

Artifactual Processing of Penicillin-Binding Proteins 7 and 1b by the OmpT Protease of *Escherichia coli*

THOMAS A. HENDERSON, PATRICE M. DOMBROSKY, AND KEVIN D. YOUNG*

Department of Microbiology and Immunology, School of Medicine, University of North Dakota, Grand Forks, North Dakota 58202-9001

Received 19 August 1993/Accepted 30 October 1993

Penicillin-binding proteins (PBPs) were visualized in strains of *Escherichia coli* that carried mutations in one or more of the following protease genes: *tsp*, *degP*, *ptr*, and *ompT*. In the absence of a functional *ompT* gene, PBPs 1b α and 7 were not processed to the shortened forms 1b β and 8, respectively. Cleavage of PBPs 1b α and 7 could be restored by introduction of a plasmid carrying the wild-type *ompT* gene. These PBPs were processed only after cell lysis or after membrane perturbation of whole cells by freeze-thaw, suggesting that the cleavage was a nonspecific artifact due to contact with OmpT, an outer membrane protease, and that such processing was not biologically significant in vivo. The degradation of other PBPs during purification or storage may also be effected by OmpT.

Penicillin-binding proteins (PBPs) participate in the polymerization and restructuring of peptidoglycan of *Escherichia coli* (for a review, see reference 8). They play important roles in cell wall elongation (PBPs 1a and 1b), shape determination (PBP 2), septation (PBP 3), and peptidoglycan crosslinking (PBPs 4, 5, and 6) (8). In addition to the seven classic PBPs, other penicillin-binding proteins have been observed. Among these are PBPs 7 and 8, which are the smallest *E. coli* proteins known to bind penicillin (13), and other binding proteins smaller than PBPs 3 and 6 (13). None of these have known biological roles or enzymatic activities, though it has been suggested that PBP 7 is a potential target of the antibiotic imipenem (24).

Three of these PBPs undergo posttranslational modification. PBP 1b exists in three forms: 1b α (the full-length protein) and two smaller fragments, 1b β and 1b γ . In the first example of PBP processing, PBP 1b γ is produced by translation of the 1b α reading frame, beginning at an internal initiation codon (23). PBP 1b β is produced by removal of 24 amino acids from the N terminus of PBP 1b α , an event that occurs only after cell disruption (23). The presence of any one of these forms of PBP 1b is sufficient for bacterial viability (12). The second example of PBP processing is the removal of 11 C-terminal amino acids from PBP 3 by protease Prc (equivalent to protease Tsp [20]) (10, 17). This processing by Prc is also not essential for cell viability (10). In the third example, 20 amino acids are removed from the amino terminus of PBP 4 by an unidentified protease (16). Finally, it has been proposed that PBP 8 is a proteolytic fragment of PBP 7 (5), but there has been no definitive evidence of such a processing event.

It is of interest to know if these processed PBPs are important in the synthesis of peptidoglycan or for regulation of cell division. Therefore, we have measured the appearance of processed PBPs in *E. coli* strains that lack one or more known proteases. We report that in at least two cases the processed proteins are probably artifacts of the method of membrane preparation. In these cases, processing occurs primarily after cell disruption and depends on the activity of an outer membrane protease, OmpT.

Bacterial strains, growth, and labeling of penicillin-binding proteins. *E. coli* strains and plasmids are listed in Table 1. PMD100 and PMD101 were constructed by P1 transduction (15) of Δ *ponA* (from ED3184 Δ *ponA*) and Δ *ponB* (from ED3184 Δ *ponB*), respectively, into SF100. The wild-type *ompT* gene is contained on a 1,961-bp *PvuII*-*ScaI* DNA fragment in pML19, and plasmid pMD11 was constructed by inserting this fragment into the *ScaI* site located in the *bla* gene of pBR322. Cells were grown on minimal M9 medium plus 0.2% glucose and 0.2% Casamino Acids (15) to mid-log phase (optical density at 600 nm, 0.400 to 0.500), and pelleted whole cells were resuspended in 100 mM Tris-HCl (pH 8.0) plus 10 mM MgCl₂. Clear separation of the forms of PBP 1b was enhanced by suspension of harvested cells in buffer plus 1% Triton X-100 prior to labeling and gel electrophoresis. The PBPs of whole cells (100 μ g of protein in 15 μ l) were labeled by mixing with 3 μ l of β -mercaptoethanol (100 mM stock) and 3.5 μ l of 83.3- μ g/ml ¹²⁵I-penicillin X (833- μ Ci/ml stock) and incubating for 15 min at 37°C (14). Samples were denatured by adding 8.5 μ l of 4 \times sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (7) and boiling for 4 min. Unlabeled penicillin G (3 μ l of 100- μ g/ml stock) was added, and the samples were separated by electrophoresis through an SDS-12% PAGE gel containing 15% glycerol (7). Gels were dried and exposed to X-Omat AR film (Eastman Kodak, Rochester, N.Y.) for 3 to 7 days. Kanamycin (50 μ g/ml), chloramphenicol (50 μ g/ml, or 20 μ g/ml when selecting for P1 lysogens), spectinomycin (50 μ g/ml), streptomycin (80 μ g/ml), ampicillin (50 μ g/ml), or tetracycline (12.5 μ g/ml) was added to growth media where required for strain construction or for maintenance of the various plasmids. Restriction enzymes and T4 DNA ligase were from New England Biolabs, Inc., Beverly, Mass. Protein assays were performed with the enhanced microBCA assay (Pierce Chemical Co., Rockford, Ill.) (21).

Effect of proteases on PBP processing. Bacterial cells deficient in one of four proteases were labeled with ¹²⁵I-penicillin X to determine their effects on the pattern of PBPs in *E. coli*. As expected, when the *tsp* gene was inactive, PBP 3 was not processed and a larger gene product was observed (Fig. 1, lane 2). This was the only PBP affected by the *tsp* mutation. No PBPs were affected by the absence of the *degP*41 or *ptr*-32 gene products (Fig. 1, lanes 4 and 5). However, in bacteria in which the *ompT* gene had been mutated, PBP 8 was absent and there

* Corresponding author. Phone: (701) 777-2624. Fax: (701) 777-4790. Electronic mail address: kyoun@vm1.nodak.edu.

TABLE 1. *E. coli* strains and plasmids

| Strain or plasmid | Relevant characteristics ^a | Source or reference |
|---------------------|--|---------------------|
| Strain | | |
| KS300 | F ⁻ <i>araD139 galE galK ΔlacX74 rpsL thi recA1 ΔphoA(PvuII)</i> | 22 |
| KS272 | KS300 <i>recA</i> ⁺ | 22 |
| KS499 | KS300 <i>degP41</i> (Km ^r) | 22 |
| SF103 | KS272 <i>ptr-32::ΩCm</i> ^r | 3 |
| SF100 | KS272 <i>ΔompT</i> | 2 |
| AD202 | <i>ompT::Km</i> ^r | 1; K. Ito |
| KS1010 | <i>eda-51::Tn10 ara nalA argE(Δm) rif thi-1 F' lacI^a lac⁺ pro⁺</i> | K. R. Silber |
| KS1000 | KS1010 <i>Δtsp::Km</i> ^r | K. R. Silber |
| ED3184 | <i>his supF</i> | 6 |
| ED3184 <i>ΔponA</i> | ED3184 <i>ponA::Km</i> ^r | 25 |
| ED3184 <i>ΔponB</i> | ED3184 <i>ponB::Spc</i> ^r | 25 |
| PMD100 | SF100 <i>ponA::Km</i> ^r | This paper |
| PMD101 | SF100 <i>ponB::Spc</i> ^r | This paper |
| Plasmid | | |
| pBR322 | Ap ^r , Tc ^r | S. Detke |
| pML19 | Ap ^r , pUC19 plus a 2.2-kb <i>PstI-EcoRI ompT</i> fragment | 9 |
| pMD11 | Tc ^r , <i>PvuII-ScaI ompT</i> fragment from pML19 inserted into the <i>ScaI</i> site of the <i>bla</i> gene of pBR322 | This paper |

^a Abbreviations in designations of drug resistance phenotypes: Km, kanamycin; Cm, chloramphenicol; Spc, spectinomycin; Sm, streptomycin; Ap, ampicillin; Tc, tetracycline.

was an increase in the intensity of the amount of PBP 7 (Fig. 1, lanes 6 and 7). In other experiments, *E. coli* strains were constructed in which up to three of the four protease mutations were present simultaneously, in various combinations. No additional degradation effects were observed. PBP 3 processing was inhibited only when the *tsp* gene was inactivated, and PBP 8 failed to appear only when the *ompT* gene was inactivated, regardless of the presence of additional protease mutations (data not shown).

In *E. coli* SF100 (Fig. 1, lane 6) *ompT* and several surrounding genes are deleted (2), and in *E. coli* AD202 (Fig. 1, lane 7) the *ompT* gene is inactivated by the insertion of a kanamycin resistance cassette (1). Because multiple genes were absent in SF100 and because the AD202 insertion could potentially interrupt expression of downstream genes, it was possible that

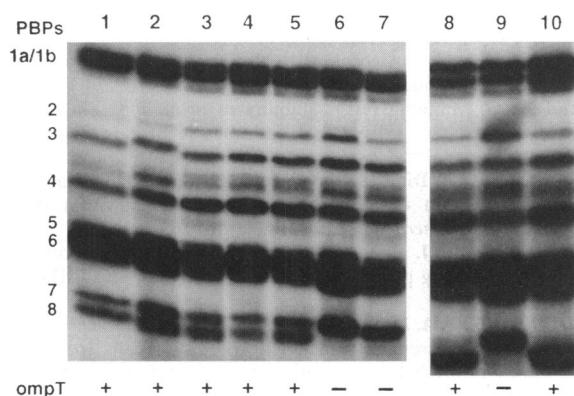


FIG. 1. Effect of protease mutations on the processing of *E. coli* PBPs. *E. coli* PBPs were labeled with ¹²⁵I-penicillin X, and the proteins were separated by SDS-PAGE. The locations of the PBPs are indicated at the left, and the presence or absence of a functional *ompT* gene is indicated by a plus or minus sign at the bottom of each lane. Lane 1, KS1010; lane 2, KS1000 (*Δtsp*); lane 3, KS300; lane 4, KS499 (*degP41*); lane 5, SF103 (*ptr-32*); lane 6, SF100 (*ΔompT*); lane 7, AD202 (*ompT::Km*^r); lane 8, KS300; lane 9, SF100 (*ΔompT*); lane 10, SF100 pMD11.

a protein other than OmpT could be responsible for the conversion of PBP 7 to PBP 8. We subcloned the wild-type *ompT* gene from pML19 (9) into pBR322, creating pMD11, in which the ampicillin resistance gene was disrupted. When pMD11 was transformed into SF100, PBP 8 reappeared (Fig. 1; compare lanes 9 and 10). Approximately equal amounts of PBPs 7 and 8 were observed in wild-type strains and in the *tsp*, *degP41*, and *ptr-32* mutants (Fig. 1, lanes 1 to 5), but when *ompT* was introduced on a multicopy plasmid, PBP 8 predominated and very little or no PBP 7 was visible (Fig. 1, lane 10). This was also true for wild-type cells into which pMD11 was introduced (data not shown). These results confirmed that the presence of OmpT was necessary for the production of PBP 8 from PBP 7 and that, in the presence of elevated levels of OmpT, PBP 7 was transformed almost entirely into PBP 8.

Processing of PBP 7 to PBP 8 depends on sample preparation. The presence of PBP 8 was not required for the viability of *E. coli*, since *ompT* mutants, which lacked PBP 8, grew normally. Therefore, we questioned whether PBP 8 was present in wild-type cells or whether it was an artifact created from PBP 7 while manipulating the samples. Although OmpT is an outer membrane protease, it can cleave a variety of intracellular proteins when bacterial cells are permeabilized or broken, thus allowing OmpT to come into contact with these normally unavailable substrates (1, 2). PBP labeling of whole *E. coli* cells is usually carried out on samples that have been stored frozen, a treatment that permeabilizes the membrane to large proteins. Therefore, the PBP profile of cells labeled immediately after growth and harvesting was compared with the profile of the same cells labeled after freezing (Fig. 2). Two results were apparent. First, the amounts of PBPs that were labeled were significantly greater (approximately 10-fold greater) in samples that had been frozen (Fig. 2, lanes 1 to 3 versus lanes 4 to 6). This is not too surprising, since access of penicillin to its targets is impeded by an intact cell envelope. The second observation is that no PBP 8 was visible in cells labeled immediately after harvest, even in cells overproducing OmpT from pMD11 (Fig. 2, lanes 1 to 3), in contrast to the large amount of PBP 8 that appeared in frozen samples (Fig. 2, lanes 5 and 6). PBP 8 also appeared if freshly grown and

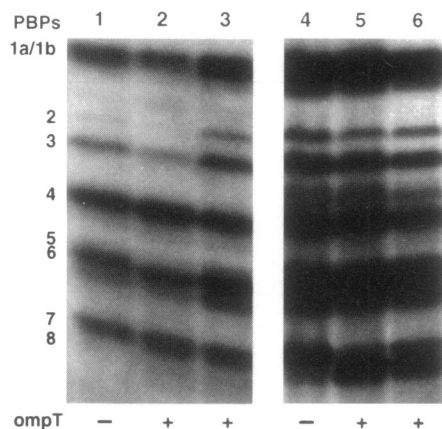


FIG. 2. Effect of freeze-thaw on labeling of PBPs in *E. coli*. Whole cells of *E. coli* were labeled with ^{125}I -penicillin X, and the proteins were separated by SDS-PAGE. PBPs were labeled immediately after harvest of freshly grown cells (lanes 1 to 3) or after one cycle of freezing at -20°C and thawing (lanes 4 to 6). Lanes 1 and 4, SF100 ($\Delta ompT$); lanes 2 and 5, SF100 pMD11; lanes 3 and 6, KS300.

harvested cells were lysed prior to labeling with ^{125}I -penicillin X, but only if a wild-type *ompT* gene was present (data not shown). Thus, PBP 8 should be considered an artifact created by exposure of PBP 7 to the OmpT protease during sample preparation.

PBP 1b α is cleaved by OmpT. It has been suggested that PBP 1b β is produced from PBP 1b α by an analogous artifactual processing during sample preparation (23). Therefore, the different forms of PBP 1b were visualized in *E. coli* strains that did or did not contain the OmpT protease (Fig. 3). PBP 1b β was visible in the parental strain KS300 (Fig. 3, lane 1) but not in strains deleted for *ompT* (Fig. 3, lanes 2 and 4). Reintroduction of the *ompT* gene via pMD11 restored to these mutant strains the ability to process PBP 1b α to PBP 1b β (Fig. 3, lanes 3 and 5). This processing was specific for PBP 1b: proteolysis was observed even when the cells lacked PBP 1a (Fig. 3, lanes 4 and 5), and strains in which PBP 1b was deleted did not show this processing pattern (Fig. 3, lanes 6 and 7). Also, PBP 1b α was processed to PBP 1b β in cells with mutations in *ptr-32* and *degP41* (data not shown). Thus, the OmpT protease is responsible for the artifactual processing that creates PBP 1b β .

Implications for PBP processing and purification. The OmpT protease cleaves proteins between a pair of positively charged amino acids (Arg-Arg, Lys-Lys, Arg-Lys, or Lys-Arg)

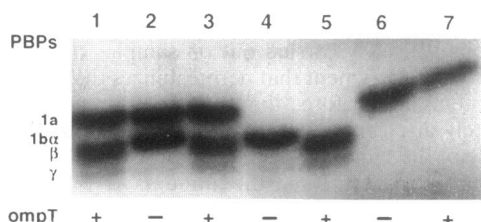


FIG. 3. Effect of protease mutations on the processing of *E. coli* PBP 1b. Whole cells of *E. coli* were solubilized with 1% Triton X-100 in Tris-HCl buffer and frozen at -20°C . PBPs were labeled with ^{125}I -penicillin X and separated by SDS-PAGE, allowing electrophoresis to proceed for 6 h instead of the normal 3 h of separation time. Lane 1, KS300; lane 2, SF100 ($\Delta ompT$); lane 3, SF100 pMD11; lane 4, PMD100 ($\Delta ponA \Delta ompT$); lane 5, PMD100 pMD11; lane 6, PMD101 ($\Delta ponB \Delta ompT$); lane 7, PMD101 pMD11.

(4). The cleavage of PBP 1b α to form PBP 1b β is known to occur between two arginine residues (23), which is consistent with the evidence presented here for the role of OmpT in PBP 1b processing. These data confirm and extend the suggestion of Suzuki et al. (23) that the production of PBP 1b β might be incidental to membrane preparation. Analogous data for PBPs 7 and 8 are not available, but we have purified a small amount of PBP 8 and have determined that its amino-terminal sequence begins with lysine (11), which is consistent with OmpT proteolysis of PBP 7. In a similar vein, when overproduced from a multicopy plasmid, PBP 3 preparations are contaminated with a 40-kDa fragment of PBP 3 that is still able to bind penicillin (19). Nagasawa et al. (17) found that the amino-terminal amino acid of this major degradation product began with the lysine at position 211 of the complete PBP 3 protein. This lysine is preceded by an arginine residue at position 210 (18) and so forms a potential cleavage site for the OmpT protease. Thus, the rapid degradation of PBP 3 into these alternate forms during purification and storage (19) may be due to exposure to OmpT, and such degradation might be avoided by expressing PBP 3 in an *ompT* mutant.

Summary. PBPs 1b and 7 are substrates for OmpT proteolysis. Cleavage of these PBPs occurs only when cells are frozen or lysed and does not occur in freshly labeled whole cells or in lysates of *E. coli* strains that contain a deletion of the *ompT* gene. Therefore, PBPs 1b β and 8 represent PBP fragments that are artificially created during membrane preparation and, as such, probably possess no *in vivo* function. Although the relationship between PBPs 7 and 8 has been suggested previously (5), this report presents the first data indicating that PBP 8 is derived from PBP 7.

This work was supported by Public Health Service grant GM40947 from the National Institutes of Health.

REFERENCES

- Akiyama, Y., and K. Ito. 1990. SecY protein, a membrane-embedded secretion factor of *E. coli*, is cleaved by the OmpT protease *in vitro*. *Biochem. Biophys. Res. Commun.* **167**:711-715.
- Baneyx, F., and G. Georgiou. 1990. *In vivo* degradation of secreted fusion proteins by the *Escherichia coli* outer membrane protease OmpT. *J. Bacteriol.* **172**:491-494.
- Baneyx, F., and G. Georgiou. 1991. Construction and characterization of *Escherichia coli* strains deficient in multiple secreted proteases: protease III degrades high molecular weight substrates *in vivo*. *J. Bacteriol.* **173**:2696-2703.
- Baneyx, F., and G. Georgiou. 1992. Expression of proteolytically sensitive polypeptides in *Escherichia coli*, p. 69-108. In T. J. Ahern and M. C. Manning (ed.), *Stability of protein pharmaceuticals, part A: chemical and physical pathways of protein degradation*. Plenum Press, New York.
- Barbas, J. A., J. Díaz, A. Rodríguez-Tébar, and D. Vázquez. 1986. Specific location of penicillin-binding proteins within the cell envelope of *Escherichia coli*. *J. Bacteriol.* **165**:269-275.
- Broome-Smith, J. K., and B. G. Spratt. 1982. Deletion of the penicillin-binding protein 6 gene of *Escherichia coli*. *J. Bacteriol.* **152**:904-906.
- Dreyfuss, G., S. A. Adam, and Y. D. Choi. 1984. Physical change in cytoplasmic messenger ribonucleoproteins in cells treated with inhibitors of mRNA transcription. *Mol. Cell. Biol.* **4**:415-423.
- Frère, J.-M., and B. Joris. 1985. Penicillin-sensitive enzymes in peptidoglycan biosynthesis. *Crit. Rev. Microbiol.* **11**:299-396.
- Grodberg, J., M. D. Lundrigan, D. L. Toledo, W. F. Mangel, and J. J. Dunn. 1988. Complete nucleotide sequence and deduced amino acid sequence of the *ompT* gene of *Escherichia coli* K-12. *Nucleic Acids Res.* **16**:1209.
- Hara, H., Y. Nishimura, J.-I. Kato, H. Suzuki, H. Nagasawa, A. Suzuki, and Y. Hirota. 1989. Genetic analyses of processing involving C-terminal cleavage in penicillin-binding protein 3 of *Escherichia coli*. *J. Bacteriol.* **171**:5882-5889.

11. Henderson, T. A., and K. D. Young. Unpublished data.
12. Kato, J.-I., H. Suzuki, and Y. Hirota. 1984. Overlapping of the coding regions for α and γ components of penicillin-binding protein 1b in *Escherichia coli*. *Mol. Gen. Genet.* **196**:449-457.
13. Leidenix, M. J., G. H. Jacoby, T. A. Henderson, and K. D. Young. 1989. Separation of *Escherichia coli* penicillin-binding proteins into different membrane vesicles by agarose electrophoresis and sizing chromatography. *J. Bacteriol.* **171**:5680-5686.
14. Masson, J. M., and R. Labia. 1983. Synthesis of a ^{125}I -radiolabeled penicillin for penicillin-binding protein studies. *Anal. Biochem.* **128**:164-168.
15. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. Mottl, H., P. Terpstra, and W. Keck. 1991. Penicillin-binding protein 4 of *Escherichia coli* shows a novel type of primary structure among penicillin-interacting proteins. *FEMS Microbiol. Lett.* **78**:213-220.
17. Nagasawa, H., Y. Sakagami, A. Suzuki, H. Hara, and Y. Hirota. 1989. Determination of the cleavage site involved in C-terminal processing of penicillin-binding protein 3 of *Escherichia coli*. *J. Bacteriol.* **171**:5890-5893.
18. Nakamura, M., I. N. Maruyama, M. Soma, J.-I. Kato, H. Suzuki, and Y. Horota. 1983. On the process of cellular division in *Escherichia coli*: nucleotide sequence of the gene for penicillin-binding protein 3. *Mol. Gen. Genet.* **191**:1-9.
19. Nicholas, R. A., J. L. Strominger, H. Suzuki, and Y. Hirota. 1985. Identification of the active site in penicillin-binding protein 3 of *Escherichia coli*. *J. Bacteriol.* **164**:456-460.
20. Silber, K. R., K. C. Keiler, and R. T. Sauer. 1992. Tsp: a tail-specific protease that selectively degrades proteins with non-polar C termini. *Proc. Natl. Acad. Sci. USA* **89**:295-299.
21. Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**:76-85.
22. Strauch, K. L., and J. Beckwith. 1988. An *Escherichia coli* mutation preventing degradation of abnormal periplasmic proteins. *Proc. Natl. Acad. Sci. USA* **85**:1576-1580.
23. Suzuki, H., J.-I. Kato, Y. Sakagami, M. Mori, A. Suzuki, and Y. Hirota. 1987. Conversion of the α component of penicillin-binding protein 1b to the β component in *Escherichia coli*. *J. Bacteriol.* **169**:891-893.
24. Tuomanen, E., and J. Schwartz. 1987. Penicillin-binding protein 7 and its relationship to lysis of nongrowing *Escherichia coli*. *J. Bacteriol.* **169**:4912-4915.
25. Yousif, S. Y., J. K. Broome-Smith, and B. G. Spratt. 1985. Lysis of *Escherichia coli* by β -lactam antibiotics: deletion analysis of the role of penicillin-binding proteins 1A and 1B. *J. Gen. Microbiol.* **131**:2839-2845.