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

Guy Condemine and Vladimir E. Shevchik

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 pilV 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 (Bleves et al., 1998). Their leader peptide is cleaved by the GspO prepilin peptidase (Nunn & Lory,
GspS proteins are outer-membrane lipoproteins that PulD and OutD (Daefler et al., 1998). The interaction requires OutS (d'Enfert & Pugsley, 1989; Schmidt & Condemine, 1992; Shevchik & Howard, 1994). GspS has only been identified in K. oxytoca, in the enterohaemorrhagic Esc. coli O157:H7 strain, in Esc. chrysanthemi and Erw. carotovora (PulS, EtpO and OutS) (d'Enfert & Pugsley, 1989; Schmidt 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 (Daeffler 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 exea forms an operon with exea. Exea 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 polygalacturionate (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"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 PeIb signal peptide. The pelB"outB" construct was then digested with BglII and XhoI and introduced into the expression vector pACT3 digested by BamHI and SalI. Finally, the outS gene was deleted from the construct by digestion with HindIII and EcoNI, treatment with the Klenow enzyme and ligation to give plasmid pABC3.

Construction of OutB–BlaM protein fusions. The outB gene was cloned between the EcoRI and KpnI sites of plasmid pJBSKpn, a derivative of plasmid pJB5633 (Broome-Smith et al., 1990) that contains an additional KpnI site upstream of the blaM gene. The resulting construct was digested with KpnI and HindIII 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' 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' 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"outB") was overproduced in the NM522/pABC3 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"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

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Genotype/description</th>
<th>Reference/origin</th>
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</thead>
<tbody>
<tr>
<td>Strain Erwinia chrysanthemi</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A350</td>
<td>lmrT lacZ</td>
<td>Hugouvieux-Cotte-Pattat &amp; Robert-Baudouy (1985)</td>
</tr>
<tr>
<td>A1524</td>
<td>lmrT lacZ pecS::MudIPR13</td>
<td>Reverchon et al. (1994)</td>
</tr>
<tr>
<td>A1903</td>
<td>lmrT lacZ outS::uidA-kan</td>
<td>Condemine et al. (1992)</td>
</tr>
<tr>
<td>A1919</td>
<td>lmr lacZ outC::uidA-kan</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>A2348</td>
<td>lmrT lacZ kdgR pecS::Cm</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>A2582</td>
<td>lmrT lacZ outB::uidA-kan</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>A2591</td>
<td>lmrT lacZ outD</td>
<td>Shevchik et al. (1997)</td>
</tr>
<tr>
<td>A3157</td>
<td>lmrT lacZ outB::uidA-kan</td>
<td>This work</td>
</tr>
<tr>
<td>A3158</td>
<td>lmrT lacZ pecS::MudIPR13 outB::uidA-kan</td>
<td>This work</td>
</tr>
<tr>
<td>A3494</td>
<td>As A2348 but also outD</td>
<td>This work</td>
</tr>
<tr>
<td>A3558</td>
<td>lmrT lacZ outD</td>
<td>This work</td>
</tr>
<tr>
<td>Esc. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM522</td>
<td>supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM5) r− k− (F' proAB lacI ΔacZM15)</td>
<td>Stratagene</td>
</tr>
<tr>
<td>K38</td>
<td>HfrC(λ) pboA4 pit-10 fhuA22 ompF627 relAI</td>
<td>Russel &amp; Model (1984)</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pACT3</td>
<td>Cm', ptac</td>
<td>Dykxhoorn et al. (1996)</td>
</tr>
<tr>
<td>pABTC2</td>
<td>pACT3 carrying the outB gene</td>
<td>This work</td>
</tr>
<tr>
<td>pABSC3</td>
<td>pACT3 carrying the pelB&lt;sup&gt;qu&lt;/sup&gt;-outB construct</td>
<td>This work</td>
</tr>
<tr>
<td>pBSAC</td>
<td>pACYC184 carrying outB and outS (1853 bp)</td>
<td>This work</td>
</tr>
<tr>
<td>pBT1</td>
<td>pT7-5 carrying outB and outS</td>
<td>This work</td>
</tr>
<tr>
<td>pET-20b (+)</td>
<td>Ap'</td>
<td>Novagen</td>
</tr>
<tr>
<td>pODN1</td>
<td>pBS carrying outD with a AvrII–MscI deletion</td>
<td>Shevchik et al. (1997)</td>
</tr>
<tr>
<td>pTdB-OD</td>
<td>pT7-6, carrying outD under the pelC promoter</td>
<td>Shevchik et al. (1997)</td>
</tr>
<tr>
<td>pJBSkpn</td>
<td>kan', blaM</td>
<td>J. C. Lazzaroni, Claude Bernard University, Villeurbanne, France</td>
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</table>

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 23489 Da. To detect the protein

cells (OD<sub>600</sub> 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 (200000 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 OD<sub>600</sub> 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.
encoded by this ORF, the 1.8 kb BclI–NsiI 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 $[^{35}S]$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 hydrophathy 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 an MIC of 2 µg ml$^{-1}$ to isolated Esc. coli colonies on GL Amp plates whereas the last two gave an MIC of 15 µg 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.
Localization of OutB. When the membranes of the MIC produced.

The same result was observed in an 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 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). KdgR is the repressor of the pectinolytic enzyme 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

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Pectate lyase</th>
<th>EGZ</th>
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<tbody>
<tr>
<td>A350</td>
<td>WT</td>
<td>−/−</td>
<td>−/−</td>
</tr>
<tr>
<td>A2582</td>
<td>outB</td>
<td>+/+</td>
<td>+/+</td>
</tr>
<tr>
<td>A3157</td>
<td>outB kdgR</td>
<td>+/−</td>
<td>+/+</td>
</tr>
<tr>
<td>A3158</td>
<td>outB pecS</td>
<td>+/−</td>
<td>+/+</td>
</tr>
<tr>
<td>A2582/pTdB-OD</td>
<td></td>
<td>+/+</td>
<td>+/+</td>
</tr>
</tbody>
</table>

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.
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

**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
detected with OutB antibodies (Fig. 7a). When the same experiment was performed with an *E. 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 gradient 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 the addition of an inducer, the pJBSkpn, to Fre-K.

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**REFERENCES**


be isolated from E. coli by repeated cycles of freezing and thawing. Bio/Technology 12, 1357–1360.


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