Published in: Biochemical Journal (1994), vol. 298, pp. 189-195.
Status: Postprint (Author's version)

Engineering and overexpression of periplasmic forms of the penicillin-binding protein 3 of *Escherichia coli*

Claudine FRAIPONT,* Maggy ADAM,* Martine NGUYEN-DISTÈCHE,* Wolfgang KECK,† Jozef VAN BEEUMEN,‡ Juan A. AYALA,§ Benât GRANIER,* Hirashi HARA‖ and Jean-Marie GHUYSEN*

**Centre d'Ingénierie des Protéines, Université de Liège, Institut de Chimie, B6, B-4000 Sart Tilman (Liège 1), Belgium, †Bioson Research Institute, Department of Biochemistry, University of Groningen, Nijenborgh, The Netherlands, ‡Vakgroep Biochemie, Fysiologie en Microbiologie, Rijksuniversiteit-Gent, K. L. Ledeganckstraat, 35, B-9000 Ghent, Belgium, §Centro de Biologia Molecular, C.S.I.C., Universidad Autonoma, Canto Blanco, E-28049 Madrid, Spain, and ‖ National Institute of Genetics, Mishima, Shizuoka-ken 411, Japan*

Abstract

Replacement of the 36 and 56 N-terminal amino acid residues of the 588-amino-acid-residue membrane-bound penicillin-binding protein 3 (PBP3) of *Escherichia coli* by the OmpA signal peptide allows export of F37-V577 PBP3 and G57-V577 PBP3 respectively into the periplasm. The modified *ftsI* genes were placed under the control of the fused *lpp* promoter and *lac* promoter/operator; expression of the truncated PBP3s was optimized by varying the copy number of the recombinant plasmids and the amount of LacI repressor, and export was facilitated by increasing the SecB content of the producing strain.

The periplasmic PBP3s (yield 8 mg/l of culture) were purified to 70 % protein homogeneity. They require the presence of 0.25 M NaCl to remain soluble. Like the membrane-bound PBP3, they undergo processing by elimination of the C-terminal decapeptide I578-S588, they bind penicillin in a 1:1 molar ratio and they catalyse hydrolysis and aminolysis of acyclic thioesters that are analogues of penicillin. The membrane-anchor-free PBP3s have ragged N-termini. The G57-V577 PBP3, however, is less prone to proteolytic degradation than the F37-V577 PBP3.

Abbreviations used: IPTG, isopropyl *β*-D-thiogalactopyranoside ; PBP3, penicillin-binding protein 3.

INTRODUCTION

The membrane-bound penicillin-binding protein 3 (PBP3) of *Escherichia coli*, the *ftsI* gene product, occurs at about 50 copies per cell, and is involved in cell septation. Processing of the precursor by removal of the C-terminal peptide I578-S588 under the action of the *prc* gene product (Nagasawa et al., 1989) gives rise to the mature 577-amino-acid-residue (M1-V577) PBP3. A membrane anchor M1-A36 (Bowler and Spratt, 1989) attaches to the outer face of the membrane, the N-terminal module F37-S259 itself being fused to the C-terminal penicillin-binding module G260-V577 (Englebert et al., 1993). This latter module possesses, at the expected places, the three motifs S307TVK (where S307 is the essential serine), S359SN and K494TGT of the penicilloyl serine transferases (Ghuysen, 1991). PBP3, FtsW, FtsQ, FtsA and FtsZ form a large protein complex, the divisome (Nanninga, 1991) or septator (Vicente et al., 1991), which encompasses the cytosol, the membrane and the cytoplasm. Penicilloylation of S307 of PBP3 causes dysfunctioning of the protein complex, cell filamentation and cell death (Houba-Herin et al., 1985; Broome-Smith et al., 1985).

Obtaining PBP3 in the form of a catalytically active water-soluble derivative suitable for X-ray analysis is an important aim of the research (Bartholomé-De Belder et al., 1988; Bowler and Spratt, 1989). In the present work, we have exploited *ompA* fusion vectors to overproduce the truncated F37-V577 and G57-V577 forms of PBP3 in the periplasm of *E. coli.* The gene fusions were inserted into plasmids downstream of the strong *lpp* promoter and the *lac* promoter/operator, and the production was optimized by varying the copy number of the OmpA-signal-peptide-truncated PBP3-encoding gene and the copy number of the Lac-repressor-encoding genes *lacI* and *lacI*^{q}. Biogenesis and export were aided by increasing the intracellular level of the chaperone SecB (Wickner, 1990; Wickner et al., 1991).

Published in: Biochemical Journal (1994), vol. 298, pp. 189-195.
Status: Postprint (Author's version)

MATERIALS AND METHODS

Bacterial strains and plasmids

E. coli K12 HB101, RR1 (a *recA*$^+$ derivative of HB101), D1210 (HB101 derivative in which the *lacI*^{q} is chromosome borne) and TGI (see the oligonucleotide-directed *in vitro* mutagenesis system version 2-1, Amersham) were used as hosts of the recombinant plasmids (Boyer and Roulland-Dussoix, 1969; De Boer et al., 1983). *E. coli* K12 SF100 and SF103 were deficient in OmpT protease and in metalloprotease III respectively (Baneyx and Georgiou, 1991). *E. coli* JE7929 (*recA1* derivative of JE7925) was deficient in *prc* gene. *E. coli* JE7928 (*recA1* derivative of JE7924) was the corresponding wild-type (Hara et al., 1991).

pWK80 was the source of the *ftsI* gene (Nakamura et al., 1983). pINIII-*ompA*-Hind (a derivative of pBR322) and pIMIIA3 (a runaway replication plasmid derived from R1) served as vectors (Masui et al., 1983 ; Rentier-Delrue et al., 1988). pINIIIA2 and pMJR1560 (derivatives of pBR322) were the sources of the *lacI* and *lacI*^{q} genes respectively (Masui et al., 1983 ; Stark, 1987). pDC2 (a gift from Dr. P. J. Bassford, University of North Carolina) and pDNAK325 (a gift from Dr. G. J. Philips, Princeton University, NJ, U.S.A.) were the sources of the *secB* gene and the *dnaK* gene respectively. pGroESL carried *GroEL* and *GroES* (Goloubinoff et al., 1989).

Recombinant plasmids

PDML214 (l*acI*$^+$) pDML215 (*lacI*$^-$), pDML218 (*lacI*^{q}) pDML219 (*lacI*$^+$) and pDML232 (*lacI*$^+$)

Figure 1(a) shows the region of the low-copy-number secretion vector pINIII-*ompA*-Hind in which the truncated *ftsI* genes were inserted. Figure 1(b) shows the environment of the F37-S588 PBP3-encoding gene in the low-copy-number pDML214 (*lacI*$^+$) and in the high-copy-number pDML215 (*lacI*$^-$), pDML218 (*lacI*^{q}) and pDML219 (*lacI*$^+$). Figure 1(c) shows the environment of the G57-S588-encoding gene in the high-copy-number pDML232 (*lacI*$^+$). The plasmids were constructed as described in Figure 2. All recombinant DNA techniques were as described (Sambrook et al., 1989).

PDML230 (*secB*) and pDML233 (*dnaK*)

The *PvuII* DNA segment carrying *secB* was excised from pDC2 and inserted into pSW1 (a derivative of pACYC184; Verschoor et al., 1989), giving rise to pDML230. The *Hin*dIII DNA segment containing *dnaK* was isolated from pDNAK325 and introduced into pACYC184, giving rise to pDML233 (*dnaK*). pDML230 (*secB*) and pDML233 (*dnaK*) were compatible with pDML219 (*lacI*$^+$) and pDML232 (*lacI*$^+$).

Published in: Biochemical Journal (1994), vol. 298, pp. 189-195.
Status: Postprint (Author's version)

Figure 1 *Organization and DNA sequences of the modified ftsI genes*
(a) pINIII-*omp*A-Hind; (b) modified F37-S588 PBP3-encoding *ftsI* gene in pDML214 (*lacI*$^+$), 215 (*lacI*$^-$), 218 (*lacI*^{q}), 219 (*lacI*$^+$); (c) modified G57-S588 PBP3-encoding *ftsI* gene in pDML232 (*lacI*$^+$). Polypeptide segments are translated. RBS, ribosome binding site.

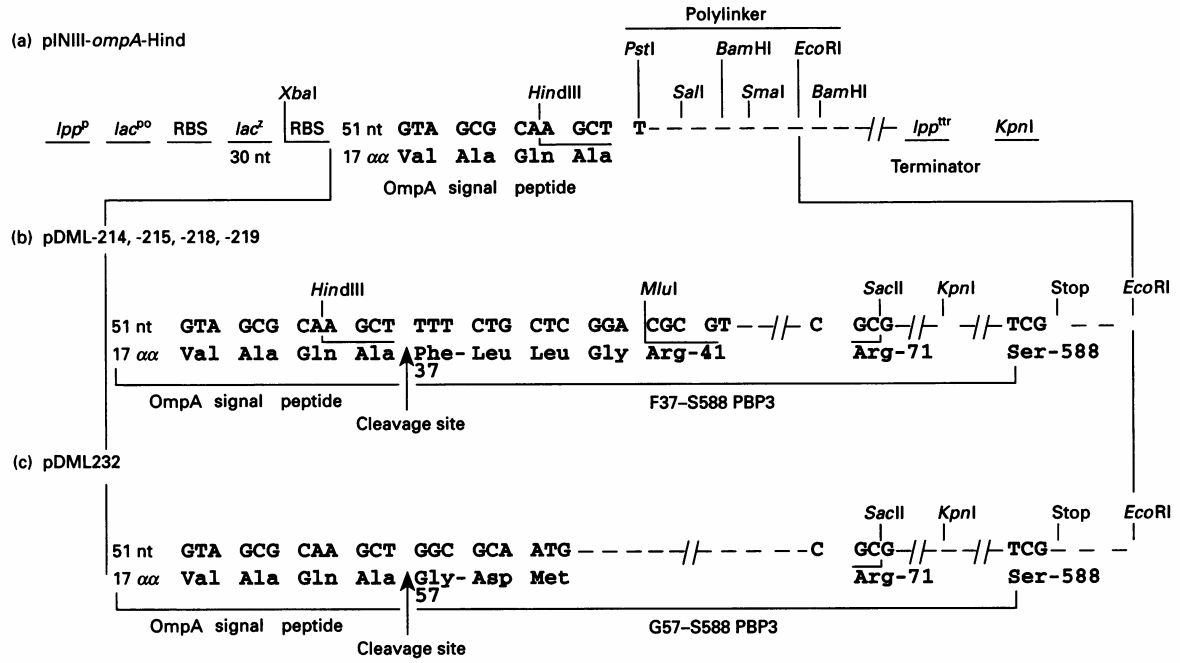
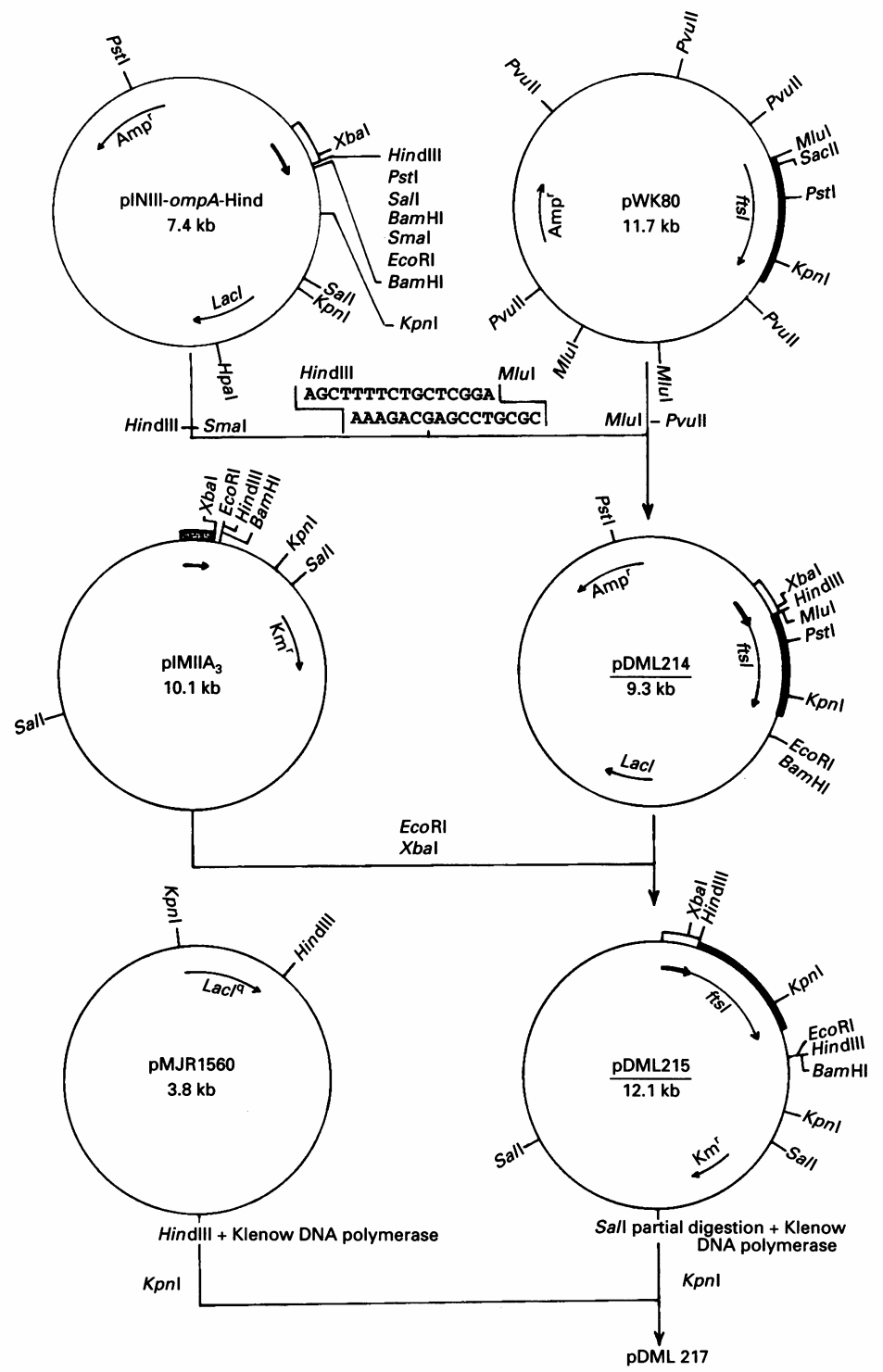
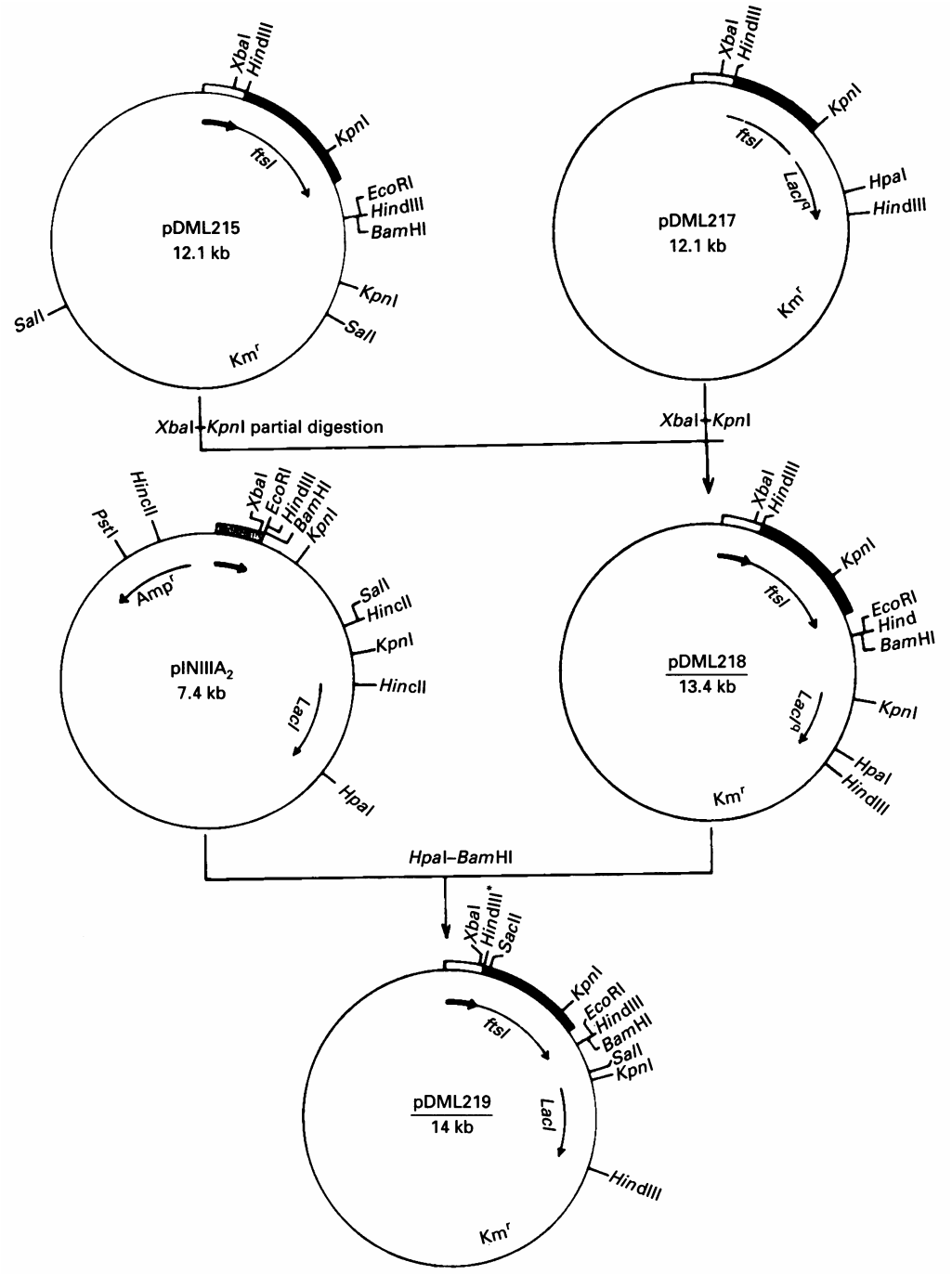


Figure 2 *Construction of plasmids pDML214 (lacI$^+$), pDML215 (lacI^q), pDML218 (lacI$^-$), pDML219 (lacI$^+$) and pDML232 (lacI$^+$)*
☐ , Fused *Ipp* promoter, *lac* promoter/operator and OmpA signal peptide. ▨, Fused *Ipp* promoter and *lac* promoter/operator (without the OmpA signal peptide). pDML214 (*lacI*$^+$): the 1.9 kb MluI-PvuII DNA segment encoding the R41-S588 PBP3 was excised from pWK80 and inserted into pINIII-ompA-Hind (downstream from the OmpA-signal-peptide-encoding gene) by using an intervening DNA duplex that encoded the tetrapeptide F37LLG40 of PBP3. pDML215 (*lacI*$^-$): the 2kb *Xba*I-*Eco*RI DNA segment of PDML214 (encoding the fused OmpA signal peptide F37-S588 PBP3) was inserted into pIMIIA3. pDML218 (*lacI*^{q}): pDML215 was cleaved with *Kpn*I and *Sal*I and the *Sal*I ends were filled with the Klenow polymerase. The 1.25 kb *Kpn*I-*Hind*III DNA segment containing *lacI*^{q} was excised from pMJR1560 and the *Hind*III ends were also filled up. Ligation yielded pDML217 which had acquired *lacI*^{q} at the expense of the 1.5 kb *Kpn*I DNA segment containing the 3' end of *ftsI*. The 2.4 kb *XbaI-KpnI* (partial digestion) DNA segment from pDML215 was inserted into DML217. pDML219 (*lacI*$^+$): the 1.45 kb *BamHI-HpaI* DNA segment (carrying *lacI*^{q}) of pDML218 was replaced by the corresponding 2 kb *Bam*HI-*Hpa*I DNA segment (containing *lac*I) of pINIIIA2. pDML232 (*lacI*$^+$) (not shown): the *Hin*dIII-*Sac*II DNA segment encoding the F37-R71 peptide of PBP3 of pDML214 was replaced by a DNA duplex encoding the G57-R71 peptide of PBP3. The 2 kb *Xba*I-*Eco*RI DNA segment of the resulting plasmid (encoding the fused OmpA signal peptide G57-S588 PBP3) was inserted into pDML219. pDML232 had the same restriction map as pDML219, except that it lacked the *Hind*III site.

Published in: Biochemical Journal (1994), vol. 298, pp. 189-195.
Status: Postprint (Author's version)



Published in: Biochemical Journal (1994), vol. 298, pp. 189-195.
Status: Postprint (Author's version)



Cell spheroplasting

The spheroplasting conditions were derived from Lindström et al. (1970). Cell suspensions (from 250 ml of cultures) made in 20 ml of 30 mM Tris/HCl, pH 8.0, containing 27 % (w/v) sucrose were supplemented with 5 mM EDTA (pH 8.0) and 2 mg of lysozyme. Depending on the strains and the growth conditions, conversion of the cells into spheroplasts was maximal after a 20-30 min incubation carried out at 20 or 4 °C. The spheroplasts, stabilized with 15 mM $CaCl_2$ and supplemented with 0.5 M NaCl, were collected by centrifugation at 30000 *g* for 15 min. The supernatant (periplasmic) fraction was supplemented with 0.1 mM phenylmethanesulphonyl fluoride. The pellet was suspended in 10 ml of 10 mM Tris/HCl, pH 8.0, containing 0.5 M NaCl, 10 mM $MgCl_2$

and 200 μg of DNAase, and the suspension was submitted to freezing and thawing. Centrifugation at 30000 *g* for 45 min at 4 °C yielded a supernatant (cytoplasmic) fraction and a pellet (membrane) fraction. After being washed, the pellet was suspended in 2 ml of 10 mM Tris/HCl, pH 8.0, containing 10% (w/v) glycerol, 0.5 M NaCl and 0.5 mM EDTA.

Dyes

Dyes (Ciba Geigy, Basel, Switzerland) coupled to Fractogel TSK HW65F were screened with respect to their ability to bind the water-soluble forms of PBP3 (Mottl and Keck, 1991, 1992; van der Linden et al., 1992). Of the 40 dyes tested, Procion Blue MW4GD (mix 1591)-Fractogel equilibrated against 10 mM Tris/HCl, pH 8.0, containing 10% glycerol and 150 mM NaCl bound PBP3, and the bound PBP3 was eluted with 1 M NaCl.

Purification of periplasmic PBP3

Step 1

The periplasmic fraction was dialysed against 10 mM Tris/HCl, pH 8.0, containing 10% (w/v) glycerol. The precipitated PBP3 was collected by centrifugation and solubilized in buffer/ glycerol/0.5 M NaCl. The yield was 70% and the PBP3 was 20 % pure. The soluble preparation was dialysed stepwise against a series of buffer/glycerol solutions containing decreasing concentrations of NaCl from 0.5 M to 150 mM. After dialysis, 70-80% of the PBP3 remained soluble.

Step 2

The dialysed solution was applied to a 20 ml column of Procion Blue MX4GD (mix 1591)-Fractogel, previously equilibrated against buffer/glycerol/150mM NaCl. On treatment with a linear NaCl gradient, from 150 mM to 1 M, PBP3 was eluted at 0.7 M NaCl (final yield 30%; purity 70%).

Acyl transfer activities on thioesters and penicillin (PBP analysis)

Hydrolysis and aminolysis of thioesters were performed as described (Adam et al., 1990, 1991). For PBP analysis, samples were incubated with 0.1 mM [^{35}S]benzylpenicillin (0.5 μCi/nmol ; New England Nuclear-Dupont de Nemours) or 0.1 mM [^{3}H]benzylpenicillin (5 μCi/nmol; The Radiochemical Centre, Amersham, Bucks., U.K.) for 10 min at 37 °C. SDS/PAGE, Coomassie Blue staining and fluorography of the gels were carried out as described (Laemmli and Favre, 1973 ; Chamberlain, 1979; Bartholomé-De Belder et al., 1988). Enzyme samples exhibiting β-lactamase activity were incubated with 50 μM β-iodopenicillanate (a gift from Dr. J. Kemp, Pfizer Research Centre, Sandwich, Kent, U.K.) for 20 min at 37 °C before labelling with radioactive penicillin.

Immunological detection of PBP3

Immunoblotting was carried out by using an anti-(membrane-bound PBP3) monoclonal antibody (a gift from Dr. U. Schwarz, Max-Planck Institut, Tübingen, Germany) purified on a Protein A-Sepharose column. The antigen-antibody complexes were detected with alkaline phosphatase coupled to goat anti-mouse serum (Bio-Rad Immunoblot alkaline phosphatase assay system). The ascites were prepared in the laboratory of Dr. B. Rentier (University of Liège, Liège, Belgium).

Protein estimation and amino acid sequencing

The proteins were estimated as described (Lowry et al., 1951; Bradford, 1976). Proteins were submitted to SDS/PAGE and electroblotted on Immobilon membranes (Millipore Corp.). Automated microsequencing was performed on a 477A pulsed liquid sequenator by using a 120A Applied Biosystems analyser (Foster City, CA, U.S.A.).

Published in: Biochemical Journal (1994), vol. 298, pp. 189-195.
Status: Postprint (Author's version)

Table 1 *Production of periplasmic forms of PBP3 in E. coli hosts harbouring various recombinant plasmids For growth conditions, see the text. Note that the same yields were obtained when E. coli HB101 (recA$^-$) was used as host instead of E. coli RR1 (recA$^+$).*

***E. coli* hosts**	**Plasmids**	**PBP3 forms**	**Yield (mg/l of culture)**
D1210 (*lacI*^{q})	PDML214 (*lacI*$^+$)	F37-S588	0.5-1
D1210 (*lacI*^{q})	PDML215 (*lacI*$^-$)	F37-S588	(Granules)
RR1	PDML218 (*lacI*^{q})	F37-S588	≦1
RR1	PDML219 (*lacI*$^+$)	F37-S588	3
RR1	PDML232 (*lacI*$^+$)	G57-S588	3
RR1	PDML219 (*lacI*$^+$) + pDML230(*secB*)	F37-S588	8
RR1	pDML232 (*lacI*$^+$) + pDML230(*secB*)	G57-S588	8

RESULTS

Optimized production of periplasmic F37-V577 PBP3 and G57-V577 PBP3

The *E. coli* strains used as hosts and the recombinant plasmids carrying the modified *ftsI* genes are shown in Table 1. Growth of *E. coli* D1210 (*lαcI*^{q}) harbouring the low-copy-number pDML214 (*lαcI*$^+$) was carried out at 37 °C in Luria-Bertani medium containing 50 μg of ampicillin/ml until an absorbance (600 nm) of 0.6 was reached. The culture was supplemented with 2 mM isopropyl β-D-thiogalactopyranoside (IPTG) and incubated for another 3 h period. The cells contained a novel approx. 60 kDa PBP in the periplasm, presumably the C-terminal processed F37-V577 PBP3. The level of production, 0.5-1 mg of protein/litre of culture, was approx. 100-fold amplified compared with the membrane-bound PBP3 of *E. coli* D1210. Regulation of the synthesis was at the transcriptional level and IPTG-dependent. Uninduced cultures produced only trace amounts of periplasmic PBP3.

Growth of the *E. coli* harbouring the high-copy-number pDML215 (*lacI*$^-$), pDML218 (*lacI*^{q}) and pDML219 (*lacI*$^+$) was carried out at 30 °C in LB medium containing 25 μg of kanamycin/ml until an absorbance of 0.05 was reached, at which time the temperature was shifted to 37 °C. After 2 h at this temperature, IPTG was added and growth continued. The cells of *E. coli* D1210 (*lacI*^{q})/pDML215 (*lacI*$^-$) became blistered and lysed, and insoluble granules accumulated and were released into the medium, suggesting that the amount of repressor expressed by the chromosome-borne *lacI*^{q} was too low to maintain the production of the truncated PBP3 at a level compatible with cell growth. The cells of *E. coli* RR1/pDML218 (*lacI*^{q}) and *E. coli* RRl/pDML219 (*lacI*$^+$) did not produce inclusion bodies and had a normal morphology. With *E. coli* RR1/pDML218 (*lacI*^{q}), the amount of periplasmic PBP3 (produced 3 h after induction) was 1 mg/l of culture or less. Addition of IPTG up to 10 mM had no effect. With *E. coli* RR1/pDML219 (*lacI*$^+$), the amount of periplasmic PBP3 was at least 3-fold higher (3 mg/l of culture).

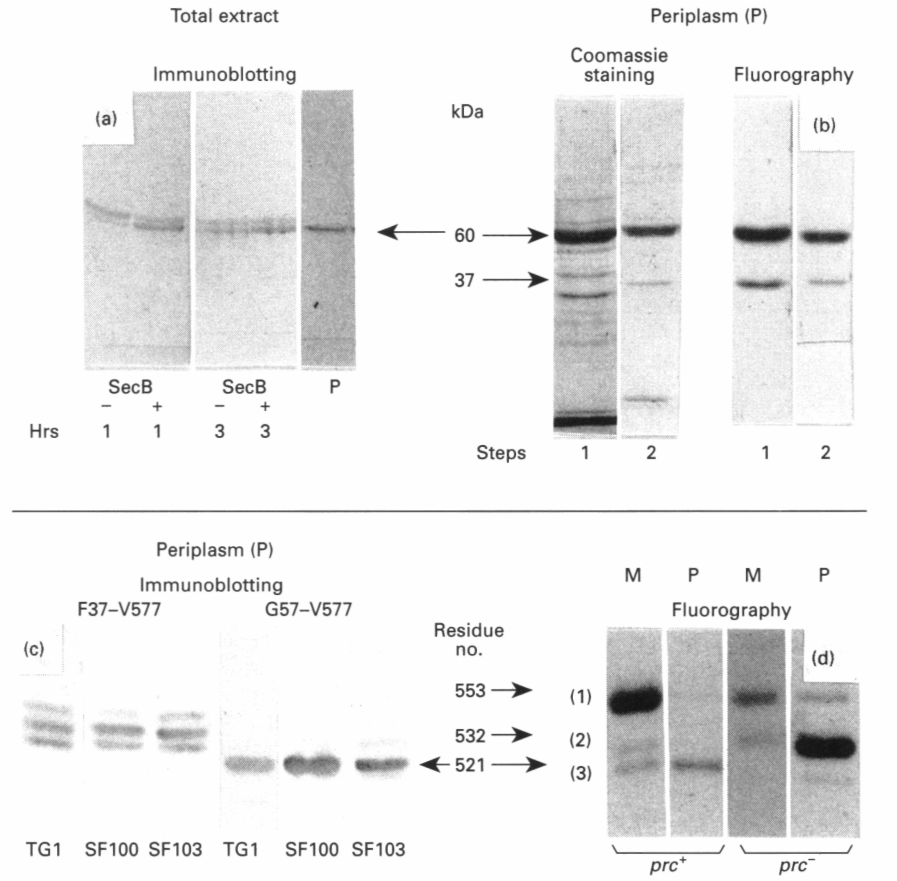
Cell fractionation of the *E. coli* RR1/pDML219 (*lacI*$^+$) showed that the periplasmic PBP3 represented only 60% of the total amount of PBP3 produced. Equivalent amounts, about 20 % each, remained cytoplasmic and membrane-bound. Moreover, the synthesized PBP occurred in the form of two immunochemically related species, presumably the unprocessed OmpA-signal-peptide-truncated PBP3 and the processed (i.e. periplasmic) truncated PBP3 (see below). Attempts to aid biogenesis and export were made by increasing the SecB, GroEL/ES or DnaK content of the host cells. When compared with the control, i.e. *E. coli* RR1 harbouring pDML219 (*lacI*$^+$) and pSW1 (see the Materials and methods section), *E. coli* harbouring pDML219 (*lacI*$^+$) and pDML230 (*secB*) had a 2.6-fold increased capacity of producing the periplasmic PBP3 (yield 8 mg/l of culture). Moreover, the amount of the processed truncated PBP3 increased at the expense of the unprocessed OmpA-signal-peptide-truncated PBP3 precursor (Figure 3a). Similar effects were not observed when the PBP3-producing host cells contained pGroESL or pDML233 (*dnaK*) instead of ρDML230 (*secB*).

pDML232 (*lacI*$^+$) contained the G57-V577 PBP3-encoding gene (see the Materials and methods section). pDML232 (*lacI*$^+$) gave results comparable with those obtained with pDML219 (*lacI*$^+$) with regard to the level of production of truncated PBP3 and the enhancing effects of SecB.

Published in: Biochemical Journal (1994), vol. 298, pp. 189-195.
Status: Postprint (Author's version)

Figure 3 *Low-resolution (7 cm; a and b) and high-resolution (25 cm; c and d) SDS/PA6E of the truncated PBP3s*

Detection was as indicated. (a) Total extracts of *E. coli* cells overproducing F37-V577 PBP3 in the presence (+) or absence (-) of increased amounts of SecB. Samples were collected after 1 h of culture at 37 °C without induction (1) and after 3 h of IPTG (2 mM) induction (3). They were suspended in dissolving buffer and boiled. The volumes of the analysed samples were adjusted so that each sample corresponded to 25 μl of a culture at an absorbance of 1.0. P, partially purified periplasmic F37-V577 PBP3 (541 amino acid residues; 0.5 pmol) used as standard of reference. (b) Periplasmic F37-V577 PBP3 after step 1 (10 μg of total protein; 33 pmol of PBP3) and step 2 (2 μg of total protein; 23 pmol of PBP3) of purification. (c) Freshly prepared periplasmic fractions of F37-V577 PBP3 and G57-V577 PBP3 produced in E. coli T61, SF100 (OmpT protease-deficient) and SF103 (metalloprotease-deficient) respectively. (d) Freshly prepared membrane (M) and periplasmic (P) fractions of G57-V577 PBP3 produced in £ coli JE7928 (*prc*$^+$) and JE7929 (*prc*$^-$) respectively. In (c) and (d), the cells were grown at 37 °C in Luria-Bertani medium to an absorbance of 0.5. IPTG (1 mM) was added and the culture continued for 50 min. All the analysed samples corresponded to 15 µl of cultures at an absorbance of 0.5. For an explanation of (1), (2) and (3), see the text.



Properties of periplasmic F37-V577 PBP3 and G57-V577 PBP3

The periplasmic PBP3s were partially purified as described in the Materials and methods section. They bound penicillin in a 1:1 molar ratio as determined by microdensity measurements of fluorograms and Coomassie Blue-stained gels (Bartholomé-De Belder et al., 1988). On this basis, the periplasmic PBP3s were 70 % pure. They required the presence of at least 150 mM NaCl to remain soluble. They were stored in 250 mM NaCl.

The truncated PBP3s showed the same thermostability as the membrane-bound enzyme (Bartholomé-De Belder et al., 1988).

They reacted with benzylpenicillin with a second-order rate constant of penicilloylation of 4000 $M^{-1}\cdot s^{-1}$ at 37 °C, and catalysed hydrolysis and aminolysis (with D-alanine as acceptor) of thioesters such as C_6H_5-CONH-$CHCH_3$-CO-S-CH_2-COO^-(Adam et al., 1991). In the absence of D-alanine, hydrolysis of the thioester (reaction product C_6H_5-CONH-$CHCH_3$-COO^-) proceeded with a $k_{cat.}/K_m$ value of 80 $M^{-1}\cdot s^{-1}$. In the presence of 5 mM D-alanine, the rate of aminolysis (reaction product C_6H_5-CONH-$CHCH_3$-CONH(D-Ala)-COO^-) was five times that of hydrolysis. On filtration on Superdex 200 (Pharmacia) in 10 mM Tris/HCl, pH 8.0, containing 10% glycerol and 0.5 M NaCl, the truncated PBPs each behaved as a single 60 kDa protein. However, SDS/PAGE revealed the presence of small amounts of a 37 kDa radioactive PBP (Figure 3b). As derived from amino acid sequencing,

this low-molecular-mass PBP was a degradation product of PBP3 resulting from cleavage of the R210-K211 peptide bond.

High-resolution (25 cm) SDS/PAGE of freshly prepared periplasmic fractions of the F37-V577 PBP3 revealed the presence of three forms of PBP3 (Figure 3c). Chromatography of the periplasmic fraction on the cationic exchanger Mono S in 150 mM cacodylate, pH 6.5, containing 10% glycerol and 150 mM NaCl and elution with increasing NaCl concentrations yielded two fractions. Both fractions bound penicillin and catalysed acyl transfer reactions from thioester substrates. Fraction I, eluted at 470 mM NaCl, contained at least three forms of the approx. 60 kDa PBP, two of which had 148 and D51 as N-terminal amino acid residues. Fraction II, eluted at 540 mM NaCl, contained a single PBP with D58 as the N-terminal amino acid residue. The presumed proteolysis still occurred in *E. coli* strains deficient in OmpT protease and metalloprotease III (Figure 3c). Freshly prepared periplasmic fractions of the G57-V577 PBP3 contained only a single PBP (Figure 3c). After purification, however, the PBP also had several Gly-57, Arg-63, Val-64 and X(?) ragged N-termini (not shown).

Maturation of the truncated PBP3s involved removal of the C-terminal 11 amino acid residues. As shown in Figure 3(d), experiments with freshly prepared membrane and periplasmic fractions of *E. coli prc*$^+$ and *E. coli prc*$^-$ harbouring pDML232 (*lacI*$^+$) led to the conclusion that the fused OmpA signal peptide G57-S588 PBP3 (preproprotein 1 ; 553 amino acid residues) was present in the membranes of both *E. coli prc*$^+$ and *prc*$^-$; the OmpA-signal-peptide-free G57-S588 PBP3 (proprotein 2; 532 amino acid residues) accumulated in the periplasm of *E. coli prc*$^-$*;* and the OmpA-signal-peptide-free G57-V577 PBP3 (mature protein 3; 521 amino acid residues) accumulated in the periplasm of *E. coli prc*$^+$.

DISCUSSION

The secretion vectors described above allow the truncated F37-V577 PBP3 and G57-V577 PBP3 to be exported in the periplasm of *E. coli.* F37 occurs near the end of the hydrophobic segment F4-G40 seven amino acid residues downstream from C30, the putative site of lipid modification of the membrane-bound PBP3. G57 is located at the amino end of the proposed N-terminal module of the protein (Englebert et al., 1993). As a consequence of the removal of the membrane anchor, the truncated PBPs have ragged N-termini. The G57-V577 PBP3, however, is less susceptible to proteolytic degradation than the F37-V577 PBP3.

The truncated PBPs catalyse concomitant hydrolysis and aminolysis of thioester carbonyl donors and bind penicillin in a 1:1 ratio. The value of the second-order rate constant of penicilloylation (4000 $M^{-1}\cdot s^{-1}$ at 37 °C) is about 10-fold larger than that observed with the membrane-bound PBP3 (300 $M^{-1}\cdot s^{-1}$). This difference may be due to variations in the enzyme environment (cell envelope versus aqueous medium) and to the fact that binding to membranes is a competition between all the PBPs present. A small number of copies of the truncated PBPs undergo proteolysis by cleavage of the R210-K211 peptide bond (as observed with the membrane-bound PBP3). On chromatography under non-denaturing conditions, the two fragments remain associated with each other and behave like the uncleaved protein, suggesting the existence of a nick between the N-terminal module upstream from R210 and the C-terminal module downstream from K211.

SecB facilitates biogenesis and export of the truncated PBPs in agreement with a previous report showing that SecA and SecY are probably involved in the synthesis of the membrane-bound PBP3 (Gomez et al., 1993). In contrast, increased concentrations of the chaperones DnaK (Kumamoto and Beckwith, 1985) and GroEL/ES (Goloubinoff et al., 1989) have no effects, although GroEL/ES facilitates β-lactamase export (Kusakawa et al., 1989 ; Kumamoto, 1991). Using the secretion vectors/host cells described above, regulation of the synthesis of the truncated PBPs is at the transcriptional level and IPTG-dependent. Unexpectedly, however, *E. coli* RR1/pDML219 (*lacI*$^+$)/pDML230 (*secB*) and *E. coli* RR1/pDML232 (*lacI*$^+$)/pDML230 (*secB*) produce the truncated PBPs in the periplasm without IPTG induction when grown in kanamycin-containing 'terrific broth' (Tartof and Hobbs, 1987) (results not shown). The PBPs appear at the beginning of the stationary phase and, after 10 h of culture, they accumulate to about 15 mg/l of culture. The underlying mechanism is not understood.

Acknowledgement

This work was supported in part by the Belgian programme on Interuniversity Poles of Attraction initiated by the Belgian State, Prime Minister's Office, Science Policy Programming (PAI no. 19), the Fonds de la Recherche Scientifique Médicale (contract no. 3.4531.92), a Convention tripartite between the Région wallonne, SmithKline Beecham, U.K. and the University of Liège, an Action Concertée with the Belgian Government

(convention 89/94-130) and the Geconcerteerde Onderzoeksactie (contract 12052293). J.A.A. was supported by a grant from the Direccion General de Investigacion Cientifica y Tecnica (Spanish Government).

REFERENCES

Adam, M., Damblon, C., Plaitin, B., Christiaens, L. and Frère, J.-M. (1990) Biochem. J. 270, 525-529

Adam, M., Damblon, C., Jamin, M., Zorzi, W., Dusart, V., Galleni, M., El Kharroubi, A., Piras, G., Spratt, B. G., Keck, W., Coyette, J., Ghuysen, J.-M., Nguyen-Distèche, M. and Frère, J.-M. (1991) Biochem. J. 279, 601-604

Baneyx, F. and Georgiou, G. (1991) J. Bacteriol. 173, 2696-2703

Bartholomé-De Belder, J., Nguyen-Distèche, M., Houba-Herin, N., Ghuysen, J.-M., Maruyama, I. N., Hara, H., Hirota, Y. and Inouye, M. (1988) Mol. Microbiol. 2, 519-525

Boyer, H. W. and Roulland-Dussoix, D. (1969) J. Mol. Biol. 41, 459-472

Bowler, L. D. and Spratt, B. G. (1989) Mol. Microbiol. 3, 1277-1286

Bradford, M. M. (1976) Anal. Biochem. 72, 248-254

Broome-Smith, J. K., Hedge, P. J. and Spratt, B. G. (1985) EMB0 J. 4, 231-235

Chamberlain, J. P. (1979) Anal. Biochem. 98, 132-135

De Boer, H. A, Comstock, L. J. and Vasser, M. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 21-25

Englebert, S., Piras, G., El Kharroubi, A., Joris, B., Coyette, J., Nguyen-Distèche, M. and Ghuysen, J.-M. (1993) In Bacterial Growth and Lysis: Metabolism and Structure of the Bacterial Sacculus (de Pedro, M. A., Höltjic, J. V. and Löffelhardt, W., eds.), pp. 319-333, Plenum Press, New York

Ghuysen, J.-M. (1991) Annu. Rev. Microbiol. 45, 37-67 Goloubinoff, P., Gatenby, A. A. and Lorimer, G. H. (1989) Nature (London) 337, 44-47

Gomez, M. J., Ayala, J. A., Desviat, L. R. and Merchante, R. (1993) in Bacterial Growth and Lysis: Metabolism and Structure of the Bacterial Sacculus (de Pedro, M. A., Höltjic, J. V. and Löffelhardt, W., eds.), pp. 309-318, Plenum Press, New York

Hara, H., Yamamoto, Y., Higashitani, A., Suzuki, H. and Nishimura, Y. (1991) J. Bacteriol. 173, 4799-4813

Houba-Herin, N., Hara, H., Inouye, M. and Hirota, Y. (1985) Mol. Gen. Genet. 201, 499-504

Kumamoto, C. A. (1991) Mol. Microbiol. 5, 19-22

Kumamoto, C. A. and Beckwith, J. (1985) J. Bacteriol. 163, 267-274

Kusakawa, N., Yura, T., Ueguchi, C, Akiyama, Y. and Ito, K. (1989) EMB0 J. 8, 3517-3521

Laemmli, U. K. and Favre, M. (1973) J. Mol. Biol. 80, 575-599

Lindstrom, E. B., Boman, H. G. and Steele, B. (1970) J. Bacteriol. 101, 218-231

Lowry, O. H., Rosebrough, N. J., Farr, A. L and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275

Masui, Y., Coleman, J. and Inouye, M. (1983) in Experimental Manipulation of Gene Expression (Inouye, M., ed.), pp. 15-32, Academic Press, New York

Mottl, H. and Keck, W. (1991) Eur. J. Biochem. 200, 767-773

Mottl, H. and Keck, W. (1992) Protein Expression Purif. 3, 403-409

Nagasawa, H., Sakagami, Y., Suzuki, A, Suzuki, H., Hara, H. and Hirota, Y. (1989) J. Bacteriol. 171, 5890-5893

Nakamura, M., Maruyama, I., Soma, M., Kato, J., Suzuki, H. and Hirota, Y. (1983) Mol. Gen. Genet. 191, 1-9

Published in: Biochemical Journal (1994), vol. 298, pp. 189-195.
Status: Postprint (Author's version)

Nanninga, N. (1991) Mol. Microbiol. 5, 791-795

Rentier-Delrue, F., Swennen, D. and Martial, J. A. (1988) Nucleic Acids Res. 16, 8726

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Stark, M. J. R. (1987) Gene 51, 255-267 Tartof, K. D. and Hobbs, C. A. (1987) Bethesda Res. Lab. Focus 9, 12-15

van der Linden, M. P. G., Mottl, H. and Keck, W. (1992) Eur. J. Biochem. 204, 197-202

Verschoor, E J., Luirink, J., de Waard, S., de Graaf, F. K. and Oudega, B. (1989) Antonie van Leeuwenhoek 55, 325-340

Vicente, M., Palacios, P., Dopazo, A., Garrido, T., Pla, J. and Aldea, M. (1991) Res. Microbiol. 142, 253-257

Wickner, S. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 2690-2694 Wickner, W., Driessen, A. J. and Hartl, F. U. (1991) Annu. Rev. Biochem. 60, 101-124