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Activity of Penicillin-Binding Protein 3 from *Escherichia coli*†

ANTONIO G. PISABARRO, ROSA PRATS, DAVID VÁZQUEZ, AND ALFREDO RODRÍGUEZ-TÉBAR‡*

Instituto de Biología Molecular, Consejo Superior de Investigaciones Científicas, Universidad Autónoma de Madrid, Cantoblanco, E-28049 Madrid, Spain

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The activity of penicillin-binding protein 3 of *Escherichia coli* has been studied both in vivo and in ether-permeabilized cells. The peptidoglycan transpeptidase activity of penicillin-binding protein 3 appears to use either nascent or exogenously added UDP-*N*-acetylmuramyl tripeptide-derived substrates as acceptors. By means of a defilamentation system which elicited the activity of penicillin-binding protein 3 in vivo, the structure of peptidoglycan made by this enzyme has been elucidated. This peptidoglycan, very probably of septal location, contained increased amounts of cross-linked peptidoglycan as well as a higher ratio of tripeptide-containing cross-linked subunits.

Penicillin-binding proteins (PBPs) are minor components located in intermembrane structures within the *Escherichia coli* envelope (1, 25). PBPs are actual enzymes that catalyze the last steps of peptidoglycan biosynthesis and are specifically inhibited by β-lactam antibiotics (for a review, see reference 36). Since the discovery of PBPs (4) extensive work has been done to elucidate the role of each PBP in the physiology of the bacterium. Mutations affecting a particular PBP and selective blockage by β-lactams were useful tools in ascertaining, to a certain extent, the function of a given PBP. High-molecular-weight PBPs are peptidoglycan transpeptidases responsible for the cross-linking of the peptidoglycan net, whereas low-molecular-weight PBPs are DD-carboxypeptidases (36). Nevertheless, in vitro assays of the activity of PBPs have encountered serious difficulties. Currently, only DD-carboxypeptidase and DD-endopeptidase activities have been unequivocally demonstrated in vitro with purified enzymes. In vitro transpeptidase assays have shown that both PBPs 1a and 1b act as transpeptidases (20). Additional critical questions emerge about how the in vitro transpeptidase activity can be compared with that in living cells. Recent work by Kraus et al. (13) shows that the structure of peptidoglycan synthesized by either ether-treated *E. coli* cells or isolated envelopes was different from that of peptidoglycan synthesized by the living cell.

It is well known that PBP 3 from *E. coli* participates in cell division. Mutations affecting PBP 3 or selective inhibition of this PBP by β-lactams impairs cell septation and produces filamentation of *E. coli* cells (29). Botta and Park, by selective inhibition of PBP 3 during defilamentation (a period of active septation), demonstrated that PBP 3 was involved in septal peptidoglycan synthesis (5). It is now believed that PBP 3 synthesizes, at least in part, the septal peptidoglycan. So far, little or nothing is known about either the activity of this PBP or the structure of septal peptidoglycan. A recent study by Olijhoek et al. (22) has shown that the degree of peptidoglycan cross-linkage was higher during septation. From these data, it was inferred that septal peptidoglycan is of a more cross-linked nature, with a higher ratio of both dimeric and trimeric subunits (12, 13).

In this communication, we present biochemical data on the activity of PBP 3 both in vivo and in vitro, with regard to the substrates it uses and the products yielded, as well as an outline of the structure of the septal peptidoglycan of *E. coli*. A partial account of this study has appeared elsewhere (26).

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* PA3092 (F⁻ *thr-1 leu-6 thi-1 argH1 thy his-1 trp-1 str-9 lacY1 malA1 xyl-7 mtl-2 mel fhuA2 supE44*) and PAT84 (like PA3092, but *dapA lysA sulB* [27a]). Cells were grown aerobically in L broth (16) supplemented with D-glucose (4 mg/ml) and, for PAT84 strain, 2,6-*meso*-diaminopimelic acid (5 mg/liter). Other conditions varied from one experiment to another and are defined below.

Detection of PBPs in cell extracts and cell envelopes. Usually, PBPs were detected in whole-cell extracts. Cells were collected from the cultures by rapid cooling and centrifugation at low speed. Pelleted cells were suspended in a small volume of ice-cold 20 mM sodium phosphate buffer, pH 7.0. Suspended cells (usually about 0.5 ml) were subjected to a 10-s burst of sonication in a 150-V sonic oscillator (MSE Instruments, Crawley, U.K.). Sonic treatment was carried out with a 1/8-in. (3 mm) probe at an amplitude of 6 μm (peak to peak at the source-probe interface) and at 0°C. Unbroken cells were removed by centrifugation at 5,000 × *g* for 10 min at 2°C. Supernatants were collected and used for detection of PBPs. In some cases PBPs were detected in isolated envelopes. For this purpose, cell extracts were centrifuged at 100,000 × *g* for 90 min at 2°C. Pelleted envelopes were suspended in the same phosphate buffer.

PBPs were detected in either whole-cell extracts or cell envelopes basically by the procedure of Spratt (29). The radioactive antibiotic used was the radioiodinated Bolton and Hunter derivative of ampicillin (^{125}I-ampicillin) (28). Binding experiments were carried out at either 30 or 41°C. The concentration of ^{125}I-ampicillin was 250 nM, enough to saturate nearly 100% of the PBP 3. Labeled PBPs were analyzed in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (14). Gels were stained and extensively destained as described previously (7) and then dried and exposed on prefogged Kodak X-Omat X-ray films (15). The amount of PBP 3 present was quantified by cutting out PBP 3-containing rectangles from the dried gel and counting their radioactivity in a gamma counter.

* Corresponding author.

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‡ Present address: Max-Planck Institut für Psychiatrie, Abteilung Neurochemie, D-8033 Martinsried, Federal Republic of Germany.

Labeling of PBP 3 from intact *E. coli* cells. Cells were grown at 30°C in L medium to an A_{550} of 0.20. At this point, aztreonam at concentrations from 0 to 2.0 μg/ml was added to the cultures. After either 30 or 90 min the cells were collected by cooling and centrifugation. Pelleted cells were suspended in ice-cold 20 mM sodium phosphate buffer, pH 7.0. The cells were centrifuged again, and the washing procedure was repeated twice. Cell extracts were obtained, and their PBPs were detected with ^{125}I-ampicillin as described above.

Irreversibility of the binding of aztreonam to PBP 3. *E. coli* envelopes were labeled with aztreonam at a concentration of 1.0 μg/ml, able to saturate only PBP 3 (see Results). Binding was performed at 30°C for 15 min. The reaction was terminated by cooling and centrifuging the mixture at 100,000 × *g* for 90 min at 2°C. Pelleted envelopes were centrifuged again as before and finally suspended in phosphate buffer. The suspension was incubated at 30°C for up to 90 min. At regular intervals, samples were taken and used for detection of PBP 3 with ^{125}I-ampicillin as described above.

Labeling of PBP 3-produced peptidoglycan. *E. coli* PAT84 was grown at 30°C to an A_{550} of 0.20. Then, aztreonam was added to the culture at a final concentration of 1.0 μg/ml. This concentration ensured the blockage of 91% of PBP 3, leaving the rest of the PBP unaffected. Incubation of the culture was continued, and the formation of filaments was followed by light microscopy. The increase in the A_{550} was exponential up to 1.3 to 1.4 units. At this point, the absorbance of the culture remained constant for at least 12 h. After 60 min of steady absorbance, two 10-ml culture samples were taken, and each was then filtered in a 10-cm-diameter nitrocellulose filter (Millipore Corp.). The filtration device was first warmed to 30°C. The filaments retained in each filter were washed with 10 ml of medium warmed to 30°C. Then the two filters were divided into two equal parts. The filaments retained in one part of one filter were suspended in 50 ml of fresh prewarmed (30°C) supplemented medium containing 1 μCi of 2,6-*meso*-[^{3}H]diaminopimelic acid per ml. The filaments retained in the other part of one filter were suspended in 50 ml of the same medium which additionally contained aztreonam (1.0 μg/ml). The filaments retained in one part of the second filter were suspended in 50 ml of supplemented L medium containing 1 μCi of 2,6-*meso*-[^{3}H]diaminopimelic acid and 50 μg of cefsulodin per ml. Filaments retained in the other half of the second filter were suspended in the same radioactive medium containing both cefsulodin (50 μg/ml) and aztreonam (1 μg/ml). The filtration and suspension procedures took about 2 min. The cultures containing the suspended filaments were incubated at 30°C with stirring. At regular intervals, samples were taken from the cultures and used for either detection of PBPs or quantification of SDS-insoluble peptidoglycan.

When peptidoglycan produced by PBP 3 was to be used for analysis by high-pressure liquid chromatography (HPLC), the procedure was as follows. PAT84 cells were grown to an A_{550} of 0.2. Then, aztreonam was added to a final concentration of 0.075 μg/ml. This concentration produced only an 85% blockage of PBP 3 but caused clear filamentation of all cells observable by light microscopy (see Results). The A_{550} of the culture increased exponentially up to 1.3 to 1.4 units and then stopped, as mentioned above. After 60 min, filaments were diluted 15-fold in (i) prewarmed (30°C) supplemented medium containing 5 μCi of 2,6-*meso*-[^{3}H]diaminopimelic acid per ml and (ii) prewarmed (30°C) supplemented medium containing 5 μCi of 2,6-*meso*-[^{3}H]diaminopimelic acid plus aztreonam (0.075 μg/ml, final concentration). The final volume of both cultures was 50 ml. Incubation of the diluted filaments was continued. After 7 min of incubation, the cultures were stopped by addition of 10 ml of a 10% (wt/vol) SDS solution and rapid boiling of the mixtures. Boiled cultures were centrifuged at 100,000 × *g* for 2 h at 30°C. The pellets were collected, and peptidoglycan was isolated and prepared for analysis. Parallel cultures of diluted filaments were also made. From these cultures, 2-ml samples were taken at regular intervals up to 30 min. From these samples, SDS-insoluble labeled peptidoglycan was quantified.

Quantification of labeled peptidoglycan. Samples of *E. coli* cells labeled with 2,6-*meso*-[^{3}H]diaminopimelic acid were collected from the cultures and mixed with an equal volume of 10% (wt/vol) hot SDS and incubated in a boiling-water bath. Mixtures were filtered on HWPO filters (0.45-μm pore size; Millipore Corp.). After extensive washing of the filters with hot water, the radioactivity retained was monitored in a toluene-base scintillation liquid.

Analysis of labeled peptidoglycan. Peptidoglycan from 2,6-*meso*-[^{3}H]diaminopimelic acid-labeled cells was isolated as described previously (11). Deproteinized peptidoglycan was hydrolyzed with muramidase from *Chalaropsis* spp. as described previously (10). Peptidoglycan subunits were analyzed by HPLC as described before (8, 23). Radioactive peaks were monitored in a Berthold HPLC radioactive monitor (model LB505).

Transpeptidation assays. Transpeptidation assays were done with PAT84 cells that had been grown at 30°C. Cells were rendered permeable by treatment with ether, strictly following the procedure of Vosberg and Hoffmann-Berling (35). Exogenous substrate precursors for transpeptidase reactions were UDP-[^{14}C]acetylglucosamine and either UDP-*N*-acetylmuramyl-pentapeptide or UDP-*N*-acetylmuramyl-tripeptide. These two precursors were purified from *Bacillus cereus* cells treated with either bacitracin (for UDP-*N*-acetylmuramyl-pentapeptide) or D-cycloserine (for UDP-*N*-acetylmuramyl-tripeptide). The procedures used were those described by Barnett (2). Transpeptidase assays were done basically by the method of Mirelman et al. (19). The concentration of UDP-*N*-acetyl-[^{14}C]glucosamine was 500 μM (2,000 cpm/μl of assay solution). The penta- or tripeptide precursors were also added at a concentration of 500 μM. The final volume of the assay was 200 μl. When antibiotics were used they were added 15 min before the reaction was triggered by the addition of the substrates. The temperature of the reaction was 30°C, and only radioactive SDS-insoluble peptidoglycan was quantified.

The quantitative data described in Results were obtained from the same preparation of ether-treated cells and represent the means of quadruplicate experiments.

Chemicals. UDP-*N*-acetyl-[^{14}c]glucosamine (125 Ci/mmol) and ^{125}I-labeled Bolton and Hunter reagent (2,000 Ci/mmol) were purchased from Amersham International, Amersham, U.K. 2,6-*meso*-[^{3}H]diaminopimelic acid (40 Ci/mmol) was from the Commissariat à l'Energie Atomique, Gif-sur-Yvette, France. Aztreonam, cefsulodin, and bacitracin were generous gifts from E. R. Squibb and Son, Inc., Princeton, N.J., Takeda Chemical Industries, Ltd., Osaka, Japan, and Liade, Madrid, Spain, respectively. D-Cycloserine was from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Binding of aztreonam to PBP 3. We first carried out control experiments to know the reversibility of the binding of

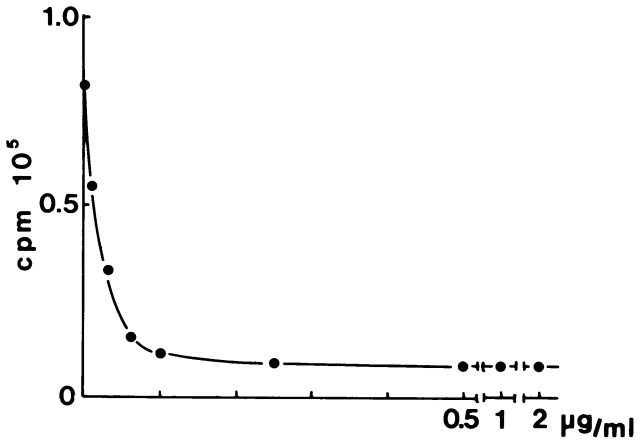

FIG. 1. Labeling of PBP 3 from growing *E. coli* cells by aztreonam. PAT84 cells were grown to an A_{550} of 0.2. Aztreonam at different concentrations was added to the cultures, and incubation was continued for 30 min. Cells were collected and disrupted by sonication. Cell extracts (all containing the same amount of protein as measured by their A_{280}) were incubated with 250 mM ^{125}I-ampicillin at 30°C for 15 min. Samples were then prepared for SDS-PAGE. Gels were autoradiographed, the portions of the dried gels containing the PBP 3 were cut out, and their radioactivity was measured in a gamma counter.

aztreonam to PBP 3. The experimental conditions are described in Materials and Methods. After binding of aztreonam to membranes under conditions in which only PBP 3 was labeled, free antibiotic was removed by centrifugation at high speed. Incubation at 30°C of membranes containing aztreonam-bound PBP 3 for up to 90 min and subsequent labeling of the PBPs with saturating concentrations of ^{125}I-ampicillin revealed that PBP 3 could not be labeled with the radioactive antibiotic. Parallel experiments demonstrated that aztreonam-unlabeled PBP 3 was stable and did not lose its ability to bind ^{125}I-ampicillin under the same conditions (not shown). We therefore concluded that the complex of aztreonam and PBP 3 is stable under the conditions used here. To know the characteristics of the binding of aztreonam to whole cells, the antibiotic was added at different concentrations to exponentially growing cells. After 30 and 90 min of incubation, the cells were broken and aztreonam-unlabeled PBP 3 was labeled and elicited with ^{125}I-ampicillin. Figure 1 shows the results of this set of experiments. The affinity of aztreonam for the PBP 3 of growing cells was very high because half-saturation was produced by 0.02 μg of antibiotic per ml. In the range of concentrations used, aztreonam appeared not to bind to other PBPs. In Fig. 1, the cells had been incubated with aztreonam for 30 min. Incubation for 90 min yielded similar results (not shown). Before the cultures were harvested, the cells were observed under light microscopy. Filaments were only observed in cells treated with aztreonam at a concentration of 0.03 μg/ml or higher. This means that blockage of only 60% of the total PBP molecules is sufficient to induce cell filamentation.

Action of D-cycloserine plus aztreonam on *E. coli* cells. It is known that D-cycloserine inhibits the synthesis of D-alanyl-D-alanine. The eventual result is the inhibition of the biosynthesis of the peptidoglycan precursor UDP-*N*-acetylmuramyl-pentapeptide and the accumulation of UDP-*N*-acetylmuramyl-tripeptide in the cytoplasm (21). We first explored the action of D-cycloserine on the growth and morphology of *E. coli* cells. D-Cycloserine up to a concentration of 10 μg/ml scarcely affected the growth of PAT84 cells (Fig. 2). However, D-cycloserine at the concentrations used (from 5 to 15 μg/ml) produced a shorter and round-shaped cells (not shown). Aztreonam also caused filamentation of the cells, as previously reported (31). Nevertheless, aztreonam did not produce any variation in the increase in the A_{550} in PAT84 cultures (Fig. 1). However, the addition of aztreonam to a culture of cells previously treated with D-cycloserine eventually caused cell growth to stop (Fig. 2). Examination of the cells treated with both D-cycloserine and aztreonam revealed the existence of both individual cells and two-cell filaments. These small filaments contained a bulge at the middle, where presumably a septum would have formed (not shown).

Combined action of D-cycloserine and temperature. The effect of aztreonam on D-cycloserine-treated cells can be mimicked by increasing the temperature of the culture of an *sulB* mutant. As shown in Fig. 2B, increasing the temperature to 41°C stopped the growth of PAT84 cells previously treated with a sublethal concentration of D-cycloserine. This effect could be associated with the *sulB* mutation, as no effect was seen in the parental strain PA3092. Several pieces of work on the *sulB* gene have recently appeared. The division cluster *sulB*, *ftsA*, *ftsQ* resides at min 2 on the *E. coli* chromosome, close to the *mur* genes, and to the *pdpB* gene responsible for coding PBP 3 (for a review, see reference 34). All these genes are involved in both peptidoglycan biosynthesis and cell division. In particular, the FtsA protein is one of the components of bacterial septum (33). The *sulB* mutation of PAT84 cells treated with D-cycloserine had an effect on cell growth similar to that caused by inhibiting PBP 3 with aztreonam. Indeed, in temperature filaments of PAT84, the amount of PBP 3 that could be labeled with ^{125}I-ampicillin was about 40% of that detectable in normal cells (Fig. 2C). At present, we do not know the relationship between the *sulB* mutation and the amount of PBP 3 detectable with ^{125}I-ampicillin, but should this decrease in PBP 3 be real, it may be responsible for the lack of septation of PAT84 cells grown at 41°C because, as shown above, blockage of half the PBP 3 by aztreonam caused filamentation.

Biosynthesis of peptidoglycan produced by PBP 3. We first took advantage of cell filamentation to see peptidoglycan produced by PBP 3. Aztreonam from cultures containing nongrowing filaments was filtered off, and the filaments were suspended in fresh medium with or without aztreonam (see Materials and Methods for details). Figure 3A shows the result of a typical experiment. There was a rapid appearance of PBP 3, and the level was maintained throughout the experiment. We believe that this PBP 3 is a de novo protein, as the old protein should remain blocked by aztreonam due to the stability of the aztreonam-PBP 3 complex (see above). Two or three minutes after the appearance of PBP 3, insoluble peptidoglycan formed a clear shoulder (Fig. 3A) which was not seen when PBP 3 remained blocked by aztreonam. The addition of the β-lactam cefsulodin to filtered and suspended filaments produced curious effects. This β-lactam at the concentration used, 50 μg/ml, binds to most PBP 1a and 1b molecules (6, 26). Cefsulodin did not produce any observable effect on SDS-insoluble peptidoglycan synthesis in aztreonam-produced filaments. However, when PBP 3 was allowed to act, the incorporation of 2,6-*meso*-[^{3}H]diaminopimelic acid into SDS-insoluble peptidoglycan was normal until the PBP 3-associated shoulder was reached, but later the amount of radioactive peptidoglycan decreased, accompanied by cell lysis. We believe that

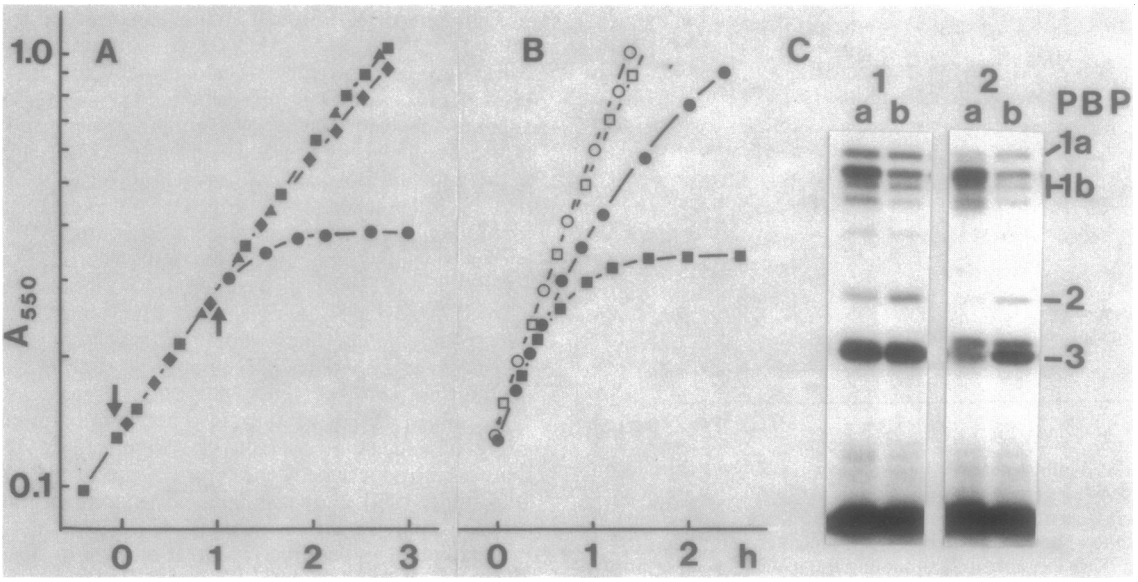

FIG. 2. (A) Action of D-cycloserine and aztreonam on the growth of *E. coli* PAT84 cells. Cells were grown at 30°C. D-Cycloserine (downward arrow) and aztreonam (upward arrow) were added. Symbols: ■, no antibiotic; ▲, aztreonam (1.0 μg/ml); ◆, D-cycloserine (10 μg/ml); ●, D-cycloserine (10 μg/ml) plus aztreonam (1.0 μg/ml). (B) Action of D-cycloserine on the growth of PAT84 and PA3092 *E. coli* cells. Cells were grown at 30°C to an A_{550} of 0.14, when D-cycloserine (10 μg/ml) was added and the temperature of the culture was shifted to 41°C. Symbols: ○, PA3092 with no D-cycloserine; □, PA3092 treated with D-cycloserine (10 μg/ml); ●, PAT84 without D-cycloserine; ■, PAT84 treated with D-cycloserine (10 μg/ml). (C) Autoradiographical pattern of PBPs from PAT84 (lanes a) and PA3092 (lanes b). Cells were grown at either 30°C (panel 1) or 41°C (panel 2). PBPs were detected in cell extracts by labeling with ^{125}I-ampicillin.

cell division in filaments was necessary before the cell could grow and become sensitive to cefsulodin.

The experiment shown above (Fig. 3A) was attempted 29 times and was reproducible on only 16 occasions. Often, the filtration procedure failed to bring about reproducible results. We later tried a gentler method for decreasing drastically the amount of aztreonam in filament cultures. Filament formation was induced with a lower concentration of aztreonam, 0.075 μg/ml. After the filaments stopped growing, the culture was diluted so that the concentration of aztreonam fell to 0.005 μg/ml. This concentration did not produce filaments and could barely label an insignificant amount of PBP 3. Incorporation of 2,6-*meso*-[^{3}H]diaminopimelic acid into SDS-insoluble peptidoglycan followed (Fig. 3B). Different amounts of radioactive peptidoglycan can be seen in the filaments suspended in medium with and without aztreonam. We believe that the difference observed was due to the synthesizing action of PBP 3. A large sample was taken at min 7, and the cells were processed for isolation and analysis of de novo-synthesized peptidoglycan.

Transpeptidase activity of PBP 3. Transpeptidase activity in ether-treated cells was studied by using exogenously added peptidoglycan precursors, namely UDP-*N*-acetylglucosamine and either UDP-*N*-acetylmuramyl-pentapeptide or UDP-*N*-acetylmuramyl-tripeptide. When UDP-*N*-acetylmuramyl-pentapeptide was used as the substrate precursor, 3,766 pmol was incorporated into SDS-insoluble peptidoglycan per mg of cell protein per h. Aztreonam (1 μg/ml) inhibited pentapeptide precursor-mediated transpeptidation by 20% (the activity fell to 3,006 pmol/mg of protein per h). These data are in agreement with those of previous work in which the β-lactam furazlocillin was used as a selective inhibitor of PBP 3 (27). The antibiotic cefsulodin, at a concentration of 50 μg/ml, produced 85% inhibition of pentapeptide-mediated transpeptidation (an activity of 426 pmol/mg of protein per h was detected). At this concentration the antibiotic blocked nearly all PBP 1a and 1b and about 20% of PBP 3 (data not shown). Therefore, we suggest that the strong inhibition of pentapeptide-mediated transpeptidase activity caused by cefsulodin was due to the inhibition of PBPs 1a and 1b.

On the other hand, when UDP-*N*-acetylmuramyl-tripeptide was used for transpeptidase assays, we also obtained incorporation of the radioactive precursor into SDS-insoluble peptidoglycan. However, the activity was 906 pmol/mg per h, i.e., about four times less than that obtained with the pentapeptide precursor. Aztreonam at a concentration of 1 μg/ml, at which only PBP 3 was blocked, strongly inhibited tripeptide precursor-mediated transpeptidation, since the activity fell to 322 pmol/mg per h. However, cefsulodin at a concentration of 50 μg/ml did not produce any noticeable effect on tripeptide-mediated transpeptidation. The data described above support the idea that PBP 3 participates in either pentapeptide or tripeptide precursor-mediated transpeptidation, although it appears that the tripeptide is the preferred substrate.

Analysis of peptidoglycan in aztreonam- and D-cycloserine-treated cells. To ascertain the peptidoglycan-making activity of PBP 3, we obtained, as a first approach, the primary structure of nascent peptidoglycan from cells treated with either aztreonam or D-cycloserine or both. Cells were labeled with 2,6-*meso*-[^{3}H]diaminopimelic acid, and only SDS-insoluble peptidoglycan was analyzed. The results are shown in Table 1, and Table 2 presents the same results with the peptidoglycan subunits grouped into families. As can be observed, aztreonam, under conditions in which only PRP 3 was affected, did not induce large alterations in the structure of de novo-synthesized peptidoglycan. There was, nevertheless, one small but clear difference that should be pointed out: the degree of peptidoglycan cross-linking decreased somewhat after aztreonam treatment (Table 2). Table 1 shows that the decrease of bis(disaccharide tetrapeptide),

along with the parallel increase of disaccharide tetrapeptide, was the main factor responsible for the decrease of peptidoglycan cross-linking. D-Cycloserine induced more definite alterations in cell peptidoglycan. The amount of disaccharide tripeptide was largely increased (Table 1). In our opinion, this fact strongly supports the idea that accumulated UDP-*N*-acetylmuramyl-tripeptide can be translocated in the bactoprenol cycle and integrated into SDS-insoluble peptidoglycan by the action of transglycosylases. Moreover, the data in Table 1 also show that very probably, the peptidoglycan-integrated tripeptide participated as an acceptor in peptidoglycan transpeptidase reactions. Indeed, comparison of columns C and A in Table 1 reveals that peptidoglycan from D-cycloserine-treated cells contained an increased amount of both trimers and dimers (i.e., cross-linked subunits) built from the tripeptide subunit. Actually, the decrease of bis(disaccharide tetrapeptide) in D-cycloserine-treated cells was compensated by the increase of tripeptide-

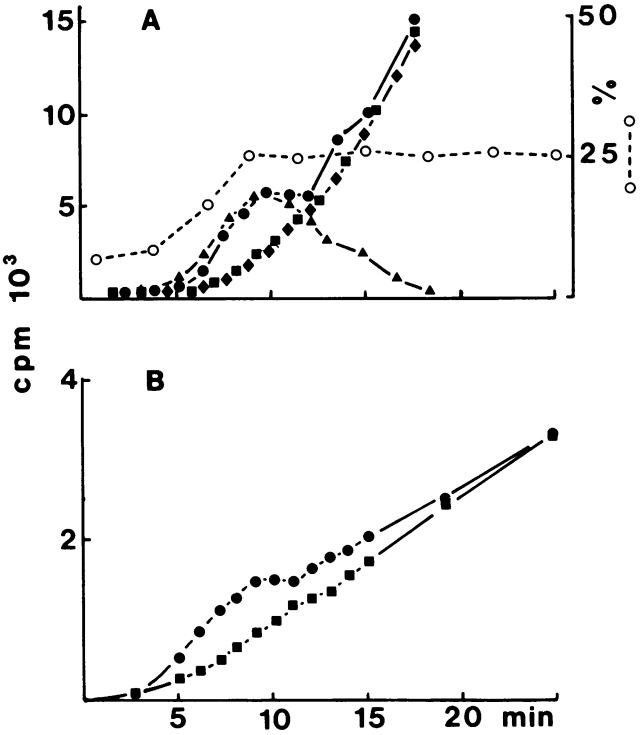

FIG. 3. (A) Levels of PBP 3 appearance and de novo SDS-insoluble peptidoglycan synthesis from PAT84 filaments reverted to normal conditions. PAT84 filaments induced by aztreonam (1.0 μg/ml) were filtered and suspended in supplemented L medium containing 1 μCi of 2,6-*meso*-[^{3}H]diaminopimelic acid per ml and incubated further. Samples were taken at different times from the fresh culture and used for determination of SDS-insoluble radioactive peptidoglycan (●). Other filament samples were suspended in the same medium containing aztreonam (1.0 μg/ml) (■), cefsulodin (50 μg/ml) (▲), or aztreonam (1.0 μg/ml) plus cefsulodin (50 μg/ml) (◆). The samples taken from the antibiotic-free culture were also used for quantitative detection of PBP 3 (○, right ordinate). These results were expressed as the percentage of PBP 3 found in normal cells growing exponentially at 30°C. (B) Appearance of de novo-synthesized SDS-insoluble peptidoglycan from PAT84 filaments restored to normal conditions. Filamentation was induced by aztreonam (0.075 μg/ml). The action of aztreonam was stopped by 15-fold dilution in supplemented medium containing 50 μCi of 2,6-*meso*-[^{3}H]diaminopimelic acid in either the absence (●) or presence (■) of aztreonam (0.075 μg/ml).

TABLE 1. Composition of peptidoglycan synthesized by *E. coli* PAT84 cells either untreated or treated with azthreonam, D-cycloserine, or both[a]

Peptidoglycan subunit[b]	Molar fraction (× 10^2) with [c]:			
	A	B	C	D
1. Tri	4.63	5.03	16.29	18.99
2. Tetra (Gly)	1.87	1.72	2.46	1.57
3. Tetra	64.41	65.06	42.85	44.82
4. Penta (Gly)	ND[d]	ND	ND	ND
5. Dipeptide	ND	ND	ND	ND
6. Penta	1.86	2.01	2.62	2.83
7. Tri-Lys-Arg	3.07	3.52	5.01	4.54
8. Tetra-tri (Gly)	ND	ND	0.22	ND
9. Tri-tri (DAP-DAP)	0.28	0.21	0.94	0.53
10. Tetra-tri	0.97	1.15	1.46	1.06
11. Tetra-tri (DAP-DAP)	2.85	3.50	7.27	6.80
12. Tetra-tetra (Gly)	ND	ND	1.69	ND
13. Tri (anh)	ND	ND	ND	ND
14. Tetra-penta (Gly)	ND	ND	ND	ND
15. Tetra-tetra	13.18	11.84	9.40	10.60
16. Tetra-penta	0.69	0.67	1.78	1.01
17. Tri-tri (DAP-DAP)-Lys-Arg	0.96	0.71	1.27	0.87
18. Tetra (anh)	0.27	ND	ND	0.23
19. Tetra-tetra-tri (DAP-DAP)	0.36	0.32	0.59	0.47
20. Tetra-tetra-tetra	0.60	0.69	0.71	0.98
21. Tetra-tri (DAP-DAP)-Lys-Arg	0.90	1.03	1.06	1.49
22. Tri-tri (DAP-DAP) (anh)	ND	ND	0.20	ND
23. Tetra-tri-tri (DAP-DAP)-Lys-Arg	0.11	0.17	0.23	0.12
24. Tetra-tri (anh)	0.27	0.25	0.15	0.14
25. Tetra-tri (DAP-DAP) (anh)	0.75	0.62	1.60	1.33
26. Tetra-tetra (anh)	1.20	1.03	1.21	0.95
27. Tetra-tetra-tri (anh)	0.19	0.06	0.39	0.06
28. Tetra-tetra-tri (DAP-DAP) (anh)	ND	0.13	0.35	0.24
29. Tetra-tetra-tetra (anh)	0.34	0.20	0.26	0.16
30. Tri-tri (DAP-DAP)-Lys-Arg (anh)	0.26	0.09	ND	0.20

[a] Cells were labeled 30 min after the addition of aztreonam and harvested 15 min later.

[b] In this nomenclature the disaccharide part is omitted for the purpose of clarity. The prefixes di-, tri-, tetra-, and penta- mean disaccharide di-, tri-, tetra-, and pepntapeptide, respectively. (Gly) indicates that the last amino acid of the peptide is glycine substituting to D-alanine. Lys-Arg indicates a lysyl-arginine residue which comes from lipoprotein that was bound to the subunit before treatment of the peptidoglycan with pronase. (DAP-DAP) indicates the existence of a diaminopimelyl-diaminopimelyl interpeptide bridge. (anh) indicates an anhydro sugar subunit which constitutes the terminal component of a glycan chain. The ratio of nonanhydro to anhydro subunits gives the length of the glycan chain.

[c] A, Untreated; B, aztreonam (1 μg/ml); C, D-cycloserine (10 μg/ml); D, aztreonam (1 μg/ml) plus D-cycloserine (10 μg/ml).

[d] ND, Not detected.

containing subunits. The overall result is the production of a somewhat more cross-linked peptidoglycan in D-cycloserine-treated cells (Table 2). The effect of selective blockage of PBP 3 by aztreonam in D-cycloserine-treated cells can be seen in columm D of Tables 1 and 2. Aztreonam produced a less cross-linked peptidoglycan due to the decrease in nearly all dimers and trimers containing the tripeptide. Also, the aztreonam produced a peptidoglycan which contained a higher amount of disaccharide tripeptide. It seems that, to some extent, aztreonam reversed some effect induced by D-cycloserine. Comparison of columns C and D in both Table 1 and 2 revealed that the blockage of PBP 3 impaired transpeptidation generated by the excess of tripeptide present in D-cycloserine-treated cells.

Analysis of peptidoglycan produced by PBP 3. Under the conditions for defilamentation described above, we managed to obtain labeled de novo peptidoglycan, to a great extent due to the action of PBP 3. Indeed, 45% of the peptidoglycan from cells collected at min 7 (Fig. 3B) was due to the action

TABLE 2. Composition of peptidoglycan synthesized by *E. coli* PAT84 cells either untreated or treated with aztreonam, D-cycloserine, or both[a]

Subunit family	Molar fraction (× 10^2) with:			
	A	B	C	D
1. Monomers[b]	76.1	77.3	69.2	73.0
2. Dimers[b]	22.3	21.1	28.2	25.0
3. Trimers[b]	1.6	1.6	2.5	2.0
4. Bridges[c]	25.5	24.2	33.3	29.0
5. DAP-DAP[d]	6.5	6.8	7.6	7.2
6. DAP-DAP/total bridges	25.3	27.9	40.6	41.4
7. Lipoprotein[e]	5.3	5.5	7.6	7.2
8. Lipoprotein D-T[f]	42.0	36.2	33.8	37.1
9. Anhydro compounds[g]	3.3	2.4	4.2	3.3
10. Glycan chain length[g]	30.6	42.2	24.0	30.1

[a] See Table 1, footnotes *a*, *b*, and *c*.
[b] Total amount of monomers (disaccharide peptides), dimers [bis(disaccharide peptides)], and trimers[tri(disaccharide peptides)].
[c] Total amount of dimers plus trimers, i.e., those subunits containing interpeptide bridges.
[d] Total amount of dimers and trimers which have one diaminopimelyl-diaminopimelyl interpeptide bridge.
[e] Total amount of subunits containing one terminal lysyl-arginine residue derived from hydrolyzed lipoprotein.
[f] Percentage of cross-linked subunits (dimers and trimers [D-T]) containing lipoprotein (100% = total subunits containing lipoprotein).
[g] Anhydro compounds are the terminal subunits of a glycan chain. The ratio of nonanhydro to anhydro compounds estimates the chain length, i.e., the number of disaccharide subunits per chain.

of PBP 3. The possibility that at least some of this peptidoglycan was a constituent of the septum has been described above and is discussed below. Analyses of this peptidoglycan are shown in Tables 3 and 4. Qualitative results can be obtained by substractive comparison of the data. Such a comparison reveals that PBP 3 was responsible for the higher levels of cross-linking observed in septating cells. Table 4 shows that PBP 3 was responsible for the higher amount of both dimers and trimers. It can be observed that there were more cross-linked subunits containing tripeptide in untreated filaments (6.22 versus 4.87% of the total). In addition, bis(disaccharide tripeptide) was also increased when aztreonam was omitted. It can also be observed that in this system the disaccharide tripeptide subunit was not detected. We believe that the tripeptide contained in the cross-linked subunits came from stepwise degradation of nascent pentapeptide. PBP 3, as in D-cycloserine-treated cells, used tripeptide, although from a different source. PBP 3 also appears to produce bis(disaccharide tetrapeptide), but it seems that this subunit was derived from pentapeptide-derived nascent disaccharide pentapeptide, which was used here as an acceptor (see Discussion). Other data on *E. coli* septum structure through indirect analysis agree with those presented here, i.e., the septum appeared to be more cross-linked (12, 13, 22).

DISCUSSION

PBP 3 as supporter of bacterial growth under limitation of UDP-*N*-acetylmuramyl-pentapeptide. In part of this study, *E. coli* cells were manipulated to limit the cytoplasmic production of UDP-*N*-acetylmuramyl-pentapeptide. This effect was achieved with the antibiotic D-cycloserine, which was used at sublethal concentrations so that it allowed normal growth. Under these artificial conditions it appeared that PBP 3 was the main factor responsible for bacterial viability. Indeed, the selective blockage of PBP 3 by aztreonam caused a cessation in cell growth. It seems that PBP 3 used the tripeptide precursor as a substrate both in vivo and in vitro. Indeed, it appeared that UDP-*N*-acetylmuramyl-tripeptide was used as a precursor for peptidoglycan biosynthesis. According to published data, the translocase enzyme can accept as substrate the tripeptide precursor in *Staphylococcus aureus* and in "*Gaffkya homari*," although at a rate much lower than that for the pentapeptide precursor (9). On the other hand, analysis of peptidoglycan from D-cycloserine-treated cells (Table 1) shows that the disaccharide-tripeptide subunit is very abundant, and in addition, dimers and trimers containing tripeptide are also increased. It seems, therefore, that the tripeptide precursor can be utilized for peptidoglycan synthesis, and this process is in fact mediated by PBP 3. Moreover, PBP 3 also used the tripeptide precursor in the in vitro transpeptidase assays with ether-treated cells. It has recently been demonstrated that in transpeptidase in vitro assays with UDP-*N*-acetylmuramyl-pentapeptide as a substrate, the substrate was used by ether-treated cells as a transpeptidation acceptor and not as a donor (13). It is not therefore surprising that the tripeptide precursor can be used in vitro as an acceptor for transpeptidation. This acceptor did not appear to be used by PBPs 1a and 1b because their blockage by cefsulodin did not affect tripeptide-mediated transpeptidation. This precursor was used by PBP 3, although possibly other PBPs are also able to use that substrate.

Some previously reported data suggest that tripeptide might be involved in peptidoglycan septation (12). Indeed, it

TABLE 3. Composition of radioactive peptidoglycan synthesized by *E. coli* PAT84 during defilamentation

Peptidoglycan subunit[a]	Molar fraction (× 10^2)	
	Treated[b]	Untreated
1. Tri	ND[c]	ND
2. Tetra (Gly)	ND	ND
3. Tetra	78.88	75.63
4. Penta (Gly)	ND	ND
5. Dipeptide	ND	ND
6. Penta	1.24	1.54
7. Tri-Lys-Arg	0.62	0.49
8. Tetra-tri (Gly)	0.09	ND
9. Tri-tri (DAP-DAP)	0.04	ND
10. Tetra-tri	0.47	0.56
11. Tetra-tri (DAP-DAP)	2.61	2.91
12. Tetra-tetra (Gly)	ND	ND
13. Tri (anh)	ND	ND
14. Tetra-penta (Gly)	ND	ND
15. Tetra-tetra	13.76	15.33
16. Tetra-penta	0.11	ND
17. Tri-tri (DAP-DAP)-Lys-Arg	0.25	0.34
18. Tetra (anh)	ND	ND
19. Tetra-tetra-tri (DAP-DAP)	0.13	0.31
20. Tetra-tetra-tetra	0.33	0.46
21. Tetra-tri (DAP-DAP)-Lys-Arg	0.42	0.69
22. Tri-tri (DAP-DAP) (anh)	ND	ND
23. Tetra-tri-tri (DAP-DAP)-Lys-Arg	0.05	0.09
24. Tetra-tri (anh)	0.01	0.14
25. Tetra-tri (DAP-DAP) (anh)	0.27	0.43
26. Tetra-tetra (anh)	0.61	0.71
27. Tetra-tetra-tri (anh)	ND	ND
28. Tetra-tetra-tri (DAP-DAP) (anh)	ND	ND
29. Tetra-tetra-tetra (anh)	0.12	0.11
30. Tri-tri (DAP-DAP)-Lys-Arg (anh)	ND	0.26

[a] See Table 1, footnotes *a* and *b*.
[b] Cells treated with aztreonam (0.075 μg/ml).
[c] ND, Not detected.

appears that the levels of both DD-carboxypeptidase and LD-carboxypeptidase activity are higher just before septation (3). This would mean that before septation, the emerging pentapeptide precursor would be rapidly degraded to tetra- and tripeptide, and they could be used as a precursor for cell septation. Besides, the role of PBP 5 has been emphasized in this process (17), PBP 5 (or 6) being the DD-carboxypeptidase responsible for the rapid degradation of the emerging pentapeptide just before septation. However, the role of PBPs 5 and 6 in septation is far from clear, as mutants lacking both PBPs septate normally. However, from our data, it can by no means be deduced that some sort of regulation of cell septation can take place at the cytoplasmic level, i.e., by modulating the ratio of tripeptide and pentapeptide precursors available in the cytoplasm. Data from van Heijenoort and his colleagues (18) do not support this idea. Indeed, the level of the tripeptide precursor in the *E. coli* cytoplasm is so low compared with that of pentapeptide that it is dubious they could play a relevant role in septation.

There are other questions concerning the nonseptal activity of PBP 3. In our artificial system, i.e., in D-cycloserine-treated cells, as mentioned above, PBP 3 supports cell life. Data about the exchangeability of PBPs do exist (12, 30, 35). Under severe conditions PBP 3 could also perform nonseptal activities. Two possible explanations can be brought forward. First, under pentapeptide precursor limitation, PBP 3 may be the one that displays a higher affinity for the precursor. Second, PBP 3 can use the available tripeptide for nonseptal transpeptidation. The possibility also exists that there are two populations of PBP 3, only one being engaged in septal activity. This idea is hinted by the fact that inhibition of only 60% of PBP 3 produced filamentation. The septal activity of PBP 3 could be modulated by other proteins such as the SulB (see above) and FtsA proteins (32).

Peptidoglycan produced by PBP 3. The experiments shown in Fig. 3 clearly show that a significant part of the de novo-synthesized peptidoglycan obtained was produced by PBP 3. Another question is whether this is septal peptidoglycan. We believe that at least part of the PBP 3-produced peptidoglycan is septal. Data not shown here demonstrated that the β-lactam cefsulodin produced lysis in growing PAT84 aztreonam-induced filaments. However, cefsulodin did not produce any effect on nongrowing filaments (Fig. 3A). Nevertheless, when aztreonam was removed from exhausted filaments, cefsulodin did produce lysis just after PBP 3-made peptidoglycan was elicited. Cefsulodin only killed growing cells, and therefore septation had to occur before the killing action of cefsulodin could be displayed. Although a morphological and statistical study would be necessary to prove the filaments septate at the time of the appearance of PBP 3-made peptidoglycan, there are data which indicate that temperature-induced PAT84 filaments double their number when they are restored to a permissive temperature in the presence of chloramphenicol (24).

Structure of PBP 3-woven peptidoglycan. Both in vivo and in vitro data indicate that PBP 3 can use disaccharide tripeptide for transpeptidation. However, the tripeptide used came either exogenously (in transpeptidation assays with ether-treated cells) or when its concentration was forcibly increased by D-cycloserine. Despite this fact, when aztreonam-induced filaments were restored to permissive conditions, the peptidoglycan, most probably of a septal nature, also contained an increased amount of cross-linked subunits containing tripeptide. Also, in restored filaments, PBP 3 appeared to synthesize bis(disaccharide tetrapeptide), which is the most abundant cross-linked subunit in *E. coli* peptidoglycan. These data suggest that PBP 3 could use either nascent tripeptide or pentapeptide subunits. There is no doubt that, as pointed out above, disaccharide tripeptide, which could be used later as a substrate for PBP 3, did not under normal conditions come from UDP-*N*-acetylmuramyl-tripeptide but from a pentapeptide precursor previously hydrolyzed by DD- and LD-carboxypeptidases whose activities are largely increased just before septation takes place (27). Thus, under conditions in which the amount of pentapeptide precursor was limited while that of the tripeptide was increased, PBP 3 could support the viability and growth of the cells because this PBP could use both penta- and tripeptide precursors as acceptors for transpeptidase reactions. Such reactions not only produced normal cell septation in D-cycloserine-treated cells, but also limited amounts of lateral peptidoglycan enough to support cell growth. The effect of D-cycloserine on cell morphology resembles very much that of either selective blockage or mutation of PBP 2 (29) and also that yielded by hyperproduction of PBP 5 (17). In both cases the normal utilization of pentapeptide was impaired and the cells did not maintain their normal rod morphology.

TABLE 4. Composition of radioactive peptidoglycan synthesized by *E. coli* PAT84 during defilamentation

Subunit family[a]	Molar fraction (× 10^2)	
	Treated[b]	Untreated
1. Monomers	80.7	77.7
2. Dimers	18.6	21.4
3. Trimers	0.6	1.0
4. Bridges	19.9	23.3
5. DAP-DAP	3.8	5.0
6. DAP-DAP/total bridges	18.9	21.6
7. Lipoprotein	1.3	1.9
8. Lipoprotein D-T	53.6	74.0
9. Anhydro compounds	1.0	1.6
10. Glycan chain length	99.8	61.0

[a] See Table 2, footnotes *b* through *g*.
[b] Cells treated with aztreonam (0.075 μg/ml).

Septal peptidoglycan produced by PBP 3 is certainly different from that produced in the absence of PBP 3 activity, but from its building block composition little can be known about the spatial structure of the septum. Moreover, PBP 3 is probably responsible for the making of only one part of the septum, the last one, and not for the total septal structure. The data presented are therefore related to the septal activity of PBP 3.

We can make a final reflection on the validity of the data presented in this article. In some experiments, *E. coli* cells have been subjected to very artificial conditions that may be the source of artifacts. However, all our attempts have led to the isolation and elicitation of the activity of PBP 3 in whole cells. It is presumed, therefore, that the activity of this PBP is the same as or similar to that performed in "normal" *E. coli* cells. It should be pointed out that most of what is known about the action of PBPs was learned from manipulation of the cells (such as genetic manipulation) or from selective blockage by β-lactam antibiotics. Moreover, the activity of PBPs in simplified in vitro systems has only been clearly demonstrated for DD-carboxypeptidases. Other in vitro systems for transpeptidase assays have failed to assign a clear activity to PBP 2 and 3 despite the fact that these enzymes are essential for peptidoglycan elongation and

septation, respectively. We believe that the experimental systems used here can be used to learn some of the relevant features of PBP 3 activity, including the septal one.

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