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► **To cite this version:**

Frédéric H. Login, Markus Fries, Xiaohui Wang, Richard W. Pickersgill, Vladimir Shevchik. 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*. *Molecular Microbiology*, 2010, 76 (4), pp.944. 10.1111/j.1365-2958.2010.07149.x . hal-00552630

HAL Id: hal-00552630

<https://hal.science/hal-00552630>

Submitted on 6 Jan 2011

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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*.

Journal:	<i>Molecular Microbiology</i>
Manuscript ID:	MMI-2009-09558.R2
Manuscript Type:	Research Article
Date Submitted by the Author:	17-Mar-2010
Complete List of Authors:	<p>Login, Frédéric; Université Lyon1, MAP, UMR 5240 CNRS-Université Lyon1, INSA Lyon, BCS Fries, Markus; Queen Mary University of London, School of Biological and Chemical Sciences Wang, Xiaohui; INSA de Lyon, MAP, UMR 5240 CNRS-Université Lyon1-Insa Lyon-BCS Pickersgill, Richard; Queen Mary University of London, School of Biological and Chemical Sciences Shevchik, Vladimir; vladimir.shevchik@insa-lyon.fr, MAP, UMR 5240</p>
Key Words:	type II secretion system, secretin, GspC, GspC-GspD interaction

1 **A 20 residue peptide of the inner membrane protein OutC mediates interaction with two**
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3 **type II secretion system in *Erwinia chrysanthemi*.**

4
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16
17 **Running title:** Peptide of OutC interacts with secretin

18

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20

1 **Summary**

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

1 Introduction

2 The type II secretion system (T2SS) is widely exploited by proteobacteria to secrete various
3 proteins involved in pathogenesis and environmental survival (Filloux 2004; Cianciotto,
4 2005; Evans *et al.*, 2008). Secretion by the T2SS is a two-step process. The proteins to be
5 secreted (exoproteins) are synthesized with an N-terminal signal sequence and they cross the
6 cytoplasmic membrane, either by the Sec or by the Tat translocon (Pugsley *et al.*, 1991;
7 Voulhoux *et al.*, 2001). Once in the periplasm, the exoproteins are folded and then translocated
8 across the outer membrane by the T2SS machinery that is composed of 12 to 15 proteins, also
9 referred to as Gsp (general secretory pathway). The individual T2SS components are integrated
10 in, or associated with, either the cytoplasmic or the outer membrane. The phytopathogenic
11 bacterium *Erwinia chrysanthemi* possesses two independent T2SS, Out and Stt (Bouley *et al.*,
12 2001; Ferrandez and Condemine, 2008). At least ten pectinases and a cellulase are secreted by
13 the Out system (Kazemi-Pour *et al.*, 2004). Two proteins, OutD and OutC, have been shown
14 to be involved in the specificity of the secretion system, probably by direct recognition of
15 exoproteins (Lindeberg *et al.*, 1996; Shevchik *et al.*, 1997; Bouley *et al.*, 2001).

16 The secretin OutD is the integral outer membrane component of the T2SS. Secretins
17 are involved in several systems of macromolecular transport through the outer membrane
18 (Genin and Boucher, 1994). This channel-forming protein forms toroidal structures composed
19 of 12 to 14 subunits (Linderoth *et al.*, 1997; Bitter *et al.*, 1998; Nouwen *et al.*, 2000; Burghout
20 *et al.*, 2004). The periplasmic N-terminal domain of the secretins is more variable and confers
21 specificity to the secretion (Daefler *et al.*, 1997; Bouley *et al.*, 2001). Direct interaction of the
22 secretin OutD with the secreted exoproteins has also been demonstrated (Shevchik *et al.*,
23 1997). The N-terminal domain of the secretin from enterotoxigenic *E. coli* (strain H10407)
24 (45% amino acid identity with OutD) consists of four subdomains (N0 to N3) (Korotkov *et*
25 *al.*, 2009). The N0 subdomain has the same fold as the signalling domain of TonB-dependent

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

8 OutC is the bitopic inner membrane protein possessing a large periplasmic region with
9 a C-terminally located PDZ domain (Bleves *et al.*, 1996; Thomas *et al.*, 1997; Korotkov *et al.*,
10 2006). The PDZ domain of OutC appears to be involved in substrate recognition, as shown by
11 swapping the PDZ domains of OutC from *E. chrysanthemi* and *E. carotovora* that switches
12 the specificity of the secretion system (Bouley *et al.*, 2001). The transmembrane segment
13 (TMS) of OutC drives the protein multimerization (Login and Shevchik, 2006). GspC appears
14 to be a less conserved component of the T2SS, only a part of the periplasmic region, the so-
15 called homology region (HR) being highly conserved (Gérard-Vincent *et al.*, 2002).

16 How the individual components of the T2SS interact to form a highly organized
17 multiprotein machine is not well understood. It has been suggested that GspC and GspD could
18 interact to provide the structural and functional integrity of the secretion machinery across the
19 two membranes of the Gram-negative bacterium (Lindeberg *et al.*, 1996; Shevchik *et al.*,
20 1997). Such an interaction could also be involved in substrate recognition, the triggering of
21 pilus formation, and pore opening. Previous studies in *Klebsiella oxytoca* and *Pseudomonas*
22 *aeruginosa* indirectly implicated a GspC-GspD interaction, but failed to detect it directly
23 (Possot *et al.*, 1999; Bleves *et al.*, 1999). An interaction between XpsD and XpsN (GspD and
24 GspC of *Xanthomonas campestris*) has been observed by cross-linking and pull-down assays
25 (Lee *et al.*, 2000; Lee *et al.*, 2005). The C-terminal region of XpsD appears to be involved in

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

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

19

20 **Results**

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

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

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

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

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

14 To test if OutC and OutD interact in the absence of other components of the T2SS, *in vitro*
15 pull-down assays were employed. OutD₂₈₋₇₁₀ was produced in *E. coli* C41(DE3)/pLysS and
16 purified with Ni-NTA agarose. As with all OutD constructs used here, the signal sequence
17 was deleted and a His tag was fused to the C-terminus (Fig. 1). GST-OutC was expressed in
18 *E. coli* BL21 (DE3) and immobilized on Glutathione Sepharose. OutD₂₈₋₇₁₀ bound to GST-
19 OutC but not to GST alone (Fig. 2A). To map this interaction, a series of GST-OutC
20 truncations were used. OutD₂₈₋₇₁₀ bound to all the fusions tested except OutC₁₆₁₋₂₇₂, consisting
21 of the PDZ domain only (Fig. 2A, lane 5). This reveals that OutD does not interact with the
22 PDZ domain. OutD₂₈₋₇₁₀ did however bind to the periplasmic region of OutC corresponding
23 approximately to the HR region (residues 40 to 172) (Fig. 2A, lane 6). In addition, a larger
24 amount of OutD₂₈₋₇₁₀ bound to fusions including the TMS (Fig. 2A, lanes 2, 7 and 8). Since
25 deletions within the two latter fusions (OutC Δ ₁₀₃₋₁₇₂ and OutC Δ ₄₁₋₁₂₇) fully cover the HR (Fig.

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

3 To dissect these putative interacting sites within OutC, we used OutD derivatives
4 consisting of the N-terminal region, which apparently protrudes into the periplasm (Chami *et*
5 *al.*, 2005). In pull-down assay, OutD₂₈₋₃₅₇ interacted with the N-terminal region of OutC,
6 carrying the TMS, OutC₁₋₄₂ (Fig. 2B, lane 3). Since, OutD₂₈₋₃₅₇ did not bind to GST-OutM₂₋₄₆,
7 which comprises the TMS of OutM (Fig. 2B, lane 4), it is plausible that OutD interacts
8 specifically with the TMS of OutC or region proximal to the TMS. When we probed the
9 periplasmic region of OutC, OutD₂₈₋₂₈₅ bound to OutC₁₂₈₋₂₇₂, but not to OutC₁₆₁₋₂₇₂ (Fig. 2C,
10 lanes 1 and 6). This binding site can therefore be mapped to the region between residues 128
11 to 160 of OutC. Since OutD₂₈₋₂₈₅ bound to OutC_{60-99/124-272}, but not to OutC_{40-102/173-272}, (Fig.
12 1A and 2C, lanes 5 and 3), no other interacting sites could be detected within the periplasmic
13 region of OutC from residue 40 to 272.

14

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

17 NMR spectroscopy was used to investigate the interaction between the periplasmic regions of
18 OutC and OutD in solution. Two constructs of OutC were used, the first (OutC₆₀₋₂₇₂)
19 comprises the periplasmic domain and the second (OutC₁₆₁₋₂₇₂) corresponds to the PDZ
20 domain only (Fig. 1). The 2D ¹⁵N-HSQC spectra of the PDZ domain (Fig. 3A spectra in red)
21 reveals well dispersed peaks which do not change in position when compared with the 2D
22 ¹⁵N-HSQC spectra of the entire periplasmic region of OutC (Fig. 3A spectra in green). This
23 result suggests that the PDZ domain makes no or very few contacts with other domains
24 present in the periplasmic region of OutC and is consistent with a “beads on a string model”
25 of the domain architecture of the periplasmic region of OutC in the absence of other proteins.

1 The two spectra were then recorded again but this time in the presence of unlabelled OutD₂₈₋
2 ₂₈₅ at a molar ratio of 2:1 (0.5mM PDZ domain or 0.5 mM periplasmic region of OutC to 0.25
3 mM OutD₂₈₋₂₈₅). The signal from the PDZ domain is unaffected by the presence of OutD
4 (compare the almost identical red spectra in Fig. 3A and B) showing that it is not involved in
5 an interaction with OutD. The observed major loss of intensity of the peaks from the non-PDZ
6 region of OutC (the signal strength of the green spectra in Fig. 3B is reduced compared to Fig.
7 3A but only significantly for the non-PDZ peaks) indicates that it is the non-PDZ region
8 (mainly corresponding to HR) that interacts with the N-domain of OutD forming a complex
9 which leads to peak broadening and intensity loss. The intensity loss of the non-PDZ peaks
10 may be best appreciated by inspection on Fig. 3C which shows the effect of adding OutD
11 (green) compared to OutC in the absence of OutD (red).

12

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

14 To map the interaction site(s) within OutD, we divided the N-domain into two
15 derivatives, OutD₂₈₋₁₁₂ and OutD₁₁₆₋₂₈₅, one carrying the subdomain N0 and the other
16 comprising the subdomains N1, N2 and the beginning of N3 (Fig. 1B). In co-purification
17 assays, both derivatives behaved like the entire N-domain (OutD₂₈₋₂₈₅) being co-purified on
18 GST-OutC₆₀₋₂₇₂, but not on GST-OutC₁₆₁₋₂₇₂ (Fig. 4, lanes 1, 5 and 6 and Fig. S2). This
19 suggests that two distinct sites of OutD, one located in the N0 subdomain and another located
20 within the N1-N2-N3' subdomains of OutD, interact with the periplasmic region of OutC. To
21 focus in on the second binding site, the N1 subdomain was removed from OutD₁₁₆₋₂₈₅ (Fig. 4,
22 lane 7). This did not apparently affect the binding of the resulting construct OutD₁₉₂₋₂₈₅.
23 Conversely, removing of the C-terminal half of N2 and the beginning of N3 prevented the
24 binding of OutD₁₁₆₋₂₂₁ to GST-OutC₆₀₋₂₇₂ (Fig. 4, lane 8). This suggests that the second
25 binding site of OutD involves residues 222 to 285.

1 Next, we examined, if these two putative binding sites of OutD interact with the two
2 putative binding sites identified within OutC. In pull-down assays, GST-OutC₁₋₄₂ bound
3 OutD₂₈₋₁₅₄ but not OutD₁₁₆₋₂₈₅ (not shown), suggesting that the N-terminal region of OutC
4 interacts with the N0 subdomain of OutD. Conversely, both OutD₂₈₋₁₁₂ and OutD₁₁₆₋₂₈₅ bound
5 specifically to GST-OutC₁₃₉₋₂₇₂ but not to GST-OutC₁₆₁₋₂₇₂ (Fig. 5A and 5B lanes 1 and 2).
6 This suggests that the two distinct regions of the N-domain of OutD interact with the same
7 segment of OutC, comprising residues 139 to 160. This segment was named OutCsip (secretin
8 interacting peptide).

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

11 We next explored whether the OutCsip peptide alone can interact with OutD. Three peptides,
12 OutC₁₃₁₋₁₅₈, OutC₁₃₉₋₁₆₂ and OutC₁₄₄₋₁₅₈ (Fig. S3), were fused to GST and probed in co-
13 purification assays with OutD₂₈₋₁₁₂ and OutD₁₁₆₋₂₈₅. OutD₂₈₋₁₁₂ bound to all these peptides
14 (Fig. 5A, lanes 3, 5 and 6). Conversely, OutD₁₁₆₋₂₈₅ did not bind to the shorter peptide,
15 OutC₁₄₄₋₁₅₈, whereas it bound to OutC₁₃₁₋₁₅₈ and OutC₁₃₉₋₁₆₂ (Fig. 5B, lanes 3, 5 and 6). These
16 results show that the two distinct sites of OutD interact *in vitro* with the 20 residue stretch of
17 OutC (residues 139 to 158). Moreover, the binding site of OutD₂₈₋₁₁₂ (the N0 subdomain) can
18 be mapped to a 15 residue peptide (residues 144 to 158).

19 To further compare the binding of OutCsip to the two sites of OutD, the three proteins
20 were co-expressed from the *tac* promoter of pGEX-6P-3 in the order GST-OutC₁₂₈₋₂₇₂,
21 OutD₂₈₋₁₁₂ and OutD₁₁₆₋₂₈₅. The ratio between the three proteins was similar to that in the
22 pairwise co-expressions (Fig. 5C). In co-purification assay with the three proteins, a lower
23 amount of OutD₂₈₋₁₁₂ was bound, in comparison with that observed with pairwise co-
24 expressed GST-OutC₁₂₈₋₂₇₂ and OutD₂₈₋₁₁₂ (Fig. 5C, lanes 1 and 3), indicating that OutD₂₈₋₁₁₂
25 competes with OutD₁₁₆₋₂₈₅ for the binding to OutC₁₂₈₋₂₇₂. Conversely, the amount of OutD₁₁₆₋

1 ²⁸⁵ bound to OutC₁₂₈₋₂₇₂ was two-fold higher than that observed with pairwise co-expressed
2 GST-OutC₁₂₈₋₂₇₂ and OutD₁₁₆₋₂₈₅ (Fig. 5C, lanes 1 and 2). This indicates that OutD₂₈₋₁₁₂
3 enhances the binding of OutD₁₁₆₋₂₈₅ to OutC_{sip}.

4

5 ***A single amino acid substitution in the OutC_{sip} region blocks secretion in *E. chrysanthemi****

6 To examine the functional relevance of the OutC_{sip} region, two C-terminal truncations of
7 OutC were generated, one at the beginning of OutC_{sip} (OutC₁₋₁₄₁) and another just after it
8 (OutC₁₋₁₅₉) (Fig. S3B). OutC₁₋₁₅₉ restored the secretion of PemaA in a complementation test
9 with *E. chrysanthemi* $\Delta outC$ strain (Fig. 6B). In contrast, OutC₁₋₁₄₁ was non-functional,
10 indicating that OutC_{sip} is essential for the function of the protein. We further substituted
11 several residues in the OutC_{sip} region, namely V143A, V144A, G149A, E152A, Y157A,
12 S137D, R142I and R150L. Immunoblotting with OutC-antibodies revealed all the mutant
13 proteins except OutC^{G149A} once expressed in *E. chrysanthemi* $\Delta outC$ strain (Fig. 6A).
14 OutC^{G149A} was not detected and probably degraded. In a complementation test, all the mutant
15 proteins, except OutC^{G149A}, fully restored secretion of the pectinases to the wild type level
16 (Fig. 6B). In addition, OutC^{V143A}, whose quantity was not apparently affected, only partially
17 restored secretion of pectinases (Fig. 6). Among three putative β strands predicted in OutC_{sip}
18 by Jpred algorithm (Cole *et al.*, 2008), one overlaps Val143 (Fig. S3). Thus, Val143 was
19 replaced by serine, which is considered to be less compatible with such a secondary structure
20 (Chou and Fasman, 1978). The quantity of OutC^{V143S} was not affected (Fig. 6A), but no
21 pectinase was secreted by A3618 expressing this mutant protein (Fig. 6B).

22

23 ***The substitution V143S prevents interaction of OutC_{sip} with OutD₁₁₆₋₂₈₅***

24 To examine the effect of the V143S mutation on the OutC-OutD interaction, this substitution
25 was introduced into several GST-OutC derivatives. GST-OutC₁₂₈₋₂₇₂^{V143S} was completely

1 degraded (not shown), indicating a strong deleterious effect of this mutation on protein
2 stability. In contrast, GST-OutC₁₃₁₋₁₅₈^{V143S} was stable (Fig. 5A and 5B, lane 4). In co-
3 purification assays, a similar amount of OutD₂₈₋₁₁₂ was bound to GST-OutC₁₃₁₋₁₅₈ and to GST-
4 OutC₁₃₁₋₁₅₈^{V143S} (Fig. 5A, lanes 4 and 5). Conversely, OutD₁₁₆₋₂₈₅ was not bound to the mutant
5 fusion protein (Fig. 5B and S3). Thus, the V143S substitution affects the interaction of
6 OutC₁₃₁₋₁₅₈ with only one interaction site of OutD located in N1-N3' subdomains.

7

8 **Discussion**

9 In this article, we provide strong evidence that the secretin OutD interacts with OutC, an inner
10 membrane component of the T2SS. We discovered that the N-terminal (periplasmic) domain
11 of OutD interacts with the short segment of OutC (residues 139 to 158), named OutC_{sip}
12 (secretin interacting peptide). OutC_{sip} is proximal to the PDZ domain and is located at the C-
13 terminus of the so-called homology region (HR) (Gérard-Vincent *et al.*, 2002). The PDZ of
14 OutC (residues 175 to 272) plays a specific role and is necessary for the secretion of pectate
15 lyases, although it is fully dispensable for the secretion of certain other proteins (Bouley *et al.*,
16 2001). The short segment located between OutC_{sip} and PDZ appears to be not essential, since
17 OutC_{Δ163-169} is secretion competent (Fig. S3). In contrast, the OutC_{sip} region is indispensable
18 for the function of OutC. Indeed, OutC₁₋₁₅₉ possessing this region allows for the secretion of
19 PemA, but OutC₁₋₁₄₁, lacking this peptide, does not.

20 Site directed mutagenesis of the OutC_{sip} region reveals that the V143S substitution
21 fully blocks secretion while the V143A substitution only partially affects it. Val143 of OutC
22 may be directly involved in a hydrophobic interaction with OutD which is partly mimicked by
23 the Ala substitution but not by the Ser substitution. Indeed, V143S mutation prevents
24 interaction of the OutC₁₃₁₋₁₅₉ peptide with OutD₁₁₆₋₂₈₅, the N1-N3' region of OutD.
25 Alternatively, Val143 may promote a conformation of OutC needed for binding, a

1 conformation that is significantly distorted by the Ser substitution. Introduction of the V143S
2 substitution in GST-OutC₁₂₈₋₂₇₂ provokes degradation of the protein consistent with increased
3 flexibility and susceptibility to cleavage. The functional relevance of OutCsip was further
4 shown by interference of this peptide with the pectinase secretion (Fig. S5). A similar
5 phenomenon has been observed by expression of the entire periplasmic domain of the *P.*
6 *aeruginosa* GspC (Bleves *et al.*, 1999).

7 We showed that the OutCsip peptide interacts *in vitro* with two distinct regions of
8 OutD: one, located in the N0 subdomain and another, involving the N2 and N3' subdomains.
9 Our results suggest that these two binding sites of OutD, (OutD₂₈₋₁₁₂ and OutD₁₁₆₋₂₈₅
10 respectively) either interact with two overlapping subsites within OutCsip, or have different
11 modes of binding to the same binding site. Indeed, both OutD₂₈₋₁₁₂ and OutD₁₁₆₋₂₈₅ bind to
12 OutC₁₃₁₋₁₅₈ and OutC₁₃₉₋₁₆₂, but only OutD₂₈₋₁₁₂ binds to OutC₁₃₁₋₁₅₈^{V143S} and to OutC₁₄₄₋₁₅₈
13 (Fig. S3C). These data suggest that the minimal binding site of OutD₂₈₋₁₁₂ but not of OutD₁₁₆₋
14 ₂₈₅ can be mapped within a 15 residue stretch (residues 144 to 158). The three-partner binding
15 experiments showed two phenomena indicative of different binding modes of OutD₂₈₋₁₁₂ and
16 OutD₁₁₆₋₂₈₅ to OutCsip: i) a lower binding of OutD₂₈₋₁₁₂ in the presence of OutD₁₁₆₋₂₈₅ that
17 suggests a competition between OutD₂₈₋₁₁₂ and OutD₁₁₆₋₂₈₅ for binding to OutCsip and ii) a
18 higher binding of OutD₁₁₆₋₂₈₅ in the presence of OutD₂₈₋₁₁₂ that suggests a synergistic binding
19 of OutD₁₁₆₋₂₈₅ in the presence of OutD₂₈₋₁₁₂ (Fig. 5C).

20 Dissection of OutC showed that, in addition to OutCsip, another site, which involves
21 the TMS of OutC and/or the proximal region (residues 1 to 42), interacts *in vitro* with the N-
22 domain of OutD. Detailed mapping of the interacting sites was compromised by a low
23 solubility of GST-OutC₁₋₄₂ when it was co-expressed with the tested OutD derivatives (not
24 shown). However, pull-down assay indicates that the N0 subdomain of OutD is involved in
25 this interaction. It seems unlikely that the soluble folded N0 subdomain of OutD comes into

1 contact with the TMS of OutC buried in the inner membrane. The second binding site of
2 OutC is likely to be in the periplasmic region close to the TMS. Therefore, the N0 subdomain
3 of OutD interacts *in vitro* with two distinct regions of OutC, while the OutC_{sip} segment
4 interacts with two distinct regions of OutD.

5 In various T2SSs, the genes encoding GspC and GspD are located together as part of
6 the same operon (Cianciotto, 2005; Evans *et al.*, 2008). One reason for such a gene
7 organization may be that GspC and GspD act in partnership. The two proteins can only be
8 exchanged pair-wise between evolutionary distant T2SSs (de Groot *et al.*, 2001; Michel *et al.*,
9 2007). This implies the presence of species-specific GspC-GspD interactions necessary for
10 the correct functioning of the protein pair. The interactions between OutC and OutD we
11 discovered could be a part of these interactions.

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

1 were unstable (Fig. S4B). Together, OutD and OutC may take the shape of a flexible cage-
2 like structure spanning the periplasm and stabilizing the complex across the two membranes,
3 reminiscent of a model suggested by Filloux et al. (1998). The periplasmic regions of the
4 secretin and of OutC possess several disordered regions, probably acting as flexible linkers
5 (Korotkov *et al.*, 2006, Korotkov *et al.*, 2009). This structural flexibility can facilitate their
6 interactions with protein partners during secretion. Since both OutC and OutD are involved in
7 the recognition of secreted proteins (Lindeberg *et al.*, 1996; Shevchik *et al.*, 1997; Bouley *et*
8 *al.*, 2001), it is plausible that the interaction between OutC and OutD would modulate their
9 affinity for exoproteins. We found that two distinct regions of OutD interact either with the
10 same site or with two partially overlapping sites located within the OutC_{sip} segment.
11 Moreover, the binding of one interacting region (OutD₂₈₋₁₁₂) to OutC_{sip} increases the binding
12 of the other interacting region (OutD₁₁₆₋₂₈₅). Conversely, the binding of OutD₁₁₆₋₂₈₅ to OutC_{sip}
13 reduces the binding of OutD₂₈₋₁₁₂ to OutC_{sip}. Considering that *in vitro* OutD₂₈₋₁₅₄ interacts
14 also with the region proximal to the TMS of OutC, we can suggest a functional implication of
15 these phenomena. In the course of secretion, the N0 subdomain of OutD (OutD₂₈₋₁₁₂) could
16 interact with the OutC_{sip} region. This interaction increases affinity of OutC_{sip} to the other
17 interaction site located in OutD₁₁₆₋₂₈₅ and promotes its binding to OutC_{sip}. Subsequently, the
18 binding of OutD₁₁₆₋₂₈₅ to OutC_{sip} provokes the dissociation of N0 from OutC_{sip} and its
19 binding to the region proximal to the TMS of OutC. Therefore, in the course of secretion, the
20 OutC_{sip} peptide could move between the two interaction sites of the N-domain of OutD and,
21 thus, switch the T2SS machinery between two functional states. Hence, OutC and OutD can
22 act together as an allosterically cooperative system (Whitty, 2008); the binding of a protein
23 partner to one site alters the binding affinity of another site on the protein. Future studies will
24 elucidate the detailed molecular architectures and residues involved in these interactions.

25

1 **Experimental procedures**

2 *Bacterial strains and growth conditions*

3 The bacterial strains and plasmids used in this study are listed in Table S1. The bacteria were
4 usually grown in Luria-Bertani (LB) or M9 minimal medium at 30°C with shaking at 150
5 rpm. If necessary, antibiotics were added at the following final concentrations: ampicillin, 150
6 $\mu\text{g ml}^{-1}$; kanamycin, 100 $\mu\text{g ml}^{-1}$ and chloramphenicol, 50 $\mu\text{g ml}^{-1}$.

7

8 *Plasmid construction*

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

22

23 *Protein purification and analysis*

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

1 were grown in M9 medium supplemented with glucose, 10 g l⁻¹, and ¹⁵NH₄Cl, 1 g l⁻¹. At an
2 OD₆₀₀ of 0.7, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 1 mM, and the
3 cultures were grown for an additional 5 h. The cells were broken with a French press and the
4 protein was purified on Glutathione Sepharose as described previously (Login and Shevchik,
5 2006). Protein was dialysed against 20 mM Tris-HCl pH 9, 1 mM EDTA and loaded onto a
6 Hi Trap Q-FF column (GE Healthcare) equilibrated with the same buffer. The pure protein
7 was eluted by NaCl gradient, concentrated and washed in 10 mM Tris-HCl pH 7, 0.1 mM
8 EDTA with centricon YM-10 or YM-3 (Millipore).

9 OutD_{His} derivatives used in pull down assays were produced in *E. coli* C41(DE3)/pLys
10 cells carrying a corresponding pET-oD_{X-Y} plasmid (oD_{X-Y} for any OutD derivative, as in
11 Table S1). Because of a strong growth-static effect provoked by expression of OutD₂₈₋₇₁₀,
12 induction time was limited to 45 min. The proteins were purified using Ni-NTA agarose, as
13 described (Login and Shevchik, 2006). The recombinant proteins were solubilized in 100 mM
14 sodium phosphate, 10 mM Tris-HCl, 100 mM NaCl pH 8.0, then bound to the resin, washed
15 with the same buffer at pH 6.8 and eluted with a linear gradient of histidine. Purification of
16 OutD₂₈₋₇₁₀ was performed in the presence of 1% Triton X-100. For NMR experiments, an
17 additional purification on a Hi Trap Q FF column (GE Healthcare) was performed as above.

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

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

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

3

4 *Protein-protein interaction assays*

5 *In vitro* GST pull-down assays were performed as described previously (Login and Shevchik,
6 2006). Equal amounts of GST or GST-OutC derivatives were immobilized on Glutathione
7 Sepharose beads. The beads with bound GST-OutC derivatives have been systematically
8 checked by SDS-PAGE prior to the binding assay and then diluted if necessary with non-
9 charged resin to reach a similar amount of various fusion proteins. Next an equal amount of
10 prey protein (OutD_{His} derivative) was loaded onto the resin with immobilized GST fusions,
11 incubated for 1 h and then washed. The bound proteins were eluted with Laemmli sample
12 buffer, separated by SDS-PAGE and probed by immunoblotting with either OutD-antibodies,
13 or Ni-NTA conjugated with peroxidase, or Tetra-His-antibodies (Quiagen).

14 For co-purification assays, the couples of GST-OutC and OutD_{His} derivatives were co-
15 expressed in *E. coli* BL21(DE3) carrying one of the pGX-oC_{X-Y}-oD_{X-Y} plasmids (oC_{X-Y} and
16 oD_{X-Y} for any OutC and OutD derivatives, as in Table S1). Bacteria were grown and induced
17 with 1 mM IPTG for 3 h. Cells were resuspended in 50 mM Tris-HCl pH 8.0, 100 mM NaCl,
18 1 mM EDTA (Buffer B) supplemented with either 1% or 0.1% (v/v) Triton X-100 and broken
19 by sonication. Unbroken cells were eliminated at 6000 g for 10 min and the cell lysates were
20 incubated with Glutathione Sepharose for 1 h. Next, the resin was washed 3 times with buffer
21 B supplemented with Triton X-100 and for an additional 3 times with buffer B without
22 detergent. The bound proteins were eluted with Laemmli sample buffer, separated by SDS-
23 PAGE and probed by immunoblotting with either OutD-antibodies, or Ni-NTA conjugated
24 with peroxidase, or Tetra-His-antibodies (Quiagen).

1 *In vivo* co-purification of proteins bound to GST or GST-OutC during secretion was
2 performed from *E. chrysanthemi* A3618 cells carrying either pGEX-6P-3 or pTdB-GoC
3 plasmid. The bacteria were grown in LB to an OD₆₀₀ of 0.7 and the proteins bound to GST or
4 GST-OutC were co-purified, as described above at the presence of 1% Triton X-100, and
5 analyzed by 2D-GE.

6

7 *NMR experiments*

8 NMR spectra were recorded at 303K on a Bruker AvanceII spectrometer operating at a proton
9 frequency of 600.13 MHz, equipped with a triple resonance HCN probe head and actively-
10 shielded z-gradients. ¹H spectra were acquired using excitation sculpting with gradients for
11 water suppression (Hwang and Shaka, 1995). ¹H/¹⁵N 2D correlation experiments (Palmer *et*
12 *al.*, 1991) were acquired via double INEPT transfer using sensitivity improvement. Phase
13 sensitive detection in the indirect dimension used echo/antiecho-TPPI gradient selection, with
14 decoupling during acquisition. Water was suppressed using a soft flip-back pulse. Data
15 acquisition and processing were performed using the Bruker Topspin program. Chemical
16 shifts were referenced to internal DSS (2,2-Dimethyl-2-silapentane-5-sulfonic acid) for ¹H
17 directly and indirectly for ¹⁵N data. All samples for NMR spectroscopy were made up in 10
18 mM Tris/HCl, pH 7.0, 10 mM CaCl₂, 10% D₂O, 100 μM DSS. When recording 2D ¹⁵N-
19 HSQC spectra, ¹⁵N-labelled proteins were used at concentrations of 0.5 and 0.2 mM;
20 unlabelled proteins were present at molar ratios of 2:1 and 1:1 (0.25mM and 0.2 mM,
21 respectively). All spectra were kindly recorded by Dr H. Toms.

22

23 **Acknowledgments**

24 We are grateful to G. Condemine and other members of the Erwinia team for reading the
25 manuscript, and for their valuable discussions. This work was supported by grants from the

1 Centre National de la Recherche Scientifique (CNRS) and the Franco-British programme
2 Alliance 12109QK.

3

4 **References**

5 Bitter, W., Koster, M., Latijnhouwers, M., de Cock, H., and Tommassen, J. (1998) Formation
6 of oligomeric rings by XcpQ and PilQ, which are involved in protein transport across the
7 outer membrane of *Pseudomonas aeruginosa*. *Mol Microbiol* **27**: 209-219.

8 Bleves, S., Lazdunski, A., and Filloux, A. (1996) Membrane topology of three Xcp proteins
9 involved in exoprotein transport by *Pseudomonas aeruginosa*. *J Bacteriol* **178**: 4297-4300.

10 Bleves, S., Gérard-Vincent, M., Lazdunski, A., and Filloux, A. (1999) Structure-function
11 analysis of XcpP, a component involved in general secretory pathway-dependent protein
12 secretion in *Pseudomonas aeruginosa*. *J Bacteriol* **181**: 4012-4019.

13 Bouley, J., Condemine, G., and Shevchik, V.E. (2001) The PDZ domain of OutC and the N-
14 terminal region of OutD determine the secretion specificity of the type II Out pathway of
15 *Erwinia chrysanthemi*. *J Mol Biol* **308**: 205-219.

16 Brok R., Van Gelder P., Winterhalter, M., Ziese, U., Koster, A.J., de Cock, H., et al. (1999)
17 The C-terminal domain of the *Pseudomonas* secretin XcpQ forms oligomeric rings with
18 pore activity. *J Mol Biol* **294**: 1169-1179.

19 Burghout, P., Beckers, F., de Wit, E., van Boxtel, R., Cornelis, G.R., Tommassen, J., and
20 Koster, M. (2004) Role of the pilot protein YscW in the biogenesis of the YscC secretin in
21 *Yersinia enterocolitica*. *J Bacteriol* **186**: 5366-5375.

22 Burghout, P., van Boxtel, R., Van Gelder, P., Ringler, P., Müller, S.A., Tommassen, J., and
23 Koster, M. (2004) Structure and electrophysiological properties of the YscC secretin from
24 the type III secretion system of *Yersinia enterocolitica*. *J Bacteriol* **186**: 4645-4654.

- 1 Chami, M., Guilvout, I., Gregorini, M., Rémigy, H., Müller, S.A., Valerio, M., et al. (2005)
2 Structural insights into the secretin PulD and its trypsin resistant core. *J Biol Chem* **280**:
3 37732-37741.
- 4 Cianciotto, N.P. (2005) Type II secretion: a protein secretion system for all seasons. *Trends*
5 *Microbiol* **13**: 581-588.
- 6 Chou, P.Y., and Fasman, G.D. (1978) Empirical predictions of protein conformation. *Annu*
7 *Rev Biochem* **47**: 251-276.
- 8 Cole, C., Barber, J.D., and Barton, G.J. (2008) The Jpred 3 secondary structure prediction
9 server. *Nucleic Acids Res* **36**: W197–W201.
- 10 Condemine, G., and Shevchik, V.E. (2000) Overproduction of the secretin OutD suppresses
11 the secretion defect of an *Erwinia chrysanthemi* *outB* mutant. *Microbiology* **146**: 639-647.
- 12 Daefler, S., Russel, M. and Model, P. (1997) Module swaps between related translocator
13 proteins pIV(f1), pIV(IKe) and PulD: identification of a specificity domain. *J Mol Biol*
14 **266**: 978-992.
- 15 de Groot, A., Koster, M., Gérard-Vincent, M., Gerritse, G., Lazdunski, A., Tommassen, J.,
16 and Filloux, A. (2001) Exchange of Xcp (Gsp) secretion machineries between
17 *Pseudomonas aeruginosa* and *Pseudomonas alcaligenes*: species specificity unrelated to
18 substrate recognition. *J Bacteriol* **183**: 959-967.
- 19 Douet, V., Loiseau, L., Barras, F., and Py, B. (2004) Systematic analysis, by the yeast two-
20 hybrid, of protein interaction between components of the type II secretory machinery of
21 *Erwinia chrysanthemi*. *Res Microbiol* **155**: 71-75.
- 22 Evans, F.F., Egan, S., and Kjelleberg, S. (2008) Ecology of type II secretion in marine
23 *gammaproteobacteria*. *Environ Microbiol* **10**: 1101-1107.
- 24 Ferrandez, Y, Condemine, G. (2008) Novel mechanism of outer membrane targeting of
25 proteins in Gram-negative bacteria. *Mol Microbiol* **69**: 1349-1357.

- 1 Filloux, A. (2004) The underlying mechanisms of type II protein secretion. *Biochim Biophys*
2 *Acta* **1694**: 163-179.
- 3 Genin, B., and Boucher, C.A. (1994) A superfamily of proteins involved in different secretion
4 pathways in gram-negative bacteria: modular structure and specificity of the N-terminal
5 domain. *Mol Gen Genet* **243**: 112-118.
- 6 Gérard-Vincent, M., Robert, V., Ball, G., Bleves, S., Michel, G.P., Lazdunski, A., and
7 Filloux, A. (2002) Identification of XcpP domains that confer functionality and specificity
8 to the *Pseudomonas aeruginosa* type II secretion apparatus. *Mol Microbiol* **44**: 1651-1665.
- 9 Guilvout, I., Hardie, K.R., Sauvonnet, N., Pugsley, A.P. (1999) Genetic dissection of the outer
10 membrane secretin PulD: are there distinct domains for multimerization and secretion
11 specificity? *J Bacteriol* **181**: 7212-7220.
- 12 Guilvout, I., Chami, M., Engel, A., Pugsley, A.P., and Bayan, N. (2006) Bacterial outer
13 membrane secretin PulD assembles and inserts into the inner membrane in the absence of
14 its pilotin. *EMBO J* **25**: 5241-5249.
- 15 Guilvout, I., Chami, M., Berrier, C., Ghazi, A., Engel, A., Pugsley, A.P., and Bayan, N.
16 (2008) *In vitro* multimerization and membrane insertion of bacterial outer membrane
17 secretin PulD. *J Mol Biol* **382**: 13-23.
- 18 Hardie, K.R., Lory, S., and Pugsley, A.P. (1996) Insertion of an outer membrane protein in
19 *Escherichia coli* requires a chaperone-like protein. *EMBO J* **15**: 978-988.
- 20 Hwang, T.-L., and Shaka, A.J. (1995) Water suppression that works: excitation sculpting
21 using arbitrary waveforms and pulse field gradients. *J Magn Reson* **112**: 275-279.
- 22 Kazemi-Pour, N., Condemine, G., and Hugouvieux-Cotte-Pattat, N. (2004) The secretome of
23 the plant pathogenic bacterium *Erwinia chrysanthemi*. *Proteomics* **4**: 3177-3186.

- 1 Korotkov, K.V., Krumm, B., Bagdasarian, M., and Hol, W.G. (2006) Structural and
2 functional studies of EpsC, a crucial component of the type 2 secretion system from *Vibrio*
3 *cholerae*. *J Mol Biol* **363**: 311-321.
- 4 Korotkov, K.V., Pardon, E., Steyaert, J., and Hol, W.G. (2009) Crystal structure of the N-
5 terminal domain of the secretin GspD from ETEC determined with the assistance of a
6 nanobody. *Structure* **17**: 255-265.
- 7 Lee, H.M., Chen, J.R., Lee, H.L., Leu, W.M., Chen, L. Y., and Hu, N.T. (2004) Functional
8 dissection of the XpsN (GspC) protein of the *Xanthomonas campestris* pv. *campestris* type
9 II secretion machinery. *J Bacteriol* **186**: 2946-2955.
- 10 Lee, M.S., Chen, L.Y., Leu, W.M., Shiau, R.J., and Hu, N.T. (2005) Associations of the major
11 pseudopilin XpsG with XpsN (GspC) and secretin XpsD of *Xanthomonas campestris* pv.
12 *campestris* type II secretion apparatus revealed by cross-linking analysis. *J Biol Chem* **280**:
13 4585-4591.
- 14 Lee, H.M., Wang, K.C., Liu, Y.L., Yew, H.Y., Chen, L.Y., Leu, W.M., et al. (2000)
15 Association of the cytoplasmic membrane protein XpsN with the outer membrane protein
16 XpsD in the type II protein secretion apparatus of *Xanthomonas campestris* pv. *campestris*.
17 *J Bacteriol* **182**: 1549-1557.
- 18 Lindeberg, M., Salmond, G.P.C., and Collmer, A. (1996) Complementation of deletion
19 mutants in a cloned functional cluster of *Erwinia chrysanthemi* *out* genes with *Erwinia*
20 *carotovora* *out* homologues reveals OutC and OutD as candidate gatekeepers of species-
21 specific secretion of proteins via the type II pathway. *Mol Microbiol* **20**: 175-190.
- 22 Linderoth, N.A., Simon, M.N., and Russel, M. (1997) The filamentous phage pIV multimer
23 visualized by scanning transmission electron microscopy. *Science* **278**: 1635-1638.

- 1 Login, F.H., and Shevchik, V.E. (2006) The single transmembrane segment drives self-
2 assembly of OutC and the formation of a functional type II secretion system in *Erwinia*
3 *chrysanthemi*. *J Biol Chem* **281**: 33152-33162.
- 4 Michel, G.P., Durand, E., and Filloux, A. (2007) XphA/XqhA, a novel GspCD subunit for
5 type II secretion in *Pseudomonas aeruginosa*. *J Bacteriol* **189**: 3776-3783.
- 6 Nouwen, N., Stahlberg, H., Pugsley, A.P., and Engel, A. (2000) Domain structure of secretin
7 PulD revealed by limited proteolysis and electron microscopy. *EMBO J* **19**: 2229-2236.
- 8 Palmer, A.G., Cavanagh, J., Wright, P.E. and Rance, M. (1991) Sensitivity improvement in
9 proton-detected two-dimensional heteronuclear correlation NMR spectroscopy. *J Magn*
10 *Reson* **93**: 151-170.
- 11 Possot, O.M., Gérard-Vincent, M., and Pugsley, A.P. (1999) Membrane association and
12 multimerization of secretin component PulC. *J Bacteriol* **181**: 4004-4011.
- 13 Possot, O.M., Vignon, G., Bomchil, N., Ebel, F., and Pugsley, A.P. (2000) Multiple
14 interactions between pullulanase secretin components involved in stabilization and
15 cytoplasmic membrane association of PulE. *J Bacteriol* **182**: 2142-2152.
- 16 Pugsley, A.P., Poquet, I., and Kornacker, M.G. (1991) Two distinct steps in pullulanase
17 secretion by *Escherichia coli* K12. *Mol Microbiol* **5**: 865-873.
- 18 Shevchik, V.E., Robert-Baudouy, J., and Condemine, G. (1997) Specific interaction between
19 OutD, an *Erwinia chrysanthemi* outer membrane protein of the general secretory pathway,
20 and secreted proteins. *EMBO J* **16**: 3007-3016.
- 21 Shevchik, V.E., and Condemine, G. (1998) Functional characterization of the *Erwinia*
22 *chrysanthemi* OutS protein, an element of a type II secretion system. *Microbiology* **144**:
23 3219-3228.

1 Thomas, J.D., Reeves, P.J., and Salmond, G.P. (1997) The general secretion pathway of
2 *Erwinia carotovora* subsp. *carotovora*: analysis of the membrane topology of OutC and
3 OutF. *Microbiology* **143**: 713-720.

4 Voulhoux, R., Ball, G., Ize, B., Vasil, M.L., Lazdunski, A., Wu, L.F., and Filloux, A. (2001)
5 Involvement of the twin-arginine translocation system in protein secretion via the type II
6 pathway. *EMBO J* **20**: 6735-6741.

7 Whitty, A. (2008) Cooperativity and biological complexity *Nat Chem Biol* **4**: 435-439.

8 9 **Figure legends**

10

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

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

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

19 * The sequences of the OutC_{sp} derivatives are shown in Fig. S3.

20

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

22 **A.** OutD interacts with two distinct regions of OutC. Equal amounts of GST-OutC derivatives
23 (indicated on top) were immobilized on Glutathione Sepharose beads, as checked by SDS-
24 PAGE prior to the binding assay. Next, an equal amount of OutD₂₈₋₇₁₀ was loaded onto these
25 matrices, incubated for 1 h and washed. Bound proteins were eluted with Laemmli sample

1 buffer, separated by SDS-PAGE and either stained (upper panels) or probed with OutD-
2 antibodies (lower panels). GST-fused proteins are indicated by asterisks and OutD derivatives
3 by triangles. Lower species, reacting with GST-antibodies, are GST-OutC degradation
4 products generated during the incubation. Amounts of bound OutD₂₈₋₇₁₀ were estimated by
5 densitometry (indicated as a percentage of that to GST-OutC on bottom of the left panel).

6 **B.** N-domain of OutD interacts with the N-terminal region of OutC. Pull-down experiments
7 were performed as in (A). OutD₂₈₋₃₅₇ was used in place of OutD₂₈₋₇₁₀.

8 **C.** N-domain of OutD interacts with the HR region of OutC. GST-OutC derivatives (indicated
9 on top) and OutD₂₈₋₂₈₅ were co-expressed in *E. coli* BL21(DE3) from the single plasmids and
10 then used in co-purification assay on Glutathione Sepharose.

11

12 **Fig. 3. 2D ¹⁵N-HSQC NMR analysis of the interaction of OutC and OutD.**

13 **A and B.** 2D ¹⁵N-HSQC spectrum of the ¹⁵N-labelled periplasmic region of OutC (OutC₆₀₋₂₇₂
14 spectra in green) overlaid with the 2D ¹⁵N-HSQC spectrum of the ¹⁵N-labelled PDZ domain
15 (OutC₁₆₁₋₂₇₂ spectra in red). In panel (A) the spectra are recorded in the absence of OutD and
16 in panel (B) in the presence of 0.25 mM OutD (unlabelled OutD₂₈₋₂₈₅). The concentration of
17 the OutC derived proteins was 0.50 mM. The PDZ domain spectra (red) in panel A is almost
18 identical to that in panel B showing there is no interaction with OutD. The loss of intensity for
19 the non-PDZ region (HR) of the spectra in panel B compared to panel A (green spectra)
20 shows it is the HR region that interacts with OutD.

21 **C.** S proximate superimposition of the ¹⁵N-HSQC spectra for OutC₆₀₋₂₇₂ in the absence (red)
22 and presence (green) of unlabelled OutD₂₈₋₂₈₅. The broadening of some of the peaks in the
23 presence of OutD (green) is clear and confirms the interaction between OutC and OutD. The
24 broadened peaks are not those assigned to the PDZ domain and hence the interaction between
25 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-OutC₆₀₋₂₇₂ 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-OutC₆₀₋₂₇₂ is indicated by asterisk and OutD derivatives by triangles.

Fig. 5. Two distinct regions of OutD, OutD₂₈₋₁₁₂ and OutD₁₁₆₋₂₈₅, interact with the OutC_{sip} peptide.

A and B. Dissection of the OutC_{sip} region interacting with OutD₂₈₋₁₁₂ (A) and OutD₁₁₆₋₂₈₅ (B). The GST-OutC derivatives (indicated at the top) were co-expressed with either OutD₁₁₆₋₂₈₅ or OutD₂₈₋₁₁₂ (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 OutD₂₈₋₁₁₂ and OutD₁₁₆₋₂₈₅ to GST-OutC₁₂₈₋₂₇₂. GST-OutC₁₂₈₋₂₇₂ was co-expressed with either OutD₂₈₋₁₁₂ or OutD₁₁₆₋₂₈₅ or the three proteins were all expressed together and used in co-purification assays as above. GST-OutC derivatives are indicated by asterisks, OutD₂₈₋₁₁₂ by open triangles and OutD₁₁₆₋₂₈₅ by filled triangles. Relative amounts of the proteins were estimated by densitometry and indicated as percentage of those with pairwise 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 OutC_{sip} region.

1 **A.** Stability of OutC mutant proteins (indicated on top) expressed in *E. chrysanthemi* A3618
 2 $\Delta outC$ cells. Immunoblotting of whole cell extracts with OutC-antibodies. In the right panel,
 3 three times more of material was loaded and the membrane was overexposed to detect low
 4 abundance products. OutC, OutC $_{\Delta 163-169}$ and OutC $_{\Delta 173-256}$ are indicated with a triangle. The
 5 species of the molecular weight slightly larger than OutC is a cross-reaction product. Lower
 6 molecular weight species in the OutC and OutC $_{\Delta 163-169}$ lanes are degradation products.

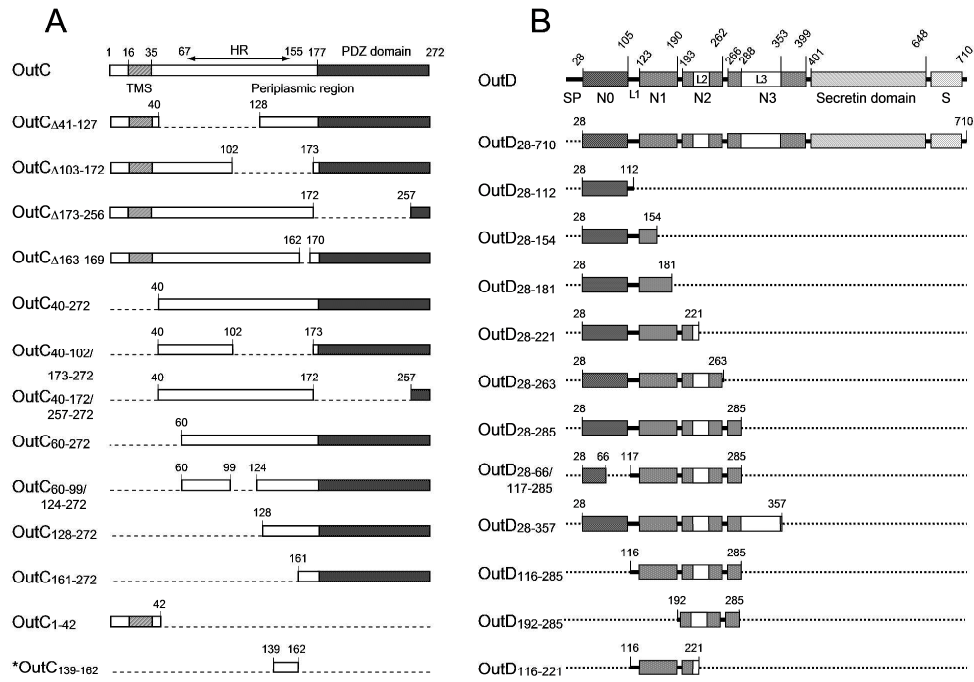
7 **B.** Complementation ability of OutC mutant proteins. The supernatant (S) and cell extract (C)
 8 of *E. chrysanthemi* A3618 $\Delta outC$ carrying a pTdB-oC plasmid with the corresponding mutant
 9 *outC* (indicated on top) were analyzed by immunoblotting with PemA-antibodies.

10

11 **Table 1. Strains used in this study**

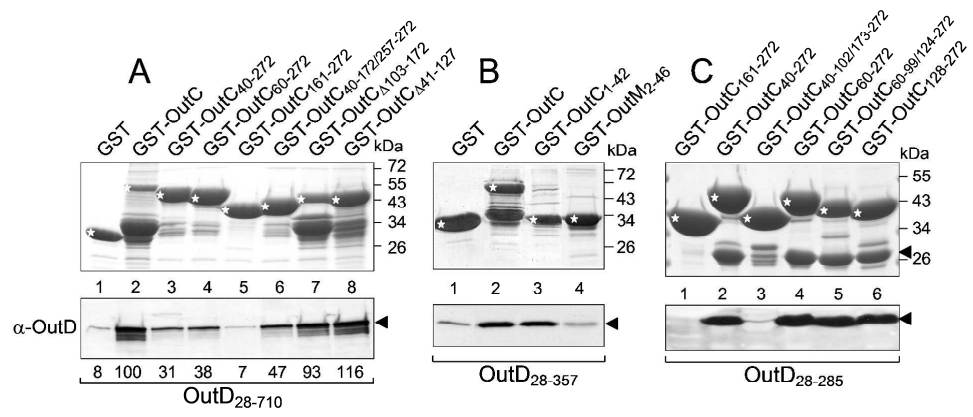
12

13 Strain	Genotype/phenotype	Reference
14		
15 <i>Escherichia coli</i>		
16 BL21(DE3)	F ⁻ <i>dcm ompT hsdSB</i> (r _B ⁻ , m _B ⁻) <i>gal lon</i> λ (DE3)	Studier <i>et al.</i> , 1990
17 BL21(DE3)C41	uncharacterized mutant of BL21(DE3)	Miroux & Walker, 1996
18 NM522	<i>supE thi-1</i> Δ (<i>lac-proAB</i>) Δ (<i>mcrB-hsdSM</i>)5	
19	(r _K ⁻ m _K ⁺) [F' <i>proAB lacI</i> ^q Z Δ M15]	Stratagene
20 <i>Erwinia chrysanthemi</i>		
21 A350	<i>lacZ2</i>	Laboratory collection
22 A1077	<i>lacZ2 kdgR::Mu Cm</i>	Laboratory collection
23 A3618	<i>lacZ2</i> $\Delta outC$ <i>kdgR::Mu Cm</i>	Bouley <i>et al.</i> , 2001



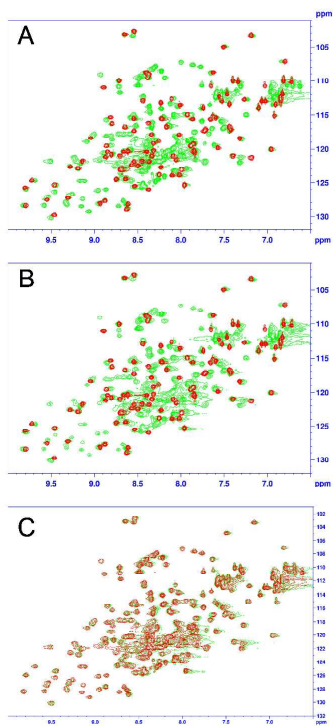
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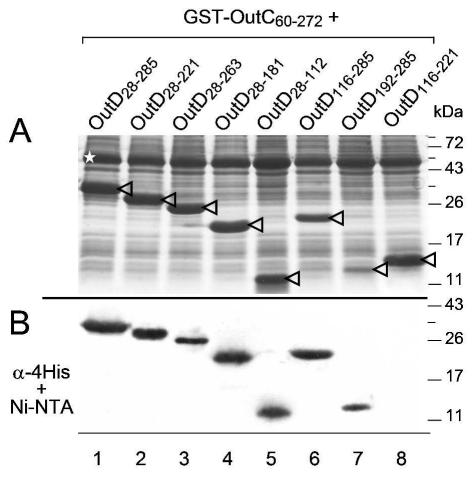
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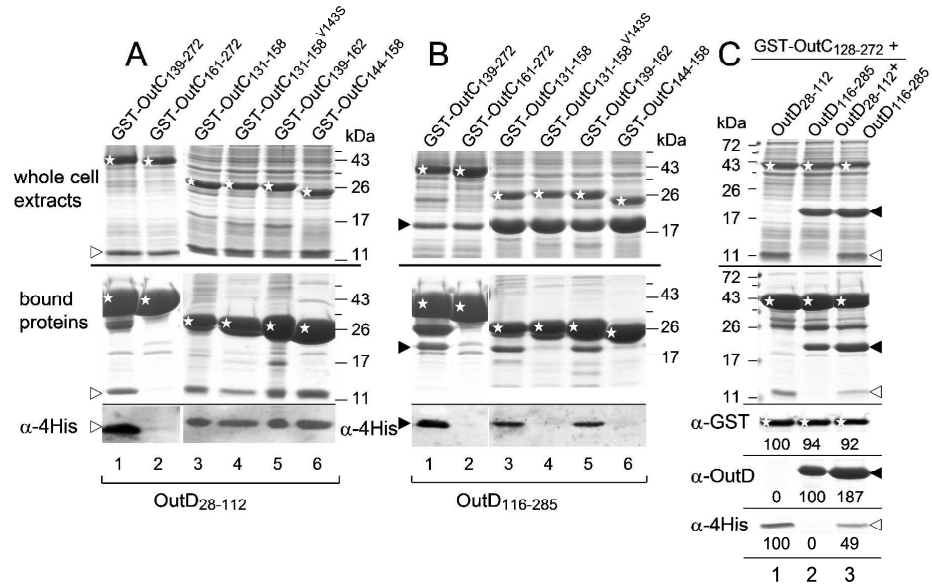
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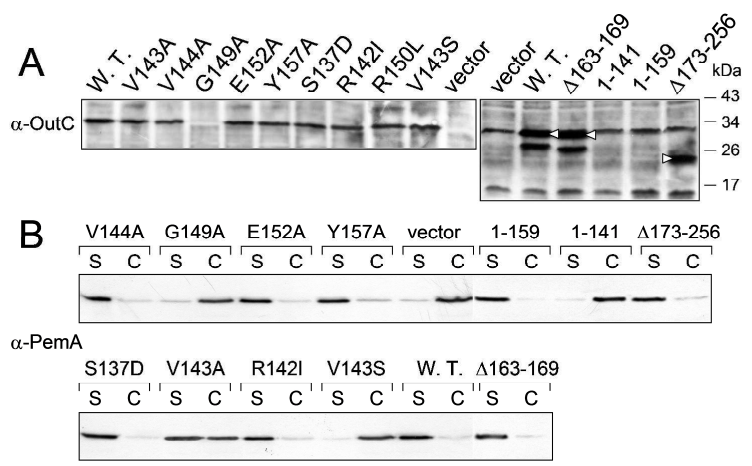
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