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

Vladimir E. Shevchik and Guy Condemine

Author for correspondence: Guy Condemine. Tel: +33 472 43 80 88. Fax: +33 472 43 87 14.
e-mail: condemin@insa.insa-lyon.fr

Laboratoire de Génétique
Moléculaire des Micro-
organismes et des
Interactions Cellulaires,
CNRS-UMR 5577, INSA Bat
406, 20 Av Albert Einstein,
69621 Villeurbanne, France

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 (Daeffler *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.

insertion into the outer membrane (Daefler *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; Condemine *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 polygalacturonate, 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*–*XhoI* fragment from pBA3 containing *outS* was cloned between the *SacI* and *XhoI* sites of pET20. The *outS* signal sequence was deleted and the *outS* coding sequence was put in-frame with the *pelB* signal sequence (*PelB*^{sp}) of pET20, after digestion of the plasmid with *SgrAI* and *EcoRV* and treatment with the Klenow enzyme. The *pelB*^{sp}–*outS* construct was then digested with *BglII* and *XhoI*, 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 OutD, using a 4-amino-acid junction of the pUC118 polylinker (an *XbaI*–*SmaI* fragment). A *MamI*–*NheI* fragment, from pN715 containing *pelD*, was cloned into the *EheI* and *XbaI* 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*–*outD* fusion.

Hydroxylamine mutagenesis. This was performed as described by Yang *et al.* (1993). Forty microlitres of hydroxylamine chlorhydrate, 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 polygalacturonate + 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 toluenized 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 (OD₆₀₀ 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 (200 000 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 vols 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_2 -macroglobulin (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 [³H]palmitate (Amersham). For the ligand-blotting experiments, ³⁵S-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 8 to 15% (w/v) and from 0.2 to 0.4% 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 antiserum. *PelB*^{sp}–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*^{sp}–OutS

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Genotype/description	Reference/origin
<i>Er. chrysanthemi</i>		
A350	<i>lmrT^c lacZ</i>	Laboratory collection
A837	<i>lmrT^c lacZ kdgR</i>	Laboratory collection
A1903	<i>lmrT^c lacZ outS::uidA-kan</i>	Condemine <i>et al.</i> (1992)
<i>E. coli</i>		
K38	HfrC(λ) <i>phoA4 pit-10 tonA22 ompF627 relA1</i>	Russel & Model (1984)
BL21	<i>F⁻ dcm ompT hsdS gal</i> , T7 polymerase gene under the <i>lacUV5</i> promoter	Tabor & Richardson (1985)
MC3	<i>pspA-lacZ</i>	Bergler <i>et al.</i> (1994)
NM522	<i>supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5 r_k⁻ m_k⁻ F' proAB lacI^q ΔlacZM15</i>	Stratagene
Plasmids		
pET20	Ap ^r	Novagen
pACT3	Cm ^r	Dykxhoorn <i>et al.</i> (1996)
pT7-5	Ap ^r	Tabor & Richardson (1985)
pT7-6	Ap ^r	Tabor & Richardson (1985)
pUC118	Ap ^r	Sambrook <i>et al.</i> (1989)
pGP1-2	<i>cl857</i> , T7 polymerase gene under the λ P _L promoter; Km ^r	Tabor & Richardson (1985)
pACTS1	pACT3 containing <i>pelB^{sp}-outS</i>	This work
pBA3	pBluescript containing <i>BclI</i> - <i>AatII</i> fragment carrying <i>outS</i>	This work
pBA3-37	As pBA3 with <i>outS</i> L37F	This work
pBA3-110	As pBA3 with <i>outS</i> R110C	This work
pCHAP378	pBR322 carrying <i>pulS</i>	d'Enfert & Pugsley (1987)
pVLTS	pVLT31 carrying <i>outS</i>	Shevchik <i>et al.</i> (1997)
pACOS	pACYC184 carrying <i>outS</i>	Shevchik <i>et al.</i> (1997)
pT7OS	pT7-5 carrying <i>outS</i>	This work
pACOD	pACYC184 carrying <i>outD</i>	Shevchik <i>et al.</i> (1997)
pTdB-OD	pT7-6 carrying <i>outD</i>	Shevchik <i>et al.</i> (1997)
pTdB-ODC1	pT7-6 carrying <i>outD</i> Δ 648-710	Shevchik <i>et al.</i> (1997)
pTdB-ODC2	pT7-6 carrying <i>outD</i> Δ 382-710	Shevchik <i>et al.</i> (1997)
pODN1	pBluescript carrying <i>outD</i> Δ 66-116	Shevchik <i>et al.</i> (1997)
pODN	pBluescript carrying <i>outD</i> Δ 1-285	Shevchik <i>et al.</i> (1997)
pODN2	pET20 carrying <i>pelB^{sp}-outD</i> Δ 1-285	Shevchik <i>et al.</i> (1997)
pMMB-OD	pMMB207 (de Lorenzo <i>et al.</i> , 1993) carrying <i>outD</i> , Cm ^R	This work
pN715	pBR322 containing <i>pelD</i>	Hugouvieux-Cotte-Pattat <i>et al.</i> (1992)
pTPLD-'62D	pT7-6 carrying <i>pelD</i> '-' <i>outD</i> fusion	This work

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 14336 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 pT7OS). 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 *Er. chrysanthemi* and an *E. coli* strain producing OutS, by flotation on a sucrose

OutS MHVSSLKVVLFVGVCCSLAACQTPAPVKNTASRSAASVPANEIQISQLASLVAAASKYLRVQCERSDLPDDGTILKTAV
PulS MFRFILFPMNAVLLSGC-----QQNRPTTLPVAVGGQAQLEQLASVAAAGARYLKNKCNRSDDLPADEAINRAAI

OutS 78 NVAVQKQWDTGRYQSLPQLSENLYQGLLKDGTPKATQCSSFNRTMTPLDAMRTVR
PulS 70 NVGKKRGWANIDANLLSQRSQAQLYQQLQDDSTFEATKCSQFNRLAPFIDSLRNK

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 *outsS* 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. chrysanthemi* 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.

Fig. 4. Effect of OutS and PelB^{SP}-OutS on the stability and cellular localization of PelD-62D and OutD. (a) Proteins were separated by SDS-PAGE and analysed by immunoblotting with PelD antibodies: total cell lysates of *E. coli* NM522/pTPLD-62D/pACT3 (OutS⁻) (lane 1), NM522/pTPLD-62D/pACTS1 (PelB^{SP}-OutS⁺) (lane 2), NM522/pTPLD-62D/pACOS (OutS⁺) (lane 3) or the periplasmic (lane 4) and the osmotic-shocked cell (lane 5) fractions of NM522/pTPLD-62D/pACTS1 (PelB^{SP}-OutS⁺). (b) Proteins were separated by SDS-PAGE and analysed by immunoblotting with OutD antibodies: total cell lysates of *E. coli* NM522/pTdB-OD/pACT3 (OutS⁻) (lane 6), NM522/pTdB-OD/pACTS1 (PelB^{SP}-OutS⁺) (lane 7), NM522/pTdB-OD/pACOS (OutS⁺) (lane 8) or the periplasmic (lane 9) and the osmotic-shocked cell (lane 10) fractions of NM522/pTdB-OD/pACTS1 (PelB^{SP}-OutS⁺) were analysed. (c) Gel-filtration chromatography analysis of the periplasmic proteins of *E. coli* NM522/pTdB-OD/pACTS1 (OutD⁺, PelB^{SP}-OutS⁺). The OutD (filled triangle) and OutS (filled star) positions are indicated. The column was calibrated with (1 and 2) α_2 -macroglobulin (340 and 170 kDa – non-reduced and reduced forms, respectively), (3) fructose-6-phosphate kinase (85 kDa), (4) BSA (67 kDa), (5) chymotrypsinogen A (25 kDa) and (6) RNase A (13.7 kDa).

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 ³⁵S-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 in-

teraction 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

Fig. 5. OutS helps in the maturation of OutD. Crude membrane fractions of *E. coli* BL21/pTdB-OD/pBluescript (OutS⁻) or BL21/pTdB-OD/pBA3 (OutS⁺) were separated by SDS-PAGE and detected with OutD antibodies. The OutD precursor (p) and mature (m) forms are indicated.

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^{SP}-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⁻ 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^{SP}-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*^{SP}-*outS* construct was unable to complement an *Er. chrysanthemi* OutS⁻ mutant. Moreover, it inhibited secretion when introduced into the wild-type strain. However, it was able to protect OutD, since coexpression of *pelB*^{SP}-*outS* with *outD* led to an increase in the amount of OutD in the cells (Fig. 4 b, lanes 6 and 7). The recombinant protein seemed to stabilize OutD as a periplasmic intermediate: in an *E. coli* strain expressing OutD with PelB^{SP}-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*^{SP}-*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. *E. coli* K38/pGP1-2/pT7dB-OD/pVLT31 (OutD⁺, OutS⁻) (a) and K38/pGP1-2/pT7dB-OD/pVLT5 (OutD⁺, OutS⁺) (b) cells were induced and labelled with [³⁵S]methionine and [³⁵S]cysteine. An excess of unlabelled methionine and cysteine was added 2 min later and the cells were incubated for an additional 60 min. Culture samples were removed during the incubation. Proteins were separated by SDS-PAGE and autoradiographed.

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⁺, PelB^{SP}-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 PelB^{SP}-OutS. Ligand-blotting experiments showed that PelB^{SP}-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 PelB^{SP}-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 PelB^{SP}-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.

OutD	GspS	β -Galactosidase activity
—	—	98 $\pm$ 14
+	—	1970 $\pm$ 130
+	OutS	510 $\pm$ 113
+	PulS	157 $\pm$ 26
+	PelB ^{SP} -OutS	1880 $\pm$ 120
+	OutS A32T	1640 $\pm$ 237
+	OutS L37F	6030 $\pm$ 605
+	OutS R43C	2700 $\pm$ 187
+	OutS A60V	3090 $\pm$ 573
+	OutS A60T	4200 $\pm$ 640
+	OutS S77N	4670 $\pm$ 370
+	OutS R110C	1600 $\pm$ 315
+	OutS81	2160 $\pm$ 223

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 PelB^{SP}-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; Missiakas *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 called secretins, 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 (Roine *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 unmaturation 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 dissipation 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 pilotin 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^{sp}–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.

ACKNOWLEDGEMENTS

We thank A. Pugsley for anti-OutS antibodies and pCHAP378, Dr F. Turnowsky for providing a *pspA*–*lacZ* strain, and N. Cotte-Pattat, S. Reverchon, W. Nasser and V. James for reading the manuscript. This work was supported by grants from the Fondation pour la Recherche Médicale, the CNRS and the DRED.

REFERENCES

- Bergler, H., Abraham, D., Aschauer, H. & Turnowsky, F. (1994). Inhibition of lipid biosynthesis induces the expression of the *pspA* gene. *Microbiology* **140**, 1937–1944.
- Brisette, J. L. & Russel, M. (1990). Secretion and membrane integration of a filamentous phage-encoded morphogenetic protein. *J Mol Biol* **211**, 565–580.
- Chen, R. & Henning, U. (1996). A periplasmic protein (Skp) of *Escherichia coli* selectively binds a class of outer membrane proteins. *Mol Microbiol* **19**, 1287–1294.
- Chen, L.-Y., Chen, D.-Y., Miaw, J. & Hu, N.-T. (1996). XcpD, an outer membrane protein required for protein secretion by *Xanthomonas campestris* pv. *campestris*, forms a multimer. *J Mol Biol* **271**, 2703–2708.
- Condemine, G., Dorel, C., Hugouvieux-Cotte-Pattat, N. & Robert-Baudouy, J. (1992). Some of the *out* genes involved in the secretion of pectate lyases in *Erwinia chrysanthemi* are regulated by *kdgR*. *Mol Microbiol* **6**, 3199–3211.
- Daefler, S., Guilvout, I., Hardie, K. R., Pugsley, A. P. & Russel, M. (1997a). The C-terminal domain of the secretin PulD contains the binding site for its cognate chaperone, PulS, and confers PulS dependence on pIV^{tr} function. *Mol Microbiol* **24**, 465–475.
- Daefler, S., Russel, M. & Model, P. (1997b). Module swaps between related translocator proteins pIV^{tr}, pIV^{ike} and PulD: identification of a specificity domain. *J Mol Biol* **266**, 978–992.
- Drake, S. L. & Koomey, M. (1995). The product of the *pilQ* gene is essential for the biogenesis of the type IV pili in *Neisseria gonorrhoeae*. *Mol Microbiol* **18**, 975–986.
- Drake, S. L., Sandstedt, S. A. & Koomey, M. (1997). PilP, a pilus biogenesis lipoprotein in *Neisseria gonorrhoeae*, affects expression of PilQ as a high-molecular-mass multimer. *Mol Microbiol* **23**, 657–668.
- Dykxhoorn, D. M., St Pierre, R. & Furlong, C. E. (1996). A set of compatible *tac* promoter expression vectors. *Gene* **177**, 133–136.
- d'Enfert, C. & Pugsley, A. P. (1987). *Klebsiella pneumoniae* *pulS* gene encodes an outer membrane lipoprotein required for pullulanase secretion. *J Bacteriol* **171**, 3673–3679.
- Genin, S. & Boucher, C. A. (1994). A superfamily of proteins involved in different secretion pathways in Gram-negative bacteria: modular structure and specificity of the N-terminal domain. *Mol Gen Genet* **243**, 112–118.
- Hardie, K. R., Lory, S. & Pugsley, A. P. (1996a). Insertion of an outer membrane protein in *Escherichia coli* requires a chaperone-like protein. *EMBO J* **15**, 978–988.
- Hardie, K. R., Seydel, A., Guilvout, I. & Pugsley, A. P. (1996b). The secretin-specific, chaperone-like protein of the general secretory pathway: separation of proteolytic protection and piloting functions. *Mol Microbiol* **22**, 967–976.
- Hugouvieux-Cotte-Pattat, N., Dominguez, H. & Robert-Baudouy, J. (1992). Environmental conditions affect transcription of the pectinase genes of *Erwinia chrysanthemi* 3937. *J Bacteriol* **174**, 7807–7818.
- Johnson, B. H. & Hecht, M. H. (1994). Recombinant proteins can be isolated from *E. coli* cells by repeated cycles of freezing and thawing. *Bio/Technology* **12**, 1357–1360.
- Kleerebezem, M., Crielaard, W. & Tommassen, J. (1996). Involvement of stress protein PspA (phage shock protein A) of *Escherichia coli* in maintenance of the proton motive force under stress conditions. *EMBO J* **15**, 162–171.
- Koster, M., Bitter, W., de Cock, H., Allaoui, A., Cornelis, G. R. & Tommassen, J. (1997). The outer membrane component, YscC, of the Yop secretion machinery of *Yersinia enterocolitica* forms a ring-shaped multimeric complex. *Mol Microbiol* **26**, 789–797.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Linderoth, N. A., Simon, M. A. & Russel, M. (1997). The filamentous phage pIV multimer visualized by scanning transmission electron microscopy. *Science* **278**, 1635–1638.
- de Lorenzo, V., Eltis, L., Kessler, B. & Timmis, K. N. (1993). Analysis of *Pseudomonas* gene products using *lacI*^q/P_{trp}–*lac* plasmids and transposons that confer conditional phenotypes. *Gene* **123**, 17–24.

- Matsuyama, S.-I., Tajima, T. & Tokuda, H. (1995). A novel periplasmic carrier protein involved in the sorting and transport of *Escherichia coli* lipoproteins destined for the outer membrane. *EMBO J* 14, 3365–3372.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Missiakas, D., Betton, J.-M. & Raina, S. (1997). New components of protein folding in extracytoplasmic compartments of *Escherichia coli* SurA, FkpA, and Skp/OmpH. *Mol Microbiol* 21, 871–884.
- Model, P., Jovanovic, G. & Dworkin, J. (1997). The *Escherichia coli* phage-shock-protein (*psp*) operon. *Mol Microbiol* 24, 255–261.
- Moran, F., Nasuno, S. & Starr, M. P. (1968). Extracellular and intracellular polygalacturonic transeliminases of *Erwinia carotovora*. *Arch Biochem Biophys* 123, 298–306.
- Noonan, B. & Trust, T. J. (1995). Molecular analysis of an A-protein secretion mutant of *Aeromonas salmonicida* reveals a surface layer-specific protein secretion pathway. *J Mol Biol* 248, 316–327.
- Roine, E., Nunn, D. N., Paulin, L. & Romantschuk, M. (1996). Characterization of genes required for pilus expression in *Pseudomonas syringae* pathovar *phaseolicola*. *J Bacteriol* 178, 410–417.
- Russel, M. & Kazmierczak, B. (1993). Analysis of the structure of the subcellular location of filamentous phage pIV. *J Bacteriol* 175, 3998–4007.
- Russel, M. & Model, P. (1984). Replacement of the *fip* gene of *Escherichia coli* by an inactive gene cloned on a plasmid. *J Bacteriol* 159, 1034–1039.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Shevchik, V. E., Condemine, G., Hugouvieux-Cotte-Pattat, N. & Robert-Baudouy, J. (1996). Characterization of pectin methyl-esterase B, an outer membrane lipoprotein of *Erwinia chrysanthemi* 3937. *Mol Microbiol* 19, 455–466.
- Shevchik, V. E., Robert-Baudouy, J. & Condemine, G. (1997). Specific interaction between OutD, an *Erwinia chrysanthemi* outer membrane protein of the general secretory pathway, and secreted proteins. *EMBO J* 16, 3007–3016.
- Tabor, S. & Richardson, C. (1985). A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc Natl Acad Sci USA* 82, 1074–1078.
- Yamaguchi, K., Yu, F. & Inouye, M. (1988). A single amino acid determinant of the membrane localization of lipoproteins in *E. coli*. *Cell* 53, 423–432.
- Yang, J., Ganesan, S., Sarsero, J. & Pittard, A. J. (1993). A genetic analysis of various functions of the TyrR protein of *Escherichia coli*. *J Bacteriol* 175, 1767–1776.

Received 25 June 1998; accepted 27 July 1998.