Functional characterization of the Erwinia chrysanthemi OutS protein, an element of a type II secretion system

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Secretion of pectate lyases and a cellulase occurs in Erwinia chrysanthemi through a type II secretion machinery, the Out system. Proper insertion of the secretin OutD in the outer membrane requires the presence of OutS. OutS is an outer-membrane lipoprotein that interacts directly with OutD. Using ligand-blotting experiments, it has been shown that this interaction requires at least the 62 C-terminal amino acids of OutD. When this domain was added to the C-terminal extremity of the secreted pectate lyase PelD, the construct was stabilized by OutS but not inserted into the outer membrane. Thus, this domain is sufficient to interact with OutS but it is unable to confer the ability to be inserted into the outer membrane in the presence of OutS. A screen for outS mutants unable to secrete pectate lyases gave only mutants unable to properly localize OutD in the outer membrane and no mutant in the protection function. Thus, the interaction between OutS and OutD can probably not be abolished by the mutation of a single amino acid, and the insertion of OutD in the outer membrane may require additional proteins.

Keywords: Gsp protein, secretion, chaperone, lipoprotein

INTRODUCTION

Translocation of macromolecules across the outer membrane of Gram-negative bacteria can occur by several processes. Some of them require a member of the secretin family of outer-membrane proteins. Secretins are involved in the biogenesis of type IV pili (Drake & Coomey, 1995), in the secretion of the A-protein of the surface layer of Aeromonas salmonicida (Noonan & Trust, 1995) and in type III secretion (Genin & Boucher, 1994). Their role has been extensively studied in two systems: the morphogenesis of the filamentous phages such as f1 and M13 and the main terminal branch (MTB) of the general secretory pathway (GSP), or type II secretion. The filamentous phage f1 protein pIV forms multimers of 14 subunits in the outer membrane, and may form a pore through which the phage could be extruded (Linderoth et al., 1997). The C-terminal half of the protein is necessary for membrane insertion and the N-terminal third forms a periplasmic domain (Russel & Kasmierczak, 1993; Brissette & Russel, 1990). The N-terminal part of the protein is responsible for the specificity of the secreted phage, since a chimeric protein containing the first 149 amino acids of the phage f1 pIV protein exchanged with the same region of the related protein of phage IKe supports f1 and not IKe assembly (Daeflher et al., 1997b).

The secretins of the MTB of the GSP contain at least two functional domains. The N-terminal part of the various GspD proteins is variable and seems to be located in the periplasm. The Erwinia chrysanthemi GspD protein, OutD, can bind secreted proteins via its N-terminal domain but cannot bind the related proteins of Erwinia carotovora (Shevchik et al., 1997). Thus, the N-terminal part of GspD proteins may be the key to the specificity of type II secretion machineries. The C-terminal part has been proposed as being required for the formation of multimeric structures in the outer membrane (Chen et al., 1996; Hardie et al., 1996a; Shevchik et al., 1997). While the C-terminal half of all secretins is relatively well conserved, their extreme C-terminal end is variable. However, sequence homology can be found between the last 60 amino acids of Klebsiella oxytoca PulD, Er. chrysanthemi OutD and Er. carotovora OutD. This part of the secretin interacts with a small protein (PulS and OutS, respectively) that stabilizes it and helps its

Abbreviations: GSP, general secretory pathway; MTB, main terminal branch.
The accession number for the sequence reported in this paper is X65265.

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insertion into the outer membrane (Daehler et al., 1997a; Shevchik et al., 1997). The chaperone and pilotin functions of PulS have been studied by Hardie et al. (1996b). Outs is the only characterized homologue of PulS (d’Enfert & Pugsley, 1987; Condehine et al., 1992).

We show in this article that Outs is a lipoprotein that interacts directly with the C-terminal end of OutD. Addition of this OutD domain to the end of another protein confers the capacity to be protected by Outs, but not the capacity to be directed to the outer membrane. The lipoprotein acylated N-terminal part of Outs is necessary for its pilotin function, but not for the protector effect with regard to OutD.

**METHODS**

**Bacterial strains, plasmids and culture conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. Er. chrysanthemi and Escherichia coli cells were grown at 30 °C and 37 °C, respectively, in LB medium or M63 minimal medium (Miller, 1972) supplemented with a carbon source (0.2%) (except polygalacturionate, 0.4%) and, when required, antibiotics at the following concentrations: 100 μg ampicillin ml⁻¹, 50 μg kanamycin ml⁻¹, 20 μg chloramphenicol ml⁻¹.

**Recombinant DNA techniques and generation of constructs.** Standard recombinant DNA procedures were performed essentially as described by Sambrook et al. (1989). DNA sequences were determined by the chain termination method with T7 DNA polymerase (Pharmacia).

To construct the periplasmic Outs derivative, a SacI–Xhol fragment from pB3A containing outs was cloned between the SacI and Xhol sites of pET26. The outs signal sequence was deleted and the outs coding sequence was put in-frame with the pelB signal sequence (PelB⁺) of pET20, after digestion of the plasmid with SgrAI and EcoRV and treatment with the Klenow enzyme. The pelB⁺-outs construct was then digested with BglII and Xhol, and introduced into the expression vector pACT3, digested with BamHI and Sall. To create the pelD⁻-62D hybrid protein, the C-terminal 12 amino acids of PelD were replaced with the C-terminal 62 amino acids of Outs, using a 4-amino-acid junction of the pUC118 polylinker (an Xbal–SmaI fragment). A Maml–Nhel fragment, from pN715 containing pelD, was cloned into the Ehel and Xbali sites of pUC118. A PvuII–SmaI fragment from this plasmid was cloned into the EcoRV and NruI sites of the pTDB-OD, to give an in-frame pelD⁺-outs fusion.

**Hydroxylation mutagenesis.** This was performed as described by Yang et al. (1993). Forty microlitres of hydroxylation chloride hydrate, pH 6.0, was added to 5 μg plasmid DNA in 60 μl 100 mM phosphate buffer, pH 6.0. The mixture was incubated at 75 °C for 90 min, dialysed against water and the DNA was recovered by ethanol precipitation. The outs mutant strain A1903 was transformed by electroporation with the mutagenized plasmids. The ability of transformants to secrete pectate lyases was tested on polygalacturate + glycerol plates (Hugouvieux-Cotte-Pattat et al., 1992). The plasmid was extracted from each non-secreting transformant and purified in E. coli.

**Enzyme assays.** Pectate lyase activity was assayed on the supernatant or on the tolenuzied cell fraction as described by Moran et al. (1968). β-Galactosidase activity was determined by following the degradation of o-nitrophenyl-β-D galactoside into o-nitrophenol, measured at 420 nm (Miller, 1972).

**Isolation and analysis of cell fractions.** Exponentially grown cultures (OD₆₅₀ 0.8–1.0) were usually used for the cell fractionation. Release of periplasmic proteins was performed by osmotic shock, and cell membrane fractionation was performed by sucrose gradient centrifugation, as described previously (Shevchik et al., 1996). Crude membrane fractions were isolated by centrifugation (20000 × g for 2 h) after French press disintegration of cells and redissolved in 50 mM Tris/HCl, pH 8.0. Gel-filtration chromatography of the periplasmic proteins was performed using a Superose 12 HR 10/30 (Pharmacia) column pre-equilibrated with 50 mM Tris/HCl, pH 8.0, 150 mM NaCl. The periplasmic fraction (0.3 ml) adjusted with this buffer was applied to the column and eluted with the same buffer. Fractions of 0.2 ml were collected, analysed for A₂₈₀ and proteins were precipitated with 5 vol. ethanol. Protein patterns were analysed by SDS-PAGE followed by immunoblotting with anti-OutD and anti-OutS antibodies. The column was calibrated with the following molecular mass standards: α-fetoprotein (340 kDa) and 170 kDa – non-reduced and reduced forms, respectively, fructose-6-phosphate kinase (85 kDa), BSA (67 kDa), chymotrypsinogen A (25 kDa) and RNase A (13.7 kDa).

**Protein labelling.** Overexpression and exclusive labelling of plasmid-encoded proteins was carried out using the T7 promoter/T7 polymerase system of Tabor & Richardson (1985). Labelling was performed with [³⁵S]methionine and [³⁵S]cysteine (Promix, Amersham) or [³⁵S]cysteine (Promix, Amersham) or [³⁵S]cysteine (Promix, Amersham). For the ligand blotting experiments, P⁵⁺-labelled Outs was extracted from cells with Triton X-100. Exclusive labelling was performed over a period of 20 min, then cells were washed in 20 mM Tris/HCl, pH 8.0, and incubated in 1% (v/v) Triton X-100, 20 mM Tris/HCl, pH 8.0, for 30 min. The cells were removed by centrifugation and the amount of labelled Outs in the supernatant was estimated by SDS-PAGE.

**Gel electrophoresis, immunoblotting and ligand blotting.** SDS-PAGE was usually performed according to Laemmli (1970). Concentrations of acrylamide and bisacrylamide varied from 5 to 15% (w/v) and from 0.5 to 2.0% respectively, depending on the experiment. Proteins were transferred onto nitrocellulose in a semi-dry apparatus, and the membrane was incubated with antibodies and developed with the ECL detection kit (Amersham), as described previously (Shevchik et al., 1997). The primary antibodies used were anti-PelD diluted 1:5000, anti-MBP diluted 1:10000 (Biolabs), anti-OutS diluted 1:6000, anti-PulS diluted 1:5000 (provided by A. Pugsley, Institut Pasteur, Paris, France) and anti-OutD diluted 1:3000.

For the ligand blotting experiments, the crude membrane fractions containing OutD or its derivatives were boiled for 3 min in the Laemmli buffer, separated by SDS-PAGE and blotted onto nitrocellulose. The membrane was saturated with 3% (w/v) BSA, incubated for 1 h with labelled Outs, washed three times for 10 min, dried and exposed to film. The incubations were usually performed at room temperature with agitation in 20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20.

**Preparation of Outs antisera.** PelB⁺-OutS was overproduced in E. coli NM522 containing pACTS1. Cells from 100 ml culture were pelleted and the periplasmic proteins were extracted by the freezing-thawing method of Johnson & Hecht (1994). The supernatant was loaded onto an 18% preparative SDS-PAGE gel. The band containing PelB⁺-OutS
was cut out and the protein was extracted with three washes with 10 mM Tris/HCl, pH 8.0, 0.1% SDS. The protein was concentrated with Centricon 3 and injected into a rabbit. Serum was used with no purification at the dilutions indicated.

**RESULTS**

**OutS is an outer-membrane lipoprotein**

Re-examination of the previously published outS sequence (X65265; Condemine et al., 1992) showed that a C residue had been omitted at position 365. The resulting frameshift led to the identification of an erroneous start codon. In the corrected sequence, the outS ORF begins with an ATG codon located at nucleotide 272, which is preceded by a Shine–Dalgarno sequence (AAGA) at nucleotides 255–258 (data not shown). This ORF encodes a putative protein of 133 amino acids with a deduced molecular mass of 14,336 Da. The 20 N-terminal amino acids of OutS show the characteristics of a lipoprotein signal sequence, with the cleavage site of the lipoprotein signal peptidase (LAAC) (Fig. 1). Thus, OutS could be processed and fatty-acylated on the N-terminal cysteine. To confirm this hypothesis, outS was placed under the control of the T7 promoter in pT7-5 (to give pT70S). When the proteins encoded by the plasmid were exclusively labelled with [35S]methionine, a 12 kDa product was detected. When the labelling was performed with [3H]palmitate, a protein of the same size was detected (Fig. 2). This confirmed that the 12 kDa protein is the processed form of the lipoprotein OutS. Fractionation of the membranes of *Erwinia chrysanthemi* and an *E. coli* strain producing OutS, by flotation on a sucrose density gradient (Fig. 3), showed that the lipoprotein is associated with the outer leaflet of the outer membrane.
**Fig. 1.** Alignment of the sequences of OutS and PuLS. The PuLS sequence is from d'Enfert & Pugsley (1987). The position of the lipoprotein signal peptidase cleavage site is indicated by a black arrowhead. Identical amino acids are indicated by asterisks. The amino acids changed by the hydroxylamine mutagenesis are shown in bold. The numbering is that of the mature protein.

**Fig. 2.** Exclusive expression of outS in the T7 RNA polymerase system. T7 RNA polymerase synthesis was induced in *E. coli* K38/pGP1-2/pT7-6 (OutS-) (lane 1) and K38/pGP1-2/pT7OS (OutS+) (lane 2) cells. Cells were collected after 2 h expression (a) or treated with rifampicin and labelled with either [35S]methionine and [35S]cysteine (b) or [3H]palmitate (c). Proteins were separated by 15% SDS-PAGE and gels were stained with Coomassie blue (a) or autoradiographed (b, c).

**Fig. 3.** In vitro interaction of OutS with OutD and OutD derivatives. Proteins from crude membrane fractions of *Er. chrysantheni* A837/pMMB-OD (lanes 1 and 2), or *E. coli* BL21 carrying pTdB-OD (lanes 3 and 4), pTdB-ODC1 (lanes 7 and 8), pODN (lanes 9 and 10) or pODN2 (lanes 11 and 12), pTdB-ODC2 (lanes 13 and 14), or *E. coli* NM522/pODN1 (lanes 5 and 6) were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The nitrocellulose membrane was incubated either with OutD antibodies (odd-numbered lanes) or with labelled OutS (even-numbered lanes). The arrows indicate the positions of OutD and OutD derivatives.
density gradient allowed for the detection of OutS in the outer-membrane fraction (data not shown). Thus, OutS is an outer-membrane lipoprotein.

**Interaction between OutS and OutD requires the last 62 amino acids of OutD**

We have shown that OutD is protected when co-expressed in *E. coli* with OutS, and that this protection requires the 62 C-terminal amino acids of OutD to be present (Shevchik et al., 1997). The protection of OutD by OutS observed in vivo led us to suppose that there is a direct interaction between these two proteins. Ligand-blotting experiments were performed to confirm in vitro the potential OutD–OutS interactions. The membrane proteins of the *Er. chrysanthemi* strain A837/pMMB-OD and of an *E. coli* strain overproducing OutD were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. When the nitrocellulose membrane was incubated with $^{35}$S-labelled OutS, binding of radioactive proteins was observed at a position corresponding to OutD (Fig. 3, lanes 2 and 4). No interaction with any other protein was observed, suggesting that OutD is the only *Er. chrysanthemi* protein able to interact with OutS. This experiment was then performed with deletion derivatives of OutD, lacking either the N-terminal domain (OutDN, OutDN1 and OutDN2) or the C-terminal domain (OutDC1 and OutDC2). OutS binding was not observed to the OutDC1 and OutDC2 derivatives lacking 62 and 328 amino acids, respectively (Fig. 3, lanes 8 and 14). These results indicate that the interaction between OutD and OutS occurs via the C-terminal part of OutD.

To test whether the 62 C-terminal amino acids of OutD are sufficient to confer the ability to interact with OutS, a fusion between the secreted protein PelD, deleted of its 12 C-terminal amino acids, and the 62 C-terminal amino acids of OutD was constructed (pTPLD–62D). The fusion protein was unstable in *E. coli*, but was stabilized by the coexpression of OutS (Fig. 4a, lanes 1 and 3). A cell fractionation experiment showed that PelD–62D was neither extracted by osmotic shock with the periplasmic proteins (Fig. 4a, lanes 4 and 5) nor localized in the outer-membrane fraction, but remained at the bottom of the sucrose gradient (data not shown), probably in aggregated form. Thus, the presence of the 62 C-terminal amino acids of OutD is sufficient to protect the fusion but it is not sufficient to localize it to the outer membrane.

**OutS assists in the maturation and stabilization of OutD**

When *outD* was overexpressed in *E. coli*, resulting in a large proportion of OutD being unprocessed, co-expression of *outs* significantly increased the amount of mature OutD (Fig. 5). OutS was bound to unprocessed OutD (with its signal sequence) and to the OutD derivative located in the cytoplasm (OutDN) in ligand-blotting experiments (Fig. 3, lanes 3, 4, 9 and 10), suggesting that it could bind to the unprocessed OutD
Outs helps in the maturation of OutD. Crude membrane fractions of *E. coli* BL21/pTdB-OD/pBluescript (Outs-) or BLZ1/pTdB-OD/pBA3 (Outs+) were separated by SDS-PAGE and detected with OutD antibodies. The OutD precursor (p) and mature (m) forms are indicated.

**Fig. 5.** Outs helps in the maturation of OutD.

precursor *in vivo.* We have no evidence that Outs can interact with OutD in the cytoplasm, but Outs could interact with the translocation intermediate of OutD from the periplasmic side and help its translocation through the Sec machinery. This effect could explain, in part, the increased quantity of OutD found in strains expressing outs. However, it may result from a protection of OutD by Outs after its translocation through the inner membrane. To confirm that the interaction between OutD and Outs results in an increased stability of mature OutD, pulse–chase experiments were performed. OutD stability was investigated in *E. coli* K38, either expressing or not expressing outs. While OutD alone had a half-life of 30 min in the presence of Outs, no decrease in the quantity of OutD was observed after 1 h (Fig. 6). Moreover, in the absence of Outs, a significant amount of OutD precursor, present at time 0, stayed visible during the experiment, confirming the role of Outs in OutD maturation.

**Functional analysis of Outs mutant proteins**

To analyse whether protection of OutD by Outs can occur independently from its insertion into the outer membrane, we tried to separate the two functions of Outs (protection of OutD and its insertion into the outer membrane), by constructing or isolating mutants of the protein. The lipoprotein signal sequence of Outs was replaced by the PelB signal sequence to create PelB<sup>op-</sup>-Outs. A set of mutants was obtained by hydroxylamine mutagenesis of the outs gene present on pBA3. After transformation by electroporation of an *Er. chrysanthemi* Outs<sup>−</sup> mutant, plasmids unable to restore pectinase secretion were screened. The outs gene present on these plasmids was sequenced to identify the mutation. Four nonsense and seven missense mutations were obtained (Fig. 1). The four nonsense mutants gave predicted mature derivatives of 22, 61, 70 and 81 amino acids. The missense mutants are A32T, L37F, R43C, A60T, A60V, S77N and R110C. The PelB<sup>op</sup>-Outs protein was located in the periplasm, while two mutants, L32F and R110C, were still outer-membrane located (data not shown). The ability of these mutants to stabilize *OutD in vivo* and to help its insertion in the outer membrane was tested.

The pelB<sup>op</sup>-outs construct was unable to complement an *Er. chrysanthemi* Outs<sup>−</sup> mutant. Moreover, it inhibited secretion when introduced into the wild-type strain. However, it was able to protect OutD, since coexpression of pelB<sup>op</sup>-outs with outD led to an increase in the amount of OutD in the cells (Fig. 4b, lanes 6 and 7). The recombinant protein seemed to stabilize OutD as a periplasmic intermediate: in an *E. coli* strain expressing OutD with PelB<sup>op</sup>-Outs, about 50% of the total OutD could be released by osmotic shock, as were other periplasmic proteins (Fig. 4b, lanes 9 and 10). Determination, by centrifugation on a sucrose gradient, of the OutD position in a strain expressing outD with pelB<sup>op</sup>-outs showed that, although most of OutD was present in the soluble fraction, part of it could be found

![Fig. 6. Post-translational stabilization of OutD by Outs.](Image)
Erwinia chrysanthemi Outs protein

Fig. 7. Mutated Outs proteins are unable to localize OutD to the outer membrane. French press lysates of E. coli NM522/pTdB-OD/pACOS (OutD', Outs') (a), NM522/pTdB-OD/pACTS1 (OutD', PeLBsp-OutS') (b), NM522/pACOS/pBA3-37 (OutD', L37F Outs') (c), NM522/pACOS/pBA3-110 (OutD', R110C Outs') (d) were fractionated in a flotation sucrose gradient. Aliquots from each fraction were analysed by SDS-PAGE and immunoblotting with OutD antibodies.

in the outer-membrane fraction [Fig. 7 (b)]. We have observed previously (Shevchik et al., 1997) that a small amount of OutD can insert into the outer membrane, even in the absence of Outs. Thus, the presence of OutD in the outer membrane may not result from the presence of PeLBsp-OutS. Ligand-blotting experiments showed that PeLBsp-OutS is able to interact in vitro with OutD, although with a lower affinity than Outs (data not shown). To determine whether the periplasmic OutD (in the presence of PeLBsp-OutS) is multimeric, we performed gel-filtration analysis of the periplasmic fraction of E. coli strain NM522/pTdB-OD/pACTS1. OutD was eluted only at a retention time close to that of fructose-6-phosphate kinase (85 kDa) and of BSA (67 kDa) (Fig. 4c). This result indicates that OutD stays monomeric in the periplasmic fraction. Surprisingly, no Outs was detected in the OutD fractions. This protein was completely eluted in low-molecular-mass protein fractions (about 10–15 kDa) (Fig. 4c). The PeLBsp-OutS/OutD complex was probably not stable in the periplasm or was dissociated during the gel filtration.

None of the nonsense mutants were able to protect or to help insertion of OutD into the outer membrane. Contrarily, in vivo and in vitro experiments showed that all the missense mutants were able to protect and to bind OutD like the wild-type Outs (data not shown). However, despite their outer-membrane location, none of them was able to help OutD insertion in the outer membrane (shown for mutants L37F and R110C in Fig. 7c and d). Thus, only missense mutants affected in the pilotin function have been isolated.

Induction of pspA by OutD in the presence of Outs mutants

Expression of the phage shock protein PspA is induced by overexpression of outer-membrane proteins. Among them, protein IV of filamentous phages and its homologues, the GspD proteins, are very good inducers. It has been proposed that the process of insertion of these proteins into the outer membrane, or Sec machinery jamming, could trigger pspA induction (Model et al., 1997). Induction of pspA by PulD can be prevented by the presence of PulS, indicating that although the quantity of PulD increases, its insertion is improved (Hardie et al., 1996a). Overexpression of OutD in E. coli also induced pspA expression, measured by the assay of a pspA–lacZ fusion (Table 2). The effect of outs and its mutant derivatives was tested. Coexpression of Outs with OutD reduced pspA induction (Table 2), although
Table 2. Induction of a pspA-lacZ fusion by OutD in the presence of different GspS proteins

Strain MC3 was grown in LB medium. The results reported [as nmol product liberated min⁻¹ (mg bacterial dry weight)⁻¹] are the mean of at least three independent experiments.

<table>
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<th>OutD</th>
<th>GspS</th>
<th>β-Galactosidase activity</th>
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<tr>
<td></td>
<td>—</td>
<td>98 ± 14</td>
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<td></td>
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<td>1970 ± 130</td>
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<tr>
<td>+</td>
<td>—</td>
<td>510 ± 113</td>
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<tr>
<td>+</td>
<td>PulS</td>
<td>157 ± 26</td>
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<tr>
<td>+</td>
<td>PelB²⁰-OutS</td>
<td>1880 ± 120</td>
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<tr>
<td>+</td>
<td>OutS A32T</td>
<td>1640 ± 237</td>
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<tr>
<td>+</td>
<td>OutS L37F</td>
<td>6030 ± 605</td>
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<tr>
<td>+</td>
<td>OutS R43C</td>
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<td>+</td>
<td>OutS A60V</td>
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<tr>
<td>+</td>
<td>OutS A60T</td>
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<td>+</td>
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<td>+</td>
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<td>+</td>
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less than PulS, which is able to complement an outS mutant (data not shown). However, the reduction observed varied considerably, depending on the stoichiometry of the two GspS and GspD proteins. When PelB²⁰-OutS or the OutS point mutants were co-expressed with OutD, not only was no decrease in the expression of the fusion observed, but in some cases, an increase was noted (Table 2). This may result from the reduced efficiency of these mutants to assist in the release of OutD from the inner membrane, or from the increased amount of stabilized, but incorrectly localized, OutD.

DISCUSSION

To reach their final cellular location, outer-membrane proteins have to travel across the periplasm prior to their correct incorporation into the outer membrane. Recent studies suggest that, for some proteins, these processes require specific chaperones that help them cross the periplasmic space and/or insert into the membrane. The Skp protein facilitates the insertion of OmpA, OmpC, OmpF and LamB in the outer membrane and forms complexes with these proteins during translocation across the periplasm (Chen & Henning, 1996; Misiakas et al., 1997). p20 is a periplasmic protein that carries the lipoprotein Lpp from the inner membrane to the outer membrane (Matsuyama et al., 1995). The outer-membrane components of the Gsp secretory pathway, seem to require specific lipoproteins to direct them to the outer membrane: in Pseudomonas syringae and Neisseria gonorrhoeae, PilP is required for the insertion of PilQ (Roiné et al., 1996; Drake et al., 1997). The VirG lipoprotein is required for efficient targeting of YscC to the outer membrane (Koster et al., 1997). The chaperone-like role of PulS has been well documented (Hardie et al., 1996a, b; Daefler et al., 1997).

We show here that the lipoprotein OutS is a specific chaperone that interacts with OutD to stabilize it and to help its insertion into the outer membrane. The protection effect of OutS is due to the direct interaction of the two proteins. Ligand-blotting experiments showed that interaction between OutD and OutS does not require additional proteins. While only the 62 C-terminal amino acids of OutD are necessary for the interaction to occur, no region of OutS specific for this interaction has been found. The observation that when OutD is overproduced, coexpression with OutS strongly diminishes the quantity of unnaturated OutD could be explained by intervention of OutS in the OutD translocation. OutS could interact from the periplasmic side with the OutD translocation intermediate and help its translocation through the Sec machinery. The slow translocation of OutD in the absence of OutS could explain the induction of pspA. Kleerebezem et al. (1996) suggested that the accumulation of protein precursors in the Sec machinery could lead to the dissolution of the proton-motive force. This would in turn induce pspA expression. A diminution of the efficiency of the OutS missense mutants and the periplasmic form of OutS to help OutD translocation could confirm this hypothesis and explain the high level of pspA expression observed. However, we were unable to obtain strong data supporting this hypothesis. No difference in the OutD precursor maturation efficiency was detected in the presence of the OutS missense mutants and the periplasmic form of OutS (data not shown).

The presence of a lipoprotein-type signal sequence is necessary for the piloting function of OutS toward OutD. Its replacement by a type I peptide signal localizes OutD in the periplasm. However, the localization of OutS in the outer membrane does not determine the destination of the protein with which it interacts. Although PelD-62D interacts with, and is stabilized by, OutS, it is not localized in the outer membrane. Thus, it seems likely that it is the intrinsic properties of OutD that confer on it the outer-membrane location. On the other hand, the function of OutS might not be to insert OutD into the outer membrane, but to draw it into contact with this membrane so that its insertion is facilitated. The partial presence in the outer membrane of OutD, or its truncated derivative OutDC1 (which lacks the 62 C-terminal amino acids and thus is incapable of interacting with OutS), in the absence of OutS, confirms that this insertion can occur spontaneously, albeit with low efficiency (Shevchik et al., 1997). However, there are no data to indicate that spontaneously inserted OutD is in a functional state. A spontaneous insertion of a PulD-PhoA hybrid, containing the N-terminal region of PulD, into the outer membrane has also been observed (Hardie et al., 1996a).

Analysis of OutS mutants showed that the two functions of OutS can be separated: protection of OutD and assistance of its insertion into the outer membrane. All
the non-functional outS point mutants isolated conserve their protection function, but are unable to localize OutD to the outer membrane. During the screening, we retained only mutants that gave less than 5% of the wild-type level of secretion. In doing this, we have excluded mutations giving a less stringent phenotype. Interestingly, the mutated amino acids are all conserved in OutS and PulS (Fig. 1). Conservation of these residues in two proteins that show only 40% identity suggests that they might play a direct role in the pilotin function of these proteins. The lack of mutants with an altered protection function suggests that mutation of a single amino acid is not sufficient to abolish OutS–OutD interaction. The four outS nonsense mutations obtained gave unstable truncated proteins and were of no use in testing the effect of shorter proteins on OutD. It would be interesting to look for other types of OutS mutants (in-frame deletions, insertions, hybrids) that are unable to interact with OutD, to identify the basis of this interaction. There is no obvious reason for the pilotin-deficient phenotype of the outS missense mutants. The lipoprotein sequence signal and the second amino acid after the cleavage site, which is important for the final membrane localization of lipoproteins (Yamaguchi et al., 1988), were not modified. Moreover, at least two of the OutS missense mutants, R110C and L37F, have been shown to be outer-membrane located in both E. coli and Er. chrysanthemi (data not shown). The fact that the protection of OutD by the OutS mutants is efficient, but that OutD insertion in the outer membrane does not occur normally led us to suppose that these mutations prevent one of the last steps of the process of insertion/multimerization of OutD in the outer membrane. OutD multimerization does not occur when it is located in the periplasm in the presence of PelB–OutS. Thus, the mutations might have modified the conformation of OutS and its ability to draw OutD into contact with the outer membrane or the binding capacity of the OutD–OutS complex to an hypothetical outer-membrane receptor and/or prevented the ability of OutD to form stable multimers.

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