Overproduction of the secretin OutD suppresses the secretion defect of an *Erwinia chrysanthemi* *outB* mutant

Guy Condemine and Vladimir E. Shevchik

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

Unité Microbiologie et
Génétique Composante
INSA, UMR CNRS-INSA-UCB
5577, INSA Bat 406, 20 Av
Einstein, 69621
Villeurbanne, France

OutB is a component of the *Erwinia chrysanthemi* Out secretion machinery. Homologues of OutB have been described in two other bacteria, *Klebsiella oxytoca* and *Aeromonas hydrophila*, but their requirement in the secretion process seems to be different. Study of OutB topology with the BlaM topology probe suggests that it is an inner-membrane protein with a large periplasmic domain. However, fractionation experiments indicate that it could be associated with the outer membrane through its C-terminal part. The secretion deficiency of an *Erw. chrysanthemi* *outB* mutant can be reversed by the addition of an inducer of the *kdgR* regulon. It was shown that this effect results from the increased expression of the secretin OutD and that secretion can be restored in an *outB* mutant by introducing the *outD* gene on a plasmid. Several experiments suggest an interaction between OutB and OutD. In *Erw. chrysanthemi*, the presence of OutD stabilizes OutB. OutD expressed in *Escherichia coli* can be protected from proteolytic degradation by the coexpression of OutB. This effect does not require the N-terminal, transmembrane segment of *outB*. OutB can be cross-linked with OutD by formaldehyde. These results indicate that OutB could act with OutD in the functioning of the Out secretion machinery.

Keywords: general secretory pathway, protein secretion, protein–protein interaction, secretin

INTRODUCTION

Pectinases, the main virulence factors of the phytopathogenic bacteria *Erwinia chrysanthemi*, are secreted by a type II secretion system. Such type II machineries [or the main terminal branch of the general secretory pathway (GSP)] are found in several other Gram-negative bacteria that secrete toxins or lytic enzymes in the outer medium: *Erwinia carotovora*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, *Klebsiella oxytoca* and *Vibrio cholerae* (Pugsley, 1993). Secreted enzymes are synthesized with an N-terminal signal sequence, which is cleaved during the Sec-dependent translocation across the inner membrane. In the absence of the GSP machinery, these proteins remain in the periplasm. Biogenesis and functioning of the various GSP machineries requires the products of a minimum of 12

genes (*gspC–M* and *gspO*) in *P. aeruginosa* but this number can go up to 15 in *Erw. chrysanthemi* or *Erw. carotovora*. Little is known about the function of their products in protein secretion. GspD is a member of a family of outer-membrane proteins, named secretins, involved in the traffic of macromolecules across the outer membrane. The filamentous phage f1 pIV protein, *Yersinia enterocolitica* YscC and *P. aeruginosa* XcpQ and PilQ are secretins that form homomultimeric rings which could constitute the pore through which phages or proteins would cross the outer membrane (Linderoth *et al.*, 1997; Bitter *et al.*, 1998). The N-terminal part of the secretins could also play a role in the selection of proteins to secrete. *Erw. chrysanthemi* OutD interacts, through its N-terminal domain, with secreted proteins and this interaction could be the key to the specificity of the secretion mechanism (Shevchik *et al.*, 1997). The N-termini of GspG, H, I, J and K resemble that of the type IV pilin subunit and thus these proteins are designated pseudopilins (Blevins *et al.*, 1998). Their leader peptide is cleaved by the GspO prepilin peptidase (Nunn & Lory,

Abbreviations: CMC, carboxymethylcellulose; GSP, general secretory pathway; PGA, polygalacturonate

1991). GspE is an ATP-binding protein with a kinase activity (Sandkvist *et al.*, 1995). The bitopic inner-membrane proteins GspC, L and M have a large periplasmic domain and GspF is a polytopic inner-membrane protein (Reeves *et al.*, 1994; Thomas *et al.*, 1997). Some possible interactions between components of the GSP machinery have been described: OutE–OutL, EspE–EspL, XcpT–XcpR, XcpY–XcpZ and EpsL–EpsM (Py *et al.*, 1999; Sandkvist *et al.*, 1995, 1999; Kagami *et al.*, 1998; Michel *et al.*, 1998).

Besides this conserved core of 12 proteins, some GSP proteins are found in only a few bacteria. GspN has been found in *Erw. carotovora* but not in *Erw. chrysanthemi* (Lindeberg & Collmer, 1992). GspA, a protein with putative ATPase activity, has only been detected in *Escherichia coli* and *A. hydrophila* (*exeA*) (Francetic & Pugsley, 1996; Jahagirdar & Howard, 1994). GspS has only been identified in *K. oxytoca*, in the entero-haemorrhagic *Esc. coli* O157:H7 strain, in *Erw. chrysanthemi* and *Erw. carotovora* (PulS, EtpO and OutS) (d'Enfert & Pugsley, 1989; Schmidt *et al.*, 1997; Condemine *et al.*, 1992; Lindeberg *et al.*, 1996). These GspS proteins are outer-membrane lipoproteins that stabilize the secretins PulD and OutD and help their insertion in the outer membrane (Hardie *et al.*, 1996; Shevchik & Condemine, 1998). The interaction requires a 62 aa domain present at the C-terminal extremity of PulD and OutD (Daefer *et al.*, 1997; Shevchik & Condemine, 1998). This domain is also present in the *Erw. carotovora* OutD protein but not in other secretins. Thus, GspS probably does not exist in other bacteria. GspB has been described in three bacteria: *K. oxytoca*, *Erw. chrysanthemi* and *A. hydrophila* (d'Enfert & Pugsley, 1989; Condemine *et al.*, 1992; Jahagirdar & Howard, 1994). It probably also exists in *Erw. carotovora* (Lindeberg *et al.*, 1996). In the first two bacteria, the gene *gspB* is clustered with *gspS*, whereas in *A. hydrophila* *exeB* forms an operon with *exeA*. ExeB forms a complex with the ATPase ExeA (Schoenhofen *et al.*, 1998) and is required for efficient secretion of aerolysin in this bacterium. By contrast, a *K. oxytoca* *pulB* mutant has no phenotype. In *Erw. chrysanthemi*, the absence of OutB leads to an intermediate phenotype, with secretion of pectate lyase reduced by 30%. In this study, we analysed the cellular localization of OutB and showed that a mutation in *outB* can be suppressed by overproduction of OutD. Our results suggest that OutB interacts with OutD.

METHODS

Bacterial strains, plasmids, culture conditions and plate assays. Bacterial strains are described in Table 1. *Erw. chrysanthemi* and *Esc. coli* cells were grown at 30 °C and 37 °C, respectively, in Luria–Bertani (LB) medium or M63 minimal medium (Miller, 1972), supplemented with a carbon source [0.2 %, except polygalacturonate (PGA) 0.08 %] and, when required, with amino acids (40 µg ml⁻¹) and antibiotics at the following concentrations: ampicillin, 100 µg ml⁻¹; kanamycin, 50 µg ml⁻¹; chloramphenicol, 20 µg ml⁻¹. LB medium was solidified with 12 g agar l⁻¹ to give GL plates. Pectate

lyase activity was revealed by flooding PGA-containing agar plates with a saturated solution of copper acetate (Andro *et al.*, 1984). Degraded PGA formed a clear halo around the colonies. Cellulase activity was detected on carboxymethyl-cellulose (CMC)-containing plates by flooding with 0.1 % Congo red for 10 min followed by bleaching with 1 M NaCl (Wood, 1980).

Recombinant DNA techniques. Preparation of plasmid DNA, restriction, ligation, DNA electrophoresis, transformation and electroporation were carried out as described by Sambrook *et al.* (1989). Nucleotide sequence analysis was performed using the Pharmacia T7 sequencing kit. To construct the periplasmic derivative of OutB (PelB^{sp}–OutB), the *EcoRV*–*EcoRI* fragment from plasmid pBT1 carrying *outB* and *outS* was cloned between the *EcoRV* and *EcoRI* sites of pET-20b(+) under the PelB signal peptide. The *pelB*^{sp}–*outB* construct was then digested with *Bgl*III and *Xho*I and introduced into the expression vector pACT3 digested by *Bam*HI and *Sal*I. Finally, the *outS* gene was deleted from the construct by digestion with *Hind*III and *Eco*NI, treatment with the Klenow enzyme and ligation to give plasmid pABSC3.

Construction of OutB–BlaM protein fusions. The *outB* gene was cloned between the *Eco*RI and *Kpn*I sites of plasmid pJBSKpn, a derivative of plasmid pJBS633 (Broome-Smith *et al.*, 1990) that contains an additional *Kpn*I site upstream of the *blaM* gene. The resulting construct was digested with *Kpn*I and *Hind*III that cleaves the plasmid downstream of *outB* to produce exonuclease III-insensitive and sensitive sites, respectively. Deletions in *outB* were generated with the Exonuclease III deletion kit (USB). After ligation and transformation, the Kan^r clones obtained were first replicated on GL plates containing Amp (50 µg ml⁻¹) to identify those producing in-frame OutB–BlaM fusion proteins. Four Amp^r clones were retained. For these, the MIC of ampicillin required to prevent colony formation by a single cell was determined and nucleotide sequencing was performed to characterize the *outB*–*blaM* junction.

OutB purification. The periplasmic soluble form of OutB (PelB^{sp}–OutB) was overproduced in the NM522/pABSC3 strain. Cells were grown in 100 ml LB containing chloramphenicol. At an OD₆₀₀ of 0.5, IPTG was added to 1 mM and, after 2 h additional growth, the cells were harvested by centrifugation for 5 min at 5000 g and frozen at –80 °C. The overproduced protein was extracted from cells by three cycles of freeze-thawing (Johnson & Hecht, 1994). The supernatant was loaded onto a 15 % (w/v) preparative SDS-PAGE. The band containing PelB^{sp}–OutB was cut out and the protein was extracted by three washes with 10 mM Tris/HCl pH 8.0, 0.1 % SDS. The protein was concentrated and injected into a rabbit for antibody production by Valbex (Villeurbanne).

Protein labelling. Overexpression and exclusive labelling of plasmid-encoded proteins was carried out using the T7 promoter/T7 polymerase system of Tabor & Richardson (1985).

Gel electrophoresis and immunoblotting. SDS-PAGE was usually performed according to Laemmli (1970). 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.*, 1996). The primary antibodies used were anti-OutB diluted 1:5000, anti-BlaM diluted 1:5000, anti-OutD diluted 1:3000 and anti-EGZ diluted 1:3000.

Isolation and analysis of cell fractions. Exponentially growing

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Genotype/description	Reference/origin
Strain		
<i>Erw. chrysanthemi</i>		
A350	<i>lmrT^c lacZ</i>	Hugouvieux-Cotte-Pattat & Robert-Baudouy (1985)
A837	<i>lmrT^c lacZ kdgR</i>	Condemine & Robert-Baudouy (1987)
A1524	<i>lmrT^c lacZ pecS::MudIPR13</i>	Reverchon <i>et al.</i> (1994)
A1903	<i>lmrT^c lacZ outS::uidA-kan</i>	Condemine <i>et al.</i> (1992)
A1919	<i>lmrT^c lacZ outC::uidA-kan</i>	Laboratory collection
A2348	<i>lmrT^c lacZ kdgR pecS::Cm</i>	Laboratory collection
A2582	<i>lmrT^c lacZ outB::uidA-kan</i>	Laboratory collection
A2591	<i>lmrT^c lacZ outD</i>	Shevchik <i>et al.</i> (1997)
A3157	<i>lmrT^c lacZ kdgR outB::uidA-kan</i>	This work
A3158	<i>lmrT^c lacZ pecS::MudIPR13 outB::uidA-kan</i>	This work
A3494	As A2348 but also <i>outD</i>	This work
A3558	<i>lmrT^c lacZ outD</i>	This work
<i>Esc. coli</i>		
NM522	<i>supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5 (r_k⁻ m_k⁻)(F' proAB lacI^a ΔlacZM15)</i>	Stratagene
K38	HfrC(λ) <i>phoA4 pit-10 fhuA22 ompF627 relA1</i>	Russel & Model (1984)
Plasmids		
pT7-5	Ap ^r , ϕ10 promoter	Tabor & Richardson (1985)
pACT3	Cm ^r , <i>ptac</i>	Dykxhoorn <i>et al.</i> (1996)
pABTC2	pACT3 carrying the <i>outB</i> gene	This work
pABSC3	pACT3 carrying the <i>pelB^{sp}-outB</i> construct	This work
pBSAC	pACYC184 carrying <i>outB</i> and <i>outs</i> (1853 bp)	This work
pBT1	pT7-5 carrying <i>outB</i> and <i>outs</i>	This work
pET-20b (+)	Ap ^r	Novagen
pODN1	pBS carrying <i>outD</i> with a <i>AviII</i> - <i>MscI</i> deletion	Shevchik <i>et al.</i> (1997)
pTdB-OD	pT7-6, carrying <i>outD</i> under the <i>pelC</i> promoter	Shevchik <i>et al.</i> (1997)
pJBskpn	kan ^r , <i>blaM</i>	J. C. Lazzaroni, Claude Bernard University, Villeurbanne, France

cells (OD₆₀₀ 0.8–1.0) were used for cell fractionation. Cell-membrane fractionation was performed by sucrose gradient centrifugation, as previously described (Shevchik *et al.*, 1996). NADH oxidase was assayed as described by Osborn *et al.* (1972). Crude membrane fractions were isolated by centrifugation (200 000 g for 2 h) after French press disintegration of cells and resolubilized in 50 mM Tris/HCl pH 8.0.

Cross-linking experiments. Bacteria were grown in LB medium to an OD₆₀₀ of 1.0. An aliquot of 1 ml of culture was centrifuged, the bacteria were washed in 10 mM potassium phosphate buffer pH 6.8 and resuspended in the same volume of the buffer. The cells were incubated with 1% formaldehyde at room temperature for 30 min, washed in phosphate buffer, and resuspended in SDS sample buffer for analysis by SDS-PAGE and immunoblotting.

RESULTS

OutB encodes a protein with an apparent molecular mass of 29 kDa

During the course of our work, we noticed that a C residue had been omitted at position 1235 of the published sequence of *outB* (GenBank accession number X65265; Condemine *et al.*, 1992). The resulting frame-shift has led to the assignment of an erroneous start codon. Considering the corrected sequence, the *outB* ORF begins at a GTG codon at nucleotide 1370 which is preceded by a putative Shine–Dalgarno sequence (GAGG) at nucleotides 1377–1380. This ORF encodes a putative protein of 220 amino acids with a deduced molecular mass of 23 489 Da. To detect the protein

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OutB  -----MKNTPEVKASP---QTG--YRIPG----YLLV-VYALLLFTLGWFGHQRWAD- 42
PulB  ----MLVRPQEPYPQSEPPA-AVGRMVQIP-----YVTVPLYAALLIALGWFGGEQWRNK 50
ExeB  MSTLLKALRRAEQPQFTPHIPAMGLPVTQEEEQNRRWIWLLAPLALLMGAGANYGWHLL 60
      : * * * : * * : *
      * * * * *

OutB  -ISPIPLSTSAIAAPPTK-VGMVPASTAVTADENHPGSLHAAENSATQTAASGSQTS 100
PulB  -PEPQPMRQSVAHAA-----LVPL-----NQP-----AVK-AAVAPVNAGPEIQ 87
ExeB  NNRPIEKTVEVKEVTPPFVRVEPRPMITRPLPPLPEPVVRPRVTPNDSAPAANGSQGL 120
      * * * * *

OutB  ASSQEATPDTPKPAKLVGTGQTAKP-----GELPYIAFSAHVYTSAPDKRSV 146
PulB  AEP-EIAIDED-----N-LPPLRYSAHVYASLADKRSI 118
ExeB  AERIMNALNSTPLMEETAPQAQSESQAMPISALPLELKQRPVPLAYGSHVFSSNPAKRAV 180
      * : : : * : : * : * : * : * : * : *

OutB  TLNGERYREGDSPYQGLVIEQIEQDMVIFSFNGEFFILDSLDQWPGGKPGDDAAQGNQE 206
PulB  VLNGQSWKEGDSPLANLVIEHIQDQDLTVFSFNGKTFTLAALDDWPG---G-----AIEES 170
ExeB  MLNGREFREGSEVAPGVTLIAIAQDYIILQVAGQNVSLKALQDWRG----- 226
      *** : : * : : * * * : : * : * : * *

OutB  PTSKPEQTVRRTTK 220
PulB  PQAE----- 174
ExeB  -----

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Fig. 1. Alignment of the sequences of OutB, PulB and ExeB with the CLUSTAL program. The stars indicate conserved residues, double dots indicate chemically equivalent residues. The sequence of PulB is from d'Enfert & Pugsley (1989) and the sequence of ExeB from Jahagirdar & Howard (1994).

Fig. 2. Topological analysis of OutB. Hydrophobicity profile of OutB using the method of Kyte & Doolittle (1982). The position of Amp^s (open circles) and Amp^r (filled circles) BlaM fusion points are shown beneath the hydrophobicity profile with the number of the corresponding residue. The MIC for each fusion is indicated.

encoded by this ORF, the 1.8 kb *Bcl*I–*Nsi*I fragment containing this ORF was cloned under the T7 promoter of plasmid pT7-5. Exclusive labelling of the proteins encoded by this DNA fragment with [³⁵S]methionine-cysteine revealed a protein with an apparent molecular mass of 29 kDa (data not shown). The anomalous migration of OutB could result from its amino acid composition since it contains 9% of proline, which could modify its mobility in SDS-polyacrylamide gels. Such an aberrant mobility has been described for ExeB (12% proline) and the proline-rich protein TonB (16% proline) (Howard *et al.*, 1996).

Topological analysis of OutB

Alignment of the sequence of OutB with those of PulB and ExeB shows that the three proteins present a low homology throughout their length with some more highly conserved regions in their C-terminal part (Fig. 1). Their hydropathy profiles, analysed by the method of

Kyte & Doolittle (1982), are also very similar. In their N-terminal parts, they contain, after a short hydrophilic segment, a highly hydrophobic region of about 20 residues which does not present the features of a signal sequence but which could anchor the protein in the cytoplasmic membrane (Fig. 2). To elucidate the OutB topology, we constructed in-frame fusions between OutB and the topology probe β -lactamase. Four in-frame OutB–BlaM fusions were obtained with the fusion at residues 5, 6, 41 and 128 of OutB. The first two gave a MIC < 2 μ g ml⁻¹ to isolated *Esc. coli* colonies on GL Amp plates whereas the last two gave an MIC of 15 μ g ml⁻¹. This suggests that OutB possesses a short N-terminal cytoplasmic extremity, a transmembrane segment and a large C-terminal periplasmic domain. The same MICs were obtained when the constructs were introduced into *Erw. chrysanthemi* strain A350, in an *outS* mutant (A1903), or in a polar *outC* mutant (A1919) that lacks all the other Out proteins, confirming that the topology of the OutB–BlaM fusions is the same in *Erw.*

Fig. 3. Cellular localization of OutB. The membrane fraction of *Erw. chrysanthemi* strain A350 was separated in a flotation sucrose gradient. An aliquot from each fraction was analysed by SDS-PAGE and immunoblotting with anti-OutB or assayed for NADH oxidase activity. The position of the porins, visualized by Ponceau red staining of the membrane, was used to determine the position of outer-membrane-containing fractions.

chrysanthemi as in *Esc. coli* and shows that no other Out protein is able, by interacting with the fusions, to modify the MIC produced.

Other experiments were performed to confirm the localization of OutB. When the membranes of the *Erw. chrysanthemi* strain A350 were separated by sucrose density gradient centrifugation, either by flotation or by sedimentation, OutB was not found in the NADH-oxidase-containing fractions (inner membrane) but in fractions corresponding to the outer membrane (Fig. 3). The same result was observed in an *outS* mutant, in a mutant containing a polar mutation in *outC*, or when OutB was produced in *Esc. coli* (data not shown), indicating that no other Out protein is required for the proper localization of OutB. A large degradation product of OutB (20 kDa) was found in the periplasmic fraction during cell fractionation experiments (data not shown). Taken together, the results obtained with the BlaM topology probe and with membrane fractionation suggest that OutB is an inner-membrane protein with a large periplasmic domain that could interact with the outer membrane. The interaction with the outer membrane seems to be stronger since it resists cell breakage and membrane separation.

Overproduction of OutD phenotypically suppresses an *outB* mutation

The effect of an *outB* mutation on pectate lyase secretion varies depending on the growth conditions used, mainly the presence or absence of pectate lyase synthesis inducers. To clarify this effect, we studied the secretion of the cellulase EGZ, which is constitutively expressed, and of pectate lyases, whose synthesis strongly increases in the presence of galacturonate or PGA. These compounds also induce the synthesis of OutC–O proteins (Condemine & Robert-Baudouy, 1995). However, PGA has to be degraded by pectinases produced at a basal

Table 2. Secretion of pectate lyases and EGZ in *outB* mutants

Secretion was estimated by the size of substrate degradation haloes on 0.08% PGA + glycerol or LB + CMC agar plates after 16 h growth. Halo size: +/–, <1 mm; +, 1–3 mm; ++, 3–5 mm; +++, >5 mm. Galacturonate (0.2%) was added as inducer.

Strain	Genotype	Pectate lyase		EGZ		
		Inducer ...	—	+	—	+
A350	WT		+++	+++	+++	+++
A2582	<i>outB</i>		+	+++	+ / —	+++
A3157	<i>outB kdgR</i>		+++	+++	+++	+++
A3158	<i>outB pecS</i>		+++	+++	+++	+++
A2582/ pTdB-OD			+++	+++	+++	+++

level by the bacteria to generate inducing molecules. Thus, induction by PGA is strongly concentration dependent. Using a low concentration of PGA in the plates (0.8 g l^{–1}), we could detect pectate lyase activity without inducing too strongly their synthesis. On glycerol + PGA plates, a small halo generated by secreted pectate lyase was seen around the *outB* mutant A2582 (Table 2). On glycerol + galacturonate + PGA plates, an *outB* mutant had a wild-type phenotype for pectate lyase secretion (Table 2). To eliminate the problems of the pectate lyase induction level, we also tested secretion of the cellulase EGZ, whose synthesis is independent of the presence of PGA and galacturonate. EGZ secretion by the *outB* mutant was almost undetectable on LB + CMC plates. Addition of galacturonate to this medium restored EGZ secretion to a wild-type level (Table 2). This led us to suppose that addition of galacturonate induced the synthesis of a protein which could suppress the *outB* mutation phenotype. KdgR is the repressor of the pectinolytic enzyme genes which responds to 2-keto-3-deoxygluconate, the inducer formed from galacturonate. In a *kdgR outB* mutant (A3158), EGZ secretion was at the wild-type level on LB + CMC medium (Table 2). PecS is another regulator controlling pectinolytic enzyme gene expression. In a *pecS outB* mutant, EGZ secretion was also restored. Thus, the increased expression of a gene regulated by *kdgR* and *pecS* can suppress the effect of an *outB* mutation. These two regulators co-repress the expression of the *outC–O* operon [each by a factor of three (Condemine & Robert-Baudouy, 1995)] and of pectate lyase genes. Since it seemed unlikely that overexpression of pectate lyases could restore EGZ secretion, we introduced plasmids containing various *out* genes into an *outB* mutant. Plasmids bearing *outD* (e.g. pTdB-OD, Table 2) were able to restore EGZ secretion on LB + CMC plates. Analysis of EGZ secretion in liquid medium cultures confirmed these results. Whilst it is fully secreted in the wild-type strain A350, half of EGZ was retained in the cell fraction in the

Fig. 4. Secretion of EGZ in *outB* mutants. Bacteria were grown to an OD₆₀₀ of 1 in LB medium, centrifuged and resuspended in the same volume of medium. The supernatant (S) and the cell fraction (C) were loaded into a SDS-PAGE and immunoblotted with anti-EGZ (top panel) or anti-BlaM (bottom panel).

Fig. 5. Amount of OutB in various *Erw. chrysanthemi* *out* mutants. Cells were grown overnight in LB medium, proteins were separated by SDS-PAGE and analysed by Western blotting using OutB antibodies. Lanes: 1, A350 (wild-type); 2, A1919 (*outC*); 3, A3558 ($\Delta outD$); 4, A1903 (*outS*).

outB mutant A2582 (Fig. 4). Complete secretion of EGZ by this strain could be restored by the introduction of a *kdgR* mutation or of a plasmid bearing *outD* (Fig. 4). Secretion of EGZ in a strain overexpressing OutD did not result from non-specific leakage, since no β -lactamase was found in the culture supernatant (Fig. 4). The fact that PGA induces OutD synthesis explains why an Out⁻ phenotype can not be seen for an *outB* mutant on medium containing the usual concentration of PGA (4 g l⁻¹).

Mutual stabilization of OutD and OutB

Interactions between proteins often lead to their mutual stabilization. To investigate whether OutB interacts with any other Out protein, the amount of OutB in the wild-type strain A350 and in various *out* mutants was estimated by Western blotting. The quantity of OutB was reduced in the polar *outC* mutant A1919 (Fig. 5), indicating that some components of the Out machinery stabilize OutB. Since a similar reduction was observed in the non-polar *outD* mutant A3558, this component might be OutD. To confirm this hypothesis, we checked

Fig. 6. Stabilization of OutD by OutB. Immunological detection of OutD in *Esc. coli* NM522 strain carrying pTdB-OD (*outD*) and either pACT3 (lane 1), pABTC2 (*outB*) (lanes 2 and 3), or pABSC3 (*pelB^{sp}-outB*) (lane 4). Where indicated, IPTG was added.

the presence of OutB in an *outs* mutant. In the absence of its chaperone OutS, the amount of OutD is very reduced in *Erw. chrysanthemi*. The amount of OutB in the *outs* mutant A1903 was reduced and equivalent to that in the *outD* mutant (Fig. 5). Remarkably, the amount of OutB in a polar *outC* mutant was not restored by the introduction of a plasmid bearing *outD* (results not shown). Thus, the presence of a functional Out machinery might be necessary for OutD to be in a conformation that allows its interaction with OutB. No decrease in the amount of OutD was observed in an *outB* *Erw. chrysanthemi* strain, probably because OutD is very stable once inserted in the outer membrane. However, our previous experiments have shown that OutD is very sensitive to proteolytic degradation when expressed in *Esc. coli* and that it can be stabilized by interaction with the outer-membrane chaperone OutS or with one of the secreted proteins (Shevchik *et al.*, 1997). To test if OutB could protect OutD, we used an *Esc. coli* strain containing plasmid pABTC2 in which *outB* is expressed from the inducible *tac* promoter. The amount of OutD increased in the presence of OutB (Fig. 6). This effect was increased when the quantity of OutB was raised by addition of IPTG to the culture. To test if the periplasmic domain of OutB was sufficient to stabilize OutD, we constructed a derivative in which the hydrophobic transmembrane domain of OutB was replaced by the PelB signal sequence. A stabilization of OutD was also observed when the periplasmic form of OutB, PelB^{sp}-OutB, was produced *in trans*, indicating that the hydrophobic N-terminal segment of OutB is not required for this effect (Fig. 6, lane 4). Attempts at ligand blotting experiments (binding of labelled OutB to OutD bound onto a nitrocellulose membrane) were unsuccessful, probably because the mutual affinity of the two proteins is not high enough.

Cross-linking of OutB and OutD

The interaction between OutB and OutD was confirmed by cross-linking experiments. When an *Esc. coli* strain containing a plasmid bearing *outB* was treated with formaldehyde, only one protein of the size of OutB was

Fig. 7. Detection of formaldehyde-cross-linked OutB by SDS-PAGE and immunoblotting with anti-OutB or anti-OutD. (a) The strains used were NM522(pBSAC/pT7-6) (OutB⁺, OutD⁻) and NM522(pBSAC/pTdB-OD) (OutB⁺, OutD⁺). (b) The strains used are *Erw. chrysanthemi* A2349 (*kdgR pecS*) and A3494 (*kdgR pecS outD*). The arrows indicate the position of OutB, OutD and of the cross-linked products.

detected with OutB antibodies (Fig. 7a). When the same experiment was performed with an *Esc. coli* strain containing the genes *outB* and *outD*, additional products reacting with both OutB and OutD antibodies, which could correspond to OutB–OutD cross-linking products, were detected (Fig. 7a). One of these products was detected in the wells of the gel. OutD, like other secretins, forms multimers that are not dissociated in SDS sample buffer unless the samples are boiled for a long time and that stay in the wells of the gel (Shevchik *et al.*, 1997). The same profile was obtained when cross-linking experiments were performed with an *Erw. chrysanthemi* *kdgR pecS* mutant A2348 (to maximize OutD synthesis) but not with the *outD* derivative A3494 (Fig. 7b). Thus, formaldehyde seems able to cross-link OutB with OutD.

DISCUSSION

In this study, we have analysed the membrane localization and topology of OutB and shown its necessity for type II secretion in *Erw. chrysanthemi*. Comparison of the hydropathy profile of OutB with those of its two homologues PulB and ExeB, showing an N-terminal hydrophobic segment, led us to suppose that they may have a bitopic conformation with a large periplasmic domain. Some of the experiments presented here confirm this hypothesis. Study of OutB topology with the BlaM topology probe indicated that the N-terminal part of OutB is anchored in the inner membrane and has a small N-terminal cytoplasmic region whilst the region extending after the hydrophobic domain is periplasmic. The low level of resistance observed for the cytoplasmic and the periplasmic fusions (2 µg ml⁻¹ and 15 µg ml⁻¹, respectively) does not result from instability of the fusions but probably from the low level of expression of the *outB* gene from the plasmid used. The topology of OutB is the same as that proposed for ExeB on the basis of PhoA fusion topology analysis (Howard *et al.*, 1996).

However, OutB presents some unusual properties. When the membranes were separated by sucrose gradi-

ent fractionation, OutB was not found in the cytoplasmic-membrane fractions but in higher density fractions that contain outer-membrane proteins. This interaction is strong enough to survive the fractionation procedures. These results may indicate that OutB is, *in vivo*, anchored in the inner membrane but that its large periplasmic domain allows for an interaction of its C-terminal part with the outer membrane. This interaction would not require an additional Out protein since the separation of OutB with the outer-membrane fractions is not modified by the presence or absence of other Out proteins. Such an association with the outer membrane of a protein predicted to be anchored in the inner membrane has been described for the pseudopilin PulG (Pugsley & Possot, 1993), PulC (Possot *et al.*, 1999) and for ExeB (Howard *et al.*, 1996). The repeated observation of the presence of ExeB in the outer-membrane fractions has been considered as a contamination (Howard *et al.*, 1996) but this could be a characteristic of the members of the GspB family. The presence of some GSP components, such as PulC and PulG, in both membrane fractions is not totally surprising since it seems normal that some of the GSP proteins will link the inner-membrane and outer-membrane components of the machinery. The energy-transducing protein TonB, which is involved in the transport of molecules across the outer membrane, has also been found associated with both membranes (Letain & Postle, 1997). Some properties of ExeB (sequence similarity, topology, a proline-rich segment, a high pI) are similar to those of TonB. Although they are rather rich in proline (9%), there is no proline domain in OutB or PulB (Fig. 1), their theoretical pIs are 7.4 and 7.1, respectively, and they have no obvious homology with TonB. Thus, the homology between ExeB and TonB may not reflect a general feature of members of the GspB family.

An interesting point of this study is that an *outB* mutation can be suppressed by the overexpression of *outD* either from the chromosomal copy or from a plasmid. This observation could explain the discrepancy observed between the phenotypes of *exeB*, *outB* and *pulB* mutants. The effect of the *outB* mutation on

pectate lyase secretion was previously tested (Condemine *et al.*, 1992). However, to induce pectate lyase synthesis, galacturonate or polygalacturonate had to be added to the culture medium. Their presence also induced the *outC*-O operon, increasing the level of OutD synthesis. In these conditions, secretion of pectate lyase was only slightly reduced. When an *outB* mutant was tested for EGZ secretion, which does not necessitate the addition of an inducer, the *outB* mutant appeared to be secretion deficient. Aerolysin secretion can be tested in *A. hydrophila* without addition of an inducer, leaving the expression of the *exeC*-N operon at its basal level (Jiang & Howard, 1991). The *exeB* mutant appears to be deficient for secretion. On the other hand, the *K. oxytoca pulB* mutant did not appear to be affected for pullulanase secretion. The phenotype of the *pulB* mutant was tested in the presence of maltose, which induced both pullulanase and PulD synthesis. It is probable that if pullulanase synthesis could be made independent from maltose induction, a *pulB* mutant would be secretion deficient.

The suppression of the *outB* phenotype by over-expression of *outD* led us to look for evidence of interactions between the two proteins. In *Erw. chrysanthemi*, OutB was stabilized by OutD. Coexpression of OutB and OutD in the same strain increased the quantity of OutD detectable in the bacteria. We have previously used this type of experiment to show the interactions between OutD and OutS, and between OutD and the secreted proteins (Shevchik *et al.*, 1997). However, the OutD stabilization obtained with OutB was less efficient than that observed with OutS or PelB, suggesting that the interaction might be weaker. This lesser effect prevented us from testing the protection by OutB of truncated derivatives of OutD: most of them are unstable and could not be stabilized by OutB to a level allowing for their detection. Protection of OutD by PelB^{sp}-OutB shows that the anchoring of OutB in the inner membrane is not required for the OutB-OutD interaction.

What might the role of OutB be? It has been proposed that ExeB could function with the putative ATPase ExeA to transduce metabolic energy to the opening of the secretion pore (Schoenhofen *et al.*, 1998). However, the presence of OutB and PulB in two strains in which no ExeA homologue has been found and the presence of GspA in *Esc. coli* that does not contain a GspB make this hypothesis doubtful in the case of OutB. The results presented here suggest that OutB is necessary for the proper functioning of the Out machinery, probably by interaction with OutD. The presence of *outB* and *pulB* next to *outS* and *pulS* could indicate that the two proteins may collaborate to pilot OutD and PulD to the outer membrane. Elucidation of the role of OutB requires further investigation.

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