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Location of Some Proteins Involved in Peptidoglycan Synthesis and Cell Division in the Inner and Outer Membranes of *Escherichia coli*

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Inner and outer membranes of *Escherichia coli* were separated by isopycnic centrifugation in sucrose gradients and analyzed for the presence of penicillin-binding proteins. All penicillin-binding proteins—except penicillin-binding protein 3, which is found almost exclusively in the cytoplasmic membrane and is involved in septum formation—are also found in gradient fractions corresponding to the outer membrane. Our results support the hypothesis that approximately half of the total amount of penicillin-binding proteins may be sacculus-located proteins linked to the outer membrane, probably through peptidoglycan bridges.

Some of the enzymes catalyzing the last steps of peptidoglycan biosynthesis in bacteria bind β-lactam antibiotics which inactivate their catalytic activities (see reference 34 for a recent review). These penicillin-binding proteins (PBPs) are minor proteins located in the bacterial cell envelope. Because these proteins are essential in the control of cell division, morphology, and elongation of the bacteria, radiolabeled β-lactams are useful tools in the determination of their location and function. It has been widely accepted that, in gram-negative bacteria, PBPs are located exclusively in the cytoplasmic membrane on the basis of Sarkosyl solubility (31). This idea was based on the observation that all proteins from *Escherichia coli* inner membrane (IM) were soluble in 1.0% (wt/vol) sodium lauroyl sarcosinate (Sarkosyl) under certain experimental conditions, whereas proteins from the outer membrane (OM) were all Sarkosyl insoluble under the same conditions (10). Because PBPs from *E. coli* were soluble in Sarkosyl, they were classified as IM proteins. However, later work showed that some OM proteins could be extracted by this detergent (4, 11). Indeed, 1% Sarkosyl also extracts a part of *ompA* and *ompF* from OMs after digestion of the peptidoglycan by lysozyme (Barbas et al., manuscript in preparation). In the present communication, we used the widely accepted method of Osborn et al. (24) for the isolation of both IM and OM of *E. coli* and studied the presence of the PBPs in these fractions. Previously, Koyasu et al. (16) separated IMs and OMs from *E. coli* cells by the method of Osborn et al. (24), and they recovered PBP material not only in the IM but also in the OM fractions although in lesser amounts. In addition, it was demonstrated that PBP 4 in *Caulobacter crescentus* was only present in the OM, whereas PBPs 1a and 3 were only detected in the IM. PBPs 1b and 2 were found in both IM and OM fractions (17, 18).

From the data presented here, we conclude that all PBPs studied except PBP 3 are also found in fractions corresponding to the OM. The significance of this finding is discussed.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* W7 (*dap*$^-$ *lys*$^-$) (12) and PAT 84 (*dap*$^-$ *lys*$^-$ *ftsZ*) (13) strains were used throughout. They were grown in L medium (21) supplemented with 10 μg of *meso*-2,6 diaminopimelic acid per ml, 40 μg of L-lysine per ml, and 2 mg of glucose per ml under forced aeration at either 30, 37, or 42°C, depending on the conditions needed for a particular experiment (see below as well as the figure legends).

Filamentous cells of *E. coli* W7 were obtained by selective inhibition of PBP 3 with the β-lactam azthreonam (E. R. Squibb & Sons, Inc., Princeton, N.J.) (32). Cells were grown at 37°C in L medium as described above (t = 22 min) up to an absorbance at 550 nm of 0.6. A culture sample was removed and diluted fivefold in fresh prewarmed L medium (37°C) containing azthreonam (0.5 μg/ml [final concentration]). The formation of filaments was followed by light microscopy.

Filamentous cells from *E. coli* PAT 84 were obtained by changing the temperature of the culture. Cells were grown in L medium at 30°C as described above (t = 45 min) up to an absorbance at 550 nm of 0.6. A sample was then removed and diluted fivefold into fresh L medium that was prewarmed at 42°C. Cells were allowed to filament at this temperature, and the formation of filaments was followed by light microscopy.

Labeling of PBPs from *E. coli*. We followed essentially the procedure described by Spratt (31), replacing benzyl [^{14}C]penicillin with a [^{125}I]ampicillin derivative described by Schwarz et al. (30). The [^{125}I]ampicillin derivative was obtained by coupling radioiodinated Bolton and Hunter reagent (2,000 Ci/mmol; Amersham International, Amersham, United Kingdom). Kinetic studies were performed to elucidate the concentrations of [^{125}I]ampicillin derivative that saturate all of the PBPs. Concentrations of as low as 250 nM produce at least a 90% saturation of standard PBPs. Therefore, we used this concentration in our binding experiments.

PBPs were analyzed from the fractions of the sucrose gradients (see below) by incubating a sample of each fraction with the [^{125}I]ampicillin derivative for 15 min at 37°C. Samples of the fractions were previously made to 20 mM sodium phosphate (pH 7.0) by the addition of concentrated buffer. Incubation of the mixture was stopped by the addition of a 1:4 (vol/vol) ratio sample-denaturing buffer (0.25 M Tris-hydrochloride buffer [pH 7.2] containing 0.14 M 2-mercaptoethanol, 35% [vol/vol] glycerol, 0.03% [wt/vol] bromophenol blue, 5.0% [wt/vol] sodium dodecyl sulfate [SDS]). Samples were then heated in a boiling-water bath for 5 min, and electrophoresis was as described below. Control

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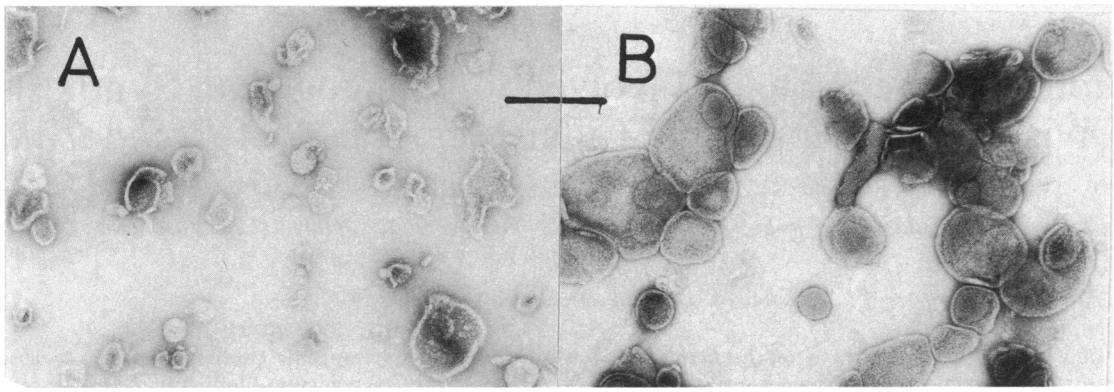

FIG. 1. Electron micrographs of vesicles from IMs (A) and OMs (B) from *E. coli* W7 cells growing exponentially. Cells were treated and fractionated as described in the experimental section. Fractions analyzed from the sucrose gradients containing either the IM or the OM were pooled. A sample from each pool was adhered to carbon-coated collodion grids and negatively stained with 2% uranyl acetate. Micrographs were taken on a Jeol JEM 100B electron microscope at a magnification of ×30,000. Bar, 0.25 μm.

experiments demonstrated that neither sucrose nor EDTA (both were used in the gradients of the method of Osborn et al. [24] for envelope fractionation; see below) interfered with the binding of the radioiodinated β-lactam to the PBPs.

Fractionation and detection of PBPs from *E. coli* by SDS-PAGE. SDS-polyacrylamide gel electrophoresis (PAGE) was performed as previously described (19), with the modifications introduced by Spratt (31). The concentration of starting acrylamide was usually 10% (wt/vol). The total distance of electrophoretic separation was 15 to 16 cm. Gels were run overnight at a constant voltage of 60 to 90 V. The thickness of the gels was 2.0 mm. After electrophoresis, the gels were fixed, stained, and destained as previously described (9). They were then dried under vacuum and exposed on prefogged Kodak X-Omat X-ray film as previously described (20). Exposure time varied from 0.5 to 7 days.

Isolation of IMs and OMs from *E. coli*. *E. coli* cells grown under various conditions (see below as well as the figure legends) were harvested by rapid cooling and centrifugation at low speed, and IMs and OMs were separated by isopycnic centrifugation in discontinuous sucrose gradients. The procedure of Osborn et al. (24) was strictly followed; full details are therefore omitted. Spheroplast breakage was accomplished by one 30-s pulse of sonic oscillation (MSE 150 W ultrasonic desintegrator MK2; MSE Scientific, Crawley, United Kingdom). Sucrose gradients were fractionated into 30 to 40 samples by means of an automatic gradient analyzer.

Residual peptidoglycan in the OM. Cells were labeled with 5 μCi of [^{3}H]2,6-*meso*-diaminopimelic acid per ml (30 Ci/mmol; CEA, Gif-sur-Ivette, France) in L medium. They were then harvested, and IMs and OMs were separated as described above. Fractions containing the OM were pooled, diluted with 50 mM sodium phosphate buffer, and centrifuged at 100,000 × *g* for 2 h. The resultant pellets were suspended, the centrifugation was repeated, and the final pellet was resuspended in potassium phosphate buffer (pH 4.9) containing 5 mg of protein per ml. The OM suspension was then incubated with "*Chalaropsis*" lysozyme (provided by M. A. de Pedro) at a concentration of 10 μg/ml for 16 h at 37°C. Control experiments were run without "*Chalaropsis*" lysozyme. The muramidase-treated OM was then centrifuged in a sucrose gradient as previously described (24). Gradients were analyzed, and radioactivity from each fraction was counted directly in Bray fluid after the appropriate dilution was added. A sample of each fraction was precipitated with 5% cold trichloroacetic acid and filtered through a glass paper filter, and the radioactivity was counted in a toluene-based scintillation liquid. PBPs were also studied in the fractions (see above).

Criteria for purity of membrane fractions. The following criteria were used for assessing the purity of IM and OM fractions: (i) measurement of succinic dehydrogenase activity in all fractions from the gradients as a marker for the IM; enzyme assays were performed as previously described (8) by using 3,5 dichlorophenol indophenol as a final electron acceptor; (ii) determination of β-NADH oxidase activity in all of the fractions from the gradients as a marker for the IM; enzyme assays were performed as previously described (25); (iii) measurement of the content of 2-keto-3-deoxyoctanoic acid as a marker for OM in all of the fractions from the gradients by the procedure described earlier (25); (iv) identification by fractionation in SDS-PAGE of the polypeptides contained in each fraction from the gradients and fractions containing the major OM proteins; and (v) transmission electron microscopy of negatively stained vesicles from pools of fractions that tentatively belonged to the IM and OM (see the legend to Fig. 1).

Chemicals. Apart from those specified above, all other chemicals used in the present work were of the highest purity commercially available.

RESULTS

Purity of IMs and OMs. The study of the membrane markers revealed the absence of cross-contamination between the IM and OM fractions by the standard method of separation (24). In all cases, succinic dehydrogenase and β-NADH oxidase activities in the OM never represented more than 5% of the total amount. Electron micrographs of both membrane fractions showed differences in the morphology of the membrane vesicles. Those from the IM (Fig. 1A) were irregular in size but generally smaller than those from the OM (Fig. 1B) which were more uniform and spherical.

PBPs in the cell envelope of *E. coli*. Figure 2 shows the PBP patterns of 25 fractions of a discontinuous sucrose gradient (24) to separate IMs and OMs from a culture of *E. coli* W7 growing exponentially at 37°C. Separation of IMs from OMs was effective because cross-contamination of membrane markers was negligible. Proteins in the fractions were also detected by gels staining (data not shown), and it was observed that the OM major proteins remained confined almost exclusively in the OM. Figure 2 clearly shows that all

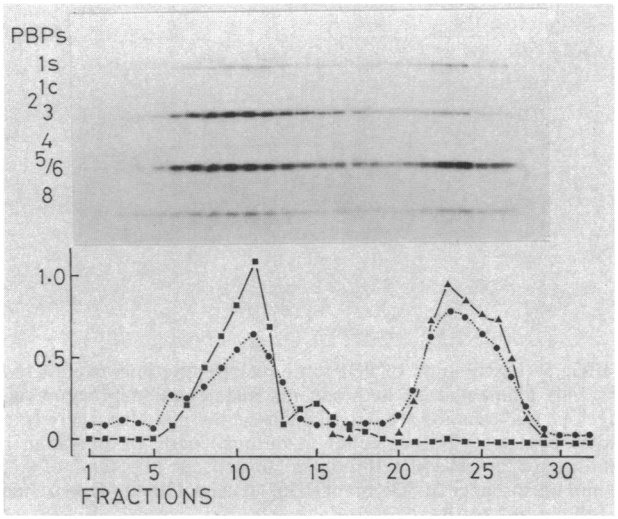

FIG. 2. Distribution of PBPs in envelope fractions from *E. coli* W7 cells growing exponentially. Exponentially growing cells in L medium at 37°C were harvested and processed for envelope fractionation (24). A total of 33 fractions were collected for the sucrose gradients. Samples from 25 fractions (those containing proteins) were analyzed for PBP content and membrane markers. Symbols: (■) succinic dehydrogenase; (▲) 2-keto-3-deoxyoctanoic acid (for the purpose of clarity symbols in which 2-keto-3-deoxyoctanoic acid was not detected are omitted); (●) protein content by measuring absorbance at 280 nm (uncorrected for light scattering). Ordinate in arbitrary units.

PBPs were also found in significant amounts in fractions corresponding to the OM. In fact, virtually all PBPs except PBP 3 were evenly distributed in the IM and the OM fractions. Only PBP 3 was mostly present in the IM, although the OM fractions still contained detectable amounts. This experiment was repeated 12 times with identical results.

Membrane separation and PBP distribution was also studied in *E. coli* W7 growing exponentially at 30 and 42°C. Results are not shown, but growth temperature did not appear to affect either envelope fractionation or PBP distribution in the various fractions.

However, a different distribution pattern of PBPs was obtained when a lower concentration of lysozyme was used for spheroplast formation. Thus, Fig. 3 shows the PBP patterns in the fractions analyzed from a sucrose gradient. For this experiment, *E. coli* W7 was grown in L medium. Cells were collected at the exponential phase of growth and treated with lysozyme at 25 μg/ml instead of the 100 μg/ml concentration used in the original procedure (24). It can be seen that a set of PBP-containing fractions appeared in the intermediate zone of the gradient. Furthermore, the 34.7-kilodalton band (tentatively known as PBP 8, as previously described [30]) was present exclusively in the OM. Unpublished results from our laboratory indicate that, when a lower concentration of lysozyme is used, incomplete peptidoglycan breakage occurs (in *E. coli* W7 labeled with [^{3}H]diaminopimelic acid), and a significant amount of acid-precipitable peptidoglycan remains associated with the intermediate fraction. Our data suggest that PBP 3 from the IM is closely associated with the peptidoglycan (see below). Moreover, with *E. coli* cells growing exponentially in citrate-salt medium, separation of IMs from OMs was not so well defined as in the case of cells growing in L medium. In this case, PBP 3 (and also PBP 5/6) again appeared in the intermediate fractions in significant amounts, whereas PBP 8 was only found in the OM (data not shown).

Distribution of PBPs in the envelopes from filamentous *E. coli*. Two kinds of filamentous *E. coli* cells were used: the PAT 84 strain (*ftsZ*) and the W7 strain treated with the β-lactam azthreonam at a concentration of 0.5 μg/ml, at which it selectively binds PBP 3 and impedes the complete formation of the septa, leaving the rest of the PBPs intact (32). PAT 84 cells grew normally at 30°C in L medium (t = 45 min), and their PBPs were distributed (Fig. 4A) in a way similar to that of *E. coli* W7 (Fig. 2). After the temperature shift to 42°C, formation of filaments occurred, and the subsequent location of the PBPs was studied. Figures 4B and C show the distribution of the PBPs during the process of cell filamentation. It can be seen that, in concomitance with cell filamentation, PBPs were mostly found to be associated with the IM and almost disappeared from the OM fractions. Similar phenomena were observed when cell filamentation was triggered by blocking PBP 3 with azthreonam. Figure 5A and B show the position of the PBPs from azthreonam-treated cells at various times after the addition of the antibiotic. In this case, there was a significant reduction of the PBPs in the OM fractions.

An interesting observation during the experiment (Fig. 5) was that, although the β-lactam antibiotic bound to a significant amount of PBP 3 within 5 min, the azthreonam only bound to PBP 3 of IM fractions. If it is assumed that during the time of the experiments there was no exchange of the PBP 3 molecules from the IM to the OM fractions and vice

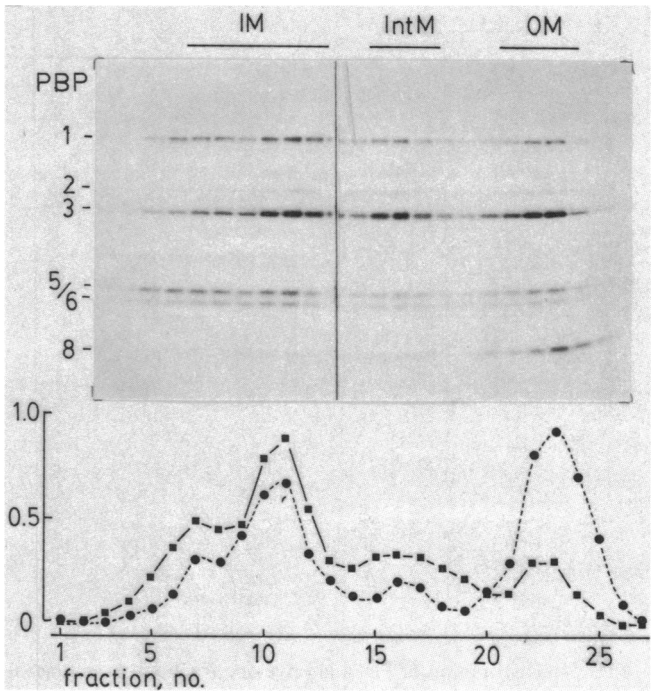

FIG. 3. Distribution of PBPs from *E. coli* W7 cells growing exponentially with low lysozyme concentrations before envelope fractionation. Conditions were the same as those described in the legend to Fig. 2, except that a concentration of 25 μg of lysozyme per ml was used instead of the 100 μg/ml concentration used in the original procedure for cell fractionation. Symbols: (■) succinic dehydrogenase activity; (●) protein content by measuring absorbance at 280 nm (uncorrected for light scattering). Ordinate in arbitrary units. IntM, Intermembrane fractions.

versa (see below), then we could postulate that PBP 3 associated with the OM was not essential for septum completion because azthreonam induced the formation of long filaments at the concentration used.

Location of the PBPs in envelopes from *E. coli* cells in the stationary phase. It was not clear from the experiments with filamentous cells described below whether the disappearance of the PBPs from the OM was due to termination of cell growth, since experiments (Fig. 4C and 5B) were performed with filamentous cells that had their growth nearly halted. Therefore, we carried out experiments to study any possible alteration in the distribution of the PBPs in the stationary phase. There was no apparent change in the distribution of the PBPs in this phase, except for PBPs 3 and 8 (Fig. 6). A previous report from our laboratory showed that PBP 3 almost disappeared from the whole envelope in the steady state (5). Strikingly, the small amount of PBP 3 associated with the OM remained, suggesting a quite different environment in the OM for this species of PBP which made it more resistant to degradation. PBP 8 also disappeared, even more rapidly than did PBP 3, and was absent from the cell envelopes 5.5 h after reaching the steady state.

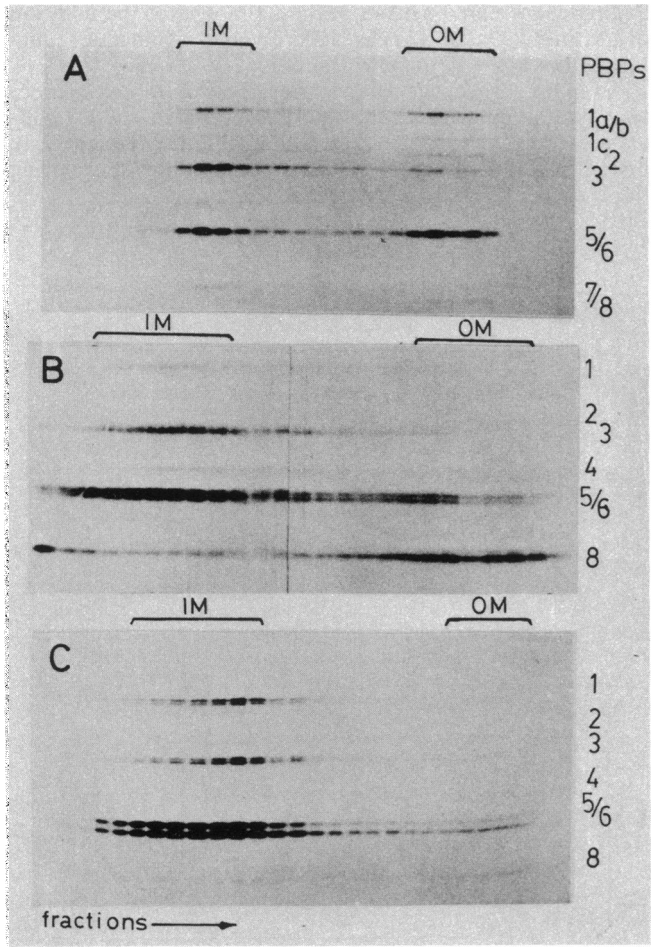

FIG. 4. Distribution of PBPs in envelope fractions from normal and filamentous cells of *E. coli* PAT 84. Cells were grown in L medium at 30°C (t = 45 min) up to an absorbance at 550 nm of 0.6. A culture sample was collected and processed as described above (see the text and the legend to Fig. 2). (A) PBP patterns of the fractions of the sucrose gradient. Another culture sample was diluted fivefold into fresh L medium prewarmed at 42°C. Cells were allowed to filament, and two samples were taken at various times; after 90 min (absorbance at 550 nm of 0.5) (B) and after 160 min (absorbance at 550 nm of 0.8) (C), and processed as described in the text.

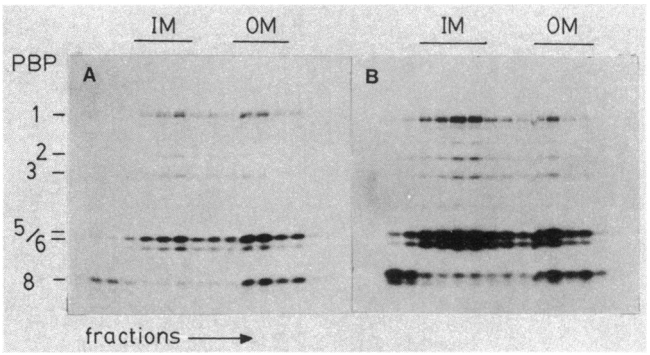

FIG. 5. Distribution of PBPs in envelope fractions from *E. coli* W7 cells filamented by azthreonam. Filamentation proceure and envelope fractionation are described in the text. Samples were taken and processed for PBP detection at various lengths of time after the exposure of the cells to azthreonam (absorbance at 550 nm of 0.12) 60 min (absorbance at 550 nm of 0.56) (A) and 130 min (absorbance at 550 nm of 1.6) (B).

OM-associated peptidoglycan. After spheroplasting, substantial amounts of peptidoglycan remained associated with the OM. When the OM was treated with "*Chalaropsis*" lysozyme (20 μg/ml) after the standard initial lysozyme treatment, ca. 10 to 20% of the residual peptidoglycan was digested. However, the position of the PBPs in the gradient did not change, suggesting that they remained attached to the OM (data not shown).

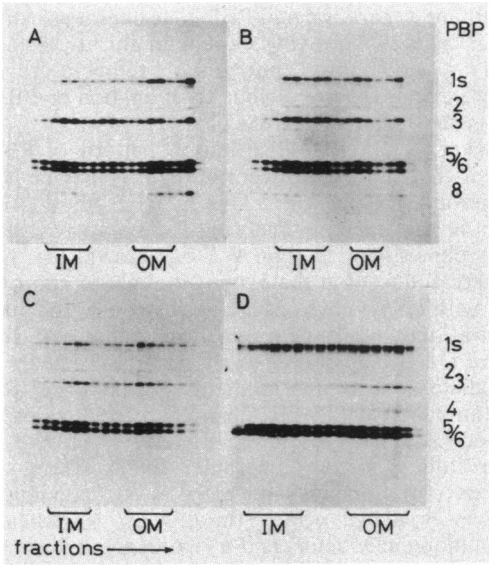

FIG. 6. PBPs in envelope fractions from *E. coli* W7 cells in the stationary phase of growth. Cells were grown at 37°C in L medium. Culture samples were taken at various times after the exponential phase was ended: 1.5 h (A), 5.5 h (B), 9.0 h (C), and 27 h (D). No cell lysis ocurred during the experiment. Cells were collected and processed for envelope fractionation and PBP detection as described in the text. 1s, PBPs 1a plus 1b.

DISCUSSION

IMs and OMs from *E. coli* and other gram-negative bacteria have been separated by using several methods, such as differential solubilization of both membrane proteins (7, 31), electrophoresis (15), and isopycnic centrifugation in sucrose gradients, based on the higher density of the OM (14, 22–25, 28). This last method has been widely used for a variety of purposes, including protein localization.

The method of membrane preparation appears to lead to differing conclusions insofar as subcellular localization is concerned. PBPs were considered to be exclusively located in the IM on the basis of their solubility in Sarkosyl (31). PBPs were evenly associated with both IMs and OMs (except PBP 8 that was mainly associated with the OM fractions) (27) by using the sucrose gradient technique of Schnaitman (28, 29). Koyasu et al. (16–18) also found PBPs to be associated with the OM from *E. coli* and *C. crescendus* by using the sucrose gradient technique of Osborn et al. (24) that we have also followed in this work.

Although we must be cautious of definitive conclusions about the location of a special protein, particularly under unusual conditions of bacterial culture (filamentation and stationary), we have followed a widely accepted method for envelope fractionation. We studied a number of membrane markers and proved that, under the standard conditions, cross-contamination was negligible and always less than 5%. However, all PBPs except PBP 3 are recovered in higher amounts with the OM fractions.

The question raised here is why PBPs would be located in the OM. We could argue that, on one hand, PBPs of *E. coli* are enzymes that catalyze energy-independent reactions. On the other hand, crystallographic analysis of the peptidoglycan layer reveals that the peptide moieties of the peptidoglycan layer are located at both sides of the glycan chains (3). Those facing the OM might be properly oriented substrates for those PBP enzymes located outside the IM.

We believe that, regardless of the nature of the links of the PBPs with the OM, a significant amount of PBP material should be considered as proper sacculus proteins that have lost their former association with the IM and can be recovered with the OM. It must be borne in mind that the spheroplasting procedure for cell breakage does not completely hydrolyze the peptidoglycan and that sizable amounts of peptidoglycan are recovered in the gradient fractions corresponding to the OM (24; unpublished data).

PBPs outside the cytoplasmic membrane could be active enzymes. They disappear from the OM in filamented cells long after their elongation has stopped. PAT 84 cells do not incorporate [^{14}C]diaminopimelic acid after 100 min at nonpermissive temperatures (26); therefore, peptidoglycan synthetic reactions may not exist in exhausted filaments.

PBP distribution in the stationary cell envelope did not seem to change apart from the disappearance of PBPs 3 and 8 as described above (5). Strikingly, the small amount of PBP 3 collected with the OM did not disappear. Doubts still remain as to whether the presence of this small amount of PBP 3 is due to actual cross-contamination or whether the protein is actually located outside the IM and is more resistant to proteolytic enzymes developed at the stationary phase of growth (6; De la Rosa, personal communication). However, when the method of Schnaitman (28) for IM and OM separation was used, around half of PBP 3 appeared to be associated with the OM (27). With this method, lysozyme is not used and peptidoglycan is broken mechanically. These data suggest that a significant amount of PBP 3 is also linked to peptidoglycan. The nature of the association of the PBPs with the sacculus could be electrostatic. Recent work has shown that the isoelectric points of the PBPs are basic or weakly acidic, ranging from 5.3 for PBP 1a to 8.3 for PBP 8 (2).

ADDENDUM

After this manuscript was written, we found that a PBP was also found in the OM of *Haemophilus influenzae* (P. M. Mendelman, D. O. Chaffin, K. D. Mack, and T. L. Stull, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, K22, p. 150).

LITERATURE CITED

1. **Arrigoni, D., and T. P. Singer.** 1962. Limitations of the fenazine methosulphate assay for succinic and related dehydrogenases. Nature (London) **193**:1256–1258.
2. **Ayala, J. A., M. A. de Pedro, and D. Vázquez.** 1984. Application of a charge/size two-dimensional gel electrophoresis system to the analysis of the penicillin-binding proteins of *Escherichia coli*. FEBS Lett. **168**:93–96.
3. **Barnickel, G., D. Naumann, H. Bradacreck, H. Labischinski, and P. Giesbrecht.** 1983. Computer aided molecular modelling of the three-dimensional structure of bacterial peptidoglycan, p. 61–66. *In* R. Hackenbeck, J.-V. Höltje, and H. Labischinki (ed.), The target of penicillin. Walter de Gruyter & Co., Berlin.
4. **Chopra, I., and S. W. Shales.** 1980. Comparison of the polypeptide composition of *Escherichia coli* outer membranes prepared by two methods. J. Bacteriol. **144**:425–427.
5. **De la Rosa, E. J., M. A. de Pedro, and D. Vázquez.** 1982. Modification of penicillin-binding proteins of *Escherichia coli* associated with changes in the state of growth of the cells. FEMS Microbiol. Lett. **14**:91–94.
6. **De la Rosa, E. J., M. A. de Pedro, and D. Vázquez.** 1983. Effect of a *rel A* mutation on the growth-dependent modification of penicillin-binding protein 3 in *Escherichia coli*. FEMS Microbiol. Lett. **19**:165–167.
7. **Diedrich, D. L., A. O. Summers, and C. A. Schnaitman.** 1977. Outer membrane proteins of *Escherichia coli*. V. Evidence that protein 1 and bacteriophage-directed protein 2 are different polypeptides J. Bacteriol. **131**:598–607.
8. **Dobrogozs, W. J.** 1981. Enzymatic activity, p. 365–392. *In* P. Gerhardt (ed.), Manual of methods for general microbiology. American Society for Microbiology, Washington, D.C.
9. **Fairbanks, G., T. L. Steck, and D. H. F. Wallach.** 1975. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry **10**:2606–2617.
10. **Filip, C., G. Fletcher, J. L. Wulff, and C. F. Earhart.** 1973. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. J. Bacteriol. **115**:717–722.
11. **Hall, M. N., and T. J. Silhavy.** 1981. Genetic analysis of the major outer membrane proteins of *Escherichia coli*. Annu. Rev. Genet. **15**:91–142.
12. **Hartmann, R., J.-V. Höltje, and U. Schwarz.** 1972. Targets of penicillin action in *Escherichia coli*. Nature (London) **235**: 426–429.
13. **Hirota, Y., A. Ryter, and F. Jacob.** 1968. Thermosensitive mutants of *E. coli* affected in the processes of DNA synthesis and cellular division. Cold Spring Harbor Symp. Quant. Biol. **33**:677–692.
14. **Ito, K., T. Sato, and T. Yura.** 1977. Synthesis and assembly of the membrane proteins in *E. coli*. Cell **11**:551–559.
15. **Joseleau-Petit, D., and A. Kepes.** 1975. A novel electrophoretic fractionation of *Escherichia coli* envelopes. Biochim. Biophys. Acta **406**:2504–2507.
16. **Koyasu, S., A. Fukuda, and Y. Okada.** 1980. The penicillin-binding proteins of *Caulobacter crescentus*. J. Biochem. **87**:363–366.
17. **Koyasu, S., A. Fukuda, and Y. Okada.** 1981. Properties of the penicillin-binding proteins of *Caulobacter*. J. Gen. Microbiol. **126**:111–121.

18. **Koyasu, S., A. Fukuda, and Y. Okada.** 1982. Penicillin-binding proteins in the soluble fraction of *Caulobacter crescentus*. J. Gen. Microbiol. **128**:1117–1124.
19. **Laemmli, U. K., and M. Favre.** 1973. Maturation of the head of bacteriophage T4. DNA packaging events. J. Mol. Biol. **80**:575–599.
20. **Laskey, R. A., and A. D. Mills.** 1975. Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. Eur. J. Biochem. **56**:335–341.
21. **Lennox, E. S.** 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1**:190–206.
22. **Miura, T., and S. Mizushima.** 1968. Separation by density gradient centrifugation of two types of membranes from spheroplast membrane of *Escherichia coli*. Biochim. Biophys. Acta **150**:159–161.
23. **Miura, T., and S. Mizushima.** 1969. Separation and properties of outer and cytoplasmic membranes in *Escherichia coli*. Biochim. Biophys. Acta **193**:268–276.
24. **Osborn, M. J., J. E. Gander, E. Parisi, and J. Carson.** 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. J. Biol. Chem. **247**:3962–3972.
25. **Osborn, M. J., and R. Munson.** 1974. Separation of the inner (cytoplasmic) and outer membranes of gram-negative bacteria. Methods Enzymol. **31**:642–653.
26. **Ricard, M., and Y. Hirota.** 1973. Process of cellular division in *Escherichia coli*: physiological study on thermosensitive mutants defective in cell division. J. Bacteriol. **116**:314–322.
27. **Rodríguez-Tébar, A., J. A. Barbas, and D. Vázquez.** 1983. Distribution of penicillin-binding proteins within the cell envelope of *Escherichia coli*, p. 427–432. *In* R. Hackenbeck, J.-V. Höltje, and H. Labischinski (ed.) The target of penicillin. Walter de Gruyter & Co., Berlin.
28. **Schnaitman, C. A.** 1970. Examination of the protein composition of the cell envelope of *Escherichia coli* by polyacrylamide gel electrophoresis. J. Bacteriol. **104**:882–889.
29. **Schnaitman, C. A.** 1970. Protein composition of the cell wall and cytoplasmic membrane of *Escherichia coli*. J. Bacteriol. **104**:890–901.
30. **Schwarz, U., K. Seeger, F. Wengenmayer, and H. Stretcher.** 1981. Penicillin-binding proteins of *Escherichia coli* identified with a ^{125}I-derivative of ampicillin. FEMS Microbiol. Lett. **10**:107–109.
31. **Spratt, B. G.** 1977. Properties of the penicillin-binding proteins of *Escherichia coli* K 12. Eur. J. Biochem. **72**:341–352.
32. **Sykes, R. B., D. P. Bonner, K. Bush, and N. H. Georgopapadakou.** 1982. Azthreonam (SQ 26,776), a synthetic monobactam specifically active against aerobic gram-negative bacteria. Antimicrob. Agents Chemother. **21**:85–92.
33. **Vogel, H. G., and D. H. Bonner.** 1956. Acetylornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218**:97–106.
34. **Waxman, D. J., and J. L. Strominger.** 1983. Penicillin-binding proteins and the mechanism of action of β-lactam antibiotics. Annu. Rev. Biochem. **52**:825–869.