

Possible Role of *Escherichia coli* Penicillin-Binding Protein 6 in Stabilization of Stationary-Phase Peptidoglycan

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Plasmids for high-level expression of penicillin-binding protein 6 (PBP6) were constructed, giving rise to overproduction of PBP6 under the control of the lambda p_R promoter in either the periplasmic or the cytoplasmic space. In contrast to penicillin-binding protein 5 (PBP5), the presence of high amounts of PBP6 in the periplasm as well as in the cytoplasm did not result in growth as spherical cells or in lysis. Deletion of the C-terminal membrane anchor of PBP6 resulted in a soluble form of the protein (PBP6^{Δ350}). Electron micrographs of thin sections of cells overexpressing both native membrane-bound and soluble PBP6 in the periplasm revealed a polar retraction of the cytoplasmic membrane. Cytoplasmic overexpression of native PBP6 gave rise to the formation of membrane vesicles, whereas the soluble PBP6 formed inclusion bodies in the cytoplasm. Both the membrane-bound and the soluble forms of PBP6 were purified to homogeneity by using the immobilized dye Procion rubine MX-B. Purified preparations of PBP6 and PBP6^{Δ350} formed a [¹⁴C]penicillin-protein complex at a 1:1 stoichiometry. The half-lives of the complexes were 8.5 and 6 min, respectively. In contrast to PBP5, no DD-carboxypeptidase activity could be detected for PBP6 by using bisacetyl-L-Lys-D-Ala-D-Ala and several other substrates. These findings led us to conclude that PBP6 has a biological function clearly distinct from that of PBP5 and to suggest a role for PBP6 in the stabilization of the peptidoglycan during stationary phase.

Three enzymes having D-alanine carboxypeptidase activity were isolated from *Escherichia coli* by Tamura et al. (27). These enzymes appeared to be identical to penicillin-binding proteins 4 (PBP4), 5 (PBP5), and 6 (PBP6). PBP5 and PBP6 are the major penicillin-binding components in the cell, accounting for 85% of the penicillin-binding capacity (23, 25). PBP5 and PBP6 are encoded by two distinct genes, *dacA* and *dacC*. Their primary structures show 62% sequence identity (5). PBP5 has been thoroughly studied with respect to membrane association, cellular location, penicillin binding, and DD-carboxypeptidase activity (8, 14, 29, 30). PBP5 (molecular mass, 41,337 Da) catalyzes the cleavage of the D-alanine-D-alanyl peptide bond in the pentapeptide side chain of the peptidoglycan polymer, resulting in a tetrapeptide (18, 25). The proposed biological function for this enzyme is the regulation of peptide cross-linkage in the peptidoglycan (17).

PBP6 (molecular mass, 40,804 Da) is translated as a preprotein and translocated across the cytoplasmic membrane by an N-terminal signal sequence 27 amino acids long. Cleavage of the signal sequence yields a mature protein of 373 residues (21). PBP6 is bound to the cytoplasmic membrane by a C-terminal membrane anchor, the bulk of the protein sticking out into the periplasm. The C-terminal 22 residues can form an amphiphilic helix associating the protein with the membrane (14). PBP6 was purified to homogeneity, and its penicillin-binding characteristics and enzymatic activity were determined by Amanuma and Strominger (1). The protein was shown to have DD-carboxypeptidase activity by using various natural and synthetic substrates. The specific activity was determined to be three- to fourfold lower than that of PBP5. However, there is some evidence that the two enzymes do not fulfill the same

function. Buchanan and Sowell described a considerable rise in expression level of PBP6 in stationary-phase cells compared with exponentially growing cells (7). Therefore, we undertook a more thorough study of PBP6.

PBP6 is present in the cell in relatively small amounts, 570 molecules per cell (23). To investigate the physiological effects of elevated levels of PBP6, the *dacC* gene was cloned into the earlier-described pPROFIT expression vector, giving rise to high-level expression of PBP6 in either the periplasmic or the cytoplasmic space (30).

In this paper, we describe the effects on the cell of high-level expression of both membrane-bound and soluble forms of PBP6. We further report on the purification and enzymatic characterization of these proteins. A possible physiological role of PBP6, clearly different from that of PBP5, is discussed.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* MC1061 was used as a host strain for subcloning and expression of proteins (19). Site-directed mutagenesis according to the method of Kunkel et al. (15) was performed with *E. coli* CJ236 (Bio-Rad Laboratories, Richmond, Calif.) and JM101 and phage M13mp18 (20). The plasmid pBS102, harboring the *dacC* gene, was a gift from B. Spratt, University of Sussex, Brighton, United Kingdom. Growth of bacteria was at 30°C in Luria-Bertani medium supplemented with kanamycin (50 μg ml⁻¹) for antibiotic-resistant strains. To induce the lambda p_R promoter, the growth temperature was shifted to 42°C at an A_{600} of 0.6.

Enzymes and radiochemicals. Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, and Klenow polymerase were used as recommended by the supplier (Boehringer). α-³⁵S-dATP used for DNA sequencing and [¹⁴C]benzylpenicillin (specific activity, 1.97 GBq mmol⁻¹) used for

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penicillin-binding assays were obtained from Amersham, Amersham, United Kingdom.

Site-directed mutagenesis and DNA sequencing. The *SmaI* and *SaII* restriction sites of M13mp18 were used to clone the 1.5-kb *AhaIII-SaII* fragment from pBS102, containing the *dacC* gene, into the phage.

The method described by Kunkel et al. (15) was used for oligonucleotide-directed mutagenesis. Primers were synthesized by Eurosequence, Groningen, The Netherlands. Sequences of the primers are given throughout the text. The desired mutations and the complete sequence of the *dacC* gene were verified by DNA sequencing according to Sanger et al. (22).

Protein purification. Cultures overexpressing PBP6 (culture volume, 2 liters; 4.5 g [wet mass] of cells) were harvested by centrifugation and resuspended in 10 ml of 10 mM Tris-HCl, pH 7.0, containing 1 mM 1,4-dithiothreitol. Cells were disrupted by passage through an Aminco French press at 68.9 MPa. The soluble and particulate fractions were separated by ultracentrifugation (45 min, 100,000 × *g*, 4°C). Membrane-bound forms of PBP6 were purified from the particulate fraction in the presence of 0.2% Triton X-100. In the purification of the soluble forms of PBP6, the detergent was omitted. The crude protein fractions were directly applied to a column (200 by 24 mm) of Procion rubine MX-B coupled to Fractogel TSK HW-65F, and the purification procedure was further carried out as described earlier for PBP5 (30).

SDS-PAGE and Western blotting (immunoblotting). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Lugtenberg et al. (16). A 10% (mass/vol) running gel and a 4.5% (mass/vol) stacking gel were used, the acrylamide/bisacrylamide ratio being 30:0.5. Proteins were electrophoretically transferred onto nitrocellulose as described by Bittner et al. (3). Rabbit antisera against PBP6 were obtained in this laboratory from rabbits immunized with purified protein. PBP6 was immunochemically detected by using antiserum, goat anti-rabbit immunoglobulin G-conjugated horseradish peroxidase (Tago, Inc., Burlingame, Calif.) and chloronaphthol-H₂O₂ (26).

Penicillin-binding assays. The penicillin-binding activity of cytoplasmically and periplasmically located soluble and membrane-bound forms of PBP6 was assayed as described earlier (30). The binding stoichiometry of the different forms of PBP6 was determined by incubating protein samples of 25 pmol each with 200 pmol of [¹⁴C]benzylpenicillin at 37°C for 10 min. The molar ratio of bound [¹⁴C]benzylpenicillin and the time course of the release of the [¹⁴C]benzylpenicilloyl moiety from PBP6 were determined as described by van der Linden et al. (30).

Carboxypeptidase activity assays. DD-Carboxypeptidase activity was determined qualitatively by thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC) detection of UDP-muramyl-tetrapeptide and D-alanine release after incubation of PBP6 with UDP-muramyl-pentapeptide. TLC was performed on Silicagel 60-kieselguhr F₂₅₄-precoated aluminum sheets (Merck) with *n*-butanol-acetic acid-H₂O (4:1:1) as a solvent. Samples of PBP6 (1 to 2 μg each) were incubated with 6.5 nmol of UDP-muramyl-pentapeptide for 10 min at 37°C in 100 mM Tris-HCl, pH 7.0. The reaction mixture was spotted on a TLC sheet, dried with a hot gun, and eluted. The peptides and released D-alanine were detected with ninhydrin. HPLC analysis was performed as follows. PBP6 (1 to 2 μg) was incubated with 6.5 nmol of UDP-muramyl-pentapeptide at 37°C for 10 min in

100 mM Tris-HCl, pH 7.0. The sample volume was adjusted to 250 μl with water, and the sample was boiled for 5 min. The pH was adjusted to 2.0 by using H₃PO₄, and the sample was applied to a Lichrosorb RP18 column (particle size, 7 μm; 250 by 4 mm), mounted in a Merck-Hitachi HPLC system. UDP-muramyl-pentapeptide and UDP-muramyl-tetrapeptide were eluted by using a 0 to 30% methanol gradient in 100 mM Na₂HPO₄ (pH 2.0). Peaks were detected at 205 nm with a Hitachi L-4200 UV-VIS detector.

Kinetic measurements. DD-Carboxypeptidase activity was quantitatively assayed by incubating 10 μg of purified PBP6 in 100 mM Tris-HCl, pH 8.0, at 37°C with various concentrations of substrates. Bisacetyl-L-Lys-D-Ala-D-Ala, bisacetyl-L-Lys-D-Ala-D-Lac, L-Ala-D-γ-Glu-L-Lys-D-Ala-D-Ala, and UDP-muramyl-pentapeptide were used as substrates. The amount of released D-alanine was determined by using D-aminoacid oxidase and *O*-dianisidine chloride as described by Frère et al. (9).

Secondary structure prediction by hydrophobic cluster analysis. Gaboriaud et al. (10) described a method for the prediction of secondary structure elements based on a two-dimensional representation of the distribution of hydrophobic residues along the protein sequence. Clustered hydrophobic residues supposed to indicate secondary structure elements are used to align weakly related primary structures.

Electron microscopy. For morphological studies, cells were fixed in 3% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h at 0°C. Postfixation was performed in 1% OsO₄ and 2.5% K₂Cr₂O₇ in the same buffer for 1 h at room temperature. Subsequently, the material was stained en bloc overnight with 1% (wt/vol) uranyl acetate in distilled water, dehydrated in a graded series of ethanol solutions, and embedded in Epon 812. Ultrathin sections were cut with a diamond knife on an LKB ultramicrotome. Sections were examined in a Philips CM10 microscope.

Other techniques. Protein concentrations were determined by using the method of Bradford (4). This assay had been calibrated by a PBP5 standard of known protein concentration as was described earlier (30).

RESULTS

Construction of plasmids for high-level expression of PBP6. The 1.5-kb *AhaIII-SaII* fragment from pBS102 containing the *dacC* gene coding for PBP6 was cloned into M13mp18 by using the *SmaI* and *SaII* restriction sites (M13mp18*dacC*). The *dacC* gene was cloned from M13mp18 into the expression vector pPROFIT by using the *EcoRI* and *PstI* restriction sites (30). In the resulting vector pPROFIT6Aⁿ, the *dacC* gene is placed under the control of the temperature-inducible lambda *p_R* promoter, giving rise to overexpression of the native membrane-bound PBP6, with the bulk of the protein sticking out into the periplasmic space (Fig. 1).

For cytoplasmic overexpression of PBP6, a *BgIII* site was created by using site-directed mutagenesis at the signal peptide cleavage point in the *dacC* gene. The primer used for this mutation was CGCTTCAACGGTTAGATC/TGCCCG GAATGCCG (mutated bases are underlined, and the *BgIII* site is indicated by a slash). Verification of the mutated gene by DNA sequencing showed no further mutations. By using the *BgIII* and *PstI* restriction sites, the mutated gene was cloned into the *BamHI* and *PstI* sites of pPROFIT, resulting in the vector pPROFIT6Cⁿ (Fig. 1). pPROFIT6Cⁿ gave rise to overexpression of the native membrane-bound PBP6, inserted with its C-terminal membrane anchor into the mem-

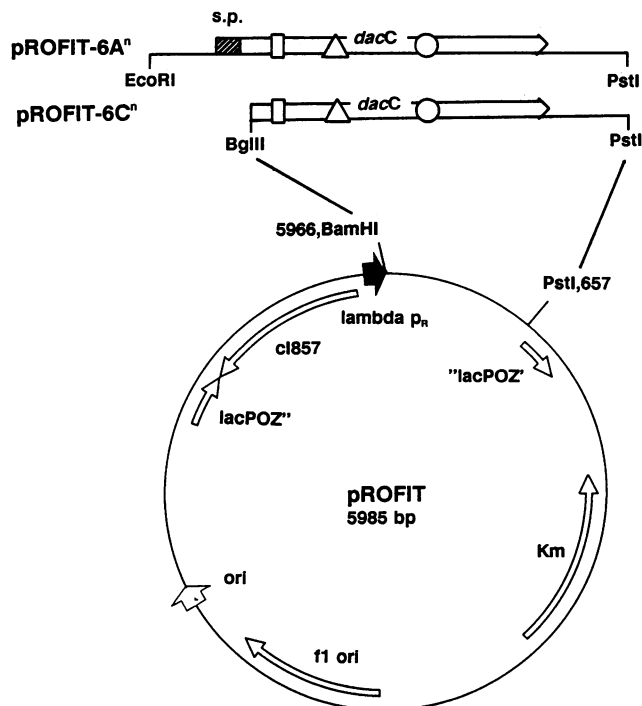


FIG. 1. Construction of plasmids for the overexpression of PBP6A. The vector pPROFIT-6Aⁿ for the accumulation of the overexpressed PBP6 in the periplasm was obtained by cloning the 1.5-kb *EcoRI-PstI* fragment containing the *dacC* gene from M13mp18*dacC* into the vector pPROFIT. A *BglII* site introduced by site-directed mutagenesis was used to clone the *BglII-PstI* fragment from M13mp18*dacC* into pPROFIT, resulting in the vector pPROFIT-6Cⁿ giving rise to overexpression of PBP6 in the cytoplasm. The fingerprints conserved in the family of active-site serine penicillin-interacting enzymes are indicated by a square (SxxK), a triangle (SxN), and a circle (KTG). s.p., signal peptide.

brane from the cytoplasmic side, with the bulk of the protein sticking out into the cytoplasmic space.

Construction of soluble forms of PBP6. The C-terminal residues 350 to 367 of PBP6, which are believed to form an amphiphilic helix serving as a membrane anchor, were deleted by the introduction of stop codons at positions Val-347 and Gly-351, respectively. The two positions were chosen on the basis of primary structure comparisons of PBP5 and PBP6 in combination with hydrophobic cluster analysis to possibly avoid destruction of secondary structure elements (Fig. 2 and reference 10). The regions P353EGN in PBP5 and E348EGG in PBP6 are located between two predicted structural elements at the C-terminal ends of the



FIG. 2. C-terminal amino acid sequence alignment of PBP5 and PBP6. The C-terminal residues of PBP5 and PBP6 were aligned. Identical residues are shaded. The boxed parts indicate secondary structure elements predicted by hydrophobic cluster analysis. In both proteins, the extreme C-terminal structural element is the amphiphilic helix serving as a membrane anchor. Stop codons introduced to create soluble forms of PBP5 and PBP6 are indicated by arrows.

proteins. The primers used to create the stop codons were GCCCTCTTCCIAATTTTCCATCAC and CCGACCAAA GAAT/CTCACTCTTCCAC, respectively (mutated bases are underlined, and the *EcoRI* cleavage site is indicated by a slash). Mutated genes were verified by DNA sequence analysis. The mutated genes were recloned into pPROFIT6Aⁿ and pPROFIT6Cⁿ, resulting in the vectors pPROFIT6A^{s346}, pPROFIT6A^{s350}, pPROFIT6C^{s346}, and pPROFIT6C^{s350}. The Val-347 mutation was recloned by using the *BamHI* and *PstI* sites. Recloning of the Gly-351 mutation by using the newly created *EcoRI* site resulted in a complete deletion of the sequence coding for the membrane anchor.

Overexpression of membrane-bound and soluble forms of PBP6. Each of the pPROFIT6A and pPROFIT6C plasmids, coding for both membrane-bound and soluble forms of PBP6, was introduced into *E. coli* MC1061. Cells were grown at 28°C to an A_{600} of 0.6. The growth temperature was then raised to 42°C to induce the lambda *p*_R promoter. The pPROFIT6A constructs gave rise to overproduction of PBP6 and accumulation in the periplasm whereas for the pPROFIT6C constructs, the overexpressed protein remained in the cytoplasm. Neither the periplasmically nor the cytoplasmically located forms of PBP6 resulted in lysis of the cells upon overexpression, and the cells could be grown at 42°C for several hours. The level of overproduction was different from one construct to another. PBP6Aⁿ was expressed to about 200 times the wild-type level as was determined by a Western blot analysis of a dilution series. Overexpression of PBP6Cⁿ was 100-fold whereas PBP6A^{s346}, PBP6A^{s350}, PBP6C^{s346}, and PBP6C^{s350} were expressed to 50 times the wild-type level.

Cells were disrupted by passage through an Aminco French press at 68.9 MPa followed by centrifugation at 100,000 × *g* for 45 min. The soluble and the particulate fractions were separated and analyzed for the presence of PBP6. PBP6Aⁿ and PBP6Cⁿ were localized exclusively in the particulate fraction. PBP6A^{s346}, PBP6A^{s350}, PBP6C^{s346}, and PBP6C^{s350} were found in the soluble fraction when analyzed on Coomassie blue-stained SDS-PAGE, by Western blot, and by penicillin-binding assay. PBP6A^{s346} and PBP6C^{s346} both appeared to be unstable, resulting in the appearance of a 30-kDa degradation product during overexpression of the proteins (Fig. 3).

Purification of soluble and membrane-bound PBP6. Purification of PBP5 by using the immobilized dye Procion rubine MX-B was described earlier (30). PBP6 appeared to have affinity for the same dye. Both the membrane-bound and the soluble forms of PBP6 were purified to homogeneity on a Procion rubine MX-B column in a one-step procedure (Fig. 4). The conditions for this purification procedure were the same as those described for PBP5, and both proteins eluted at the same NaCl concentration (30). Purified protein was obtained for PBP6Aⁿ, PBP6Cⁿ, PBP6A^{s350}, and PBP6C^{s350}. PBP6A^{s346} and PBP6C^{s346} showed no affinity for the dye and therefore could not be purified by using this method. Starting from 4.5 g (wet mass) of cells, the yield was 15 mg of pure PBP6Aⁿ. For the soluble form PBP6C^{s350}, the yield was 10 times lower, mainly because of the lower expression level.

Polyclonal antibodies against purified PBP6 were raised in rabbits in this laboratory. These antibodies showed affinity for all the various forms of PBP6 on Western blots (Fig. 3B).

Characterization of purified forms of PBP6. After purification, all the forms of PBP6 described above retained their ability to bind [¹⁴C]benzylpenicillin in a 1:1 stoichiometry. Like PBP5, PBP6 shows a rather fast release of the bound penicillin. A 50% degradation of the protein-[¹⁴C]benzylpen-

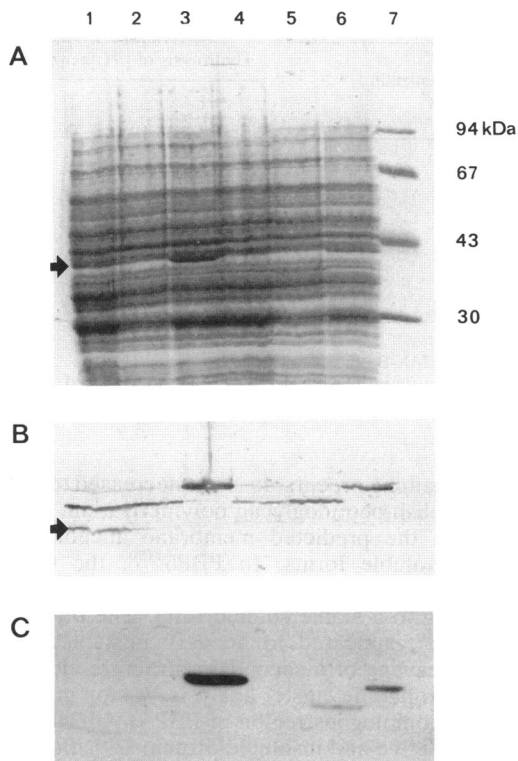


FIG. 3. Overexpression of membrane-bound and soluble forms of PBP6. Coomassie blue-stained SDS-PAGE (A), Western blot after immunostaining (B), and fluorogram after incubation with [14 C]benzylpenicillin (C) of cells overexpressing membrane-bound and soluble forms of PBP6. Cells were grown at 30°C to an A_{600} of 0.6, after which the temperature was shifted to 42°C. Total protein of the cells after 2 h of growth was applied to the gel. Lanes 1 to 6, PBP6A^{s346}-, PBP6A^{s350}-, PBP6Aⁿ-, PBP6C^{s346}-, PBP6C^{s350}-, and PBP6Cⁿ-overproducing cells, respectively; lane 7, molecular mass markers. Arrows indicate the positions of PBP6Aⁿ and PBP6Cⁿ (A) or the position of the 30-kDa degradation product of PBP6A^{s346} and PBP6C^{s346} (B).

icillin complex was observed after 8.5 min for the membrane-bound forms and after 6 min for the soluble forms of PBP6 (Table 1). Purified PBP6Aⁿ was assayed for DD-carboxypeptidase activity by using the artificial substrates bisacetyl-L-Lys-D-Ala-D-Ala, bisacetyl-L-Lys-D-Ala-D-lactate, and Ala-D- γ -Glu-L-Lys-D-Ala-D-Ala. No release of D-alanine was observed with any of these substrates. When, however, PBP5 was incubated with these substrates under exactly the same conditions, a considerable release of D-alanine could be detected (Table 1). Incubation of PBP6Aⁿ or PBP6A^{s350} with isolated UDP-muramyl-pentapeptide and subsequent analysis by HPLC or TLC, again in contrast to PBP5, did not show any appearance of UDP-muramyl-tetrapeptide or released D-alanine. DD-Carboxypeptidase activity measurements with membrane fractions from the PBP6Aⁿ overproducer, in contrast to PBP5Cⁿ membrane fractions, did not show any increase in release of D-alanine from bisacetyl-L-Lys-D-Ala-D-Ala (data not shown). These findings are in clear contradiction to the earlier-described DD-carboxypeptidase activity of purified PBP6 with the very same substrates (1, 2).

Morphological observations. Electron micrographs of thin sections of cells overexpressing membrane-bound or soluble

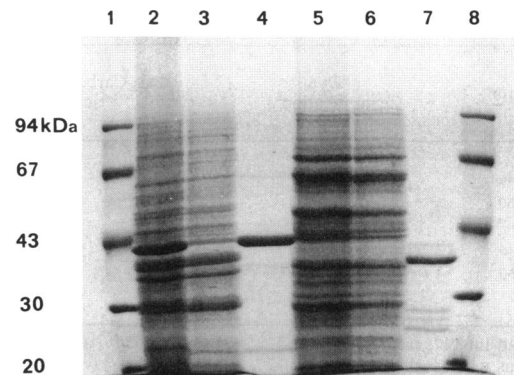


FIG. 4. Dye affinity purification of PBP6Aⁿ and PBP6C^{s350}. The membrane-bound and soluble forms of PBP6 were purified by using the immobilized dye Procion rubine MX-B. PBP6A^{s350} and PBP6Cⁿ could be purified by the same methods. PBP6A^{s346} and PBP6C^{s346} showed no affinity for the dye. Lanes 1 and 8, molecular mass markers; lane 2, Triton X-100-solubilized membrane proteins from the PBP6Aⁿ-overproducing strain; lane 3, flowthrough of the Procion rubine MX-B dye affinity column; lane 4, PBP6Aⁿ eluted from the Procion rubine MX-B column by a NaCl gradient; lane 5, soluble fraction of the PBP6C^{s350}-overproducing strain; lane 6, flowthrough of the dye affinity column; lane 7, PBP6C^{s350} eluted from the dye column by a NaCl gradient.

forms of PBP6 in either the periplasm or the cytoplasm are shown in Fig. 5. Overexpression of membrane-bound PBP6 in the cytoplasm (PBP6Cⁿ) gave rise to the formation of intracellular membrane vesicles throughout the cytoplasm of many cells (Fig. 5B). These vesicles seemed to consist of a membrane similar in general appearance and thickness to the cytoplasmic membrane. Cells overexpressing the native membrane-bound PBP6 in the periplasm (PBP6Aⁿ) do not show formation of membrane vesicles. However, a retraction of the cytoplasmic membrane at the polar tips of the cells was observed (Fig. 5A). The same phenomenon was found in cells overexpressing the periplasmically located soluble form of PBP6, PBP6A^{s350} (Fig. 5C). Overexpression of the cytoplasmically located soluble form of PBP6, PBP6C^{s350}, gave rise to intracellular structures visible as homogeneously stained areas with a distinct border with the cytoplasm. These areas most probably are cross sections of inclusion bodies formed in the cells.

DISCUSSION

Despite the accumulation of information on PBP5 and PBP6 in recent years, no evident physiological role for these two major PBPs in *E. coli* could be established. Both proteins are usually considered the major DD-carboxypeptidases and because of their similarities in primary structure and biochemical characteristics are believed to fulfill comparable functions in determining the degree of cross-linkage in the peptidoglycan polymer.

The previously described minor but nevertheless essential differences between the two proteins, like the differences in expression during exponential or stationary phase and the finding that peptidoglycan isolated from log-phase cells of a *dacC* deletion strain did not show the increase in pentapeptide side chains that was observed for peptidoglycan isolated from a *dacA* deletion strain, did not gain much attention (11). The expression of a DD-carboxypeptidase in stationary-phase cells, the peptidoglycan of which does not contain the

TABLE 1. Kinetic constants and purification results of membrane-bound and soluble forms of PBP6

Protein form	Dye purification ^b	DD-Carboxypeptidase activity ^c	[¹⁴ C]benzylpenicillin binding ^d	Hydrolysis of [¹⁴ C]benzylpenicillin ^e	
				<i>t</i> _{1/2} (s)	<i>k</i> ₃ (10 ⁻³ s ⁻¹)
PBP6A ⁿ	+	-	+	517 ± 30	1.34 ± 0.08
PBP6A ^{s346}	-	ND	+	ND	ND
PBP6A ^{s350}	+	-	+	363 ± 50	1.91 ± 0.26
PBP6C ⁿ	+	-	+	510 ± 50	1.36 ± 0.13
PBP6C ^{s346}	-	ND	+	ND	ND
PBP6C ^{s350}	+	-	+	377 ± 16	1.84 ± 0.08
PBP5C ⁿ	+	+	+	260 ± 10	2.7 ± 0.1

^a *t*_{1/2}, half-life; *k*₃, rate constant.

^b Proteins were purified (+) or could not be purified (-) with the immobilized dye Procion rubine MX-B.

^c DD-Carboxypeptidase activity was assayed as described in the text. + and -, release of alanine was or was not detected. ND, not detected.

^d Penicillin binding capacity was assayed as described in the text. +, binding ability was retained.

supposed substrate-pentapeptide side chains any more, raises again the question of its specific role in vivo (7, 12). All these observations were taken as indications for a physiological function of PBP6 distinct from that of PBP5.

To assess the differences in function, overproducers of PBP6 in either the native or the soluble form were established. Transport of the various forms of PBP6 into the periplasm does not cause the detrimental effect on the producing cells that had been observed during PBP5 overexpression (17, 30). As in the case of PBP5, high-level expression of PBP6 in the cytoplasm also did not affect cellular growth, which indicates that the amount of pen-

tapeptide containing precursors is not decreased to an extent that could abolish peptidoglycan polymerization.

Deletion of the predicted membrane anchor of PBP6 yielded two soluble forms. In PBP6^{s350}, the C-terminal α -helix predicted by hydrophobic cluster analysis was deleted, resulting in a stable soluble form. The other soluble form, PBP6^{s346}, appeared to be very unstable, probably because of cleavage of a secondary structure element predicted by hydrophobic cluster analysis. A stop codon introduced in the homologous region of PBP5 (Val-348) resulted in a totally inactive and insoluble protein (29). Both soluble forms of PBP6 still bound penicillin in a 1:1 ratio, which

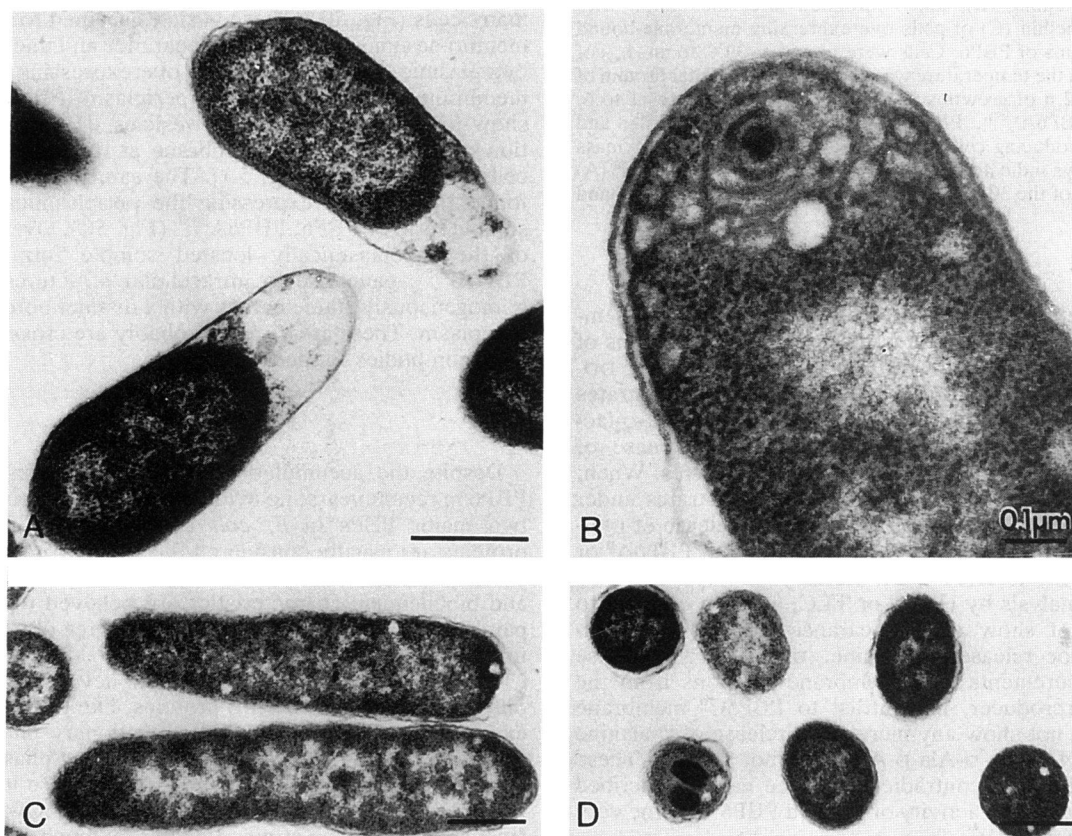


FIG. 5. Electron micrographs of ultrathin sections of PBP6-overproducing cells. Cells overproducing PBP6Aⁿ (A), PBP6Cⁿ (B), PBP6A^{s350} (C), and PBP6C^{s350} (D) are shown. Bars, 0.5 μ m unless otherwise stated.

indicates that PBP6^{s346} is structurally not completely disturbed as was the case for the analogous form of PBP5. However, the structural disturbance was such that no affinity for the dye resin was observed. The 30-kDa degradation product of PBP6^{s346} still carried the radiolabelled penicillin, indicating that it still contains the active-site serine, which means that a 10-kDa portion of the protein has been removed from the C terminus.

The unexpected tolerance of the cells for high-level expression of PBP6 led us to investigate whether we were dealing with a functional form of PBP6.

The [¹⁴C]benzylpenicillin-protein complexes of the soluble forms PBP6A^{s350} and PBP6C^{s350} have considerably shorter half-lives compared with the respective membrane-bound forms (PBP6Aⁿ and PBP6Cⁿ). We do not have an explanation for this difference, but the same effect was observed for PBP5 (30).

In contrast to results published by Amanuma and Strominger (1), we were not able to detect DD-carboxypeptidase activity for either the native membrane-bound or the soluble forms of PBP6 by using various artificial substrates as well as UDP-muramyl-pentapeptide. Samples of PBP6 purified in our laboratory from a nonoverproducing, *dacA* deletion strain (SP 5003) by conventional covalent affinity chromatography also showed no DD-carboxypeptidase activity. This is again in strong contrast to the findings of Amanuma and Strominger (2). The discrepancy between their and our results so far remains unexplained. Lack of activity in assays with membrane fractions from PBP6Aⁿ-overproducing cells proved that the proteins had not been inactivated during purification.

Resequencing of the entire *dacC* gene revealed one difference from the published sequence. At the codon corresponding to residue 204, our sequence reads GTT instead of GAA. This results in a valine rather than a glutamate at position 204. It is not likely that this difference could cause a complete inactivation of PBP6 as DD-carboxypeptidase since a valine is also found at the corresponding position in PBP5. The most likely explanation is that there has been a reading error in the original sequence (24).

These results contradict the current opinion that PBP6 is fulfilling the very same role as PBP5. Nevertheless, PBP6 might still be a DD-carboxypeptidase, being active only on a specific substrate under specific conditions. Stationary-phase cells show elevated amounts of PBP6 (7). Apparently, there is a need for PBP6 at this particular stage, since on resumption of exponential growth the amount of PBP6 falls to the original level. Nongrowing cells were found to stabilize their peptidoglycan by increasing the level of Dap-Dap cross-linkages (12), and the same effect was found for amino acid-starved cells (13). We suggest a role for PBP6 in stabilization of the peptidoglycan during stationary phase in analogy to the sporulation-specific PBP5a (PBP5*) from *Bacillus subtilis* for which, with a partially purified batch, only a very low specific activity as DD-carboxypeptidase has been detected (6, 28). The morphological alterations observed in the ultrathin sections of cells overexpressing various forms of PBP6 are obviously a direct consequence of the presence of high amounts of protein. Morphological effects of this kind were not found in PBP5-overproducing strains. However, extensive membrane formation was previously reported for cells producing large amounts of the membrane proteins fumarate reductase and F₁-F₀ proton-translocating ATPase (31, 32). Apparently, cells produce new membrane structures to accommodate an excess of membrane-bound protein. The cytoplasmically overex-

pressed soluble form of PBP6, however, is accumulating in inclusion bodies. Large amounts of either the membrane-bound or the soluble form of PBP6 in the periplasm do not result in extra membrane structures. The cause of the observed polar retraction of the cytoplasmic membrane remains unclear.

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