The Single Transmembrane Segment Drives Self-assembly of OutC and the Formation of a Functional Type II Secretion System in Erwinia chrysanthemi

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Many pathogenic Gram-negative bacteria secrete toxins and lytic enzymes via a multiprotein complex called the type II secretion system. This system, named Out in Erwinia chrysanthemi, consists of 14 proteins integrated or associated with the two bacterial membranes. OutC, a key player in this process, is probably implicated in the recognition of secreted proteins and signal transduction. OutC possesses a short cytoplasmic sequence, a single transmembrane segment (TMS), and a large periplasmic region carrying a putative PDZ domain. A hydrodynamic study revealed that OutC forms stable dimers of an elongated shape, whereas the PDZ domain adopts a globular shape. Bacterial two-hybrid, cross-linking, and pulldown assays revealed that the self-association of OutC is driven by the TMS, whereas the periplasmic region is dispensable for self-association. Site-directed mutagenesis of the TMS revealed that cooperative interactions between three polar residues located at the same helical face provide adequate stability for OutC self-assembly. An interhelical H-bonding mediated by Gln appears to be the main driving force, and two Arg residues located at the TMS boundaries are essential for the stabilization of OutC oligomers. Stepwise mutagenesis of these residues gradually diminished OutC functionality and self-association ability. The triple mutant R15V/Q29L/R36A became monomeric and non-functional. Self-association and functionality of the triple mutant were partially restored by the introduction of a polar residue at an alternative position in the interhelical interface. Thus, the OutC TMS is more than just a membrane anchor; it drives the protein self-association that is essential for formation of a functional secretion system.

The type II secretion system (T2SS) is employed by a number of pathogenic Gram-negative bacteria to secrete lytic enzymes and toxins. Secretion via this pathway is a two-step process. The proteins first cross the cytoplasmic membrane either by the Sec system or by the twin-arginine transport system, Tat (2). Once exported into the periplasm, the proteins are then secreted by the T2SS across the outer membrane into the medium. Depending on the species, the secretion machinery consists of 12–15 proteins whose exact function is still obscure for most of them. The majority of the components of T2SS are highly conserved, and most of the corresponding genes can be swapped between diverse bacterial species, except for gspC and gspD (3, 4) (gsp for general secretory pathway (5)).

The T2SS of the phytopathogenic enterobacteria Erwinia chrysanthemi, referred to as the Out system, secretes several pectinases and a cellulase (6).

Curiously, most of the proteins composing the T2SS are associated with or integrated in the inner membrane, except for OutD and OutS, which are located in the outer membrane (7, 8). This suggests that certain components of the T2SS ensure a permanent or transient junction between the two cellular membranes to allow for a functional integrity of the secretion machinery. The existence of two separate steps in the T2SS pathway assumes that the secreted proteins, once they have been exported into the periplasm, should be recognized by a special element(s) of the T2SS machinery. The inner membrane protein OutC has been suggested for the roles of signal transduction between the two cell membranes and recognition of secreted proteins (3, 6, 7, 9).

OutC consists of a short cytoplasmic sequence, a single transmembrane segment (TMS), and a large periplasmic region. A putative PDZ domain is located close to its C terminus (11). GspC proteins from certain other bacteria presumably possess a coiled-coil domain instead of a PDZ domain (12). Some algorithms also predict a coiled-coil structure for Erwinia OutC. Regardless of its structure, inter-species swapping indicated that this region of OutC directly participates in the specific recognition of the secreted proteins (6). Recently it was proposed that this region of GspC could be involved in the formation of homo-multimeric complexes (13). Genetic and biochemical studies suggested that GspC could interact with the inner membrane proteins GspM and GspL (14–16). Furthermore, the current models of the T2SS imply that GspC interacts, at least transiently, with the outer membrane protein GspD (1, 7, 9, 15, 17).

It has been shown that some components of the T2SS are assembled into homomultimeric structures. The NTPase GspE located in the cytoplasm seems to take the shape of a hexameric
TABLE 1

Plasmids used in this study

| Plasmid   | Genotype/phenotype | Reference |
|-----------|--------------------|-----------|
| Two-hybrid vectors |                    |           |
| pUT18C    | pUC19 derivative coding T18 fragment of CyaA upstream of a multicloning site, Ap<sup>a</sup> | 25        |
| pKT25     | pSU40 derivative coding T25 fragment of CyaA upstream of a multicloning site, Kn<sup>a</sup> | 25        |
| pUT-oC    | pUT18C carrying outC(aa 2–272) fused in-frame to the T18 gene | This work |
| pUT-oC<sub>A</sub>A | pUT-oC with a BsaHI-BsaHI deletion (Δ aa 100–123) | This work |
| pUT-oC<sub>A</sub> | pUT-oC with a HindII-BsrBI deletion (Δ aa 173–256) | This work |
| pUT-oC<sub>A</sub>H | pUT-oC with a HpaI<sup>a</sup>-NruI<sup>a</sup> deletion (Δ aa 61–72) | This work |
| pUT-oC<sub>A</sub>N | pUT-oC with a NaeI-HindII deletion (Δ aa 103–172) | This work |
| pUT-oC<sub>A</sub>U | pUT-oC with a Smal<sup>a</sup>-NruI<sup>a</sup> deletion (Δ aa 41–72) | This work |
| pUT-oC<sub>L</sub>L | pUT18C carrying truncated outC fused at BamHI<sup>a</sup> (aa 161–272) | This work |
| pUT-oC<sub>A</sub>S | pUT18C carrying truncated outC fused at Smal<sup>a</sup> (aa 40–207) | This work |
| pUT-oC-TMS | pUT-oC with Opal stop codon at 43 aa (Δ aa 43–272) | This work |
| pKT<sub>A</sub>C | pKT25 carrying outC (aa 2–272) fused in-frame to the T25 gene | This work |
| pKT<sub>A</sub>ΔR | pKT-oC with a HindII-BsrBI deletion (Δ aa 173–256) | This work |
| pKT<sub>A</sub>ΔN | pKT-oC with a NaeI-HindII deletion (Δ aa 103-172) | This work |
| pKT<sub>A</sub>ΔU | pKT-oC with a Smal<sup>a</sup>-NruI<sup>a</sup> deletion (Δ aa 41–72) | This work |
| pKT<sub>A</sub>ΔC | pKT25 carrying truncated outC fused at Spel<sup>a</sup> (aa 14–272) | This work |
| pKT<sub>A</sub>ΔL | pKT25 carrying truncated outC fused at BamHI<sup>a</sup> (aa 161–272) | This work |
| pKT<sub>A</sub>ΔS | pKT25 carrying truncated outC fused at Smal<sup>a</sup> (aa 40–207) | This work |
| pKT<sub>A</sub>TMS | pKT25 with Opal stop codon at 43 aa (Δ aa 43–272) | This work |
| GST fusion vectors |                    |           |
| pGE-6P-3    | GST fusion vector with PreScission protease cleavage site, Ap<sup>a</sup> | GE Healthcare |
| pGX-oC     | pGEX-6p-3 carrying outC (aa 2–272) fused in-frame to the GST gene | This work |
| pGX-oCA    | pGX-oC with a BsaHI-BsaHI deletion (Δ aa 100–123) | This work |
| pGX-oCAH   | pGX-oC with a HpaI<sup>a</sup>-NruI<sup>a</sup> deletion (Δ aa 61–72) | This work |
| pGX-oCAN   | pGX-oC with a NaeI-HindII deletion (Δ aa 103–172) | This work |
| pGX-oCAR   | pGX-oC with a HindII-BsrBI deletion (Δ aa 173–256) | This work |
| pGX-oCU    | pGX-oC with a Smal<sup>a</sup>-NruI<sup>a</sup> deletion (Δ aa 41–72) | This work |
| pGX-oCS    | pGX-6p-3 carrying truncated outC (aa 40–272) fused in-frame to the GST gene | This work |
| pGX-oCTMS  | pGX-oC with Opal stop codon at 43 aa (Δ aa 43–272) | This work |
| His<sub>A</sub> fusion vectors |                    |           |
| pQE-30     | His<sub>A</sub> fusion vectors, Ap<sup>a</sup> | Qiagen |
| pQE-32     | pQE-32 carrying His<sub>A</sub>-outC (aa 2–272) | 6         |
| pQE-HisOC  | pQE-32 carrying His<sub>A</sub>-outC (aa 2–272) | This work |
| pQE-HisOCΔL | pQE-30 carrying His<sub>A</sub>-outC (aa 161–272) | This work |
| pQE-HisOCΔS | pQE-30 carrying His<sub>A</sub>-outC (aa 40–272) | This work |
| pTDB-OC    | pTDB-OC carrying His<sub>A</sub>-outC under the petC promoter | 6         |

<sup>a</sup> Restriction endonuclease sites introduced in the outC sequence by site-directed mutagenesis.

ring-like structure (18). When overexpressed, certain pseudopili form long flexible pili comprising multiple pseudopilin subunits (19). The secretin GspD forms dodecameric rings in a lipid bilayer that could correspond to the channels in the bacterial outer membranes (20, 21). Therefore, it seems plausible that OutC, which was presumed to interact with OutD and with the inner membrane platform formed by OutE, OutF, OutL, and OutM (22), could also be assembled into multimeric structures.

The mechanisms that govern the assembly of the T2SS components into a functional multiprotein complex are still poorly understood. Certain binary interactions between soluble protein regions have been detected by using yeast two-hybrid analysis and in vitro assays (22, 23). Although specific interactions between α-helical TMS are important for the folding and oligomerization of membrane proteins (24), their role in the assembly and function of the T2SS has not been thoroughly analyzed.

Here we performed a detailed analysis of the oligomerization state of OutC. Bacterial two-hybrid, cross-linking and pull-down assays revealed that the self-association of OutC is driven by the TMS, whereas the periplasmic region is dispensable for self-association. Site-directed mutagenesis of the TMS revealed that cooperative interactions between three polar residues located at the same helical face, Gln<sup>29</sup>, Arg<sup>15</sup>, and Arg<sup>36</sup>, provide adequate stability for the OutC self-assembly necessary for the protein function. These results allowed us to revise the previous opinion that a single TMS of GspC plays a passive role assuming the anchoring of the protein into the inner membrane (15), and we demonstrated instead that the TMS drives the self-association of OutC that is essential for the formation of a functional secretion system.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The plasmids used in the study are listed in Table 1. The single and multiple mutations were introduced in the outC sequence of E. chrysanthemi 3937 by site-directed mutagenesis using the QuickChange kit (Stratagene). The primers used are listed in supplemental Table S1. The nucleotide sequences of mutant genes were systematically checked (Genome Express). The OutC truncated derivatives were constructed by using the restriction sites introduced by site-directed mutagenesis and naturally existing sites to give in frame deletions, as indicated in Table 1.

**Bacterial Two-hybrid Experiments**—The bacterial two-hybrid system, kindly provided by G. Karimova (25), was used according to the authors’ instructions. outC or its truncated derivatives were fused in phase to the C termini of gene fragments coding for the T18 and T25 domains of adenylate cyclase from E. coli strain DHP1 (F<sup>−</sup> cya glnV44<sup>AS</sup> recA1 endA1 gyrA96 (Nal<sup>+</sup> thi1 hisdR17 spoT1 rfbD1) (25) and the transformants were plated on MacConkey-
Self-assembly of OutC

![Table A]

**FIGURE 1.** A, schematic representation of OutC and its derivatives used in the study. Depending on the experiment, the N terminus of a derivative was fused to a sequence coding either for His6, GST, or one of the Cya fragments, T25 and T18. The short gray segment represents the cytoplasmic region, the black box represents the predicted TMS, and the hatched box represents the putative PDZ domain. B, deduced amino acid sequence of the predicted TMS of OutC, C, a helical wheel projection of the predicted TM α-helix of OutC. Charged residues are in bold. The residues mutated in the present study are indicated by asterisks.

maltose agar supplemented with ampicillin and kanamycin.

The color of the colonies was monitored during incubation at 30 °C for 36–48 h. β-Galactosidase assays were performed as described (26) in DH51 liquid cultures grown in Luria-Bertani (LB) medium supplemented with 1 mM isopropyl-β-d-thiogalactopyranoside and with antibiotics at 28 °C for 18 h. All assays were performed from triplicate cultures on three to four different bacterial transformants and on several different days.

**Gel Electrophoresis and Immunoblotting—**SDS-PAGE was performed according to Laemmli (27), and proteins were either stained with Coomassie G-250 or transferred onto nitrocellulose. The membrane was then incubated with antibodies and developed with the ECL detection kit (GE Healthcare) as described previously (7). The primary antibodies used were anti-OutC, anti-PelD, anti-LspA, and anti-Cel5 as described previously (6). Horseradish peroxidase-conjugated Ni-NTA (Qiagen) was diluted 1:3000.

**Complementation Test—**To test the functionality of OutC mutant proteins, the *E. chrysanthemi* ΔOutC strain A3618 (6) was transformed with a pTdB-OC derivative carrying a corresponding outC mutant gene. Exoprotein secretion was initially tested using the holo size on plate assays for pectinase and cellulase activities (6). For immunoblotting assays, *E. chrysanthemi* were grown at 28 °C in LB for 14 h until early stationary phase. Cells were pelleted by centrifugation at 10,000 × g for 2 min and resuspended at the same volume of LB. The culture supernatants and cell extracts were separated by SDS-PAGE and revealed with antibodies against diverse exoproteins.

**Protease Accessibility Assay—** *E. coli* MG1655 (F’ λ- rph-1) cells carrying a plasmid with one of the outC derivatives were grown in LB at 30 °C to an A600 of 0.6. Cells from 5 ml of cultures were pelleted and resuspended in 0.2 ml of 0.1 M Tris-HCl (pH 8.0), 0.5 M sucrose, and 1 mM EDTA. Lysozyme (5 µl of 3 mg/ml) was added, and the cells were incubated on ice for 15 min. After the incubation, 0.2 ml of ice-cold 5 mM MgSO4 was added, and the spheroplast suspension was separated into 100-µl aliquots. Two aliquots were treated with trypsin (50 µg/ml) for 15 min on ice and, before the proteolysis, 0.05% Triton X-100 was added to one of them. Proteolysis was stopped by the addition of phenylmethylsulfonyl fluoride to 2 mM, and the spheroplasts were harvested at 4000 × g for 4 min and resuspended in the same volume of Laemmli sample buffer. An untreated spheroplast aliquot was used for β-galactosidase and alkaline phosphatase activity assays (26).

**Protein Purification—** *E. coli* NM522 (New England Biolabs) cells carrying a plasmid coding for one of the His-tagged OutC proteins (Table 1) were grown in LB supplemented with ampicillin (150 µg/ml) at 30 °C. At an A600 of 0.7, isopropyl-β-d-thiogalactopyranoside was added to 1 mM, and the cultures were grown for an additional 3 h. Cells were pelleted by centrifugation and frozen at −70 °C. The cell pellet was resuspended in 100 mM sodium phosphate, 10 mM Tris-HCl, 100 mM NaCl, 1% Triton X-100 (pH 8.0) (buffer A), and broken by sonication. The recombinant proteins were purified on Ni-NTA agarose (Qiagen) at 15 °C as described by the manufacturer. Pefabloc (0.1 mg/ml) was used in all solutions.

*E. coli* BL21(DE3) (Strategene) cells carrying a plasmid coding for one of the GST-OutC derivatives were grown and stored as above. Purification was performed at 15 °C. The cell pellet was resuspended in 50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100 (pH 7.0) (buffer B), and sonicated. The lysate was centrifuged at 7000 × g for 5 min, mixed with glutathione-Sepharose 4B (GE Healthcare) equilibrated in the same buffer, and then incubated with mixing for 1 h. Unbound proteins were removed by washing 3 times for 5 min with buffer B and an additional 3 times with buffer B containing 0.1% Triton X-100. The proteins were cleaved from GST by the addition of PreScission protease (GE Healthcare) to 35 units/ml for 2 h. Eluted proteins were separated from the resin by centrifugation at 10000 × g for 2 min and mixed with a new portion of the resin to eliminate any trace of uncleaved GST fusions and the PreScission protease.

**Pulldown Assay—**GST-OUTC derivatives were purified as above except that the PreScission protease was omitted, and the fusion proteins remained immobilized on glutathione-Sepharose beads. The quantities of immobilized GST fusions were checked by SDS-PAGE before the binding assays. An equal amount (about 50 µg) of purified His-tagged OutC or 0.6 ml of BL21 lysate containing OutC mutant proteins were added to immobilized GST or GST-OutC fusions in buffer B containing 0.1% Triton X-100. After a 1-h incubation with mixing at 8 rpm and at 15 °C, the mixtures were spun for 2 min at 10000 × g, and
the pelleted beads were washed 3 times with the same buffer. The bound proteins were eluted with Laemmli sample buffer and analyzed by SDS-PAGE and immunoblotting with Ni-NTA-peroxidase or anti-OutC.

**Gel Filtration Chromatography**—A Superdex 200 10/300 GL column (GE Healthcare) was equilibrated with buffer B containing 0.1% Triton X-100 at 15 °C. The flow rate was 0.4 ml/min, and 0.2-ml fractions were collected. The fractions were analyzed by SDS-PAGE followed by immunoblotting with anti-OutC. Blue dextran and NaCl were used for the determination of the void volume ($V_v$) and the total volume ($V_t$), respectively. The elution volumes ($V_e$) of proteins of known molecular mass and Stokes radius ($R_s$) were used as the standards. The standard curve was plotted with the logarithm of $R_s$ against the $K_D$ of the standard protein ($K_D = V_e - V_v / V_t - V_v$).

**Determination of Sedimentation Coefficient**—Linear 3.8-ml sucrose gradients of 2.5–20% sucrose (w/v) in buffer B containing 0.1% Triton X-100 were prepared in either H$_2$O or D$_2$O. The sample of 60 µl, containing OutC or one of its derivatives, together with the standard proteins was loaded onto the gradients and centrifuged for 14 h (H$_2$O) or 20 h (D$_2$O) at 55,000 rpm in a Beckman SW-60 rotor at 4 °C. Then forty fractions were collected from the bottom of each gradient and analyzed by SDS-PAGE. The positions of the proteins were determined by quantitative scanning of stained gels and immunoblots. Refractive indices were determined with a Carl Zeiss refractometer. The partial specific volumes ($\rho_n$) of proteins were calculated from the amino acid composition using Sednterp software. The apparent hydration ($\delta$) was estimated from $f / f_o$ using Perrin's function ($P$) as described (31), with the apparent hydration ($\delta$) values calculated using Sednterp.

**RESULTS**

**Bacterial Two-hybrid Assays Detect OutC Homodimers**—To determine whether OutC is able to self-interact in vivo, we used the bacterial two-hybrid (BTH) system (25). In BTH the physical association of the two interacting proteins is spatially separated from the transcriptional events (via cAMP synthesis) so it is possible to analyze protein interactions that occur either in the bacterial cytoplasm or in the inner membrane. Full-length OutC fused to the C termini of T18 and T25 fragments were protease-sensitive in a spheroplast assay (Fig. 2A and not shown), indicating that the OutC moiety takes a correct N$_{term}$ C$_{term}$ topology in the inner membrane, as does intact OutC. The E. coli DHPL1 cells expressing these two fusions formed red colonies on MacConkey plates, whereas the negative controls (one of the fusions combined with an empty vector) appeared white (Table 2). A positive control (pUT18-zip and pKT25-zip) formed dark red colonies. The level of β-galactosidase activity measured with the two OutC fusions was 5–7-fold higher than that of the negative control and was comparable with the positive control (Table 2). This clearly indicates OutC self-interaction in vivo. Because the OutC moiety in the fusion acquired the correct membrane topology, the OutC homodimerization detected by BTH could be results of interactions in the cytoplasmic membrane, the periplasm, or both.

The **TMS Is Essential for the OutC Homodimerization in BTH**—To define the regions involved in homodimerization, a series of OutC deletion mutants fused to the C termini of T18 and T25 fragments was constructed (Fig. 1A and Tables 1 and 2). Immunoblotting tests with anti-OutC and anti-Cya were systematically performed throughout the BTH assays to check the amounts of truncated hybrids (not shown).

When the short cytoplasmic region was deleted (OutC U), the β-galactosidase activity was equivalent to that with the full-length fusions (Table 2), indicating that this region is not involved in OutC dimerization. The fusions carrying deletions in the periplasmic region (OutCΔA, OutCΔH, OutCΔN, OutCΔR, and OutCΔU) were sensitive to trypsin in a spheroplast assay, and thus, they were exposed in the periplasm as is full-length OutC (Fig. 2 and not shown). When these truncated fusions were co-expressed pair-wise, red colonies were formed in vivo.

**TABLE 2**

| Fused protein   | Phenytoin on MacConkey maltose | β-Galactosidase activity |
|-----------------|--------------------------------|--------------------------|
| Leucine-Zip     | White                          | 5,800 ± 500              |
| Leucine-Zip     | Dark red                       | 46,500 ± 5,400           |
| OutC            | White                          | 5,700 ± 400              |
| OutC            | Red                            | 38,000 ± 4,000           |
| OutCΔA1–13      | Red                            | 37,000 ± 3,300           |
| OutCΔA173–256   | Red                            | 23,100 ± 2,700           |
| OutCΔA103–172   | Red                            | 35,300 ± 3,800           |
| OutCΔN103–172   | Red                            | 36,000 ± 3,000           |
| OutCΔN100–123   | Red                            | 40,500 ± 3,800           |
| OutCΔN100–123   | Red                            | 22,800 ± 3,300           |
| OutCΔN173–256   | Red                            | 35,400 ± 3,600           |
| OutCΔA1–13      | Red                            | 7,500 ± 1,200            |
| OutCΔA1–160     | White/center red               | 7,800 ± 1,000            |

**FIGURE 2.** Cellular localization of OutC derivatives tested by protease sensitivity in a spheroplast assay. Each spheroplasts were prepared from MG1655 cells producing one of the OutC derivatives (indicated on top). The spheroplasts were treated (+) or not (−) with trypsin, and the whole protein lysates were analyzed by immunoblotting with anti-OutC.
Self-assembly of OutC

Alternatively, these deletions may create steric hindrance, diminishing the strength of the self-interaction mediated by another OutC region.

The portions of the periplasmic region fused to Cya fragments, OutCΔA and OutCΔL, were not sensitive to trypsin in a spheroplast assay and, thus, were not exposed to the periplasm (Fig. 2). The β-galactosidase activities observed with these fusions, lacking the TMS, were almost equivalent to those detected with empty vectors (Table 2), indicating that the periplasmic region alone is not able to dimerize.

To test if the OutC TMS self-interacts in vivo, an Opal stop codon was introduced at the beginning of the periplasmic region, creating OutC_{TMS} (Fig. 1A and Table 1). DHP1 cells co-expressing these truncated fusions formed red colonies and produced β-galactosidase activity similar to that with the full-length fusions (Table 2). Thus, the results from BTH experiments showed that the TMS is essential and the periplasmic region is nonessential for OutC homodimerization.

Chemical Cross-linking Analysis of OutC—To analyze the oligomeric state of OutC in vitro, the purified protein was subjected to cross-linking with formaldehyde. The dimeric form of OutC (60 kDa) was the major cross-linked product with some amounts of tetramers (120 kDa) and further traces of higher order species (supplemental Fig. S1A). No band corresponding to a trimer was observed.

To map the protein regions involved in self-association, cross-linking was performed with the OutC-truncated derivatives. OutCΔA and OutCΔH, lacking short segments of the periplasmic region (Fig. 1A), gave cross-linking patterns similar to that of the full-length OutC (supplemental Fig. S1B and not shown). The derivatives devoid of larger portions of the periplasmic region, OutCΔR, OutCΔN, and OutCΔU, were cross-linked less efficiently, but the dimeric forms were detected as well (supplemental Fig. S1C and not shown). Thus, none of the deletions in the periplasmic region prevented the formation of dimers. When OutCΔS lacking the TMS was subjected to cross-linking, no additional band was observed even when the protein concentration was increased 4-fold in comparison to that used with the full-length OutC (supplemental Fig. S1D). This suggests a monomeric state of OutCΔS in solution.

The TMS Is Sufficient to Drive Self-interaction of OutC in Vitro—As an alternative approach to test OutC self-interaction, we employed in vitro pulldown assays. The GST fused to full-length OutC or its truncated derivatives was immobilized on glutathione-Sepharose and used as affinity matrices (Fig. 3B). When His-OutC was incubated with this bait, it was retained on the full-length GST-OutC but not on GST alone (Fig. 3A), demonstrating a specific OutC-OutC interaction. An equivalent amount of His-OutC was bound on GST-OutCΔR, indicating that the absence of the PDZ domain does not affect OutC self-interaction. Conversely, no binding of His-OutC was detected on GST-OutCΔS, lacking the TMS (Fig. 3A), suggesting that the periplasmic region was unable to self-interact in vitro. This was confirmed in a reciprocal experiment; His-OutCΔS, used instead of His-OutC in the liquid phase, did not bind on GST-OutC and GST-OutCΔS (not shown). In contrast, OutC was specifically retained on GST-OutC_{TMS} (Fig. 4), demonstrating that the TMS of OutC self-interacts in vitro.

OutC Forms in Vitro Stable Dimers of Elongated Shape—To determine the stoichiometry of purified OutC, gel filtration in the presence of Triton X-100 was used. OutC was eluted as a single narrow peak consistent with a species of an apparent R_s of 5.11 nm (Fig. 5A). The position of this species was not affected within a range of detergent concentrations above the critical micellar concentration (0.05–1%), and protein concentrations from 0.1 to 0.5 mg/ml, indicating the presence of a protein-detergent complex of a regular stoichiometry. The R_s...
of 5.11 nm corresponds to a water-soluble globular protein of about 200 kDa. However, Triton X-100 bound to the protein can contribute significantly to the apparent size and shape of the protein-detergent complex. Therefore, to establish the exact OutC stoichiometry in the complex, we calculated the amount of detergent bound to the protein. For that purpose we determined the partial specific volume, \( \nu \), of the complex by using ultracentrifugation of purified OutC in the presence of Triton X-100 on linear 2.5–20% (w/v) sucrose gradients prepared in H\(_2\)O or in D\(_2\)O (30) (Fig. 5B). The apparent sedimentation coefficients, 2.5 S and 1.5 S respectively, were used to calculate the partial specific volume of the protein-detergent complex, \( \nu_{\text{p},\text{d}} \), (0.839 ml/mg). This value together with the value \( \nu_{\text{d}} \) of the detergent alone and \( \nu_{\text{p}} \) of the OutC moiety were used to calculate the amount of Triton X-100 bound to OutC, \( n_{\text{D}} \) (1.52 mg/mg of protein) (Table 3). The values of the sedimentation coefficient, partial specific volume, and Stokes radius were next substituted into the Svedberg equation (30), and the molecular mass of the OutC-Triton X-100 complex was calculated to be 163 kDa. Considering the ratio of Triton X-100 in the complex, the molecular mass of OutC in the protein-detergent complex was calculated to be 64.6 kDa, corresponding to a dimer. Thus, OutC in the presence of Triton X-100 forms a stable complex of two OutC molecules and about 150 molecules of detergent. The frictional ratio, \( f/f_{\text{mono}} \), of 1.96 and the axial ratio, \( a/b \), of 13.8 calculated for the OutC-Triton X-100 complex were extremely elevated. Such values are generally typical of highly elongated or extended proteins (32).

The Periplasmic Region of OutC Is Monomeric in Vitro—We next examined the hydrodynamic properties of purified OutCASN, lacking the TMS. Using gel filtration, OutCASN appeared as a single monodispersed peak with a \( R_g \) of 2.94 nm (Fig. 5A). Its position was not affected by the presence of Triton X-100, indicating that elimination of the TMS prevents binding of detergent. Therefore, the partial specific volume of OutCASN (0.718 ml/mg) was calculated directly from the protein sequence. The apparent sedimentation coefficient, 2.22 S, was determined by sucrose gradient centrifugation (Table 3). The molecular mass of OutCASN calculated using these values, 26.2 kDa, corresponds to a monomer. The same analysis of OutCAL, mainly consisting of the presumed PDZ domain, also revealed its monomeric state (Fig. 5, Table 3). The frictional ratio of 1.18 and the axial ratio of 1.4 calculated for OutCAL are typical for globular proteins and agree with a globular shape of known PDZ domains (33). In contrast, a \( f/f_{\text{mono}} \) value of 1.37 and an \( a/b \) of 3.9, determined for OutCASN, are consistent with a slightly elongated overall shape, indicating that the part of the OutC periplasmic region outside the PDZ domain adopts a more extended shape.

Disulfide-linked OutC Dimer Is Formed within a Functional Secretion System—In vivo cross-linking was ineffective for probing the oligomeric state of OutC in *E. chrysanthemi* (not shown). Therefore, we used cysteine-directed mutagenesis to test whether covalently linked OutC can be formed within a functional T2SS. The OutC-A43C mutant protein, carrying a cysteine residue at the beginning of the periplasmic region close
to the TMS, was stable and fully restored pectinase secretion in \textit{E. chrysanthemi} \textit{ΔoutC} (Fig. 6), indicating that its function was not affected. To analyze the \textit{in vivo} redox state of OutC-A43C, the free cysteine residues of the cell proteins were alkylated with iodoacetamide to prevent the spontaneous formation of disulfide bonds (34) (Fig. 7). Both dimeric and monomeric forms of OutC-A43C were detected, with the dimer being the prevalent species. The relative ratio of dimers was higher in \textit{Erwinia} than in \textit{E. coli}, suggesting that integration of OutC in the functional secretion machinery favors its dimerization. A less intense reactive species of 35 kDa, detected in both bacteria (Fig. 7), was also observed with the purified OutC (supplemental Fig. S1A) and, hence, was not a heterodimer. An efficient formation of disulfide bonds in OutC-A43C in \textit{Erwinia} and the absence of heterodimers reveal close juxtaposition of the beginning of the periplasmic region of two OutC moieties, indicating that OutC is self-associated within a functional T2SS.

**Search for Potential Dimerization Motifs in the OutC TMS**—The predicted TMS of OutC, bordered by Arg\textsuperscript{15} and Arg\textsuperscript{16} and Trp\textsuperscript{35} and Arg\textsuperscript{36} contains neither the GXXXG motif (35) nor a canonical repeated leucine zipper motif (36), arguing against these modes of self-interaction of TM α-helices (Fig. 1, B and C). A single strongly polar residue (Gln, Asn, Glu, Asp, or His) or multiple weakly polar residues can drive a strong association of model TM helices through the formation of interhelical H-bonds (37, 38, 39). We searched for potential H-bond donors in the OutC TMS by mutating the six polar residues and testing the functionality of the mutant proteins in a complementation test and their self-interaction ability using \textit{in vitro} assays.

Gln\textsuperscript{28} and Gln\textsuperscript{29} located around the center of the TMS have a good potential for forming interhelical H-bonding (40) and may indicate two alternative interfaces of self-interaction (Fig. 1C). The single substitutions of these residues with Leu were fully functional in a complementation test (Fig. 6). \textit{In vitro} self-association ability of the single Q28L mutant was not apparently affected, whereas the binding ability of the Q29L mutant on GST–OutC\textsubscript{TMS} was slightly diminished (Fig. 4 and not shown). This suggests that Gln\textsuperscript{28} is involved in self-interaction of the OutC TMS.

A single substitution of each of the three Arg residues located at both the TM boundaries and Cys\textsuperscript{27}, R15V, R16A, R36A and C27L, did not obviously affect the complementation efficiency and self-association ability of the corresponding single mutants (Fig. 6 and not shown). To test the position of Cys\textsuperscript{27} with respect to the TMS interface, oxidizing cross-linking with copper phenanthroline was performed. This oxidizing agent is able to catalyze the formation of TM disulfide bonds \textit{in vitro}, depending on the juxtaposition of the Cys residues (41). Only monomers were detected after such a treatment of the purified OutC (not shown), indicating that Cys\textsuperscript{27} was not located close to the interaction interface.

**Cooperative Interactions between Three Polar Residues Located at the Same Helical Face Drive the Self-assembly of the OutC TMS**—We tested whether self-association of the OutC TMS is stabilized by multiple interhelical bonds. Self-interaction of the Q29L mutant was slightly diminished (Fig. 4), indicating that this residue can mediate an interhelical bonding. Arg\textsuperscript{15}, Arg\textsuperscript{36}, and Gln\textsuperscript{29} occupy the α position of the heptad motif and, hence, are located at the same helical face (Fig. 1C). Double mutants combining substitutions of two of these three residues were constructed. The R15V/R36A mutant showed the same complementation efficiency as the wild type OutC (Fig. 6). In contrast, complementation efficiency of the R15V/Q29L and Q29L/R36A double mutants dropped by about 30%. The triple R15V/Q29L/R36A OutC mutant was barely func-
tional in E. chrysanthemi ΔoutC; only 10–20% of pectinases were found in the outer medium (Fig. 6). As checked by protease sensitivity in a spheroplast assay, neither protein stability nor the correct membrane topology of the triple OutC mutant was affected (Fig. 2). In vitro analysis showed that stepwise substitutions of Arg15 and Arg36 in the OutC-Q29L mutant provoked a gradual diminution of the protein self-association strength. The double mutants R15V/Q29L and Q29L/R36A bound weaker in pulldown assay and took an intermediate position between monomer and dimer in gel filtration (supplemental Fig. S2 and not shown). The triple mutant R15V/Q29L/R36A was replaced with either Asn, Gln, or Glu, and the corresponding quadruple mutants were assayed in a complementation test. Whereas the two first substitutions had a limited effect, L25E improved the complementation ability of the quadruple mutant (Fig. 6). Conversely, when Leu22 of the triple mutant R15V/Q29L/R36A was replaced with either Asn, Gln, or Glu, the complementation ability of the first quadruple mutant was notably improved (not shown). In vitro analysis showed that the self-association capacity of the quadruple mutants R15V/L22N/Q29L/R36A and R15V/L25E/Q29A/R36A was almost fully restored. Their Rg corresponded to a dimer, and their binding ability on the GST-OutCTMS was equivalent to that of wild type OutC (Fig. 4 and supplemental Fig. S2). Thus, the L22N and L25E substitutions restored the self-association ability and simultaneously improved the functionality of the corresponding quadruple mutants. This fact strongly supports the idea that functionality of OutC is directly dependent on the self-association state of its TMS.

DISCUSSION

This study provides the first detailed analysis of the role of a TM α-helix in the assembly and function of a component of the T2SS. Using the BTH assay and pulldown experiments, we found that the OutC TM α-helix tightly self-interacts, even in the absence of the periplasmic region. The periplasmic region alone was incapable of self-association, and no deletion preventing the self-interaction of OutC was identified within this region, suggesting that this region is dispensable for the self-association of OutC. We demonstrated that the TMS of OutC is more than just a membrane anchor; it actually drives the protein self-association that is essential for the formation of a functional secretion system.

Hydrodynamic studies of detergent-solubilized OutC purified to homogeneity revealed that in vitro it forms stable dimers of a very elongated shape associated through their TMSs. The globular shape of the C-terminal part of OutC, carrying the PDZ domain (OutCΔL), significantly differs from the rest of the protein. The extended overall shape of OutC and the presence of several Ser- and Pro-rich regions could provide the protein with a high flexibility, giving it easy access to protein partners.

A strong intrinsic propensity of the OutC TMS to self-interact in vivo and in vitro should allow for a tight protein self-association within the T2SS. Indeed, insertion of a Cys at the beginning of the periplasmic region (OutC-A43C) led to an efficient formation of covalently linked homodimers in Erwinia (Fig. 7), strongly supporting self-association of OutC within the T2SS. Because OutC-A43C fully complemented an E. chrysanthemi ΔoutC mutant, it seems likely that the covalently linked OutC dimer remains functional. Alternatively, it is possible that just a fraction of OutC-A43C escaping from disulfide linkage...
allowed for a functional complementation. Site-directed mutagenesis revealed a direct correlation between the self-association of OutC and its functionality. Although OutC is dimeric in vitro, it is possible that it can form a higher order homo-oligomer in the plasma membrane within the T2SS. Alternatively, it could be associated into hetero-oligomers with some other components of the T2SS.

The sequence of the predicted OutC TMS is slightly reminiscent of a leucine zipper. In artificial polyleucine segments and in some natural TMSs that adopt a leucine zipper-like conformation, interhelical interactions have been stabilized by the introduction of polar residues at helical interfaces, mediating the formation of interhelical H-bonds (37, 38, 40). A search for potential H-bond donors in the OutC TMS revealed that three polar residues, Arg\textsuperscript{15}, Gln\textsuperscript{29}, and Arg\textsuperscript{36}, located at the same helical face are crucial for OutC function and self-interaction of the TMS.

An interhelical H-bond mediated by Gln\textsuperscript{29} appears to be the main force driving the self-association of OutC. Indeed, only the double substitutions R15V/Q29L and Q29L/R36A, but not R15V/R36A, significantly diminished the self-interaction and complementation efficiency of OutC (Fig. 6). The single amino acid substitution, Q29E, strengthened self-association of OutC in vitro, whereas Q29L and Q29A decreased it (Fig. 4). However, the Gln\textsuperscript{29}, Gln\textsuperscript{29} interaction is not sufficient to define a correct self-association state of OutC because the effect of the Q29L substitution became apparent in a complementation test only in the presence of additional mutations of Arg\textsuperscript{15} and Arg\textsuperscript{36}.

The mechanism of stabilization of the OutC TMS self-association by Arg\textsuperscript{15} and Arg\textsuperscript{36} is not completely clear. The basic residues are often preferentially located at the boundaries of the TM helices, where they can interact with the negatively charged head groups of phospholipids (43). The protease sensitivity in a spheroplast assay showed that correct membrane location was not affected in the R15V/Q29L/R36A mutant (Fig. 2). This indicates that a probable role of Arg\textsuperscript{15} and Arg\textsuperscript{36} in helix-end interactions with bilayer interfaces is not essential or could be substituted by Arg\textsuperscript{16} and Trp\textsuperscript{35}. Indeed, the polar-aromatic residue tryptophan, which has a specific affinity for a region near the lipid carbonyls, is also usually located near the membrane-water interface, where it confers stability to a TM helix (44). To our knowledge the ability of arginine residues to promote the self-association of TM \(\alpha\)-helices has not been previously reported. Arginine can stabilize the TMS-TMS assembly by interaction with an acidic residue (45), which is not the case for OutC TMS. Arginine is one of the most predominant interface residues, and the guanidinium group of its side chain has a high capacity to donate H-bonds (46). Computational analysis of the membrane helical interfaces showed that Arg-Arg pairs have a high propensity for interhelical polar interactions (47). Taken together, this supports the possibility of interhelical Arg-Arg H-bonds within the OutC TMS. In addition, a side chain of arginine could be involved in inter- or intrahelical cation-\(\pi\) interactions with aromatic residues, stabilizing the protein structure (48, 49). It was found that the cationic groups of the cation-\(\pi\) pairs are often involved in intermolecular H-bonds (46). Therefore, the side chains of Arg\textsuperscript{15} and Arg\textsuperscript{36} can participate in multiple interactions, providing an adequate stability of the OutC TMS self-assembly. It has been shown that multiple weakly polar residues (Ser and Thr), interacting cooperatively across the TM helical interface, can drive oligomer formation through a series of weak H-bonds (39). We propose that cooperative interactions between three polar residues, located at the same helical face, Gln\textsuperscript{29}, Arg\textsuperscript{15}, and Arg\textsuperscript{36}, provide sufficient energy to drive the self-association of the OutC TMS.

The validity of the proposed mode of the OutC TMS self-association was proven by the introduction of polar residues at alternative positions in the proposed helical interface that restored self-association and improved the functionality of the OutC triple mutant (Figs. 4 and 7 and supplemental Fig. S2). Interestingly, the substitution of Leu\textsuperscript{22} was more efficient with Asn, whereas that of Leu\textsuperscript{25} was more efficient with Glu. The strength of interhelical H-bonds mediated by polar residues can be strongly influenced by their local sequence and packing context (50). Also, depending on the side chain length and the presence of carboxyl or amide groups, the polar residues exhibit differing abilities to promote the association of TM helices (38). Thus, the two polar substitutions found to stabilize TM helix self-association more efficiently, L22N and L25E, may reflect variations in density of interhelical packing at the mutations 22 and 25.

It seems unlikely that self-interacting TMSs of OutC form a sort of rigid clasp. Indeed, introduction of a cysteine at the beginning of the periplasmic region of the R15V/Q29L OutC mutant did not restore its function despite the formation of disulfide-linked dimers (not shown). This indicates that a covalent linkage outside of the TMS is unable to repair functional defects caused by failure of the TMS self-association. Furthermore, unusually strong self-association caused by the L22N/Q29E double substitution diminished the complementation ability of the mutant (not shown). This suggests that the OutC function depends on a particular packing density of the self-associated TMS. We can also speculate that packing density of the OutC TMS may vary during the secretion process, depending on interactions with other components of the T2SS or with the protein to be secreted, and hence, the OutC TMS may participate in signal transduction.

Contradictory reports on the role of the GspC TMS have been published. Earlier we showed that the 38-amino acid N-terminal region of OutC, including the TMS, is essential for protein function (6). Its replacement with the PelB signal peptide provoked a complete loss of protein function. Similarly, studies of GspC of Pseudomonas aeruginosa and Xanthomonas campestris demonstrated that substitution of the N-terminal region either with the first TMS of TetA or with a signal peptide completely abolished protein function (9, 14). In contrast to these data, it has been suggested that the TM region of PulC, a GspC of Klebsiella oxytoca, is not essential for any function and can be replaced with a signal sequence (15). In the present study, we demonstrated that the TMS of OutC is more than just a membrane anchor. It mediates an efficient self-association necessary for protein function. The disruption of OutC TMS self-association resulted in a loss of protein activity in vivo. This contradiction seems even more surprising because PulC is
functional in *E. chrysanthemi*, suggesting a similar mode of assembly within the T2SS. A possible explanation of this discrepancy may be provided by the fact that the signal sequences fused to the periplasmic region of PulC were not efficiently cleaved (15). Thus, an uncleaved signal peptide could partially replace the native TMS of PulC, allowing for the insertion of the protein into the inner membrane. In addition, the sequence patterns of many signal sequences are similar to the leucine zipper motif mediating the self-interaction of TMSs (36). Moreover, the polar residues added to the linker region between PulC and the signal sequence (15) may allow for the formation of interhelical H-bonds stabilizing self-association. It seems likely, therefore, that an uncleaved signal peptide fused to the periplasmic region of PulC can drive its spontaneous self-association, thus restoring the protein function. We showed that self-interaction of the OutC TMS is rather tolerant concerning the exact position and strength of the interhelical H-bond because several polar residues placed at alternative positions in the proposed helical interface (positions 22, 25, and 29) still mediated the functional self-association of the protein. Specific interactions between α-helical TMS are important for the oligomerization and folding of membrane proteins. We demonstrated that self-interaction of the OutC TMS is an essential prerequisite for protein function within the T2SS. Several non-mutually exclusive functions have been proposed for OutC and its homologues, namely recognition of the secreted proteins, interactions with the secretin D, and with the inner membrane located constituents of the T2SS and signal transduction within the T2SS (3, 6, 7, 9). We can, therefore, speculate that any of these presumed functions of OutC may be affected by the disruption of its self-association. Firstly, self-assembly of the OutC TMS could bring together the periplasmic regions of several OutC moieties otherwise incapable of self-interaction and, hence, create in the periplasm a more complex surface necessary for interaction with its protein partners. Second, the TMSs of some other inner membrane components of the T2SS may interact with the OutC TMS, and its self-association could be necessary for such TMS-TMS interactions. Finally, a correct self-associated state of the OutC TMS becomes an important prerequisite in the context of a signal transduction hypothesis. An attractive idea is that the packing density of the OutC TMS could vary depending on ligand binding to the periplasmic region. Hence, a signal may be transduced via a TMS-TMS interaction with another inner membrane component of the T2SS. Our future analysis of the T2SS will be focused on testing these ideas.

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