Separation of *Escherichia coli* Penicillin-Binding Proteins into Different Membrane Vesicles by Agarose Electrophoresis and Sizing Chromatography

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Membrane vesicles from the envelope of *Escherichia coli* were separated by electrophoresis through dilute agarose and by sizing chromatography through Sephacryl S-1000. These techniques revealed that proteins were associated with different subsets of vesicles. In particular, dilute agarose electrophoresis clearly separated the inner membrane penicillin-binding proteins (PBPs) into different vesicle groups. Vesicles containing PBPs 4, 6, 7, and 8 migrated rapidly through agarose; vesicles with PBPs 1a, 1b, 2, 3, and 5 eluted later. With the exception of PBP 4, which migrated with PBPs 1 through 5, chromatography through Sephacryl S-1000 was able to distinguish the same two vesicle sets, though the extent of separation was poorer than with agarose. The existence of these associations among vesicles and proteins suggests that there is an organization to the inner membrane of *E. coli* which is not observed when membrane vesicles are separated solely on the basis of density in sucrose gradients.

It is not known how a bacterial cell organizes proteins in its membrane so that site-specific events, such as cell division, can be localized. Distinct membrane regions or domains appear to exist which may be specially involved with cell elongation, division, or maintenance of shape (2, 5, 6, 10, 13, 16). For example, electron microscopic observations suggest two circumscribed areas within the inner membrane to which proteins might be specifically localized: zones of adhesion, points where the inner and outer membranes seem to be contiguous (4), and the periseptal annulus, predicted to be the forerunner of septal division sites (reviewed in reference 31).

Membrane vesicles from broken cells have been fractionated to study these and other possible organized domains. Inner and outer membranes from gram-negative bacterial envelopes have been separated from one another on the basis of density by sucrose gradient ultracentrifugation (20, 24, 33, 34). By using this general approach, inner membrane fragments from *Escherichia coli* exhibited asymmetries in protein and lipid composition (10, 13, 15). For example, Ishidate et al. used a two-step sucrose separation protocol with which they were able to isolate four or five vesicle populations with different protein profiles (13). With a shallow gradient, Jacoby and Young found penicillin-binding proteins (PBPs) asymmetrically distributed into more than one set of inner membrane vesicles (15).

Vesicles with different compositions but similar sucrose sedimentation properties would not be separated by the approaches described above. It is therefore desirable to supplement the classical sucrose density gradient method with techniques which separate vesicles on the basis of other, independent physical characteristics. One attempt to do this used free-flow electrophoretic fractionation stabilized by a sucrose gradient (16, 23, 25, 41). This technique

separated inner and outer membranes but required unusual equipment and has not been widely used.

We describe two additional methods of fractionating bacterial membrane vesicles: electrophoresis through dilute agarose and sizing chromatography through Sephacryl S-1000. With these techniques, we observed heterogeneity in the way proteins were associated with vesicles of *E. coli*. This was especially true for the inner membrane PBPs. Modification and use of these additional methods of vesicle fractionation furnish more evidence for substructure within the bacterial inner membrane and extend our ability to explore this organization.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* CS109 (W1485 F⁻) was provided by K. Catron and C. Schnaitman, University of Virginia, Charlottesville. *E. coli* ED20 (relevant genotype, *minB1 zcf-117::Tn10*) was supplied by P. de Boer, University of Connecticut Health Center, Farmington. *E. coli* JX2 (CS109 *minB1 zcf-117::Tn10*) was constructed by moving *minB1* and the linked *Tn10* insertion from ED20 into CS109 by P1 cotransduction (19), selecting for tetracycline resistance, and screening microscopically for the minicell phenotype (9). Cells were grown at 37°C in minimal medium consisting of M9 plus 0.2% glucose (19).

Preparation of bacterial membrane vesicles. Bacteria were grown in 400 ml of M9-glucose medium to an A_{550} of 0.4 and were harvested by centrifugation at $10,000 \times g$ in a GSA rotor (Ivan Sorvall, Inc., Norwalk, Conn.) for 20 min at 4°C. Cells were concentrated 200-fold by suspending the pellet in 2 ml of 0.05 M MES, pH 6.5 [2-(*N*-morpholino)ethanesulfonic acid; U.S. Biochemical Corp., Cleveland, Ohio]. Cells were ruptured at $16,000 \text{ lb/in}^2$ by a single passage through a chilled (4°C) French pressure cell (SLM Aminco, Urbana, Ill.). Crude lysate was cleared of cell debris and unbroken cells by centrifugation at 4°C for 4 min at $12,000 \times g$ in an Eppendorf centrifuge.

Dilute agarose electrophoresis. The procedure was adapted from that of Kedersha and Rome (17) (see schematic diagram

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Separate Vesicles By Electrical Charge (Through Agarose)

FIG. 1. Dilute agarose electrophoresis. (A) Schematic diagram (redrawn from Kedersha and Rome [17], with permission). The 0.15% agarose slab is held in place by a 1% agarose frame around the sides and bottom (not shown). Electrophoresis is toward the cathode. (B) Wells into which the vesicle sample is loaded and from which fractions are eluted. The milled acrylic block is suspended over the gel box so that the screened portion enters the 0.15% agarose. Each screen is attached to the lower posts by 1% agarose. An acrylic spacer is inserted between the screens through the top opening to exclude agarose during gelation of the 0.15% agarose. The spacer is removed, leaving the screens to form the well into which sample will be loaded.

in Fig. 1A). Melted 1% FMC SeaKem HGT agarose (FMC Bioproducts, Rockland, Maine) was poured into a horizontal agarose gel electrophoresis apparatus (R. Shadel, San Francisco, Calif.), and an acrylic mold (G. DuBuque, University of North Dakota) was inserted to create an agarose "swimming pool" frame and bottom surrounding an empty rectangular central well (3 3/8-in. [ca. 8.6 cm] length, 2 5/8-in. [ca. 6.7 cm] width, 9/16-in. [ca. 1.4 cm] height). After the 1% agarose frame had hardened, the acrylic block was removed, melted 0.15% Isogel agarose (FMC Bioproducts) was poured into the central well and allowed to solidify for 2 h at 4°C, and sample and elution troughs were cut into the 0.15% gel (4 cm between them) as described elsewhere (17) (Fig. 1A). Ficoll sample buffer (1/6 volume) (18) was added to the vesicle preparation, and 300 μ l was loaded into the trough nearest the anode. Buffer (0.05 M MES, pH 6.5) was added to both ends of the gel apparatus and to the elution trough, up to the level of the gel top. Electrophoresis was performed at 4°C at 23 V constant voltage. After 10 to 12 h of electrophoresis, buffer in each of the end reservoirs was replaced with fresh buffer, and electrophoresis was continued.

Needles (20 gauge) with attached tubing were inserted at both ends of the elution trough. A pump fed buffer through one needle and pulled buffer into the other (at 6 ml/h) to sweep material moving into the trough to a fraction collector. Typically, 30-min (3-ml) fractions were collected. In approximately 6 h, the bromphenol blue dye front moved into the elution comb, and after 36 h the membranes had moved from the gel into the elution trough. Fraction 1 was defined as that tube containing the dye front peak, as determined by A_{550} . Approximately 48 fractions were collected for analysis.

In experiments performed later in this study, we found it more convenient to modify slightly the procedure of Kedersha and Rome (17), as follows. Specially machined combs were constructed into which sample was loaded and eluted (Fig. 1B). Up to 1 ml of sample could be loaded into the rectangular space between two polypropylene screens (1-mm pore size; Fisher Scientific Co., Eden Prairie, Minn.). These screens supported the fragile 0.15% Isogel agarose during loading and suppressed erosion of the elution trough

over time. We also found it more effective to recirculate the electrophoresis buffer continuously, taking care that the buffer level did not overflow onto the surface of the gel. This was most easily achieved by implanting two glass tubes into the 1% agarose frame and pumping buffer from one reservoir to the other so that the return flow through the tubes moved toward the cathode (as pictured in reference 11).

Sephacryl S-1000 chromatography. The procedure was adapted from Reynolds et al. (28). Sephacryl S-1000 (Sigma Chemical Co., St. Louis, Mo.) was coated with phosphatidylcholine (50 mg/ml in 0.05 M MES, pH 6.5; Sigma) at room temperature for at least 2 h and washed with several changes of MES buffer. The coated Sephacryl was poured into a column (1 by 50 cm) and packed at 4°C for 48 h with 0.05 M MES, pH 6.5, at a flow rate of 10 ml/h. Membrane vesicles (500 μ l of cleared lysate) were loaded onto the column and chromatographed at 6 ml/h in 0.05 M MES (pH 6.5), and 3-ml fractions were collected. Fraction 1 was defined as the first fraction after the column void volume was discarded.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and staining. Membrane vesicles were collected from agarose or Sephacryl fractions by ultracentrifugation at 60,000 rpm (240,000 $\times$ g) for 1.25 h at 4°C in a 70.1 Ti rotor (Beckman Instruments, Inc., Palo Alto, Calif.). The pellet was rinsed twice with 3 ml of 0.05 M MES (pH 6.5), suspended in 100 μ l of 1 $\times$ SDS loading buffer, and boiled for 4 min, and 15 to 20 μ l was loaded onto 10 to 18% gradient SDS-acrylamide gels, as described by Dreyfuss et al. (12). Molecular mass markers (Sigma) were treated similarly. Samples were electrophoresed at 100 V until the samples entered the separating gel and then at 200 V until 15 min after the dye front ran off the bottom (approximately 7 h total). Gels were silver stained by the method of Morrissey (21).

Other methods. Protein concentrations were determined by the bicinchoninic acid method of Smith et al. (35), using bovine serum albumin as a standard. For enzyme assays and labeling of PBPs, fractions from agarose electrophoresis or Sephacryl chromatography were concentrated from 3 ml to 45 μ l by centrifugal filtration through Centricon-30 microconcentrators (Amicon Corp., Lexington, Mass.) (15). NADH oxidase and lactate dehydrogenase were assayed as described by Osborn et al. (24). Succinate dehydrogenase

FIG. 2. Distribution of membrane proteins in envelope fractions separated by dilute agarose electrophoresis. Membrane vesicles of *E. coli* CS109 were prepared and separated as described in Materials and Methods. Membranes in each fraction were collected by ultracentrifugation and suspended in 100 μ l of SDS sample buffer, and 20 μ l was loaded to a 10 to 18% SDS-polyacrylamide gel. Proteins were silver stained by the method of Morrissey (21). Fraction numbers appear above the lanes, each representing the material which eluted from the agarose in 30 min. Molecular mass standards (lanes Hi and Lo) are included, with their masses given in kilodaltons (Kd).

was assayed by preincubating each fraction with 25 mM succinate for 25 min (1) before continuing the procedure described by Osborn et al. (24). β -Mercaptoethanol (5 mM final concentration) was added to each concentrated fraction, and the PBPs were labeled with 125 I-penicillin X (17 μ g/ml) and visualized by autoradiography, as previously described (15). Competition of unlabeled penicillin derivatives for specific PBPs was assayed by incubation for 10 min with unlabeled ampicillin (2 μ g/ml) before labeling with 125 I-penicillin X, as previously described (15). Gels were scanned and analyzed with a GS300 densitometer and GS360 Data Systems software (Hoefer Scientific Instruments, San Francisco, Calif.).

RESULTS

Dilute agarose electrophoretic separation of membrane vesicles. Since subcellular particles from eucaryotic cells could be separated from one another by electrophoresis through dilute agarose (17), we tested the capability of the technique to separate subsets of bacterial membrane fragments. Membrane vesicles were separated by electrophoresis and pelleted by ultracentrifugation, as described in Materials and Methods. Proteins associated with each fraction were separated by SDS-PAGE and silver stained. Vesicles which eluted at different times from the agarose gel were associated with different proteins (Fig. 2). The first vesicles to elute contained proteins with molecular masses primarily from 14 to 29 kilodaltons (Fig. 2, lane 3). These were followed by a group of vesicles containing larger proteins (Fig. 2, lanes 7 to 13). Later, a group of vesicles eluted which contained what appeared to be a subset of the proteins of the second group (Fig. 2, lanes 19 to 23). Even within these three broad groups, differences could be seen in positions of particular proteins.

Three lines of evidence indicated that the silver-stained proteins of Fig. 2 were indeed membrane associated. Almost all proteins disappeared from the pellet if membranes were previously removed by ultracentrifugation. Also, almost all disappeared when Triton X-100 was used to solubilize the vesicles before electrophoretic separation. Finally, the protein profile of the fractions before ultracentrifugation differed

FIG. 3. Distribution of PBPs in envelope fractions separated by dilute agarose electrophoresis. Membrane vesicles of *E. coli* CS109 were separated through 0.15% agarose, each fraction was concentrated to 45 μ l, and 15 μ l was labeled with 125 I-penicillin X. The labeled proteins were separated by SDS-PAGE and visualized by autoradiography. Fractions are the same as in Fig. 2.

from the protein profile of pelleted membranes (see Fig. 4E and compare with Fig. 2). The results confirmed the association of silver-stained proteins with a quickly sedimenting and detergent-sensitive fraction (membrane vesicles) from which most or all cytoplasmic proteins had been removed by the centrifugation step.

Dilute agarose electrophoretic separation of vesicles containing PBPs. We previously identified at least two PBP-inner membrane vesicle populations in sucrose density gradients (15). However, the separation between these two groups was not large, and we wished to confirm their existence by an independent method. *E. coli* lysate was separated by electrophoresis through dilute agarose, and the PBPs in each fraction were labeled with 125 I-penicillin and visualized by SDS-PAGE (Fig. 3). The PBPs were distributed among different sets of vesicles (Fig. 3), and the separation between the sets was much more pronounced than when separated in sucrose gradients (15). At least two broad PBP-vesicle classes could be identified: group 1, composed of PBPs 1a, 1b, 2, 3, and 5, and group 2, composed of PBPs 4, 6, 7, and 8 (Fig. 3 and 4).

PBPs 4, 6, 7, and 8 were associated with vesicles which eluted early (Fig. 3 and Fig. 4A) but not with that group which contained the bulk of low-molecular-mass proteins (compare Fig. 3, lane 5, with Fig. 2, lane 3). There was a strong correlation between a protein-poor fraction (Fig. 2, lane 5) and the location of PBPs 4, 6, 7, and 8 (Fig. 3, lane 5). In all experiments, PBP 7 eluted first (Fig. 3, lane 3, and Fig. 4A). The only other PBPs present in significant amounts in this early fraction were PBPs 4, 6, and 8, which peaked 2 fractions later (Fig. 3, lane 5, and Fig. 4A). PBP 4 was identified by the ability of unlabeled ampicillin to competitively prevent the labeling of the protein (22). In Fig. 5, lanes 4 and 5 show the inhibitory effect of ampicillin competition on PBP 4 in fraction 7. A band of a similar molecular mass could not bind unlabeled ampicillin (Fig. 5, lanes 2 and 3) and so was judged to be a penicillin-binding fragment other than PBP 4.

PBPs 1a, 1b, 2, 3, and 5 eluted several fractions later than PBPs 4, 6, 7, and 8 (Fig. 3 and 4B and C). PBPs 1a, 1b, and 5 appeared in fractions 7 to 21 and peaked in fraction 13 (Fig. 3 and 4B). PBPs 2 and 3 appeared in fewer fractions and peaked slightly later, in fractions 15 and 17 (Fig. 3 and 4C).

This elution pattern of vesicles containing PBPs has been confirmed in over 20 separate experiments. In three experiments, membrane vesicles from a *minB1* strain of CS109 showed the same PBP distribution as did vesicles from the wild-type parent (data not shown).

Dilute agarose electrophoretic separation of membrane-

FIG. 4. Distributions of PBPs, NADH oxidase, lactate dehydrogenase, and total protein after dilute agarose electrophoresis (DAE). The PBP data are from the experiment illustrated in Fig. 3. PBP quantities were determined by densitometric scanning of the autoradiograph. The graphs depict the location of each PBP as a

FIG. 5. Identification of PBP 4 by ampicillin binding. Portions (15 μ l) of fractions 19 (lanes 2 and 3) and 7 (lanes 4 and 5) from Fig. 3 were labeled with 125 I-penicillin X with (+) or without (-) preincubation with 2 μ g of unlabeled ampicillin per ml. Lane 1 (control) is fraction 17 labeled in the absence of ampicillin.

associated enzymes. Three enzymes normally associated with the inner membrane were assayed in fractions from dilute agarose electrophoresis. CS109 vesicles exhibited maximum lactate dehydrogenase activity in fractions 11 and 13 (Fig. 4D). NADH oxidase activity peaked in fractions 13 and 15 (Fig. 4D), and succinate dehydrogenase activity peaked in fraction 13 (data not shown).

Sephacryl S-1000 chromatographic separation of membrane vesicles containing PBPs. Sephacryl S-1000 is an acrylamide-based gel which can separate membrane vesicles up to 260 nm in diameter (28). Since separation by density (15) and by agarose electrophoresis suggested the existence of a variety of inner membrane vesicles, separation by size was performed as an additional physical method of defining these groups.

Vesicles containing PBPs were not separated from one another by sizing chromatography as well as they were by electrophoresis through agarose (compare Fig. 3 with Fig. 6). Still, groupings could be identified. One vesicle group, which appeared in fractions 4 to 7 and peaked in fraction 6, included PBPs 1a, 1b, 2, 3, 4, and 5 (Fig. 6 and 7A). A second group peaked in fraction 7, tailed off into fractions 8 and 9, and included PBPs 6, 7, and 8 (Fig. 6 and 7B). These results were obtained in four separations. In two other experiments, PBP 7 peaked in fractions slightly later than either PBP 6 or 8, suggesting that PBP 7 may associate with a unique vesicle population. NADH oxidase and lactate dehydrogenase appeared in the same fractions as the high-molecular-mass PBPs (data not shown).

DISCUSSION

Historically, the primary tool used to define populations of bacterial membrane vesicles has been ultracentrifugation through sucrose density gradients. We report the application of two additional membrane separation techniques, based on physical principles other than separation by density, which confirm and extend observations about bacterial membrane organization.

A growing body of evidence suggests that the inner membrane of *E. coli* can be separated into subpopulations of

percentage of the total amount of that PBP in all fractions. The distributions of PBPs 4, 6, 7, and 8 (A), PBPs 1a, 1b, and 5 (B), PBPs 2 and 3 (C), NADH oxidase and lactate dehydrogenase (LDH) (D), and total protein (E) are shown.

FIG. 6. Distribution of PBPs in envelope fractions separated by Sephacryl S-1000 chromatography. Membrane vesicles of *E. coli* CS109 were separated as described in Materials and Methods, each fraction was concentrated to 45 μ l, and 15 μ l was labeled with 125 I-penicillin X. The labeled proteins were separated by SDS-PAGE and visualized by autoradiography. Fraction numbers above the lanes represent the material which eluted from the column in consecutive 30-min collection periods.

vesicles, each associated with a unique set of proteins (10, 13, 15, 16). This heterogeneity may reflect the existence of specific inner membrane regions having biologically discrete functions. We chose to examine the PBPs of *E. coli* since they play major roles in cell wall synthesis and because at least one (PBP 3) is involved in cell septation (7) and its location might mark a membrane domain important in that spatially restricted event.

The literature regarding the membrane location of the PBPs of *E. coli* is confusing. PBPs have been localized as classical inner membrane proteins (14, 15, 27, 37), but one laboratory has reported that they exist in the outer membrane or in a variety of combinations between the two membranes (3, 29, 30). We have shown that the classical procedure for separating inner from outer membranes includes growth media, chemical treatments, and membrane fractionation techniques which alter associations between proteins and membrane vesicles (15; unpublished observations). This may account for the reported variation. We always observe greater than 99% of the PBPs in fractions corresponding to inner membrane vesicles (15) and so have interpreted our results in the light of that finding.

The associations we observed between membrane vesicles and PBPs are summarized in Table 1. Using sucrose density gradients, there appeared to exist at least two populations of vesicles containing different sets of PBPs: PBPs 1 to 4 were found in one set, and PBPs 5 to 8 were found in another (15). PBPs 4, 6, 7, and 8 were associated with vesicles which migrated more rapidly through dilute agarose than did vesicles containing PBPs 1a, 1b, 2, 3, and 5. Surface charge and size determine mobility of vesicles through dilute agarose (17, 32); it is not known which contributed most to the mobility of PBP-containing vesicles. Sizing chromatography through Sephacryl S-1000 was less useful in separating bacterial vesicles. Even so, PBPs 1 to 5 eluted from the column just before PBPs 6 to 8, indicating only a slight difference in vesicle size. In general, the more rapidly vesicles migrated through agarose, the smaller they appeared in sizing chromatography, with the exception of vesicles associated with PBP 4.

The results from the three techniques argue strongly for some clear and some subtle differences in the way PBPs are arranged within the bacterial inner membrane. PBPs 1 to 3 and 6 to 8 were associated consistently with two separate sets of vesicles (Table 1). Because PBP 5 was prominently labeled, the evidence is quite strong that PBP 5-containing vesicles fractionated with one or the other of these sets, depending on the separation procedure. PBP 4 behaved similarly, although a contaminating penicillin-binding frag-

FIG. 7. Distributions of PBPs and total protein after Sephacryl S-1000 chromatography. The PBP data are from the experiment illustrated in Fig. 6. PBP quantities were determined by densitometric scanning of the autoradiograph. The graphs depict the location of each PBP as a percentage of the total amount of that PBP in all fractions. The distributions of PBPs 1a, 1b, and 5 (A) and PBPs 6, 7, and 8 (B), a comparison of the separation between PBP1a and PBP7 (C), and the distribution of total protein (D) are shown. PBPs 2, 3, and 4 had the same distribution as those PBPs in panel A (data not shown).

ment made it more difficult to identify. PBP 7 always eluted earlier than any other PBP in dilute agarose electrophoresis. In addition, on several occasions PBP 7 fractionated through the Sephacryl column as the last PBP.

These groupings generally parallel biological observations. The high-molecular-mass PBPs 1 to 3 are essential for

TABLE 1. PBP-membrane vesicle associations

Separation procedure	Group ^a	PBP ^b							
Sucrose density gradient ^c	1	1a	1b	2	3	(4)			
	2						5	6	7 8
Dilute agarose electro- phoresis	1	1a	1b	(2)	(3)		5		
	2					4		6	(7) 8
Sephacryl S-1000 chro- matography	1	1a	1b	2	3	4	5		
	2							6	(7) 8

^a Defined as a set of PBPs associated with vesicles which fractionate together in the procedure.

^b Parentheses denote that in some or all of the experiments the PBP appeared to migrate with vesicles slightly different from those containing the rest of the PBP group.

^c Data for the sucrose density gradients are from Jacoby and Young (15).

bacterial growth, are targets for lethal penicillin derivatives (7, 36, 39), and fractionated together in the procedures reported here. The low-molecular-mass PBPs 4 to 6 are individually dispensable (8, 39) and fractionated with vesicles other than those containing the essential PBPs. In addition, these high- and low-molecular-mass PBPs fall into the same two groupings on the basis of amino acid sequence analysis of selected areas of the proteins (38). PBP 7 has been identified as biologically unique in that it is preferentially bound by the penem class of penicillins, which have the singular property of lysing bacteria in the stationary phase (40).

Since the low numbers of PBPs per cell (26) may make electron microscopic visualization difficult or impossible, the absolute location of PBPs within the cell may not be directly demonstrable by current techniques. We have attempted the lesser goal of describing combinations of PBPs which appear to associate with one another or which reside in the same vesicles. Such combinations may or may not be located separately in the intact growing cell, but their existence will enrich our ideas about relationships among the PBPs and may be useful as protein markers for vesicles involved in cell septation.

Finally, dilute agarose electrophoretic fractionation of the inner membrane PBPs into clearly different vesicle classes illustrates that there is more to the organization of the inner membrane than can be observed by separation by density alone. We hope that the technique can be profitably applied to the localization of other membrane proteins and lipids.

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