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A 20 residue peptide of the inner membrane protein OutC mediates interaction with two distinct sites of the outer membrane secretin OutD and is essential for the functional type II secretion system in *Erwinia chrysanthemi*.

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Summary

The type II secretion system (T2SS) is widely exploited by proteobacteria to secrete enzymes and toxins involved in bacterial survival and pathogenesis. The outer membrane pore formed by the secretin OutD and the inner membrane protein OutC are two key components of the secretion complex, involved in secretion specificity. Here, we show that the periplasmic regions of OutC and OutD interact directly and map the interaction site of OutC to a 20 residue peptide named OutCsip (secretin interacting peptide, residues 139 to 158). This peptide interacts *in vitro* with two distinct sites of the periplasmic region of OutD, one located on the N0 subdomain and another overlapping the N2-N3' subdomains. The two interaction sites of OutD have different modes of binding to OutCsip. A single substitution, V143S, located within OutCsip prevents its interaction with one of the two binding sites of OutD and fully inactivates the T2SS. We show that the N0 subdomain of OutD interacts also with a second binding site within OutC located in the region proximal to the TMS. We suggest that successive interactions between these distinct regions of OutC and OutD may have functional importance in switching the secretion machine.

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Introduction

The type II secretion system (T2SS) is widely exploited by proteobacteria to secrete various proteins involved in pathogenesis and environmental survival (Filloux 2004; Cianciotto, 2005; Evans *et al.,* 2008). Secretion by the T2SS is a two-step process. The proteins to be secreted (exoproteins) are synthesized with an N-terminal signal sequence and they cross the cytoplasmic membrane, either by the Sec or by the Tat translocon (Pugsley *et al.,* 1991; Voulhoux *et al.,* 2001). Once in the periplasm, the exoproteins are folded and then translocated across the outer membrane by the T2SS machinery that is composed of 12 to 15 proteins, also referred to as Gsp (general secretory pathway). The individual T2SS components are integrated in, or associated with, either the cytoplasmic or the outer membrane. The phytopathogenic bacterium *Erwinia chrysanthemi* possesses two independent T2SS, Out and Stt (Bouley *et al.,* 2001; Ferrandez and Condemine, 2008). At least ten pectinases and a cellulase are secreted by the Out system (Kazemi-Pour *et al.,* 2004). Two proteins, OutD and OutC, have been shown to be involved in the specificity of the secretion system, probably by direct recognition of exoproteins (Lindeberg *et al.,* 1996; Shevchik *et al.*, 1997; Bouley *et al.,* 2001).

The secretin OutD is the integral outer membrane component of the T2SS. Secretins are involved in several systems of macromolecular transport through the outer membrane (Genin and Boucher, 1994). This channel-forming protein forms toroidal structures composed of 12 to 14 subunits (Linderoth *et al.,* 1997; Bitter *et al*., 1998; Nouwen *et al.,* 2000; Burghout *et al.,* 2004). The periplasmic N-terminal domain of the secretins is more variable and confers specificity to the secretion (Daefler *et al.,* 1997; Bouley *et al.,* 2001). Direct interaction of the secretin OutD with the secreted exoproteins has also been demonstrated (Shevchik *et al.*, 1997). The N-terminal domain of the secretin from enterotoxigenic *E. coli* (strain H10407) (45% amino acid identity with OutD) consists of four subdomains (N0 to N3) (Korotkov *et al.,* 2009). The N0 subdomain has the same fold as the signalling domain of TonB-dependent

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receptors. The subdomains N1 and N2 exhibit a fold similar to the KH domains that is also present in ring-forming proteins of the type III secretion system. The C-terminal domain of the secretins is more conserved and is involved in multimerization and channel formation (Brok *et al.,* 1999; Nouwen *et al.,* 2000; Chami *et al.,* 2005). In certain T2SS, some accessory components, the pilotin OutS and OutB, improve the maturation and targeting of the secretin (Hardie *et al.,* 1996; Shevchik and Condemine, 1998; Condemine and Shevchik, 2000). In the absence of the pilotin, the secretin mislocalizes to the inner membrane (Guilvout *et al.,* 2006).

OutC is the bitopic inner membrane protein possessing a large periplasmic region with a C-terminally located PDZ domain (Bleves *et al.,* 1996; Thomas *et al.,* 1997; Korotkov *et al.*, 2006). The PDZ domain of OutC appears to be involved in substrate recognition, as shown by swapping the PDZ domains of OutC from *E. chrysanthemi* and *E. carotovora* that switches the specificity of the secretion system (Bouley *et al.,* 2001). The transmembrane segment (TMS) of OutC drives the protein multimerization (Login and Shevchik, 2006). GspC appears to be a less conserved component of the T2SS, only a part of the periplasmic region, the so-called homology region (HR) being highly conserved (Gérard-Vincent *et al.,* 2002).

How the individual components of the T2SS interact to form a highly organized multiprotein machine is not well understood. It has been suggested that GspC and GspD could interact to provide the structural and functional integrity of the secretion machinery across the two membranes of the Gram-negative bacterium (Lindeberg *et al.,* 1996; Shevchik *et al.*, 1997). Such an interaction could also be involved in substrate recognition, the triggering of pilus formation, and pore opening. Previous studies in *Klebsiella oxytoca* and *Pseudomonas aeruginosa* indirectly implicated a GspC-GspD interaction, but failed to detect it directly (Possot *et al.,* 1999; Bleves *et al.,* 1999). An interaction between XpsD and XpsN (GspD and GspC of *Xanthomonas campestris*) has been observed by cross-linking and pull-down assays (Lee *et al.,* 2000; Lee *et al.,* 2005). The C-terminal region of XpsD appears to be involved in

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the formation of this complex. However, the study of *Vibrio vulnificus* T2SS demonstrated a strong interaction *in vitro* between the N-domain of GspD and the HR-domain of the periplasmic region of GspC (Korotkov *et al.,* 2006). Conversely, the yeast two-hybrid assay did not reveal any interaction between these regions of OutC and OutD (Douet *et al.,* 2004). Thus, the experimental data on the GspC-GspD interaction remains puzzling. One reason for this may be the different experimental approaches used. *In vivo*, GspC and GspD probably also interact with other components of the T2SS and with exoproteins. Some data suggest that the periplasmic region of GspC proximal to the TMS interacts with the GspL-GspM subcomplex (Lee *et al.,* 2004; Gérard-Vincent *et al.,* 2002; Possot *et al.,* 2000). An interaction between the secretin GspD and the pseudopilins GspG and GspJ has also been detected by cross-linking and by yeast two hybrid assays (Douet *et al.,* 2004; Lee *et al.,* 2005).

In this study, we examined the interaction between the secretin OutD and an inner membrane component of the T2SS, OutC, using both *in vitro* and *in vivo* approaches. We show that a short segment of the periplasmic region of OutC, which is located within the C-terminal part of the HR domain, interacts with two distinct regions of the N-domain of OutD. Mutagenesis analysis shows that this segment of OutC is critical for the function of the T2SS. We also show that OutD interacts with a second binding site within OutC located in the region proximal to the TMS

Results

Co-purification of OutD and GST-OutC from a functional T2SS

Proteins interacting with OutC *in vivo* were identified by co-purification exploiting GST-tagged OutC as affinity bait. When the pTdB-GoC plasmid expressing *gst-outC* was introduced into the *outC* deficient strain *E. chrysanthemi* A3618, pectate lyase secretion was restored to wild type level (not shown). The quantity of full-length GST-OutC in A3618 cells

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carrying pTdB-GoC was equal to that of chromosomally encoded OutC in the parental strain A1077 as estimated using immunoblotting with OutC-antibodies (not shown). We conclude that GST-OutC is correctly integrated into the T2SS to form a functional secretion complex.

E. chrysanthemi A3618 Δ*outC* cells expressing either GST-OutC or GST alone were grown to a mid exponential phase and then used for affinity chromatography on Glutathione Sepharose. Bound proteins were eluted from the resin and analyzed by 2D-gel electrophoresis (Fig. S1). As expected a large quantity of GST (Fig. S1A) or of GST-OutC and a 31 kDa species reactive with anti-GST (Fig. S1B) were detected. Importantly, a series of intense spots of about 70 kDa were also observed in A3618 expressing GST-OutC but not GST alone (Fig. S1). Mass spectrometry analysis revealed that these bands correspond to the secretin OutD. This result suggests that OutC and OutD interact within the functional T2SS.

The N-domain of OutD interacts with two regions of OutC

To test if OutC and OutD interact in the absence of other components of the T2SS, *in vitro* pull-down assays were employed. $OutD_{28-710}$ was produced in *E. coli* C41(DE3)/pLysS and purified with Ni-NTA agarose. As with all OutD constructs used here, the signal sequence was deleted and a His tag was fused to the C-terminus (Fig. 1). GST-OutC was expressed in *E. coli* BL21 (DE3) and immobilized on Glutathione Sepharose. $OutD_{28-710}$ bound to GST-OutC but not to GST alone (Fig. 2A). To map this interaction, a series of GST-OutC truncations were used. $OutD_{28-710}$ bound to all the fusions tested except $OutC_{161-272}$, consisting of the PDZ domain only (Fig. 2A, lane 5). This reveals that OutD does not interact with the PDZ domain. $OutD_{28-710}$ did however bind to the periplasmic region of OutC corresponding approximately to the HR region (residues 40 to 172) (Fig. 2A, lane 6). In addition, a larger amount of $OutD_{28-710}$ bound to fusions including the TMS (Fig. 2A, lanes 2, 7 and 8). Since deletions within the two latter fusions ($OutC_{\Delta 103-172}$ and $OutC_{\Delta 41-127}$) fully cover the HR (Fig.

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1), OutD may also bind to the remaining region of OutC (residues 1 to 40), which carries the TMS. These results suggest that OutD may interact with two distinct regions of OutC.

To dissect these putative interacting sites within OutC, we used OutD derivatives consisting of the N-terminal region, which apparently protrudes into the periplasm (Chami *et al.,* 2005). In pull-down assay, $OutD_{28-357}$ interacted with the N-terminal region of OutC, carrying the TMS, $OutC_{1-42}$ (Fig. 2B, lane 3). Since, $OutD_{28-357}$ did not bind to GST-$OutM_{2-46}$, which comprises the TMS of OutM (Fig. 2B, lane 4), it is plausible that OutD interacts specifically with the TMS of OutC or region proximal to the TMS. When we probed the periplasmic region of OutC, $OutD_{28-285}$ bound to $OutC_{128-272}$, but not to $OutC_{161-272}$ (Fig. 2C, lanes 1 and 6). This binding site can therefore be mapped to the region between residues 128 to 160 of OutC. Since $OutD_{28-285}$ bound to $OutC_{60-99/124-272}$, but not to $OutC_{40-102/173-272}$, (Fig. 1A and 2C, lanes 5 and 3), no other interacting sites could be detected within the periplasmic region of OutC from residue 40 to 272.

NMR spectroscopy reveals an interaction between the non-PDZ periplasmic region of OutC and the N-domain of OutD.

NMR spectroscopy was used to investigate the interaction between the periplasmic regions of OutC and OutD in solution. Two constructs of OutC were used, the first ($OutC_{60–272}$) comprises the periplasmic domain and the second ($OutC_{161-272}$) corresponds to the PDZ domain only (Fig. 1). The 2D ^{15}N-HSQC spectra of the PDZ domain (Fig. 3A spectra in red) reveals well dispersed peaks which do not change in position when compared with the 2D ^{15}N-HSQC spectra of the entire periplasmic region of OutC (Fig. 3A spectra in green). This result suggests that the PDZ domain makes no or very few contacts with other domains present in the periplasmic region of OutC and is consistent with a “beads on a string model” of the domain architecture of the periplasmic region of OutC in the absence of other proteins.

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The two spectra were then recorded again but this time in the presence of unlabelled $OutD_{28-285}$ at a molar ratio of 2:1 (0.5mM PDZ domain or 0.5 mM periplasmic region of OutC to 0.25 mM $OutD_{28-285}$). The signal from the PDZ domain is unaffected by the presence of OutD (compare the almost identical red spectra in Fig. 3A and B) showing that it is not involved in an interaction with OutD. The observed major loss of intensity of the peaks from the non-PDZ region of OutC (the signal strength of the green spectra in Fig. 3B is reduced compared to Fig. 3A but only significantly for the non-PDZ peaks) indicates that it is the non-PDZ region (mainly corresponding to HR) that interacts with the N-domain of OutD forming a complex which leads to peak broadening and intensity loss. The intensity loss of the non-PDZ peaks may be best appreciated by inspection on Fig. 3C which shows the effect of adding OutD (green) compared to OutC in the absence of OutD (red).

A short region of OutC interacts with the two distinct sites within the N-domain of OutD

To map the interaction site(s) within OutD, we divided the N-domain into two derivatives, $OutD_{28-112}$ and $OutD_{116-285}$, one carrying the subdomain N0 and the other comprising the subdomains N1, N2 and the beginning of N3 (Fig. 1B). In co-purification assays, both derivatives behaved like the entire N-domain ($OutD_{28-285}$) being co-purified on GST-$OutC_{60-272}$, but not on GST-$OutC_{161-272}$ (Fig. 4, lanes 1, 5 and 6 and Fig. S2). This suggests that two distinct sites of OutD, one located in the N0 subdomain and another located within the N1-N2-N3' subdomains of OutD, interact with the periplasmic region of OutC. To focus in on the second binding site, the N1 subdomain was removed from $OutD_{116-285}$ (Fig. 4, lane 7). This did not apparently affect the binding of the resulting construct $OutD_{192-285}$. Conversely, removing of the C-terminal half of N2 and the beginning of N3 prevented the binding of $OutD_{116-221}$ to GST-$OutC_{60-272}$ (Fig. 4, lane 8). This suggests that the second binding site of OutD involves residues 222 to 285.

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Next, we examined, if these two putative binding sites of OutD interact with the two putative binding sites identified within OutC. In pull-down assays, GST-OutC$_{1-42}$ bound OutD$_{28-154}$ but not OutD$_{116-285}$ (not shown), suggesting that the N-terminal region of OutC interacts with the N0 subdomain of OutD. Conversely, both OutD$_{28-112}$ and OutD$_{116-285}$ bound specifically to GST-OutC$_{139-272}$ but not to GST-OutC$_{161-272}$ (Fig. 5A and 5B lanes 1 and 2). This suggests that the two distinct regions of the N-domain of OutD interact with the same segment of OutC, comprising residues 139 to 160. This segment was named OutCsip (secretin interacting peptide).

The two binding sites of the N-domain of OutD recognise different regions of OutCsip

We next explored whether the OutCsip peptide alone can interact with OutD. Three peptides, OutC$_{131-158}$, OutC$_{139-162}$ and OutC$_{144-158}$ (Fig. S3), were fused to GST and probed in co-purification assays with OutD$_{28-112}$ and OutD$_{116-285}$. OutD$_{28-112}$ bound to all these peptides (Fig. 5A, lanes 3, 5 and 6). Conversely, OutD$_{116-285}$ did not bind to the shorter peptide, OutC$_{144-158}$, whereas it bound to OutC$_{131-158}$ and OutC$_{139-162}$ (Fig. 5B, lanes 3, 5 and 6). These results show that the two distinct sites of OutD interact *in vitro* with the 20 residue stretch of OutC (residues 139 to 158). Moreover, the binding site of OutD$_{28-112}$ (the N0 subdomain) can be mapped to a 15 residue peptide (residues 144 to 158).

To further compare the binding of OutCsip to the two sites of OutD, the three proteins were co-expressed from the *tac* promoter of pGEX-6P-3 in the order GST-OutC$_{128-272}$, OutD$_{28-112}$ and OutD$_{116-285}$. The ratio between the three proteins was similar to that in the pairwise co-expressions (Fig. 5C). In co-purification assay with the three proteins, a lower amount of OutD$_{28-112}$ was bound, in comparison with that observed with pairwise co-expressed GST-OutC$_{128-272}$ and OutD$_{28-112}$ (Fig. 5C, lanes 1 and 3), indicating that OutD$_{28-112}$ competes with OutD$_{116-285}$ for the binding to OutC$_{128-272}$. Conversely, the amount of OutD$_{116-}$

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$_{285}$ bound to OutC$_{128-272}$ was two-fold higher than that observed with pairwise co-expressed GST-OutC$_{128-272}$ and OutD$_{116-285}$ (Fig. 5C, lanes 1 and 2). This indicates that OutD$_{28-112}$ enhances the binding of OutD$_{116-285}$ to OutCsip.

A single amino acid substitution in the OutCsip region blocks secretion in E. chrysanthemi

To examine the functional relevance of the OutCsip region, two C-terminal truncations of OutC were generated, one at the beginning of OutCsip (OutC$_{1-141}$) and another just after it (OutC$_{1-159}$) (Fig. S3B). OutC$_{1-159}$ restored the secretion of PemA in a complementation test with *E. chrysanthemi* Δ*outC* strain (Fig. 6B). In contrast, OutC$_{1-141}$ was non-functional, indicating that OutCsip is essential for the function of the protein. We further substituted several residues in the OutCsip region, namely V143A, V144A, G149A, E152A, Y157A, S137D, R142I and R150L. Immunoblotting with OutC-antibodies revealed all the mutant proteins except OutC^{G149A} once expressed in *E. chrysanthemi* Δ*outC* strain (Fig. 6A). OutC^{G149A} was not detected and probably degraded. In a complementation test, all the mutant proteins, except OutC^{G149A}, fully restored secretion of the pectinases to the wild type level (Fig. 6B). In addition, OutC^{V143A}, whose quantity was not apparently affected, only partially restored secretion of pectinases (Fig. 6). Among three putative β strands predicted in OutCsip by Jpred algorithm (Cole *et al.*, 2008), one overlaps Val143 (Fig. S3). Thus, Val143 was replaced by serine, which is considered to be less compatible with such a secondary structure (Chou and Fasman, 1978). The quantity of OutC^{V143S} was not affected (Fig. 6A), but no pectinase was secreted by A3618 expressing this mutant protein (Fig. 6B).

The substitution V143S prevents interaction of OutCsip with OutD$_{116-285}$

To examine the effect of the V143S mutation on the OutC-OutD interaction, this substitution was introduced into several GST-OutC derivatives. GST-OutC$_{128-272}$^{V143S} was completely

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degraded (not shown), indicating a strong deleterious effect of this mutation on protein stability. In contrast, GST-OutC$_{131-158}^{V143S}$ was stable (Fig. 5A and 5B, lane 4). In co-purification assays, a similar amount of OutD$_{28-112}$ was bound to GST-OutC$_{131-158}$ and to GST-OutC$_{131-158}^{V143S}$ (Fig. 5A, lanes 4 and 5). Conversely, OutD$_{116-285}$ was not bound to the mutant fusion protein (Fig. 5B and S3). Thus, the V143S substitution affects the interaction of OutC$_{131-158}$ with only one interaction site of OutD located in N1-N3' subdomains.

Discussion

In this article, we provide strong evidence that the secretin OutD interacts with OutC, an inner membrane component of the T2SS. We discovered that the N-terminal (periplasmic) domain of OutD interacts with the short segment of OutC (residues 139 to 158), named OutCsip (secretin interacting peptide). OutCsip is proximal to the PDZ domain and is located at the C-terminus of the so-called homology region (HR) (Gérard-Vincent *et al.,* 2002). The PDZ of OutC (residues 175 to 272) plays a specific role and is necessary for the secretion of pectate lyases, although it is fully dispensable for the secretion of certain other proteins (Bouley *et al.,* 2001). The short segment located between OutCsip and PDZ appears to be not essential, since OutC$_{\Delta 163-169}$ is secretion competent (Fig. S3). In contrast, the OutCsip region is indispensable for the function of OutC. Indeed, OutC$_{1-159}$ possessing this region allows for the secretion of PemA, but OutC$_{1-141}$, lacking this peptide, does not.

Site directed mutagenesis of the OutCsip region reveals that the V143S substitution fully blocks secretion while the V143A substitution only partially affects it. Val143 of OutC may be directly involved in a hydrophobic interaction with OutD which is partly mimicked by the Ala substitution but not by the Ser substitution. Indeed, V143S mutation prevents interaction of the OutC$_{131-159}$ peptide with OutD$_{116-285}$, the N1-N3' region of OutD. Alternatively, Val143 may promote a conformation of OutC needed for binding, a

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conformation that is significantly distorted by the Ser substitution. Introduction of the V143S substitution in GST-OutC$_{128-272}$ provokes degradation of the protein consistent with increased flexibility and susceptibility to cleavage. The functional relevance of OutCsip was further shown by interference of this peptide with the pectinase secretion (Fig. S5). A similar phenomenon has been observed by expression of the entire periplasmic domain of the *P. aeruginosa* GspC (Bleves *et al.,* 1999).

We showed that the OutCsip peptide interacts *in vitro* with two distinct regions of OutD: one, located in the N0 subdomain and another, involving the N2 and N3' subdomains. Our results suggest that these two binding sites of OutD, (OutD$_{28-112}$ and OutD$_{116-285}$ respectively) either interact with two overlapping subsites within OutCsip, or have different modes of binding to the same binding site. Indeed, both OutD$_{28-112}$ and OutD$_{116-285}$ bind to OutC$_{131-158}$ and OutC$_{139-162}$, but only OutD$_{28-112}$ binds to OutC$_{131-158}^{V143S}$ and to OutC$_{144-158}$ (Fig. S3C). These data suggest that the minimal binding site of OutD$_{28-112}$ but not of OutD$_{116-285}$ can be mapped within a 15 residue stretch (residues 144 to 158). The three-partner binding experiments showed two phenomena indicative of different binding modes of OutD$_{28-112}$ and OutD$_{116-285}$ to OutCsip: i) a lower binding of OutD$_{28-112}$ in the presence of OutD$_{116-285}$ that suggests a competition between OutD$_{28-112}$ and OutD$_{116-285}$ for binding to OutCsip and ii) a higher binding of OutD$_{116-285}$ in the presence of OutD$_{28-112}$ that suggests a synergistic binding of OutD$_{116-285}$ in the presence of OutD$_{28-112}$ (Fig. 5C).

Dissection of OutC showed that, in addition to OutCsip, another site, which involves the TMS of OutC and/or the proximal region (residues 1 to 42), interacts *in vitro* with the N-domain of OutD. Detailed mapping of the interacting sites was compromised by a low solubility of GST-OutC$_{1-42}$ when it was co-expressed with the tested OutD derivatives (not shown). However, pull-down assay indicates that the N0 subdomain of OutD is involved in this interaction. It seems unlikely that the soluble folded N0 subdomain of OutD comes into

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contact with the TMS of OutC buried in the inner membrane. The second binding site of OutC is likely to be in the periplasmic region close to the TMS. Therefore, the N0 subdomain of OutD interacts *in vitro* with two distinct regions of OutC, while the OutCsip segment interacts with two distinct regions of OutD.

In various T2SSs, the genes encoding GspC and GspD are located together as part of the same operon (Cianciotto, 2005; Evans *et al.,* 2008). One reason for such a gene organization may be that GspC and GspD act in partnership. The two proteins can only be exchanged pair-wise between evolutionary distant T2SSs (de Groot *et al.,* 2001; Michel *et al.,* 2007). This implies the presence of species-specific GspC-GspD interactions necessary for the correct functioning of the protein pair. The interactions between OutC and OutD we discovered could be a part of these interactions.

Formation of a tight complex between the periplasmic regions of OutC and OutD *in vitro* may reflect the formation of a similar structure within the functional T2SS. Indeed, OutD was efficiently co-purified with GST-OutC from the *E. chrysanthemi* secretion system (Fig. S1). The cylindrical dodecameric structure formed by the N-terminal domain of the secretin apparently protrudes into the periplasm (Chami *et al.,* 2005, Korotkov *et al.,* 2009) and, hence, it can easily interact with the periplasmic region of OutC. The stoichiometry of the OutC-OutD complex within the T2SS remains unknown. In contrast to PulD and many other secretins, OutD does not form SDS-resistent multimers when it is expressed at physiological level (Guilvout *et al.,* 1999). Therefore, it was not possible to estimate the multimeric state of the full-length OutD co-purified with GST-OutC from the *E. chrysanthemi* T2SS. Nevertheless, the derivatives consisting of the periplasmic regions of OutC and OutD were co-eluted in an equimolar ratio from Glutathione Sepharose (Fig. S4A), suggesting a dodecameric stoichiometry of OutC *in vivo*. Size exclusion chromatography did not allow the exact stoichiometry of these complexes to be determined, since once released from GST, they

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were unstable (Fig. S4B). Together, OutD and OutC may take the shape of a flexible cage-like structure spanning the periplasm and stabilizing the complex across the two membranes, reminiscent of a model suggested by Filloux et al. (1998). The periplasmic regions of the secretin and of OutC possess several disordered regions, probably acting as flexible linkers (Korotkov *et al.,* 2006, Korotkov *et al.,* 2009). This structural flexibility can facilitate their interactions with protein partners during secretion. Since both OutC and OutD are involved in the recognition of secreted proteins (Lindeberg *et al.,* 1996; Shevchik *et al.*, 1997; Bouley *et al.,* 2001), it is plausible that the interaction between OutC and OutD would modulate their affinity for exoproteins. We found that two distinct regions of OutD interact either with the same site or with two partially overlapping sites located within the OutCsip segment. Moreover, the binding of one interacting region ($OutD_{28-112}$) to OutCsip increases the binding of the other interacting region ($OutD_{116-285}$). Conversely, the binding of $OutD_{116-285}$ to OutCsip reduces the binding of $OutD_{28-112}$ to OutCsip. Considering that *in vitro* $OutD_{28-154}$ interacts also with the region proximal to the TMS of OutC, we can suggest a functional implication of these phenomena. In the course of secretion, the N0 subdomain of OutD ($OutD_{28-112}$) could interact with the OutCsip region. This interaction increases affinity of OutCsip to the other interaction site located in $OutD_{116-285}$ and promotes its binding to OutCsip. Subsequently, the binding of $OutD_{116-285}$ to OutCsip provokes the dissociation of N0 from OutCsip and its binding to the region proximal to the TMS of OutC. Therefore, in the course of secretion, the OutCsip peptide could move between the two interaction sites of the N-domain of OutD and, thus, switch the T2SS machinery between two functional states. Hence, OutC and OutD can act together as an allosterically cooperative system (Whitty, 2008); the binding of a protein partner to one site alters the binding affinity of another site on the protein. Future studies will elucidate the detailed molecular architectures and residues involved in these interactions.

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Experimental procedures

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table S1. The bacteria were usually grown in Luria-Bertani (LB) or M9 minimal medium at 30°C with shaking at 150 rpm. If necessary, antibiotics were added at the following final concentrations: ampicillin, 150 μg ml^{-1}; kanamycin, 100 μg ml^{-1} and chloramphenocol, 50 μg ml^{-1}.

Plasmid construction

DNA cloning and manipulations were performed using standard methods. Site directed mutagenesis was performed using the QuickChange kit (Stratagene). The primers used in this study are listed in Table S2. The nucleotide sequences of mutant and amplified genes were checked (Cogenics). To generate plasmids expressing GST-OutC derivatives, corresponding gene fragments were amplified by PCR and subcloned into pGEX-6P-3 in frame with the GST-encoding sequence. Since the beginning of the *outD* gene overlaps the end of *outC*, an Amber stop-codon was created at the beginning of *outD* by the introduction of an *Xba*I site. To generate plasmids expressing the signal sequence-less $OutD_{His}$ derivatives, corresponding gene fragments were amplified by PCR and subcloned into the *Nde*I/*Hin*dII sites of pET20b in frame with a 6His-encoding sequence. To generate plasmids co-expressing GST-OutC and $OutD_{His}$ derivatives, corresponding *outD* gene fragments preceded by an RBS were subcloned from an appropriate pET20b(+) construct into *Xba*I/*Ssp*I sites of pGEX-6P-3 downstream of the *GST-outC* sequence. More details are described in supplementary information.

Protein purification and analysis

^{15}N-labelled OutC derivatives fused to GST were produced in *E. coli* BL21(DE3) carrying a corresponding pGX-oC_{X-Y} plasmid (oC_{X-Y} for any OutC derivative, as in Table S1). Bacteria

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were grown in M9 medium supplemented with glucose, 10 g l^{-1}, and $^{15}NH_4Cl$, 1 g l^{-1}. At an OD_{600} of 0.7, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 1 mM, and the cultures were grown for an additional 5 h. The cells were broken with a French press and the protein was purified on Glutathione Sepharose as described previously (Login and Shevchik, 2006). Protein was dialysed against 20 mM Tris-HCl pH 9, 1 mM EDTA and loaded onto a Hi Trap Q-FF column (GE Healthcare) equilibrated with the same buffer. The pure protein was eluted by NaCl gradient, concentrated and washed in 10 mM Tris-HCl pH 7, 0.1 mM EDTA with centricon YM-10 or YM-3 (Millipore).

$OutD_{His}$ derivatives used in pull down assays were produced in *E. coli* C41(DE3)/pLys cells carrying a corresponding pET-oD_{X-Y} plasmid (oD_{X-Y} for any OutD derivative, as in Table S1). Because of a strong growth-static effect provoked by expression of $OutD_{28-710}$, induction time was limited to 45 min. The proteins were purified using Ni-NTA agarose, as described (Login and Shevchik, 2006). The recombinant proteins were solubilized in 100 mM sodium phosphate, 10 mM Tris-HCl, 100 mM NaCl pH 8.0, then bound to the resin, washed with the same buffer at pH 6.8 and eluted with a linear gradient of histidine. Purification of $OutD_{28-710}$ was performed in the presence of 1% Triton X-100. For NMR experiments, an additional purification on a Hi Trap Q FF column (GE Healthcare) was performed as above.

SDS-PAGE and immunoblotting were performed as previously (Shevchik *et al.,* 1997, Bouley *at al.,* 2001). Monoclonal tetra-His antibody (Qiagen), peroxidase conjugated Ni-NTA (Qiagen) and anti-GST antibody (Sigma) were used as suggested by the manufacturers. 2D-GE was performed as described by Kazemi-Pour *et al.* (2004).

To test the functionality of OutC mutant proteins, complementation assays were performed with *E. chrysanthemi* A3618 Δ*outC* strain transformed with a pTdB-OC plasmid carrying a corresponding mutant *outC* gene. Efficiency of secretion was estimated by

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immunoblotting of the secreted proteins in culture supernatants and whole cell extracts, as described previously (Login and Shevchik, 2006).

Protein-protein interaction assays

In vitro GST pull-down assays were performed as described previously (Login and Shevchik, 2006). Equal amounts of GST or GST-OutC derivatives were immobilized on Glutathione Sepharose beads. The beads with bound GST-OutC derivatives have been systematically checked by SDS-PAGE prior to the binding assay and then diluted if necessary with non-charged resin to reach a similar amount of various fusion proteins. Next an equal amount of prey protein ($OutD_{His}$ derivative) was loaded onto the resin with immobilized GST fusions, incubated for 1 h and then washed. The bound proteins were eluted with Laemmli sample buffer, separated by SDS-PAGE and probed by immunoblotting with either OutD-antibodies, or Ni-NTA conjugated with peroxidase, or Tetra-His-antibodies (Quiagen).

For co-purification assays, the couples of GST-OutC and $OutD_{His}$ derivatives were co-expressed in *E. coli* BL21(DE3) carrying one of the pGX-oC_{X-Y}-oD_{X-Y} plasmids (oC_{X-Y} and oD_{X-Y} for any OutC and OutD derivatives, as in Table S1). Bacteria were grown and induced with 1 mM IPTG for 3 h. Cells were resuspended in 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA (Buffer B) supplemented with either 1% or 0.1% (v/v) Triton X-100 and broken by sonication. Unbroken cells were eliminated at 6000 g for 10 min and the cell lysates were incubated with Glutathione Sepharose for 1 h. Next, the resin was washed 3 times with buffer B supplemented with Triton X-100 and for an additional 3 times with buffer B without detergent. The bound proteins were eluted with Laemmli sample buffer, separated by SDS-PAGE and probed by immunoblotting with either OutD-antibodies, or Ni-NTA conjugated with peroxidase, or Tetra-His-antibodies (Quiagen).

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In vivo co-purification of proteins bound to GST or GST-OutC during secretion was performed from *E. chrysanthemi* A3618 cells carrying either pGEX-6P-3 or pTdB-GoC plasmid. The bacteria were grown in LB to an OD_{600} of 0.7 and the proteins bound to GST or GST-OutC were co-purified, as described above at the presence of 1% Triton X-100, and analyzed by 2D-GE.

NMR experiments

NMR spectra were recorded at 303K on a Bruker AvanceII spectrometer operating at a proton frequency of 600.13 MHz, equipped with a triple resonance HCN probe head and actively-shielded z-gradients. ^{1}H spectra were acquired using excitation sculpting with gradients for water suppression (Hwang and Shaka, 1995). $^{1}H/^{15}N$ 2D correlation experiments (Palmer *et al.*, 1991) were acquired via double INEPT transfer using sensitivity improvement. Phase sensitive detection in the indirect dimension used echo/antiecho-TPPI gradient selection, with decoupling during acquisition. Water was suppressed using a soft flip-back pulse. Data acquisition and processing were performed using the Bruker Topspin program. Chemical shifts were referenced to internal DSS (2,2-Dimethyl-2-silapentane-5-sulfonic acid) for ^{1}H directly and indirectly for ^{15}N data. All samples for NMR spectroscopy were made up in 10 mM Tris/HCl, pH 7.0, 10 mM $CaCl_2$, 10% D_2O, 100 µM DSS. When recording 2D ^{15}N-HSQC spectra, ^{15}N-labelled proteins were used at concentrations of 0.5 and 0.2 mM; unlabelled proteins were present at molar ratios of 2:1 and 1:1 (0.25mM and 0.2 mM, respectively). All spectra were kindly recorded by Dr H. Toms.

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Figure legends

Fig. 1. Schematic diagrams of OutC (A) and OutD (B) and their truncated derivatives used in the study.

The position of HR (homology region) is according to Gérard-Vincent *et al.* (2002). The subdomain structure of the N-terminal region of OutD (subdomains N0 to N3) is according to the 3D structure of peri-GspD (Korotkov *et al.,* 2009): 45% of amino acid identity among 235 residues with OutD. L1 to L3 indicate disordered linker regions; SP, signal peptide; S, S-domain; and TMS, transmembrane segment.

All the OutD derivatives used in the study possess a C-terminal His tag.

* The sequences of the OutCsip derivatives are shown in Fig. S3.

Fig. 2. Mapping of the OutC regions interacting with OutD.

A. OutD interacts with two distinct regions of OutC. Equal amounts of GST-OutC derivatives (indicated on top) were immobilized on Glutathione Sepharose beads, as checked by SDS-PAGE prior to the binding assay. Next, an equal amount of $OutD_{28-710}$ was loaded onto these matrices, incubated for 1 h and washed. Bound proteins were eluted with Laemmli sample

buffer, separated by SDS-PAGE and either stained (upper panels) or probed with OutD-antibodies (lower panels). GST-fused proteins are indicated by asterisks and OutD derivatives by triangles. Lower species, reacting with GST-antibodies, are GST-OutC degradation products generated during the incubation. Amounts of bound $OutD_{28-710}$ were estimated by densitometry (indicated as a percentage of that to GST-OutC on bottom of the left panel).

B. N-domain of OutD interacts with the N-terminal region of OutC. Pull-down experiments were performed as in (A). $OutD_{28-357}$ was used in place of $OutD_{28-710}$.

C. N-domain of OutD interacts with the HR region of OutC. GST-OutC derivatives (indicated on top) and $OutD_{28-285}$ were co-expressed in *E. coli* BL21(DE3) from the single plasmids and then used in co-purification assay on Glutathione Sepharose.

Fig. 3. 2D ^{15}N-HSQC NMR analysis of the interaction of OutC and OutD.

A and B. 2D ^{15}N-HSQC spectrum of the ^{15}N-labelled periplasmic region of OutC ($OutC_{60-272}$ spectra in green) overlaid with the 2D ^{15}N-HSQC spectrum of the ^{15}N-labelled PDZ domain ($OutC_{161-272}$ spectra in red). In panel (A) the spectra are recorded in the absence of OutD and in panel (B) in the presence of 0.25 mM OutD (unlabelled $OutD_{28-285}$). The concentration of the OutC derived proteins was 0.50 mM. The PDZ domain spectra (red) in panel A is almost identical to that in panel B showing there is no interaction with OutD. The loss of intensity for the non-PDZ region (HR) of the spectra in panel B compared to panel A (green spectra) shows it is the HR region that interacts with OutD.

C. S proximate uperimposition of the ^{15}N-HSQC spectra for $OutC_{60-272}$ in the absence (red) and presence (green) of unlabelled $OutD_{28-285}$. The broadening of some of the peaks in the presence of OutD (green) is clear and confirms the interaction between OutC and OutD. The broadened peaks are not those assigned to the PDZ domain and hence the interaction between OutC and OutD does not involve the PDZ domain.

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Fig. 4. Mapping of the OutD regions interacting with the periplasmic region of OutC.

A. Whole cell extracts of *E. coli* BL21(DE3) co-expressing GST-OutC$_{60-272}$ with various OutD derivatives (indicated on top).

B. Co-purification assay on Glutathione Sepharose using the extracts shown in (A). Bound proteins were eluted with Laemmli sample buffer. The proteins were separated by SDS-PAGE and probed with Tetra-His antibodies and then with Ni-NTA conjugated with peroxidase. GST-OutC$_{60-272}$ is indicated by asterisk and OutD derivatives by triangles.

Fig. 5. Two distinct regions of OutD, OutD$_{28-112}$ and OutD$_{116-285}$, interact with the OutCsip peptide.

A and **B**. Dissection of the OutCsip region interacting with OutD$_{28-112}$ (A) and OutD$_{116-285}$ (B). The GST-OutC derivatives (indicated at the top) were co-expressed with either OutD$_{116-285}$ or OutD$_{28-112}$ (upper panels) and used in co-purification assays on Glutathione Sepharose. Bound proteins were eluted with Laemmli sample buffer, separated by SDS-PAGE and then stained with Coomassie G250 (middle panels) or probed with Tetra-His antibodies (lower panels).

C. Simultaneous binding of OutD$_{28-112}$ and OutD$_{116-285}$ to GST-OutC$_{128-272}$. GST-OutC$_{128-272}$ was co-expressed with either OutD$_{28-112}$ or OutD$_{116-285}$ or the three proteins were all expressed together and used in co-purification assays as above. GST-OutC derivatives are indicated by asterisks, OutD$_{28-112}$ by open triangles and OutD$_{116-285}$ by filled triangles. Relative amounts of the proteins were estimated by densitometry and indicated as percentage of those with pair-wise expressions (on bottom of the corresponding panels). The experiments were repeated three times and a representative assay is present.

Fig. 6. Functional analysis of the OutC point mutants generated in the OutCsip region.

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A. Stability of OutC mutant proteins (indicated on top) expressed in *E. chrysanthemi* A3618 Δ*outC* cells. Immunoblotting of whole cell extracts with OutC-antibodies. In the right panel, three times more of material was loaded and the membrane was overexposed to detect low abundance products. OutC, $OutC_{\Delta 163\text{-}169}$ and $OutC_{\Delta 173\text{-}256}$ are indicated with a triangle. The species of the molecular weight slightly larger than OutC is a cross-reaction product. Lower molecular weight species in the OutC and $OutC_{\Delta 163\text{-}169}$ lanes are degradation products.

B. Complementation ability of OutC mutant proteins. The supernatant (S) and cell extract (C) of *E. chrysanthemi* A3618 Δ*outC* carrying a pTdB-oC plasmid with the corresponding mutant *outC* (indicated on top) were analyzed by immunoblotting with PemA-antibodies.

Table 1. Strains used in this study

Strain	Genotype/phenotype	Reference
Escherichia coli		
BL21(DE3)	F^- *dcm ompT hsdS*B(r_B^-, m_B^-) *gal lon* λ(DE3)	Studier *et al*., 1990
BL21(DE3)C41	uncharacterized mutant of BL21(DE3)	Miroux & Walker, 1996
NM522	*supE thi-1* Δ(*lac-proAB*) Δ(*mcrB-hsdSM*)*5*	
	(r_K^- m_K^+) [F′ *proAB lacI*^{q}*Z*Δ*M15*]	Stratagene
Erwinia chrysanthemi		
A350	*lacZ2*	Laboratory collection
A1077	*lacZ2 kdgR*::Mu Cm	Laboratory collection
A3618	*lacZ2* Δ*outC kdgR*::Mu Cm	Bouley *et al.,* 2001

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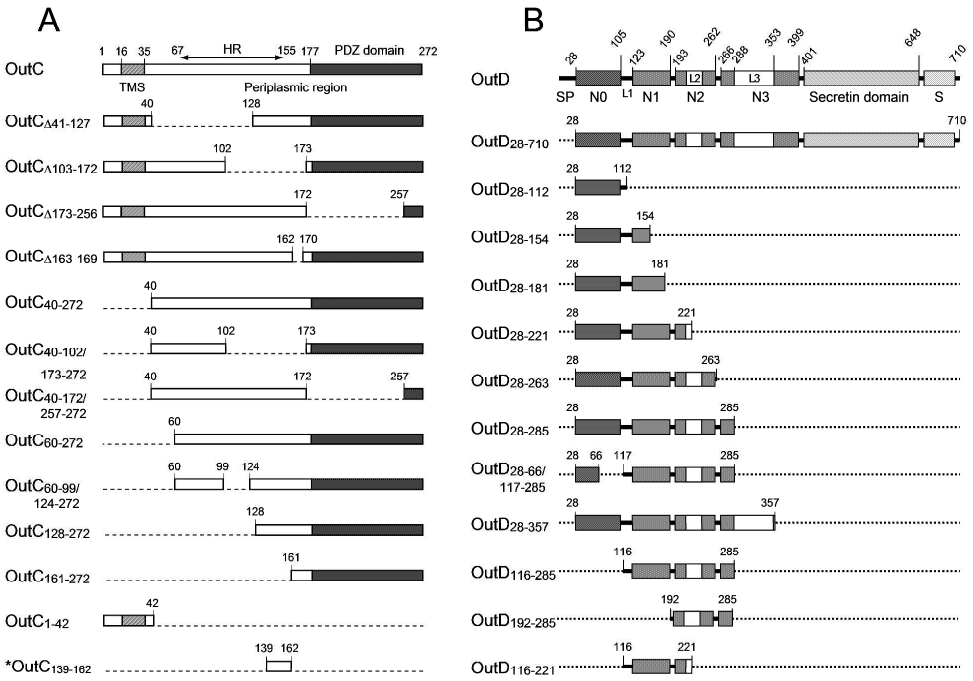

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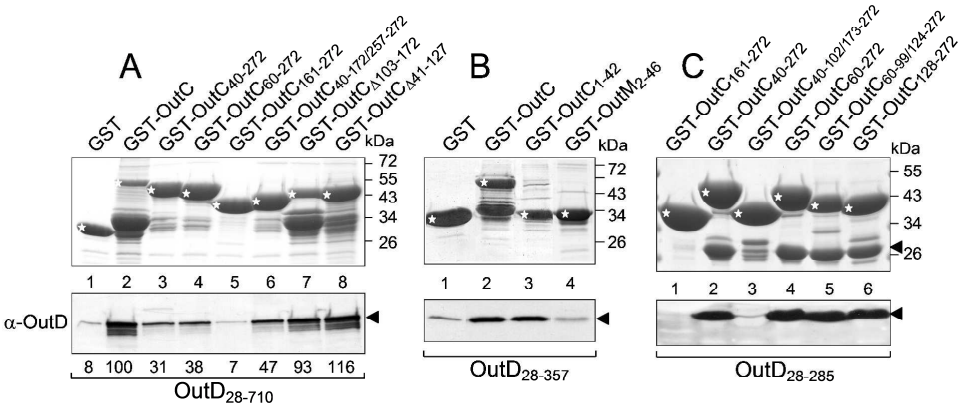

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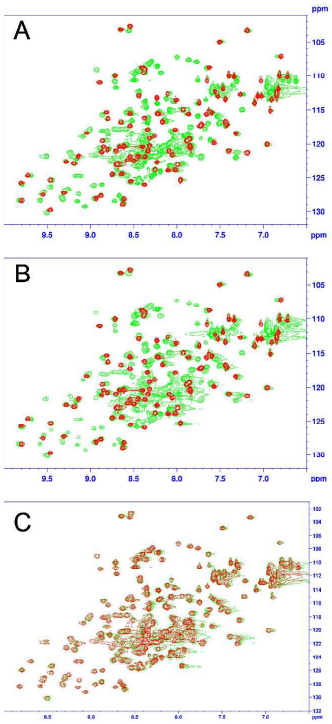

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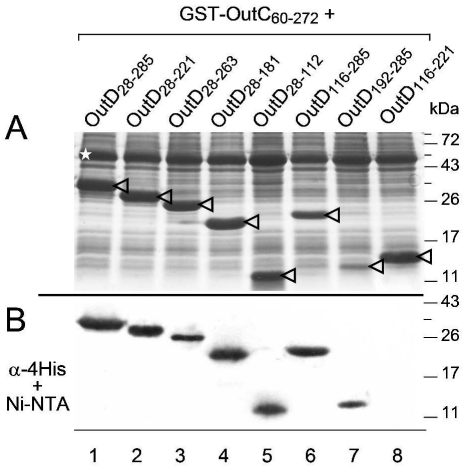

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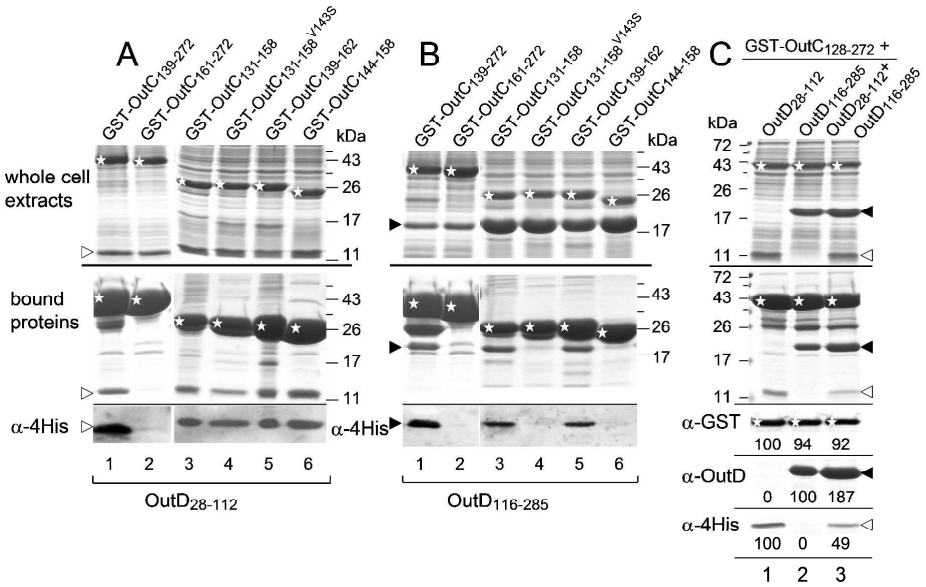

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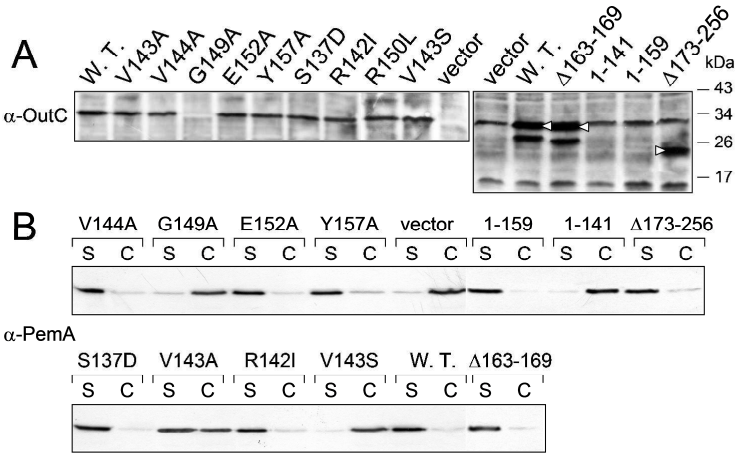

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