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Topographical Distribution of Penicillin-Binding Proteins in the *Escherichia coli* Membrane

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The penicillin-binding proteins (PBPs) found in the membranes of *Escherichia coli* X925 minicells (primarily cell ends or septa) were compared with those found in rod-shaped cells (primarily sidewalls) in an effort to determine whether certain PBPs are unevenly distributed over the bacterial cell membrane. The seven major PBPs of *E. coli* were all present in minicell membranes. PBP 1B was altered in minicells, however, appearing as two bands on sodium dodecyl sulfate-polyacrylamide gels rather than the usual three. PBP 2, which is needed for longitudinal growth of the cell but not for septum formation, was significantly reduced in minicell membranes. This observation is consistent with the fact that minicells contain very little sidewall material and raises the possibility that the specialized function of PBP 2 may be determined or regulated by its uneven topographical distribution in the membrane. None of the PBPs appeared to be selectively enriched in minicell membranes.

Membrane proteins that covalently bind penicillin have been found in a variety of bacterial species (7, 24-26, 30, 33, 38). These proteins are not major components of the cell membrane, however, and probably do not have a structural role. Genetic and biochemical evidence suggests that at least some of the penicillin-binding proteins (PBPs) could be enzymes involved in various penicillin-sensitive processes such as peptidoglycan synthesis, septum formation, and maintenance of a cell's rod shape (6, 14, 28-30, 34, 36). Hence, a thorough characterization of the PBPs should provide some important details about the mechanism and regulation of bacterial cell growth and division.

There are at least seven major PBPs located in the inner membrane of *Escherichia coli* (30). In no case has the physiological function of one of these PBPs been unambiguously determined. Biochemical analyses of partially purified PBP 1B have shown that the protein has transglycosylase as well as transpeptidase activities (23). Only the latter activity is penicillin-sensitive. Other evidence suggests that PBP 1A may compensate in vivo for the loss of PBP 1B (34-36). It is not known which of the two enzyme activities, if either, is essential for peptidoglycan synthesis.

Biochemical and genetic analyses have revealed that PBP 4 corresponds to D-alanine carboxypeptidase IB, an enzyme that has endopeptidase activity as well (17, 19, 37). PBP 5 corresponds to the major carboxypeptidase IA (20, 32). PBP 6, which copurifies with PBP 5, also may have some carboxypeptidase IA activity. Although it is likely that some carboxypeptidase activity is needed for normal wall growth or septum formation (21, 22), there is no information to suggest that the activity must be provided by any particular one of these three PBPs.

PBPs 2 and 3 have no known enzymatic activity, but significant progress has been made in determining their in vivo functions. Cells in which PBP 2 has been inactivated, either by mutation or by the specific action of the β-lactam antibiotic mecillinam, grow as round or ovoid cells (16, 31, 34). Therefore, PBP 2 must perform an essential role in determining the cell's rod shape. PBP 3, on the other hand, may be specifically required for septum formation since its inactivation by mutation or by certain β-lactam antibiotics such as cephalexin or piperacillin leads to formation of long nonseptate filaments (15, 29).

One approach to a better understanding of how the PBPs may function in vivo is to determine their topographical distribution within the cell membrane. A protein that is specifically involved in septum formation, for example, might be concentrated in the membrane at the septum. Alternatively, a protein that is more essential to the longitudinal growth or the rod shape of the cell might be relatively more abundant in the sidewalls than in the septal area. Thus, PBPs in the membranes from spherical minicells (primarily cell ends or former septa) were compared with those from rod-shaped cells (primarily sidewalls).

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* strains X925 and X975 were obtained from R. Curtiss

(10). X975 was formerly named P678. Its genotype is F⁻ *thr ara leu azi tonA lacY supE gal minB rpsL malA xyl mtl thi*. X925 was formerly named P678-54. It has the same genotype as X975 except that it is also *minA*. The combination of the *minA* and *minB* mutations leads to minicell production by X925 (10).

Both strains were grown in M9 basal salts (1) supplemented with 0.6% glucose, 10 mg of thiamine per liter, and 1.5% Casamino Acids (Difco). Usually, 3-liter cultures of each strain were grown overnight at 37°C in an incubator shaker.

Minicell and cell purification. X925 whole cells and minicells were harvested and purified essentially as described by Frazer and Curtiss (9). The initial separation of X925 whole cells (the rod-shaped cells that occasionally produce a minicell) from the minicells was by differential centrifugation. Primarily whole cells were obtained in the pellet from a 5-min centrifugation at 1,000 × *g*. The minicells in the supernatant were harvested by centrifugation for 15 min at 11,000 × *g*. The crude minicell preparation was suspended in a small volume of buffered-saline gelatin (BSG) (9), blended at top speed for 2 min in a Vortex mixer, and then layered on top of 12 32-ml sucrose step gradients (8 ml each of 5, 10, 15, and 20% sucrose [wt/vol] in BSG). The sucrose gradients were centrifuged for 15 min at approximately 2,700 × *g* in an IEC swinging-bucket rotor no. 269. The visible band of minicells which formed in the top one-third of each gradient was removed with a syringe and slowly diluted with an equal volume of BSG. This minicell preparation was concentrated by centrifugation, again resuspended in a small volume of BSG, blended vigorously in a Vortex mixer, and then layered on four 5-to-20% sucrose linear gradients which were centrifuged as before. The minicells removed from the second set of gradients were slowly diluted with an equal volume of BSG and assayed for their purity. The number of contaminating whole cells was determined by viable cell counts on nutrient agar plates. The number of minicells per milliliter was determined with a Petroff-Hausser counting chamber and a phase-contrast microscope. No minicell preparation was used which had more than one colony-forming unit per 10^6 minicells.

The purified minicells and the whole cell preparations were stored as cell pastes at −20°C for no more than 1 week.

Membrane preparation. Frozen cells were thawed and suspended in approximately 3 ml of cold 0.05 M sodium phosphate buffer, pH 7.0, containing 0.14 M 2-mercaptoethanol and 2 drops of tributyl citrate, an antifoaming agent. The cells were subjected to 30-s pulses with a model 185 Branson Sonifier until the optical density of the cold cell suspension was reduced by approximately 70%.

Intact cells were removed from the sonicated suspension by centrifugation as described previously (30). The torn cell envelopes in the supernatant were then collected by ultracentrifugation at 100,000 × *g* for 40 min in a Beckman type 42.1 rotor. The pellet was resuspended in a small volume of the above buffer (without mercaptoethanol and antifoam) to a concentration of approximately 20 mg of protein per ml and stored at −20°C.

Detection of PBPs. Benzyl [^{14}C]penicillin (Amersham Corp., 58 mCi/mmol) was bound to *E. coli* membranes as described by Spratt (30). After solubilization of the inner membrane proteins with Sarkosyl (ICN Pharmaceuticals, Inc.) (8), the proteins were separated from one another by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with the modifications described by Suzuki et al. (34). The sodium dodecyl sulfate, acrylamide, *N,N'*-methylene-bisacrylamide, and molecular weight standards were obtained from Bio-Rad Laboratories. After electrophoresis, the gel was stained and destained (6) for observation of the inner membrane protein profiles. Usually, the stained gel was photographed with Plus-X film (Kodak) and an orange filter. Finally, the slab gel was treated so that the radioactive proteins could be detected by fluorography (3).

Quantitation of PBPs. To quantitate the PBPs, the fluorograph (after 35 to 40 days of exposure to the dried gel) was scanned with a soft laser-scanning densitometer which had a built-in recorder and integrator (Biomed Instruments, Inc.). The amount of each PBP was originally expressed in arbitrary units of optical density. These values were affected by the time of exposure of the gel to the film and by any variation in the darkroom procedure, such as the age and temperature of the X-ray developing solution. Thus, it was inappropriate to compare directly the optical density values obtained from scanning two different fluorographs.

Two approaches were used to quantitatively summarize and compare the results from different fluorographs. First, the ratio of penicillin bound by a minicell PBP to the amount bound by the corresponding whole cell PBP on the same fluorograph was calculated by using the optical density of each protein's fluorographic image. The ratios from different fluorographs could then be averaged to yield a meaningful comparison of the absolute amount of penicillin bound by minicell and whole cell PBPs (Table 1).

In a second approach, the optical density data were used to calculate the amount of penicillin bound by each PBP relative to the total bound by PBPs 1A to 4. Then, the average and the standard deviation of these relative values taken from different fluorographs were determined (Table 2). Although some variation in the assays was inevitable, the standard deviations were not so great as to obscure what were probably true differences (or similarities) among the different membrane samples.

TABLE 1. *Comparison of the absolute amount of penicillin bound by each PBP*

PBP	Minicell/whole cell ratio[a]
1A	1.39 ± 0.53
1B	1.57 ± 0.57
2	0.31 ± 0.22
3	2.26 ± 1.64
4	1.87 ± 1.10

[a] Each value is the average of eight separate assays ± one standard deviation. The optical density of a minicell PBP was divided by the optical density of the corresponding whole cell PBP measured on the same fluorograph.

TABLE 2. *Relative amounts of E. coli PBPs 1A to 4*[a]

PBP	X975 parent cells ($n = 4$)[b]	X925 whole cells ($n = 11$)	X925 minicells ($n = 8$)
1A	33.2 ± 4.5	35.4 ± 2.9	34.1 ± 2.6
1B	35.0 ± 4.7	37.4 ± 4.2	42.6 ± 7.7
2	9.2 ± 1.5	8.4 ± 2.0	1.6 ± 0.7
3	1.9 ± 1.0	2.4 ± 1.4	2.9 ± 1.4
4	20.6 ± 6.0	16.1 ± 3.0	18.2 ± 8.4

[a] Percentage of total PBPs 1A to 4 ± one standard deviation.

[b] The number of separate assays is shown within parentheses.

RESULTS AND DISCUSSION

The seven major PBPs of *E. coli* were all present in minicell as well as whole cell membranes (Fig. 1). However, PBP 1B appeared to be altered in the minicell membranes since it consistently migrated as two bands on gels instead of the usual three. It is not known why PBP 1B normally appears as a triplet. In fact, there is strong genetic evidence to suggest that all three proteins are products of the same structural gene. PBP 1B mutants lose all three components simultaneously, and true revertants and transductants always recover all three (34, 36). One mutant was isolated in which the three bands of PBP 1B moved en masse to a lower spot on the gel (34). These minicell results are the first reported example in which the PBP 1B components have not been altered as a unit.

Despite the absence of a major member of the PBP 1B triplet, the actual amount of penicillin bound by the minicell's PBP 1B was the same as or even greater than the amount bound by cells having the complete set (Table 1). This suggests that the missing protein might not actually be absent, but might be altered (processed?) so that it migrates slightly further down on the gel to coincide with what is normally the second band of the triplet. Whether this change represents a loss, a gain, or no change in the enzyme activity of PBP 1B is not known. Such information could prove useful in understanding the relationship of the three PBP 1B proteins to one another.

The total amount of penicillin bound by minicell membranes was usually greater than the amount bound by membranes prepared from the whole cells that produced the minicells (Table 1 and Fig. 1). However, the relative amount of penicillin bound by the individual PBPs of the two types of membranes was quite similar, with the exception of PBP 2 (Table 2). Thus, no particular one of the PBPs was responsible for the higher penicillin-binding capacity of minicell membranes. This means that PBP 3, the protein

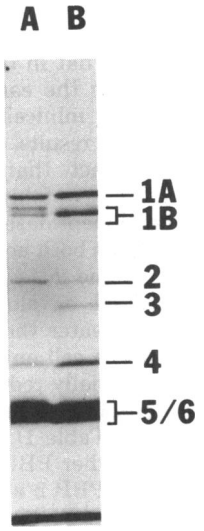

FIG. 1. *PBPs 1A to 6 of E. coli analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. Approximately the same amount of protein was added to each slot. A. X925 whole cell proteins. B. X925 minicell proteins.*

believed to be essential for septum formation, is not located exclusively or even selectively concentrated in the cell membrane of the septum. The possibility of a much greater but transient concentration of the protein in the septal region followed by its relatively rapid diffusion all around the cell surface has not been strictly excluded.

Quantitative analysis of PBPs 5 and 6 could not be done on the same piece of X-ray film with the other PBPs. There is so much more of these two proteins that it was necessary to expose the gel for a shorter time to obtain accurate measurements of their images on a fluorograph. In addition, it was usually not possible to completely separate PBP 5 from PBP 6 on most gels, so they could not be measured individually except by visual inspection. Consequently, the data for PBPs 5 and 6 have not been included with the others. In any case, no remarkable variations among the different membrane preparations were observed. The amount of penicillin bound by PBP 5 appeared to be roughly the same as that bound by PBP 6 in both the minicell and whole cell samples. The absolute amount bound was similar in the two samples as well.

Goodell and Schwarz (11) found that the specific activity of carboxypeptidase was twofold greater in minicell membranes than in rod cytoplasmic membranes. Those results cannot be correlated here with an increase in PBP 5 or PBP 6 in the minicell membranes, although these two PBPs correspond to a carboxypepti-

dase (32). On the other hand, the absolute amount of PBP 4 in minicells was found to be 1.87 times higher than that in the X925 whole cells (Table 1). Perhaps the carboxypeptidase IB activity of PBP 4 in minicells accounts for the previously reported results. This would be consistent with the fact that Goodell and Schwarz also detected a twofold increase in the specific activity of endopeptidase in minicell envelopes. Only PBP 4 has both activities (37).

Although many of the *E. coli* PBPs were present in the minicell envelope fraction in amounts equal to or greater than those which were found in the whole cell envelopes, PBP 2 appeared to be substantially reduced. This was true in terms of the absolute amount of penicillin bound by the protein (Table 1) or the amount bound relative to the other PBPs in the membrane (Table 2). Thus, PBP 2 not only seemed diminished because other PBPs in the minicell membranes were slightly enriched, whereas PBP 2 was not, but also because there was actually less of it in minicells than in rod-shaped cells. Since minicells are derived primarily from the ends (former septa) of rod-shaped cells, this finding may reflect the unequal distribution of this PBP in the ends and sidewalls of the cell. This would be consistent with data from other laboratories which suggest that the primary function of PBP 2 is maintenance of the cell's rod shape and participation in synthesis of the peripheral walls (16, 31, 34). It follows, then, that there might be less of this protein in minicell envelopes that contain little, if any, peripheral wall material.

The observation that a protein is absent or diminished in minicells must be interpreted with caution for at least two reasons. First, the purification of minicells and preparation of their membranes is not identical to the whole cell treatment. The minicells have been subjected to at least two sucrose gradients, which raises the possibility that some membrane proteins are lost due to osmotic shock. Furthermore, the minicells are more refractory than whole cells to sonication and must be sonicated two or three times longer to be disrupted. Lengthy sonication could potentially inactivate or release certain membrane proteins. In light of these possible problems, it is interesting to note that PBPs 1A, 1B, 3, and 4 are actually more abundant in minicell membranes than in an equivalent amount of whole cell membranes. If the diminished amount of PBP 2 in minicells is due to the treatment described above, it would suggest that some fraction of this protein is more loosely associated with the membrane than are the other PBPs. Although this seems unlikely, further experimentation is needed to rigorously exclude it as a possibility.

There is a second possibility that must be considered. Although minicells usually contain no DNA and never divide, they continue to be metabolically active for an indefinite length of time after their formation (2, 27). Thus, deterioration or aging of some proteins would not be unexpected. If a protein is lost from a minicell it cannot be replaced since a minicell lacks the genetic information to synthesize new copies. However, this is probably not the explanation for the reduced amount of PBP 2 in minicell membranes. When the stability of the PBPs was measured in *E. coli* cells (5), none of them was found to be uniquely unstable. Their lifetime in the membrane seems to be no shorter than that of the majority of membrane proteins. Thus, the reduced amount of PBP 2 found in minicells probably does reflect the protein's uneven topographical distribution in the *E. coli* membrane.

Although the PBPs could only be detected by fluorography, all gels were routinely stained and destained first. This provided the opportunity to observe any gross differences between the inner membrane preparations from minicells and whole cells. Some variations in the relative amounts of the proteins and even in the presence or absence of certain proteins were noted. Although most variations were not reproducible, some of the differences between the membrane protein profiles of X925 minicells and X925 whole cells were significant (compare lanes B and C in Fig. 2). The visible differences did not correspond to any of the PBPs, however, since the PBPs are very minor proteins and are not detectable on a stained gel. Goodell et al. (12) have also examined the protein composition of minicell membranes. They found that a protein with a molecular weight of approximately 48,000 was reproducibly enriched in the inner membrane of minicells. The results reported here do not confirm that finding. Instead, the most reproducible observation is that there is a major reduction in minicell membranes of a protein or protein doublet corresponding to a molecular weight of approximately 113,000 (Fig. 2). This particular difference is most striking when the cells are harvested from overnight cultures grown in minimal medium. The role this missing protein may play in normal septum location or minicell formation would be purely speculative at this time.

It should be noted that there were no major differences between the membrane proteins of *E. coli* whole cells that produce minicells and the parent cells that never produce minicells (compare lanes A and B in Fig. 2). This suggests that the *minA* mutation in the minicell-producing mutant X925 may have no effect on the

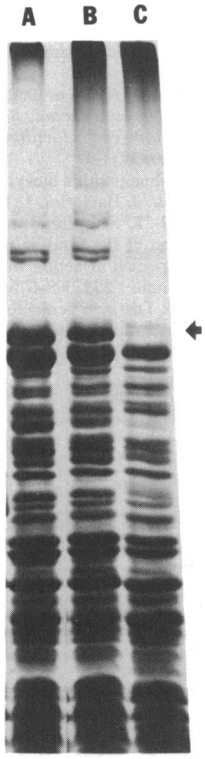

FIG. 2. *Coomassie brilliant blue-stained sodium dodecyl sulfate-polyacrylamide gel of the membrane proteins from E. coli strain X975 (lane A), X925 whole cells (lane B), and X925 minicells (lane C). The arrow points to the most major reproducible difference between the minicell membranes and the others. The indicated band has a molecular weight of approximately 113,000.*

distribution of the major proteins in the *E. coli* inner membrane. This is in sharp contrast to the effect of the *divIV-B1* mutation, which leads to minicell production and significant membrane alterations in *Bacillus subtilis* (4).

The major premise upon which this study was based is that minicell envelopes are representative of relatively pure septum material. Only one-half of the minicell is actually derived from a fresh septum; the other half corresponds to one pole of the mother cell and therefore is an old septum. There is no way to determine exactly how similar the minicell envelope composition is to the septum that normally grows across the center of a rod-shaped cell. However, given the fact that the actual synthesis, regulation of synthesis, and ultrastructure of the septum that forms the minicell are the same as those of a normally located septum, the use of minicell material as a first approximation of normal septum material seems valid (10, 18).

Although some comparisons have previously been made between *E. coli* minicell envelopes and whole cell envelopes (11–13, 39), this is the first time the PBPs have been measured in the minicell system. All of the PBPs were detectable in minicell membranes. None of them appeared to be selectively enriched in minicells, however. This does not exclude the possibility of a transient enrichment for a particular protein, which may only be evident when assays are done on synchronized cells harvested at various times in the cell cycle. Only PBP 2, which is needed for elongation of the cell but not for septum formation, was significantly reduced in minicell membranes. This result raises the possibility that the specialized function of PBP 2 may be determined or regulated by its uneven topographical distribution in the membrane. On the other hand, location of the protein primarily where it is needed could be a matter of conservation. It has been estimated that there are only 20 molecules of PBP 2 per cell (30). Unless the protein is completely free to diffuse rapidly upon demand to specific sites in the membrane, it is reasonable that there may not be very much of it bound to regions where it is probably not required.

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