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Direct Quantitation of the Number of Individual Penicillin-Binding Proteins per Cell in *Escherichia coli*†

THOMAS J. DOUGHERTY,* KAREN KENNEDY, ROBERT E. KESSLER, AND MICHAEL J. PUCCI

Department of Microbiology, Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, Connecticut 06492

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The penicillin-binding proteins (PBPs) are a set of enzymes that participate in the terminal stages of bacterial peptidoglycan assembly. As their name implies, these proteins also covalently bind and are inhibited by β-lactam antibiotics. Although many studies have examined the relative binding affinities of a number of β-lactam antibiotics, a surprisingly small number of studies have addressed the absolute numbers of each of the PBPs present in the bacterial cell. In the present study, the PBP values initially reported in *Escherichia coli* almost 20 years ago by B. G. Spratt (Eur. J. Biochem. 72:341–352, 1977) were refined. The individual PBPs from a known number of bacteria radiolabeled with [^{3}H]benzylpenicillin were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The radioactive bands were located, excised, and quantitatively extracted from the gel slices. The radioactivity was measured by scintillation counting, and the absolute disintegrations per minute were calculated. From the specific activity of the labeled penicillin, the absolute disintegrations per minute, and the CFU per milliliter, a determination of the number of each of the PBPs per cell was made. The measurements were performed on multiple samples to place statistical limits on the numbers obtained. The values for the individual PBPs found in *E. coli* deviated in several ways from the previously reported observations. Of particular significance is the higher number of molecules of PBP 2 and 3 observed, since these PBPs are known to participate in cell morphogenesis. The PBP content in both rich Luria broth medium and M9 minimal medium was determined, with the slower-growing cells in minimal medium possessing fewer of the individual PBPs per cell.

The penicillin-binding proteins (PBPs) are a set of enzymes responsible for the terminal stages of peptidoglycan biosynthesis, where they carry out transpeptidation, transglycosylation, endopeptidase, or carboxypeptidase functions. It is the transpeptidation, endopeptidase, and carboxypeptidase functions that are inhibited when these proteins are acylated by a β-lactam antibiotic (10). In all cases where this reaction has been investigated biochemically, the β-lactam ring opens and forms a covalent bond with an active-site serine residue on the PBP (10, 11). Among the most extensively investigated PBPs are those of the gram-negative bacillus *Escherichia coli*, and these have been the subject of numerous publications and reviews. It is for *E. coli* that we have perhaps the clearest understanding of the roles that the individual PBPs perform in peptidoglycan synthesis and cell morphogenesis (7, 19, 21). At the same time, however, much remains to be understood concerning the role of the PBPs in cell wall growth and their participation in cell division.

More than 18 years ago, Spratt published an estimate of the numbers of individual PBPs in an average *E. coli* cell (25). In this seminal work, the PBPs of isolated cell membranes were labeled with [^{14}C]penicillin and separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. After electrophoresis, the radiolabeled PBPs were detected by fluorography. The numbers of PBPs per cell were calculated by scanning fluorographs, obtaining the areas under the curves and calculating a percentage distribution for each of the individual PBPs. This relative distribution was then combined with a literature value for the total amount of [^{14}C]cefoxitin bound per milligram (dry weight) and a literature value for cell number per milligram (dry weight) to arrive at absolute values of individual PBP numbers per cell (20, 25). Although this was clearly meant as a rough estimate, this set of numbers has been cited repeatedly in numerous publications and several textbooks since that time (1, 7, 19, 21, 26).

Because of the importance of these proteins in β-lactam antibiotic action, cell wall assembly, and cell division, we decided to measure the numbers of the individual PBPs of *E. coli* by a direct method. In the present study, the numbers of molecules per cell of the individual PBPs were calculated directly by measuring both the cell numbers present and the radioactivity in the individual PBPs from a precise quantity of cells. Care was taken to ensure that the individual PBPs were saturated with [^{3}H]penicillin, and a sufficiently large number of samples were taken in separate experiments to allow statistical assessment of the data sets. The results obtained in the present study are in some cases significantly different from the previously published values. In addition, measurements were performed with both rapidly growing cells in Luria broth (LB) medium as well as with cells growing in M9 minimal medium. The absolute numbers of PBPs were reduced in the cells growing at a lower rate in minimal medium. The implications of these revised estimates are discussed.

MATERIALS AND METHODS

Strain and growth conditions. The strain used throughout these studies was *E. coli* MC4100 [F$^-$ *araD139* Δ(*argF-lac*)U169 *rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*]. It was chosen on the basis of its widespread availability and use in molecular biology studies. A single colony was inoculated into 5 ml of tryptone broth and grown overnight at 30°C with agitation. The next morning, a 200-fold dilution of the overnight culture was made in 40 ml of LB medium and grown at 35°C with agitation (250 rpm). Growth was monitored at 610 nm with a Sequoia-

* Corresponding author. Mailing address: Department of Microbiology (Dept. 104), Bristol-Myers Squibb Pharmaceutical Research Institute, 5 Research Parkway, Wallingford, CT 06492. Phone: (203) 284-6433.

† Dedicated to the memory of our friend and colleague, Sandra Handwerger.

Turner model 340 spectrophotometer. Experiments were started in the A_{610} range of 0.3 to 0.4, which represents approximately 2×10^8 cells per ml (see "Cell numbers" below). For experiments in which cells were growing in M9 glucose minimal medium (17), the cells were cultured overnight in M9 glucose medium with 0.5 μg of thiamine HCl (Sigma Chemical Co., St. Louis, Mo.) per ml. A 200-fold dilution was made in 40 ml of prewarmed M9 glucose medium with thiamine, and cells were grown to the A_{610} range of 0.3 to 0.4.

In vivo labeling of PBPs. For quantitative measurements, PBPs were labeled by an in vivo protocol. Exactly 1 ml of mid-logarithmic-phase cells were collected and centrifuged in microcentrifuge tubes for 2 min at 12,000 × *g*, the supernatant was removed, and the cells were resuspended in 50 μl of 50 mM sodium phosphate buffer (pH 7.0). These samples were subjected to three cycles of freeze-thaw (−80 to 37°C, 3 min at each temperature) (4). After the last thaw, 2 μg (final concentration, 40 μg/ml) of radiolabeled penicillin ([^{3}H]benzylpenicillin, ethypiperidinium salt; 27.2 Ci/mmol; DuPont NEN, Boston, Mass.) was added immediately. The samples were incubated for 20 min at 37°C, and labeling was terminated with 2 μl of 20% Sarkosyl detergent. Subsequently, 25 μl of SDS sample buffer with 2-mercaptoethanol was added (24), and the samples were placed at 100°C for 5 min. The entire volume was loaded into a sample lane for SDS gel electrophoresis.

To ensure that the freeze-thaw protocol led to quantitative labeling of the PBPs in *E. coli*, several experiments included an alternative method in which the cell pellets from 1 ml of culture were resuspended in 50 μl of 25 mM Tris-Cl (pH 8.0)–10 mM EDTA–50 mM glucose with 1 mg of lysozyme per ml and incubated for 5 min at 37°C. This led to rapid cell lysis, and 2 μg of [^{3}H]benzylpenicillin was added to these samples for 20 min at 37°C and processed for gel electrophoresis as described above.

Membrane preparations. Five-hundred-milliliter cultures of cells in LB broth were prepared at 35°C with shaking. When the cells reached an absorbance of 0.4 to 0.5, they were harvested by centrifugation (7,000 × *g*, 10 minutes at 4°C) and resuspended in sodium phosphate buffer (pH 7.0) and the membranes were prepared. The membranes were obtained by two methods. The first method involved sonication and differential centrifugation, as described previously (8). In the second method, the cells were broken with a Bead-Beater (Biospec Products, Bartlesville, Okla.). The cells were resuspended in 10 ml of phosphate buffer with 35 g of glass beads and subjected to eight cycles of agitation in an ice-jacketed chamber. The beads were separated from the broken cells with a sintered glass filter, and the membranes were collected by ultracentrifugation at 155,000 × *g* for 30 min at 4°C, as described above. Membranes from both methods were washed once with phosphate buffer at 4°C, collected by centrifugation at 155,000 × *g*, and resuspended in a final volume of 250 μl. A sample was taken for protein concentration determination by the bicinchoninic acid assay (Pierce Chemicals, Rockford, Ill.) with known concentrations of bovine serum albumin (Sigma) as a standard. In PBP assays, 200 μg of protein was used per gel lane (25).

SDS-polyacrylamide gel electrophoresis. The gels were run on a long bed (20 cm) at a constant current of 10 mA at 4°C for 16 h to maximize resolution (8). Following electrophoresis, the gels were fixed and stained as described previously (8) and prepared for fluorography with En^{3}Hance fluor (DuPont NEN) as described in manufacturer's instructions. After drying on a Bio-Rad (Richmond, Calif.) model 583 gel dryer, exposure to X-ray film (X-OMAT-AR; Kodak, Rochester, N.Y.) for 5 to 7 days revealed the positions of the radiolabeled PBPs. Shorter exposure times (1 to 3 days) were used for membrane preparations of PBPs since these samples were more concentrated. All reagents for electrophoresis were from Bio-Rad Laboratories.

Quantitation of PBPs. The PBPs were radiolabeled and separated by electrophoresis as described above. Prior to exposure of the En^{3}Hance-impregnated gel to X-ray film, an UltEmit fluorescent marker pen (DuPont NEN) was used to place orientation marks on the gel. After exposure, the orientation marks assisted in aligning the X-ray fluorograph with the proper gel lanes. The X-ray film was used as a template to identify the areas containing the radiolabeled PBPs, and these were subsequently cut out from the dried gel and placed in scintillation vials. To ensure that sufficient radioactive counts were present to give reasonable counting statistics, two adjacent lanes with identical samples of a given PBP were placed in the same vial. This resulted in all counts per minute values per vial in these experiments being above 2,000 cpm. This reduces the probable counting error to below 3% (15). When the PBP-per-cell calculations were performed, the cell numbers per sample lane were doubled to account for this procedure. In all experiments, 12 identical sample lanes were run, thus yielding six determinations for each PBP.

To each vial, 0.5 ml of distilled water was added to rehydrate the gel. After 60 min, 0.5 ml of Solvable gel solubilizer (DuPont NEN) was added to cover the rehydrated gel slice. The samples were then incubated for 3 h at 50°C. Following cooling, 10 ml of Ready Safe (Beckman Instruments, Fullerton, Calif.) scintillation fluid and 0.05 ml of glacial acetic acid were added to the samples. The samples were mixed vigorously and allowed to sit for at least 48 h before counting. In several cases, the gel slices were removed from the scintillation vials after the initial counting and the samples were recounted. In all cases, greater than 98% of the radioactivity remained, indicative of effective and quantitative extraction of the labeled proteins from the gel slices. All procedures were done as described in the DuPont NEN recommendations for gel solubilization. The radioactivity of the vials was counted in a Beckman LS5000TD liquid scintillation counter.

To obtain the absolute disintegrations per minute from the observed counts per minute values, a quench curve was constructed in the following manner. Nonradioactive areas of a gel where no sample was loaded were treated and solubilized as described above. A set of 12 nonradioactive samples were prepared in this manner, and identical quantities of a [^{3}H]water standard (DuPont NEN; 1.000 mCi/ml) were added to each vial. The [^{3}H]water had been diluted with precision microcapillary glass pipettes (Baxter Scientific, Bedford, Mass.) to 1.000 μCi/ml, and exactly 10 μl (2.2×10^4 dpm) was added to each vial. The counts per minute in the vials were determined, and the vial replicates with the 10 closest values were used. To 9 of the 10 vials, increasing amounts of a quenching agent, nitromethane (Sigma), were added. The resulting counts were used to construct a quench curve with the channel ratio method. The scintillation counter used the stored quench curve to calculate the absolute disintegrations per minute from the counts per minute for all samples in the experiments. All experimental samples were well within the limits of the constructed quench curves.

To determine relative values of PBPs from fluorographs, the X-ray films were prefogged (16), and after exposure, the film was scanned with an Ultroscan XL laser densitometer (Pharmacia LKB Biotech, Uppsala, Sweden). The area under the curve is automatically calculated by the instrument.

Cell numbers. To obtain a precise estimate of the number of individual PBPs per cell, it was necessary to determine the cell number at the time that samples were taken for PBP determinations. At the same time that the 12 samples of 1 ml each were taken for the PBP quantitations, 2 samples of 0.5 ml each were taken with precision pipettes and placed in 4.5 ml of phosphate-buffered saline (PBS). These duplicate samples were then diluted in 10-fold steps, and 0.1 ml was spread on triplicate LB agar plates of the 10^{-7}, 10^{-6}, and 10^{-5} dilutions. Since each dilution was done in duplicate and spread in triplicate, there were six plates for each dilution. After overnight incubation, the number of colonies was counted on the plates with 30 to 300 colonies, and the six determinations of a dilution were averaged. All experiments reported in this paper had values between 2.5×10^8 and 4×10^8 CFU/ml.

Cell protein determinations. Quadruplicate samples of 0.5 ml of cells in the mid-logarithmic phase were taken and centrifuged at 12,000 × *g* for 3 min. The supernatant fluid was carefully removed, and the pellet was resuspended in 0.1 ml of 20 mM Tris-Cl (pH 8.2)–1 mM EDTA–0.1% SDS (Sigma). These samples, along with a range of bovine serum albumin standards, were boiled for 5 min and cooled, and bicinchoninic acid reagent was added. After 30 min at 37°C, the A_{562} was measured in a Beckman DU-70 spectrophotometer. The amount of protein per 10^8 cells was calculated from the results.

RESULTS

In vivo labeling of *E. coli*. Most work reported on *E. coli* PBPs has used purified cell membranes, to which the PBPs are anchored, as targets for radiolabeled penicillin (24, 25). It would be extremely difficult to quantitatively prepare membranes from a known amount of cells, however, since variable losses occur during the membrane preparation process. To accurately assess the number of PBPs per cell, it was necessary to be able to label cells with [^{3}H]penicillin directly, without preliminary preparation of cell membranes. A method involving multiple freeze-thaw cycles with *E. coli* whole cells has been reported to allow access to the PBPs by radiolabeled penicillin (4). To validate that the PBPs were in fact freely accessible to the [^{3}H]penicillin, a set of preliminary experiments was devised. One-milliliter samples from a culture of *E. coli* MC4100 in the mid-logarithmic phase (A_{610} of 0.35) were collected in microcentrifuge tubes at 12,000 × *g* for 2 min. A total of eight samples were taken. Four of the samples were subjected to the freeze-thaw regimen described in Materials and Methods, whereas the other four were suspended in Tris-EDTA-glucose with lysozyme and incubated for 5 min at 37°C. The lysozyme-treated cells lysed under these conditions. Both sets of samples were then incubated for 20 min at 37°C with [^{3}H]penicillin and then processed for gel electrophoresis. Comparison of the resulting fluorographs by laser densitometry revealed that the samples had virtually identical levels of labeling for all the PBPs (data not shown). Lysozyme-treated cells were included as control lanes in several experiments during the course of this study.

A second set of studies was performed with cells in the same

TABLE 1. Comparison of the numbers of PBPs in *E. coli* determined by the present direct method with the calculations of Spratt

PBP (molecular mass [kDa])[a]	Direct measurements		Spratt values[b]	
	Molecules/cell[c]	% of total[d]	Molecules/cell[e]	% of total[d]
1a (94.5)	221 (±20)	8.9	230	8.3
1b (94.3)	127 (±13)	5.1		
2 (70.9)	120 (±14)	4.8	20	0.7
3 (63.9)	132 (±17)	5.3	50	1.8
4 (51.8)	120 (±11)	4.8	110	4.0
5 (46.9)	791 (±105)	31.9	1,800	64.7
6 (44.5)	221 (±26)	8.9	570	20.5
7 (31.2)[f]	387 (±15)	15.6		
8 (29.5)[f]	362 (±19)	14.6		

[a] PBPs are numbered by the standard nomenclature (25).
[b] Obtained from Table 1 of reference 25.
[c] PBP values are averages ± standard deviations calculated from 18 determinations for each PBP (six determinations per experiment; three experiments). Total molecules per cell, 2,481.
[d] Each PBP is represented as a percentage of the total.
[e] Total molecules per cell, 2,780. PBP 1b was not resolved in this study, and PBP 7 and 8 were not quantitated in this study.
[f] Values were estimated as described in the text.

absorbance range (i.e., 0.40) and by use of the freeze-thaw technique. In this case, increasing concentrations (0.1, 0.25, 0.5, 1.0, 2.0, and 4.0 μg) of radiolabeled penicillin were added to the 50-μl volumes of cells, and the volumes were incubated at 37°C for 20 min. After the samples had been processed, separated by gel electrophoresis, and fluorographed, laser densitometry was performed. It was found that the density of the PBP fluorograph bands leveled at a concentration of 0.5 to 1.0 μg/50 μl (equivalent to 10 to 20 μg/ml; data not shown). In all quantitation experiments, twice as much [^{3}H]penicillin was used (2.0 μg/50 μl) and cell absorbance was below 0.4 to ensure that saturation of the PBPs had occurred.

Quantitation of the PBPs in rapidly growing *E. coli*. One-milliliter samples of cells from mid-logarithmic cultures of *E. coli* MC4100 in LB medium were taken. The samples for PBP determination were immediately centrifuged and processed by freeze-thaw for PBP labeling as described above. The samples for cell number determinations were immediately diluted in PBS, and samples from the dilutions plated on LB agar for colony enumeration after incubation overnight. Each experiment yielded six values for each individual PBP along with a CFU/ml value derived from at least six plate counts of colonies. By using the information on the total number of cells per milliliter, the total dpm per lane (obtained from two lanes for counting statistics; see Materials and Methods) and the specific activity (27.2 Ci/mmol) of the radioactive penicillin were used to calculate the values of individual PBPs per cell. The results from a total of three experiments are included in Table 1, and the average and standard deviations for all 18 datum points are presented. The values previously derived by indirect methods by Spratt (25) are also included for comparison.

It was necessary to run long-bed gels for extended periods of time to achieve adequate separation of some of the PBP species to allow reliable excision of the bands from the gels. As a consequence, the low-molecular-weight PBP 7 and its proteolytic degradation product, PBP 8 (12, 13), were usually lost as they migrated off the bottom edge of the gel. In two instances, a shorter run time was used specifically to retain these PBPs and quantitate their values as described above for PBP 1 to 6. Table 1 also includes these values. These results indicate that PBP 7 and 8 make up a significant fraction of the total PBP pool in *E. coli*.

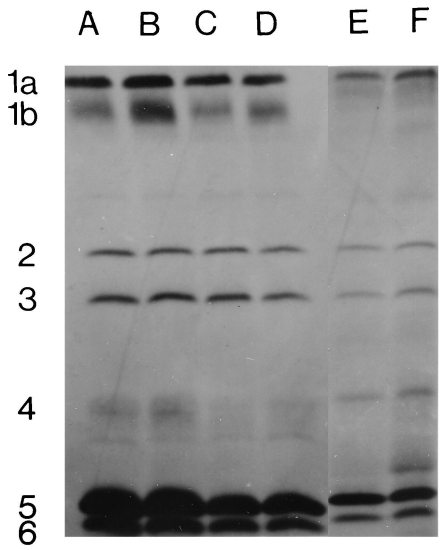

FIG. 1. Fluorograph of the PBPs of *E. coli* MC4100. The PBPs are numbered on the left by the standard nomenclature. All preparations received saturating [^{3}H]penicillin. Lanes: A and B, duplicate PBP determinations performed with membranes prepared by sonication as described by Spratt (25); C and D, duplicate membranes prepared by Bead-Beater disruption; E, in vivo PBP labeling with the freeze-thaw protocol adopted in this study; F, PBP labeling performed on EDTA-lysozyme-treated cells. It is important to note that as a result of lane width compression, lane F PBP bands appear darker than those in lane E. Extraction and scintillation counting of these two lanes yielded PBP quantities within 10% of each other.

Comparison of direct quantitation and laser densitometry. The fluorographs obtained from the two membrane preparation methods as well as those from both freeze-thaw and enzymatic lysis of whole cells were compared (Fig. 1). It is clear that the concentrated membrane preparations result in higher fluorograph densities. It is important to note in Fig. 1 that PBP 1b is spread out; it is known that PBP 1b consists of several components, and all were counted together as PBP 1b (14, 18). The results shown in Table 2 are a comparison of the relative percentages of PBPs obtained by using the generated absolute

TABLE 2. Relative percentages obtained by densitometric scanning of PBPs labeled in isolated membranes compared with those from direct determination of PBPs

PBP	Relative % determined by:		
	Densitometric scanning[a]		Direct determination[b]
	Sonicator membranes	Bead-Beater membranes	
1a	10.5	11.2	12.8
1b	6.8	10.0	7.3
2	4.5	6.3	6.9
3	4.7	7.0	7.6
4	6.5	6.2	6.9
5	52.3	43.2	45.6
6	14.6	16.1	12.8

[a] Membranes were prepared from a logarithmically growing culture of *E. coli* MC4100 as described in Materials and Methods. PBPs were labeled as described by Spratt (25).
[b] Values are from Table 1 and are derived from direct determinations of PBP labeling by scintillation counting of individual PBPs. Percentages have been recalibrated to reflect the absence of PBP 7 and 8.

TABLE 3. Comparison of the numbers of PBPs[a] from cells grown in minimal M9 glucose liquid medium with those grown in LB medium

PBP	M9 glucose medium[b]		LB medium[b]	
	No. of PBPs[c]	% of total[d]	No. of PBPs[c]	% of total[d]
1a	135 (±24)	14.3	221 (±20)	12.8
1b	123 (±19)	13.1	127 (±13)	7.3
2	58 (±8)	6.2	120 (±14)	6.9
3	63 (±12)	6.7	132 (±17)	7.6
4	84 (±14)	8.9	120 (±11)	6.9
5	317 (±69)	34.7	790 (±105)	45.6
6	161 (±18)	17.1	221 (±26)	12.8

[a] The PBP values for the PBP 1 through 6 group are presented. PBP 7 and 8 were lost off the bottom of the gel.

[b] Direct determinations were performed as described in Materials and Methods. The total number of measurements in M9 glucose medium was 12. The total amounts of cell protein for M9 glucose medium and LB medium were 46 and 64 μg/10^8 cells, respectively. The cell doubling times at 35°C for the two media were 53 and 32 min, respectively.

[c] Values are averages (± standard deviations).

[d] Percentages are for the PBPs listed; the values for PBP 7 and 8 were not determined for M9 glucose medium and are not included in the percent distribution.

values of PBPs by excision and scintillation counting with the relative values obtained by laser densitometry. It illustrates that laser densitometry yields relative percentages of PBPs similar to those obtained by direct counting determinations of individual PBPs. Also included in the table are values generated by densitometry of membranes prepared by cell breakage with either sonication or glass beads and subsequent differential centrifugation. Despite the fact that the membrane preparations are much more concentrated than the whole-cell samples, the PBP percentages are surprisingly similar to each other. Values for PBP 7 and 8 were not obtained in this experiment as a result of the prolonged electrophoresis to optimize the separations. Therefore, the direct determination percentages have been readjusted to reflect the contributions of PBP 1 to 6, for comparison purposes.

Growth conditions affect the PBP content of cells. Table 3 includes the PBP values obtained from two experiments (12 determinations in total) in which *E. coli* MC4100 was grown in M9 glucose liquid medium. When these values are compared with the LB medium-grown cells, it is readily apparent that the slower-growing cells in M9 medium contain significantly less of each PBP per cell than the LB medium-grown cells, with the notable exception of PBP 1b. Most interestingly, the ratios of the two PBPs implicated in morphogenesis and division remained equimolar, although their absolute values were roughly half that in rich LB medium. The amount of cell protein per 10^8 cells was calculated and is also indicated in the table. As expected, the M9 medium-grown cells contained less protein per unit cell than the LB medium-grown cells, indicative of their lower mass per cell and smaller size. The table also indicates the doubling times of the cells, determined by measuring A_{610}, in the two media.

DISCUSSION

The PBPs represent a group of critical enzymes both in terms of bacterial morphogenesis and as targets for the β-lactam antibiotic family. The PBPs are known to bind penicillin in a 1:1 molar ratio of drug to target protein. In the original work performed by Spratt almost two decades ago, an attempt at estimation of the numbers of molecules per cell was made (25). In the intervening period of time, this estimate has been repeatedly cited and incorporated into models of wall growth and bacterial cell division (1, 2, 7, 19, 22, 23). As recently as 1993, however, Park noted that the numbers cited were based on this single estimate and that an independent reassessment of PBP quantities was in order (21).

In the prior Spratt study, the estimate of total numbers of each of the PBPs was made for *E. coli* KN126 on the basis of the percentage distribution of each PBP against the sum total of all PBPs. These numbers result from the use of densitometric scans of fluorographs of [^{14}C]penicillin-labeled membranes prepared from cells grown in Penassay broth to establish relative PBP values. They were then converted to absolute values on the basis of a literature figure for total picomoles of [^{14}C]cefoxitin bound per milligram of dry cell weight (20). The cefoxitin was used because experiments in which total radiolabeled penicillin bound to intact cells was measured yielded lower values as a result of rapid turnover (deacylation) of penicillin from *E. coli* PBP 5 and 6. Spratt used a value that converted dry weight of bacteria to cell number of 1.95×10^9 cells per mg (dry weight) to convert picomoles of antibiotic bound to number of equivalent PBP molecules (i.e., 2,800) per cell. He then distributed these 2,800 molecules on the basis of the relative densitometric distributions (25). Since cell composition and number of cells per milligram (dry weight) are a function of growth rate (3), the precise conditions under which the dry weight determinations were made and their relationship to cell number cited in the Spratt paper (25) could influence the estimate substantially.

The rationale for the present study was to combine accurate measurements of cell number and PBPs made simultaneously on cells from the same culture. Another goal was to collect sufficiently large data sets to be able to assess by statistical means the reliability of the stated values by using data from independent replicate experiments. To ensure that an accurate estimate of cell numbers relative to PBP values were obtained, an in vivo labeling protocol was used. This allowed a simultaneous estimate of PBP and cell numbers. The alternative was to prepare cell membranes from a known number of cells and attempt to quantitate the losses that occur at each step in the membrane preparation. This would be a very difficult procedure, fraught with assumptions and experimental pitfalls.

Penetration of penicillin into intact *E. coli* cells is poor, and it is necessary to disrupt the outer membrane in some manner to achieve PBP labeling (4). Having selected the in vivo method described, it was important to verify that the multiple freeze-thaw method allowed access to all the PBPs and did not skew the results. The method was compared with a Tris-Cl-EDTA-lysozyme lysis protocol, in which cell lysis eliminates the penicillin penetration problem. The finding of virtually identical levels of PBPs with the two methods validated the freeze-thaw protocol as yielding reliable results.

An initial comparison of the Spratt data from 1977 with those of the present study for *E. coli* MC4100 cells growing in rich LB medium reveals some significant differences between the results. In the present study, the numbers of two critical PBPs, namely, PBP 2 and PBP 3, are markedly higher than those of the Spratt data (Table 1). Both of these PBPs are involved in cell morphogenesis, and several division theories have used the ratios or specific numbers in their arguments (1, 19, 23, 24). We found that in LB medium, PBP 1b, 2, 3, and 4 are virtually equimolar, with about 120 to 130 molecules of each per cell. The total numbers of PBPs per cell were not that different between the studies. Spratt estimated 2,780 molecules per cell, whereas our values indicate a total of just under 2,500.

Given the assumptions made in the calculations, the numbers are not greatly disparate. Both studies employed *E. coli* K-12 derivatives; however, strain and growth conditions are not identical. The differences observed are due almost entirely to the difference in the calculated values for PBP 5 and 6 and the addition of PBP 7 and 8. Whether the PBP 5 and 6 value differences between the studies reflect differential inactivation of PBPs during membrane preparation, strain variation, or some parameter in the current method, such as turnover (25), remains an open question. Spratt noted in his study the presence of PBP 7 and 8, although their appearance was not consistent. The recent cloning of the PBP 7 gene and the finding that PBP 8 is an OmpT protease degradation of PBP 7 firmly establishes these as bona fide PBP species (12, 13).

In the present study, the absolute values were calculated directly from cell numbers and individual PBP numbers obtained simultaneously. Since each PBP value was determined independently of the other PBPs, an error in determining the value for a given PBP has no effect on the other PBP values. This is in contrast to densitometry studies, where an error in one PBP quantitation extends to the other PBP values, since these are percentage distribution determinations. Another error that can cause potential problems in densitometry is illustrated in Fig. 1, lanes E and F. Most densitometers do not scan the entire lane width but, rather, sample a narrow central region. The narrowing or spreading of the sample lane during electrophoresis can cause density differences that are artifactual. The excision and counting of the entire radiolabeled band prevents these types of errors.

Although it is not possible to obtain absolute PBP numbers from densitometry scans of gels, relative numbers of PBPs can be readily derived. The finding (Table 2) that the relative numbers obtained by scanning can correspond well with absolute numbers determined by direct counting validates the use of densitometry scanning in PBP competition studies. This method is widely employed to study specific β-lactam antibiotic interactions with PBPs relative to untreated control cells (5, 6, 9, 27). The determination of absolute PBP numbers in the present study would allow these values to be applied to the calculation of absolute numbers in competition studies with *E. coli*, provided that the growth conditions were similar.

Examination of the number of PBPs present when cells are grown in M9 glucose minimal medium indicated that the number of PBPs present was reduced when the cells were growing at the lower rate. Since the generation time is longer and the cells are smaller in volume (3), the amount of wall that needs to be made to cover the smaller surface area per unit time is decreased. This appears to be reflected in the decreased numbers of PBPs made under slower growth conditions as well as the decreased protein per cell. Although the absolute numbers were lower, the relative amounts of two PBPs, i.e., PBP 2 and 3, involved in cell morphogenesis and division remained constant. This also indicates the necessity of carefully defining growth conditions when discussing the numbers of PBPs present per cell.

In summary, our numbers represent an attempt to measure the quantity of individual PBPs in an *E. coli* cell by direct determinations. The results indicate that overall, the total number of PBP values generated by Spratt were reasonable accurate, given the indirect method used to determine them. The most significant differences appear to be in the higher values obtained for PBP 2 and 3, enzymes known to be important in morphogenesis and cell division (7, 21). Our values also reflect the contribution of PBP 7 and PBP 8, which are present in variable amounts in membrane preparations (25). It should also be emphasized that the number of PBP molecules per cell is not a fixed amount; rather, the quantities appear to be dependent on growth conditions. The values derived in the present study can be applied to investigations of β-lactam antibiotic action and to models of bacterial cell growth and division.

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