).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. TagT, TagS and TagQ are required for activation of the H1-T6SS

A. Analysis of Hcp1 (structural component) and Tse1 (effector) export from strains containing in-frame deletions of *tag* mutants.. **B.** Cellular and secreted Hcp1 and Tse1 from strains lacking $\Delta pppA$ and indicated *tag* gene. **C.** Western blot analysis of Fha1 from indicated strains grown either in liquid or on solid medium. The band corresponding to phosphorylated Fha1 (*p*-Fha1) is indicated by an asterisk. Lower panel corresponds to the quantification of *p*-Fha1. Experiments were performed in triplicate (black=solid grown, white=liquid grown). Bac: bacteria, sup: supernatant. **D.** *P. aeruginosa* requires *tagT*, *S*, *R* and *Q* for an H1-T6SS-dependent fitness advantage against competing bacteria. The competitive index is plotted for competitions between each indicated donor strain and a H1-T6SS-susceptible recipient strain of *P. aeruginosa* (PAO1 $\Delta tse2 \Delta tsi2$) (Hood *et al.*, 2010) (white=solid grown, gray=liquid grown).

Figure 2. TagT and TagS form a membrane-bound complex harbouring ATPase activity
A. Sequence alignment of TagT and TagS with ABC-transporters of the same family in *P. aeruginosa* (Pa) and *E. coli* (Ec). Conserved features of TagT are highlighted in colours: Walker A domain (grey), "Q" motif (blue), ABC-transporter signature (red), Walker B motif (green) and "H" signature (black). The arrow indicates the conserved Lys residue mutated in the TagT^{K/A}S mutant. TagS TMHs are shown in grey background. **B.** SDS-PAGE analysis of different fractions obtained during His₆-TagTS expression, solubilization and purification. M: molecular weight marker (kDa); Sol. mb: detergent-solubilized membranes; FT: flow through; TagTS: wild-type complex eluted with 200mM imidazole; TagT^{K/A}S: Walker A mutant eluted with 200mM imidazole. **C.** Fractions collected in 200 mM imidazole elution step were analysed for ATPase activity by malachite green assay. The ATPase activity of the wild-type complex was inhibited by orthovanadate in a dose-dependent manner.

Figure 3. TagQ is an outer membrane lipoprotein that localizes independent of TagTS

A. The N-terminal sequence and lipo-box (in red) of TagQ is shown. The mutated cysteine is indicated by an arrow. Confocal microscopy images of PAO1 $\Delta retS$ -GFP producing TagQ-mCherry or TagQ Δ Cys-mCherry. TagQ-mCherry is localized at the bacterial periphery. White arrows on spheroplast images indicate the crescent-shape labelling of the OM. The bar represents 2 μ m. Bact: bacteria; Sphero: spheroplasts. The confocal image in XYZ confirms the presence of the protein in the OM. **B.** Subcellular fractionation of PAO1 $\Delta tagQ$ complemented with a plasmid encoding TagQ (WT) or TagQ Δ Cys (Δ C). Whole cells (WC), cytosol (C), membranes (M) and periplasm (P) were analyzed by Western blot using specific antibodies against TagQ. Δ Cys mutation results in partial processing of the protein (indicated with an asterisk) and its accumulation in the periplasm. **C.** Discontinuous sucrose gradient separation of inner and outer membranes in PAO1 $\Delta retS$ and PAO1 $\Delta retS \Delta tagTS$. Fractions were characterized by NADH oxidase (NADHox) activity, SDS-PAGE and Western blotting using anti-TagQ antibodies. NADH oxidase activity is represented relative to the fraction with the highest level activity (noted as 1.0). XcpY was used as an IM marker and porines visualized in Coomassie blue stained gels as an OM marker. The representative IM and OM fractions are indicated. The analysis of the whole gradient is shown in the Supplementary Figure 2.

Figure 4. Outer membrane localized TagQ is required for outer membrane anchoring of TagR
 Membrane separations and analysis were performed as described in Figure 3C. Strains used were: PAO1 (parental), PAO1 $\Delta tagQ$ and PAO1 $\Delta tagQ$ expressing either TagQ ΔCys or TagQ_{WT}. Only the representative IM and OM fractions are shown. TagR and TagQ were detected by specific antibodies.

Figure 5. *In silico* analysis of *tagTSR*-like genes

Maximum likelihood tree of TagR and TagR-like proteins (matching COG1262) with genomic context of the associated genes, together with the periplasmic PvdO protein of *P. aeruginosa* involved in pyoverdine maturation (Yeterian *et al.*, 2010) (and its genomic context) used as outgroup. Green and yellow circles indicate genomes with complete or incomplete T6SS, respectively. Inside of circles, blue points indicate the presence of *pppA*/*ppkA*/*pha1*. Colored boxes represent hits on the COG database; colors are according to Supplementary Figure 1, except for purple boxes representing hits on ABC-transporter related COGs.

Figure 6. Model for trans-membrane signaling leading to H1-T6SS activation

The H1-T6S machinery is represented as a single tunnel. Tse1-3 are T6S exported effectors. Molecular players biochemically characterized in this study are: TagQ (OM lipoprotein, *Pseudomonas* specific), TagS and TagT (ABC-transporter), TagR (OM associated protein) and TssJ1 (conserved OM lipoprotein). The Ser/Thr kinase, PpkA, and the phosphatase, PppA, as well as the phosphorylation target protein Fha1, are represented. A putative signal is designated by red stars. Proteins are represented using the same colour code as the ORFs in Figure S1, and yellow spheres indicate phosphorylation. Inner membrane (IM) and outer membrane (OM) of *P. aeruginosa* and the target bacterium are shown.