

An Amino Acid Substitution in Penicillin-Binding Protein 3 Creates Pointed Polar Caps in *Escherichia coli*

PETER E. M. TASCHNER,^{1†*} NIELS YPENBURG,¹ BRIAN G. SPRATT,² AND CONRAD L. WOLDRINGH¹
Department of Molecular Cell Biology, University of Amsterdam, Plantage Muidergracht 14, 1018 TV Amsterdam, The Netherlands,¹ and Microbial Genetics Group, School of Biological Sciences, University of Sussex, Falmer, Brighton BN1 9QG, England²

Received 22 March 1988/Accepted 20 July 1988

The *pbpB* gene product penicillin-binding protein 3 (PBP3) of *Escherichia coli* is one of the major targets of β -lactam antibiotics. At the permissive temperature, the temperature-sensitive *pbpB*^{r1} mutant, which was obtained after selection for increased resistance to cephalixin, shows a dramatic change in shape which has never been observed before; the polar caps are pointed. We show that the substitution of amino acid Asn-361 by Ser, previously shown to be responsible for increased cephalixin resistance and for temperature sensitivity, causes the pointed polar caps. However, comparison of the morphological and physiological characteristics of the *pbpB*^{r1} mutant with those of other *pbpB* mutants suggests that the formation of pointed polar caps is not correlated with temperature sensitivity or cephalixin resistance. Partial inactivation of PBP3 by subinhibitory concentrations of cephalixin, furazlocillin, and piperacillin resulted in the formation of slightly pointed polar caps, suggesting that the shape of the polar caps is correlated with PBP3 activity. The large change in the shape of the polar caps was accompanied by a small change in the kinetics of peptidoglycan synthesis and in the local rate of surface synthesis activity along the cell envelope.

It is generally assumed that the shape of *Escherichia coli* can be approximated by a cylinder with hemispherical polar caps. So far, no morphometric studies have been conducted to test the validity of this assumption. Therefore, it is possible that the shape of the polar caps is better described by ellipsoids or other three-dimensional reconstructions which have been used to represent growing septa (2, 11) or by shapes which are predicted by the surface stress theory (16).

During cell growth, the peptidoglycan layer that maintains the shape of the cell (31) is synthesized by specialized enzymes, the penicillin-binding proteins. According to the surface stress theory, the cell can divide only when the mechanism of peptidoglycan synthesis is changed at the division site in a way which locally decreases surface tension. This might be the function of penicillin-binding protein 3 (PBP3), which has a specific role in the formation of polar caps (24). As a result, the shape of the polar caps could be a reflection of the synthetic activity of PBP3. PBP3 is believed to be a bifunctional enzyme, with a transglycosylase activity and a penicillin-sensitive transpeptidase activity (13), and specifically binds several β -lactam antibiotics, such as cephalixin, furazlocillin, and piperacillin (9, 12, 22). An understanding of the interactions of β -lactam antibiotics with PBP3 requires a knowledge of its tertiary structure, but so far crystallization of purified PBP3 has not been achieved. Another approach to get information about the structure of PBP3 involved the isolation of mutants with a reduced affinity to, for instance, cephalixin (9, 10). Such mutations were mapped in the *pbpB* gene that encodes PBP3 and were characterized by Hedge and Spratt (9, 10). The amino acid substitutions resulting from these mutations were assumed to be within the active center and to result in cephalixin resistance by decreasing the affinity of PBP3 for the antibi-

otic without seriously impairing its affinity for its normal substrate. Although the amino acid substitutions defined by these studies are separated in the primary structure, they are probably close together in the tertiary structure.

Apart from information about the tertiary structure of PBP3, these *pbpB* mutants could provide additional evidence for the role of PBP3 in cell division. One of the mutations, *pbpB*^{r1}, resulted in the substitution of Asn-361 by Ser and led to a decreased thermostability of PBP3 and temperature-sensitive cell division (9). In addition, it resulted in a dramatic alteration of the shape of the polar cap at the permissive temperature. Instead of the approximately hemispherical polar caps of the parent *E. coli* strain, the *pbpB*^{r1} mutant appeared to have pointed cell poles, which are reminiscent of those of the yeast *Brettanomyces bruxellensis* (17) and which, to the best of our knowledge, have never been described for *E. coli*. This change in cell shape suggests that the Asn-361-to-Ser substitution results not only in a decreased affinity of PBP3 for cephalixin but also in some degree of impairment in the ability of PBP3 to process its normal substrate.

In this study, we demonstrate that there is a causal relationship between the *pbpB*^{r1} mutation and the formation of pointed polar caps. Furthermore, we tested the hypothesis that the shape of the polar caps is a reflection of PBP3 activity, and we determined the effect of the mutation on the overall rate of peptidoglycan synthesis and on the topography of peptidoglycan synthesis in *E. coli*.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. All strains, phages, and plasmids used in this study are shown in Table 1.

Media and growth conditions. Broth containing 1% tryptone (Difco Laboratories, Detroit, Mich.), 0.5% yeast extract (Difco), and 0.5% NaCl was used as rich medium (TY). TY agar consisted of TY broth supplemented with 1.5% agar (Difco). H agar, H top agar, and 2TY broth (18) were used

* Corresponding author.

† Present address: Department of Biochemistry, University of Leiden, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands.

TABLE 1. Bacterial strains

| Strain, phage, or plasmid | Genotypic or phenotypic characteristics | Source or reference |
|---------------------------|--|---------------------|
| <i>E. coli</i> K-12 | | |
| LMC500 | F ⁻ <i>araD139 Δ(argF-lac)U169 rpsL150 fbbB5301 ptsF25 deoC1 rbsR relA1 lysA1</i> | 29 |
| LMC502 | LMC500 <i>leu</i> ::Tn5 | 29 |
| LMC510 | LMC502 <i>leu</i> ⁺ <i>pbpB2158</i> (Ts) | 29 |
| LMC560 | LMC502 <i>leu</i> ⁺ <i>pbpB</i> ^{r1} | P1(SP1091) × LMC502 |
| LMC579 | LMC502 <i>leu</i> ⁺ <i>pbpB63</i> (Ts) | P1(SP63) × LMC502 |
| LMC580 | LMC502 <i>leu</i> ⁺ <i>pbpB</i> ^{r9} | P1(SP1125) × LMC502 |
| LMC634 | LMC502 <i>leu</i> ⁺ <i>pbpB</i> ^{r6} | P1(SP1096) × LMC502 |
| C600 | F ⁻ <i>thr thi fhuA lacY supE leu</i> | Lab collection |
| SP33 | F ⁻ <i>pbpB33</i> (Ts) | 9 |
| SP63 | F ⁻ <i>pbpB63</i> (Ts) <i>trpE9829</i> (Am) <i>tyr</i> (Am) <i>ilv supD126</i> (Ts) <i>malt</i> | 25 |
| SP1091 | C600 <i>leu</i> ⁺ <i>pbpB</i> ^{r1} | 9 |
| SP1092 | C600 <i>leu</i> ⁺ <i>pbpB</i> ^{r2} | 9 |
| SP1093 | C600 <i>leu</i> ⁺ <i>pbpB</i> ^{r3} | 9 |
| SP1096 | C600 <i>leu</i> ⁺ <i>pbpB</i> ^{r6} | 10 |
| SP1097 | C600 <i>leu</i> ⁺ <i>pbpB</i> ^{r7} | 10 |
| SP1124 | C600 <i>leu</i> ⁺ <i>pbpB</i> ^{r8} | 10 |
| SP1125 | C600 <i>leu</i> ⁺ <i>pbpB</i> ^{r9} | B. G. Spratt |
| JM101 | F ⁻ <i>proAB lacI^s ZΔM15 traD36 Δ(lac-pro) thi supE</i> | 35 |
| JE10730 | F ⁻ <i>pbpB730</i> (Ts) <i>thyA1 thr-1 leuB6 thi-1 argH1 hisG1 trp-1 lacY1 gal-6 mtlA2 xyl-7 malA1 ara-13 rpsL9 fhuA2 λ⁻ supE44 fic mel rfbD1 galP63</i> | 20 |
| Phages | | |
| P1 <i>vir</i> | | Lab collection |
| M13mp8/2-6 | | B. G. Spratt |
| Plasmids | | |
| pGG26 | <i>pbpB</i> ⁺ Ap ^r | J. R. Walker |
| pMC1 | Ap ^r Km ^r | This work |
| pBCP27 | <i>pbpB</i> ⁺ Tc ^r | R. Geerse |
| pBCP28 | Tc ^r | R. Geerse |

for transduction of JM101 with M13mp8/2-6. Minimal medium contained 3.16 g of K₂PO₄, 1.47 g of KH₂PO₄, 1.05 g of (NH₄)₂SO₄, 0.10 g of MgSO₄ · 7H₂O, 0.3 mg of FeSO₄, 7.1 mg of Ca(NO₃)₂ · 4H₂O, 1 mg of thiamine, 5 g of glucose, and 50 mg of lysine per liter and had an osmolality of 300 mosM. If necessary, tetracycline (12.5 μg/ml) and kanamycin (50 μg/ml) were added. The minimal medium was supplemented with threonine and methionine (50 μg/ml each) for diamino pimelic acid (DAP) incorporation experiments. For temperature shift experiments, cultures growing in steady state at 28°C in a water bath shaker were diluted fourfold in prewarmed medium at 42°C. After one mass doubling at 42°C, the culture was shifted to 28°C by diluting twofold in prewarmed medium. Absorbance was measured with a spectrophotometer (model 300 T-1; Gilford Instrument Laboratories, Inc.). Cell number was determined with a Coulter Counter with a 30-μm-diameter orifice.

Genetic techniques. P1 *vir*-mediated transduction was carried out as described by Miller (18). Strains were tested for temperature sensitivity on TY plates without NaCl at 42°C and on TY plates with 0.5% NaCl at 30°C. Transduction of the *pbpB*^{r1} mutation by M13mp8/2-6 was performed as follows. M13mp8/2-6 phage, isolated from a plaque on a lawn of JM101, was grown on 2 ml of a 10⁻² dilution of an overnight culture of JM101 in 2TY broth with vigorous shaking for 6 to 7 h at 37°C. A 0.1-ml sample of the infected culture was plated on TY agar containing a range of concentrations of cephalixin at 30°C. Transductants, which were able to grow on a concentration of cephalixin on which the control (uninfected JM101) did not form colonies, were tested for temperature sensitivity. A P1 lysate of a temperature-sensitive cephalixin-resistant transductant was used to transduce LMC502 to *leu*⁺. *leu*⁺ transductants were tested for temperature sensitivity and cephalixin resistance.

The CaCl₂ method of Cohen et al. (6) was used for plasmid transformations.

Preparation of sacculi. Sacculi were prepared by treatment of cells with hot sodium dodecyl sulfate as described by Wientjes et al. (32).

DAP incorporation. Cells were labeled with *meso*-[3,4,5-³H]DAP ([³H]DAP; 36.5 Ci/mmol; CEA, Gif-sur-Yvette, France; final concentration, 10 μCi/ml) to measure the kinetics of DAP incorporation, as described by Wientjes et al. (32).

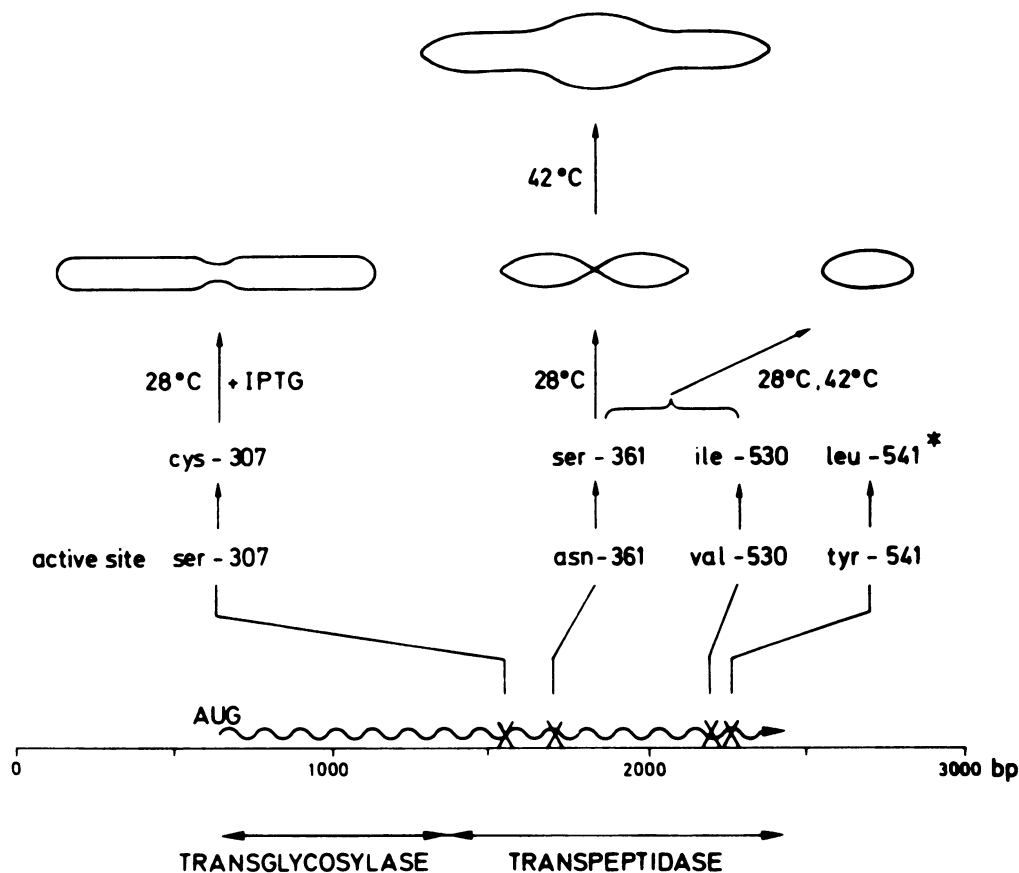
Autoradiography. Autoradiography was performed as described by Woldringh et al. (34). Instead of sacculi, whole cells were used, because the results were identical (P. Huls, unpublished observations).

Electron microscopy. Samples were fixed with 0.1% OsO₄. A one-fifth volume of 5× concentrated TY broth was added to the fixed cells to promote spreading of the cells during agar filtration (30). Cell sizes were measured from electron micrographs, as described previously (33).

RESULTS

Analysis of the mutation causing pointed polar caps. The *pbpB*^{r1} mutant SP1091 was obtained as a *leu*⁺ derivative of C600 by transduction with P1 lysates of ethyl methanesulfonate-induced temperature-sensitive cephalixin-resistant mutants (9). The *pbpB*^{r1} mutation changed Asn-361 to Ser in PBP3 (9). Phase-contrast and electron microscopy revealed the presence of pointed polar caps in this mutant at the permissive temperature (Fig. 1A). Although we have shown that *pbpB* mutants can have more tapered polar caps than *ftsZ* and *ftsA* cell division mutants (28), this dramatic change in shape was not expected of the *pbpB* mutant. The *pbpB* gene is part of the cluster of envelope genes in the 2-min

A



B

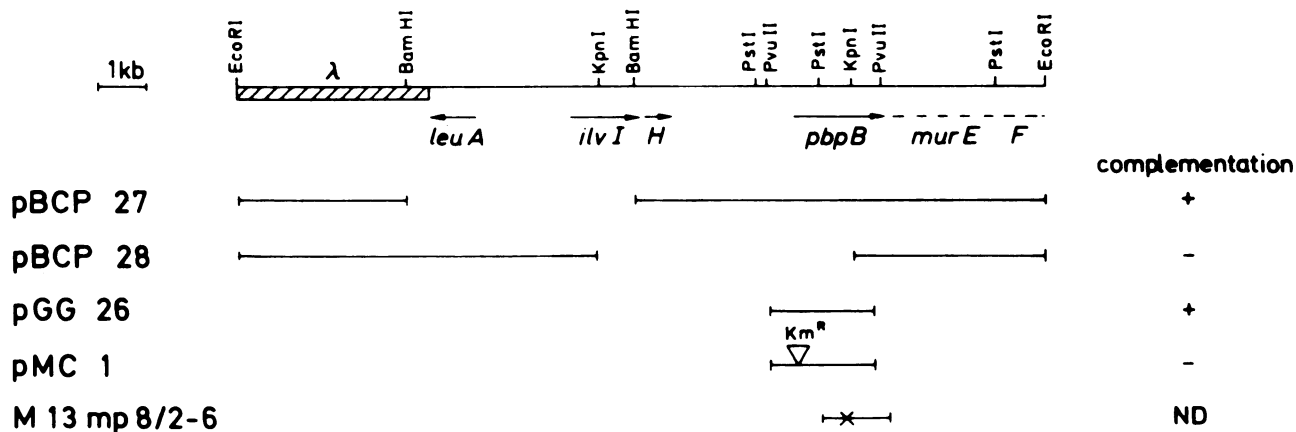


FIG. 1. (A) Effect of several amino acid substitutions in PBP3 on cell shape of *E. coli*. The positions of the amino acid substitutions (×) and the corresponding phenotypes (above the map) are shown. The substitution of Tyr-541 by Leu (*) leads, in the presence of the Ser-361 and Ile-530 amino acid residues, to the same phenotype (except for an increased level of cephalosporin resistance) as that caused by a single Ser-361 substitution. bp, Base pair; IPTG, isopropyl-β-D-thiogalactopyranoside. (B) Physical map and complementation analysis of the *leu-pbpB* region of the *E. coli* chromosome. The restriction map corresponds to part of λ *murF121* (7). Dimensions and directions of transcription of genes are indicated by arrows. Below the restriction map, chromosomal fragments present in hybrid plasmids (—) are indicated. ∇, Km^r gene of Tn903. The complementation of *pbpB*^{r1} strain LMC766 by hybrid plasmids was derived from the efficiency of plating on TY plates without salt at 42°C. kb, Kilobase; ND, not determined.

TABLE 2. Influence of different *pbpB* mutations on the shape of the polar cap, temperature sensitivity, and cephalixin resistance

| Strain(s) | <i>pbpB</i> allele | Amino acid substitutions | Characteristic shape of polar caps | Growth at 42°C ^a | Cephalixin resistance ^b | Interpretation of PBP3 activity ^c |
|-------------------------------|-----------------------------|--|------------------------------------|-----------------------------|------------------------------------|--|
| LMC500, C600 | <i>pbpB</i> ⁺ | | Hemispherical | + | - | + |
| LMC500 + cephalixin (2 µg/ml) | <i>pbpB</i> ⁺ | | Slightly pointed | + | - | + - |
| SP1091, LMC560 | <i>pbpB</i> ^{r1} | Asn-361→Ser | Pointed | - | + | - |
| SP1092 | <i>pbpB</i> ^{r2} | Thr-308→Pro | Hemispherical | - | + | + |
| SP1093 | <i>pbpB</i> ^{r3} | Val-344→Gly | Slightly pointed | + | + | + - |
| SP1096, LMC634 | <i>pbpB</i> ^{r6} | Asn-361→Ser Val-530→Ile | Slightly pointed | + | + | + - |
| SP1097 | <i>pbpB</i> ^{r7} | Asn-361→Ser Val-530→Ile | Pointed | - | ++ | - |
| SP1124 | <i>pbpB</i> ^{r8} | Tyr-541→Leu Asn-361→Ser Val-530→Ile | Slightly pointed | + - | ++ | + - |
| SP1125, LMC580 | <i>pbpB</i> ^{r9} | Tyr-541→Leu Glu-349→Lys Asn-361→Ser Val-530→Ile | Pointed | - | ++ | - |
| LMC510 | <i>pbpB</i> ²¹⁵⁸ | ? | Slightly pointed | - | + - | + - |
| SP33 | <i>pbpB</i> ³³ | ? | Slightly pointed | - | + - | + - |
| SP63, LMC579 | <i>pbpB</i> ⁶³ | ? | Hemispherical | - | - | + |
| JE10730 | <i>pbpB</i> ⁷³⁰ | ? | Hemispherical | - | - | + |

^a Symbols: +, no filamentation in TY broth, normal growth on TY plates at 42°C; + -, filamentation in TY broth, normal growth on TY plates at 42°C; -, filamentation in TY broth, no growth on TY plates at 42°C.

^b Symbols: -, no growth on 15 µg of cephalixin per ml; + -, growth on 15 µg of cephalixin per ml; +, growth on 30 µg of cephalixin per ml; ++, growth on 40 µg of cephalixin per ml.

^c Symbols: +, normal activity; + -, slightly decreased activity; -, decreased activity.

^d Undetermined mutation.

region of the *E. coli* chromosome, coding for proteins involved in the synthesis of peptidoglycan precursors and in cell division. Therefore, we determined whether the pointed polar caps could be caused by an additional mutation in the 2-min region of the chromosome. Conclusive evidence for the presence of only one mutation leading to the formation of pointed polar caps was obtained by the transduction of M13 host strain JM101 with phage M13mp8/2-6. This phage contained a *Pst*I-*Pvu*II fragment with only the last half of the *pbpB* gene and the *pbpB*^{r1} mutation (Fig. 1B). The insert has been sequenced completely by Hedge and Spratt and contained only the single base change which corresponded to the substitution of Asn-361 by Ser (9). Transduction of JM101 with M13mp8/2-6 and subsequent selection for resistance to cephalixin (30 µg/ml) yielded eight transductants, which were temperature sensitive. A P1 lysate of a temperature-sensitive cephalixin-resistant JM101 transductant was used to transduce LMC502 to *leu*⁺. All temperature-sensitive *leu*⁺ transductants were cephalixin resistant and had pointed polar caps. The plasmids pGG26 and pBCP27, which contained the wild-type *pbpB* gene, could complement the thermosensitivity and could restore cephalixin sensitivity and the shape of the polar caps of one of these transductants, designated LMC766, but the plasmids pMC1 and pBCP28, which did not contain the wild-type *pbpB* gene, could not (Fig. 1B). We conclude, that the single base change which leads to the substitution of Asn-361 by Ser in PBP3 is solely responsible for the presence of the pointed cell poles, the temperature sensitivity, and the increased cephalixin resistance of the mutant.

Effect of other *pbpB* mutations on the shape of the polar cap.

We examined the shape of the cell poles of various other *pbpB* mutants to see whether the formation of pointed polar caps is correlated with cephalixin resistance or temperature sensitivity. Two other cephalixin-resistant strains, the ther-

mosensitive *pbpB*^{r2} mutant SP1092 and the thermoresistant *pbpB*^{r3} mutant SP1093 (9) contained mutations which led to the substitution of Thr-308 by Pro and Val-344 by Gly, respectively (9). The *pbpB*^{r2} mutant had normal hemispherical cell poles, but the *pbpB*^{r3} mutant had slightly pointed cell poles (results not shown). In this group of cephalixin-resistant mutants, no correlation of characteristics seems to exist (Table 2). In a group of temperature-sensitive mutants, the mutants LMC510 and SP33 had slightly pointed cell poles and were somewhat more resistant to cephalixin than their parents. The mutants SP63 and JE10730, which had normal hemispherical polar caps, were cephalixin sensitive. In this group, the formation of slightly pointed polar caps seems to be correlated with cephalixin resistance (Table 2). From the original *pbpB*^{r1} mutant, SP1091, Hedge and Spratt (10) have produced a series of mutants that have additional mutations in the *pbpB* gene which provide increasing resistance to cephalosporins. The substitution of Val-530 by Ile to produce the *pbpB*^{r6} mutant, SP1096, resulted in a stabilization of the temperature-sensitive PBP3 with almost no effect on the level of cephalosporin resistance. This additional mutation partially suppressed the cell shape defect of the original *pbpB*^{r1} mutant, creating cell poles which were less pointed (Fig. 1A; see also Table 2). The introduction of a further mutation to produce the *pbpB*^{r7} mutant, SP1097, resulted in the substitution of Tyr-541 by Leu and in a further increased cephalosporin resistance, which was accompanied by the reappearance of temperature-sensitive cell division and the presence of pointed polar caps. The final mutant in this series (the *pbpB*^{r8} mutant SP1124) resulted from the substitution of Glu-349 by Lys, which stabilized the temperature-sensitive PBP3 but did not significantly affect the level of cephalosporin resistance. The additional mutation in SP1124 partially suppressed the cell shape defect and resulted in less pointed polar caps. However, the temperature

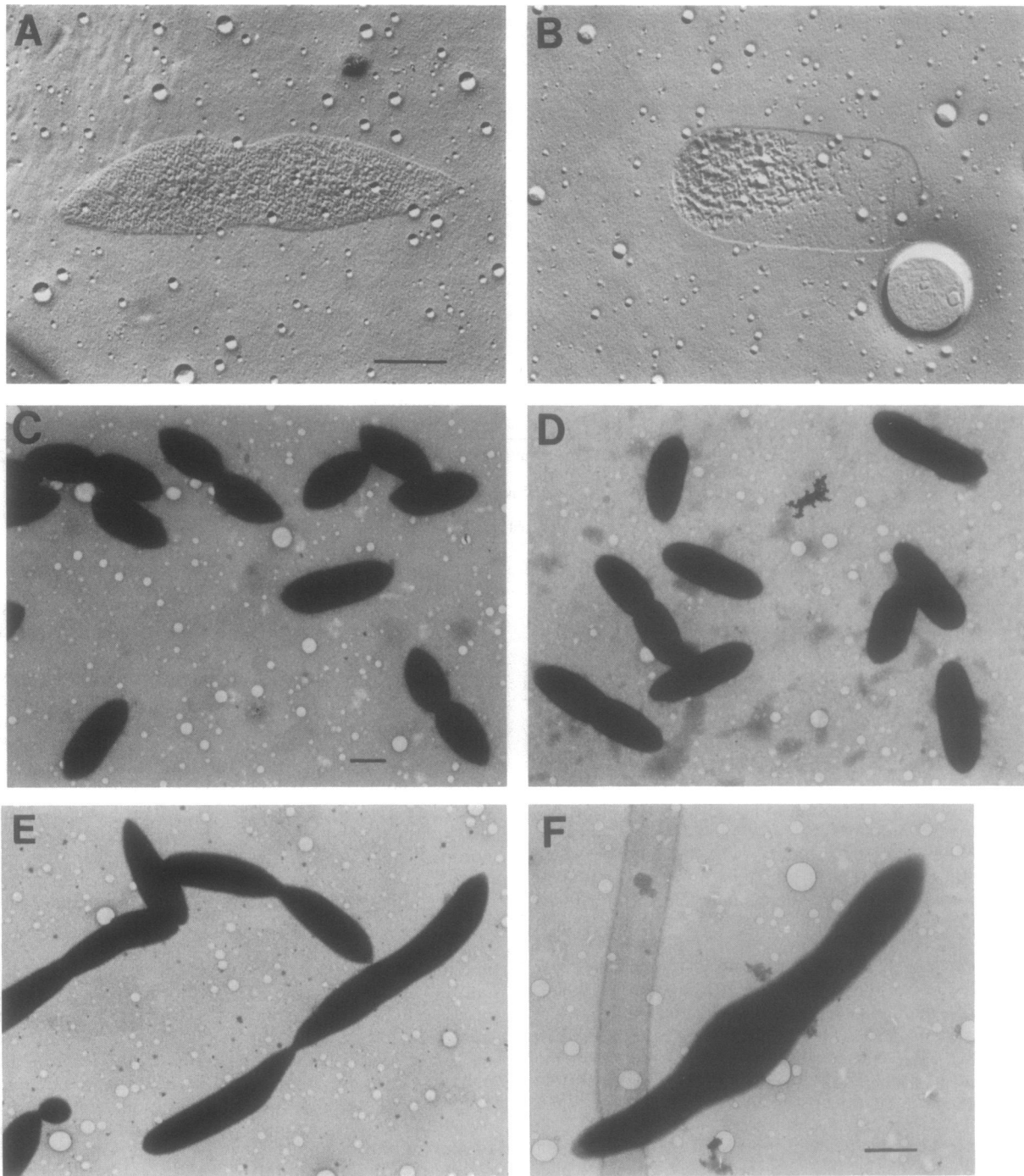


FIG. 2. Electron micrographs illustrating the differences between two *pbpB* mutants and their parent strain. Shown are isolated sacculi from *pbpB*^{r1} mutant LMC560 (A) and parent strain LMC500 (B), grown in minimal medium at 28°C; whole cells of *pbpB*^{r1} mutant LMC560 (C) and *pbpB*^{r6} mutant LMC634 (D), grown in minimal medium at 28°C; and filaments of *pbpB*^{r1} mutant LMC560, grown at 42°C in minimal medium for 1.5 doubling times (E) and in TY broth for 2 doubling times (F). Bar, 1 μ m.

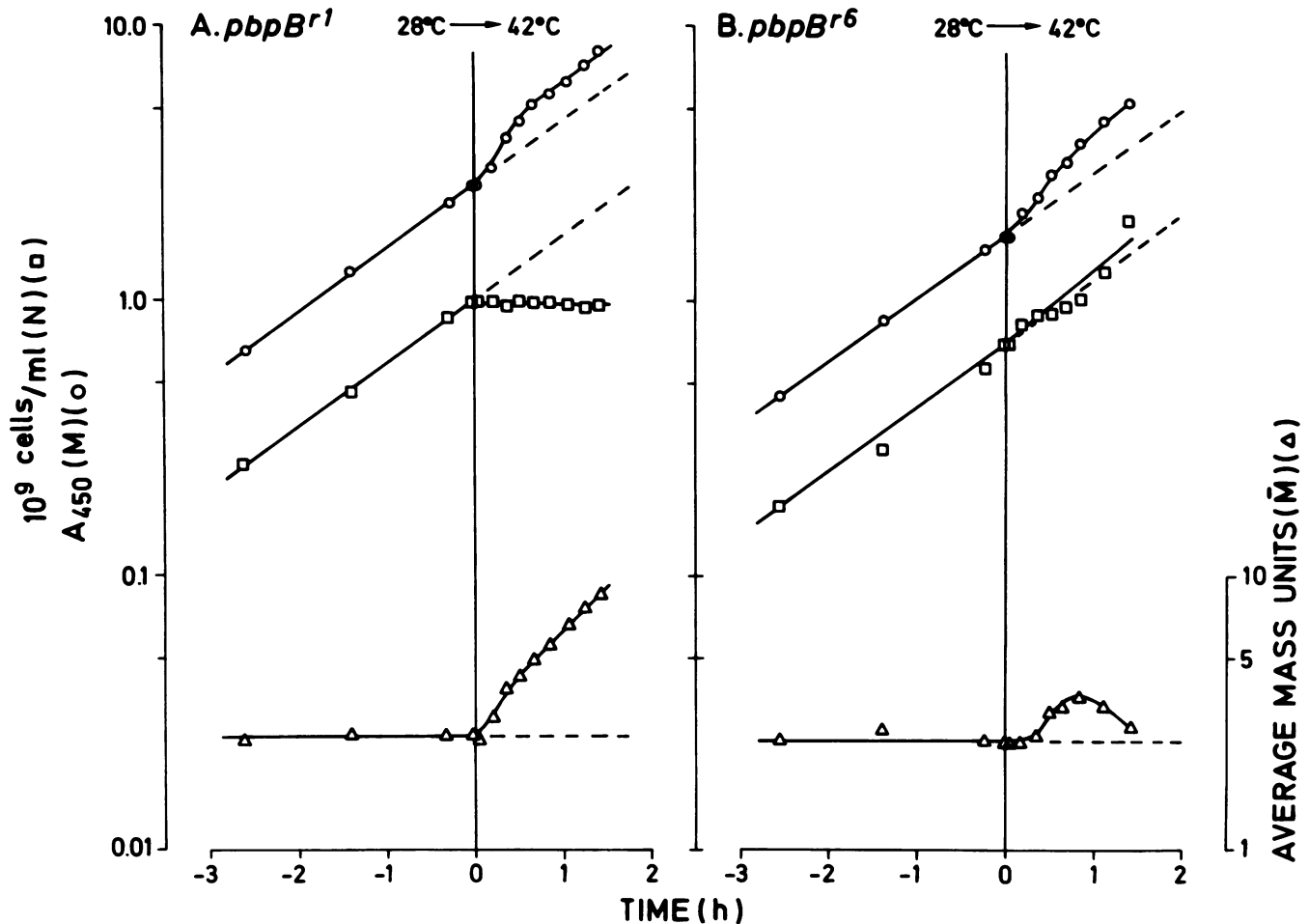


FIG. 3. Effect of a temperature shift on cell division in the *pbpB^{r1}* mutant LMC560 (A) and the *pbpB^{r6}* mutant LMC634 (B). At time zero, cultures growing at steady state at 28°C were shifted to 42°C. At intervals, absorbance (○) and cell number (□) were determined. Arbitrary units, calculated by dividing the absorbance by the cell number, indicate the average cell mass (Δ).

sensitivity of cell division was not completely restored in SP1124. A second derivative of SP1097, the *pbpB^{r9}* mutant SP1125, which has an undetermined additional mutation in PBP3, had a slightly further increased resistance to broad-spectrum cephalosporins but retained the temperature-sensitive cell division (P. J. Hedge and B. G. Spratt, unpublished results). This mutant also had the pointed polar caps of its parent strain. In this series of mutants, there is some correlation between temperature sensitivity and the formation of pointed polar caps (Table 2).

Effect of subinhibitory concentrations of cephalixin on the shape of the polar cap. If pointed polar caps are the result of partial inactivation of PBP3 at the permissive temperature, it might be possible to mimic the effect of the *pbpB^{r1}* mutation on the shape of the cell poles by concentrations of cephalixin which inhibit some of the PBP3 molecules but do not cause filamentation. Therefore, we incubated wild-type strain LMC500, grown at 37°C under steady-state conditions in minimal medium, with subinhibitory concentrations of cephalixin. After 85 min, the polar caps synthesized in the presence of 2 and 3 μg of cephalixin per ml were slightly pointed (results not shown). At lower cephalixin concentrations, no change in the shape of the cell pole occurred, whereas at higher concentrations filaments with blunt and threadlike constrictions were produced. Thus, there seems to be no difference in excluding the substrate from the active

site of some of the PBP3 molecules by occupancy with a cephalixin molecule compared with a mutational defect that presumably decreases the rate of the peptidoglycan-synthesizing reaction of every PBP3 molecule (Table 2). Similar results were obtained with subinhibitory concentrations of furazlocillin and piperacillin.

Morphological and physiological characterization of the pointed polar cap mutant. Electron micrographs of isolated sacculi from the *pbpB^{r1}* mutant LMC560 and its parent show that the shapes of the cells are maintained by the peptidoglycan layer (Fig. 2A and B). Recently, we have shown that the shape and division behavior of isogenic cell division mutants are already affected at the permissive temperature (28). We started similar experiments with this mutant but were unable to achieve steady-state growth. Since the osmolality of the minimal medium had been kept relatively low (150 mosM) to avoid salt suppression of the various *fts* mutations, we decided to increase the osmolality to 300 mosM. This allowed steady-state growth of the *pbpB^{r1}* mutant at 28°C without suppressing thermosensitivity or the formation of pointed polar caps.

Grown at 28°C and with a doubling time of 80 min, the average length of the pointed polar cap mutant LMC560 was approximately 2.6 μm, similar to that of the isogenic *pbpB^{r6}* mutant LMC634. The shapes of the polar caps of the mutants were different (Fig. 2C and D), but no significant change in

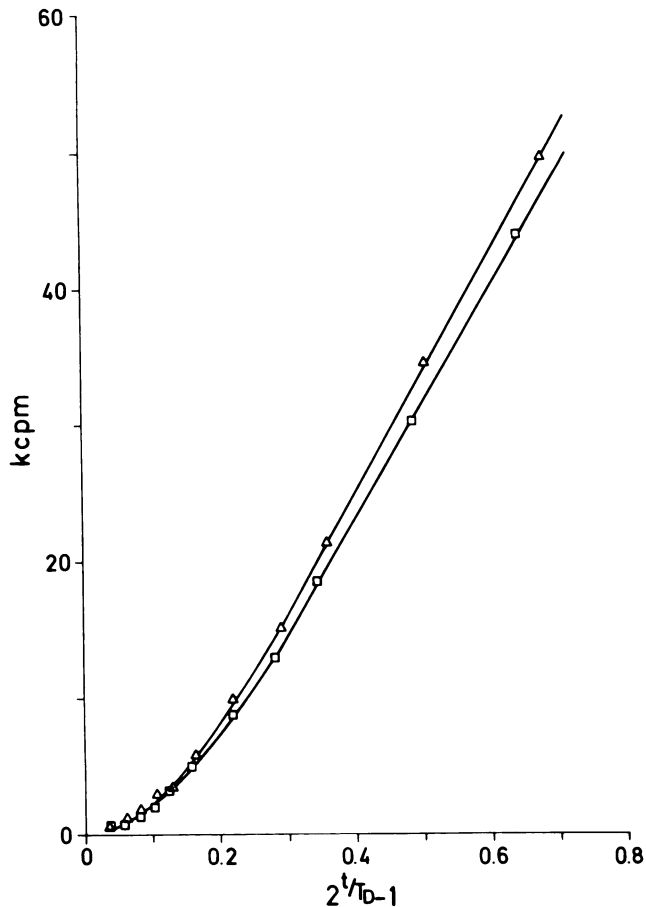


FIG. 4. The incorporation kinetics of [^3H]DAP in *pbpB*^{r1} mutant LMC560 (\square) and the parent LMC500 (Δ), grown at 28°C in minimal medium supplemented with lysine, methionine, and threonine (50 $\mu\text{g}/\text{ml}$ each). At time zero, 10 μCi of [^3H]DAP was added to 1 ml of culture. Samples (50 μl) were taken at intervals, and the radioactive DAP incorporated into sodium dodecyl sulfate-insoluble material was measured by liquid scintillation counting. The data are shown in a differential plot as described by Wientjes et al. (32), where T_D indicates mass doubling time and t indicates sample time. kcpm, Kilocounts per minute.

cell shape, as determined by the ratio between average cell length and average cell diameter, was measured. The percentage of dividing cells was 36% in the *pbpB*^{r1} mutant. As a result of the additional mutation in the *pbpB*^{r6} mutant (Table 2), its percentage of dividing cells was diminished to 29%, indicating a decrease in the duration of the constriction period from 35 min in the *pbpB*^{r1} mutant to 29 min in the *pbpB*^{r6} mutant.

We compared the cell division behavior and cell shape of the *pbpB*^{r1} and *pbpB*^{r6} mutants in temperature shift experiments (Fig. 3). After the shift from 28 to 42°C, both strains showed an increased mass synthesis. Cell division rapidly ceased in the pointed polar cap mutant (Fig. 3A). Of the constricting cells at 28°C, only 2% were able to finish cell division at 42°C. Filaments with pointed polar caps were formed (Fig. 2E). The *pbpB*^{r6} mutant continued to divide, and its behavior at 42°C was similar to that of the parent strain LMC500. We conclude that the substitution of amino acid residue Val-530 by Ile restores the thermolability of PBP3 sufficiently to allow normal cell division at 42°C. In TY broth at 42°C, the pointed polar cap mutant differed from

other thermosensitive *pbpB* mutants in forming filaments with bulges before lysis occurred (Fig. 2F).

Effect of the pointed polar cap mutation on the rate of peptidoglycan synthesis. The different shape of the sacculus of the *pbpB*^{r1} mutant suggests that the synthesis of the peptidoglycan layer is affected by the altered PBP3. For instance, the overall rate of peptidoglycan synthesis in the *pbpB*^{r1} mutant could differ from that of its parent. Therefore, we measured the incorporation of [^3H]DAP into sodium dodecyl sulfate-insoluble material at 28°C (Fig. 4). To facilitate comparison between the results for both strains, the data are presented as described by Wientjes et al. (32). After a small lag, the amount of incorporated [^3H]DAP increased in both strains in a steady-state manner, as reflected by the straight lines in Fig. 4. The incorporation of [^3H]DAP in the *pbpB*^{r1} mutant was found to be slightly lower in two independent experiments, indicating a slight effect of the *pbpB*^{r1} mutation on the overall rate of peptidoglycan synthesis.

Topography of peptidoglycan synthesis as determined by autoradiography. The alteration in shape of the polar cap could reflect changes in the local rate of peptidoglycan synthesis across the cell envelope. In individual cells, such changes could be determined by autoradiography. Because the characteristic pointed cell poles of the *pbpB*^{r1} mutant distinguished it from the parent strain, we could mix equal amounts of both strains, pulse-label them for 15 min with [^3H]DAP, and use them for autoradiography in the same preparation to obtain comparable data on the topography of the peptidoglycan synthesis. In this way, we were able to exclude experimental errors arising from differences in agar filtration, thickness of the emulsion, and background grains. Nevertheless, the number of silver grains per cell was higher in the parent strain than in the pointed polar cap mutant (Fig. 5). We assume that this was caused by the slightly higher rate of DAP incorporation in the parent strain (Fig. 4). The autoradiograms showed a diffuse pattern of silver grains in the nondividing cells and a central peak in constricting cells, indicating a relative shift of DAP incorporation from the lateral wall to the constriction site. In the largest nondividing cells, a higher grain density was already observed in the central area of the cell (Fig. 5). Thus, the rate of DAP incorporation increases at the future polar caps before constrictions become visible (34). In the parent, the peaks were higher, probably as a result of the slightly higher DAP incorporation. Apparently, the topography of peptidoglycan synthesis did not change markedly when cells formed pointed polar caps, instead of normal hemispherical cell poles.

DISCUSSION

At the permissive temperature, the *pbpB*^{r1} mutant has pointed polar caps, caused by the presence of the single substitution of Asn-361 by Ser in the presumed transpeptidase part of PBP3. This amino acid substitution probably inactivates PBP3 partially, since the introduction of a functional *pbpB* gene suppresses the formation of pointed cell poles. Other conditionally lethal *pbpB* mutants have been isolated (1, 20, 25), but none of them is known to have pointed cell poles, although we have previously shown that one of these *pbpB* mutants had somewhat pointed polar caps (28).

In addition to the formation of pointed polar caps, the *pbpB*^{r1} mutation caused temperature-sensitive cell division and increased cephalixin resistance. However, comparison of various *pbpB* mutants did not show a clear correlation

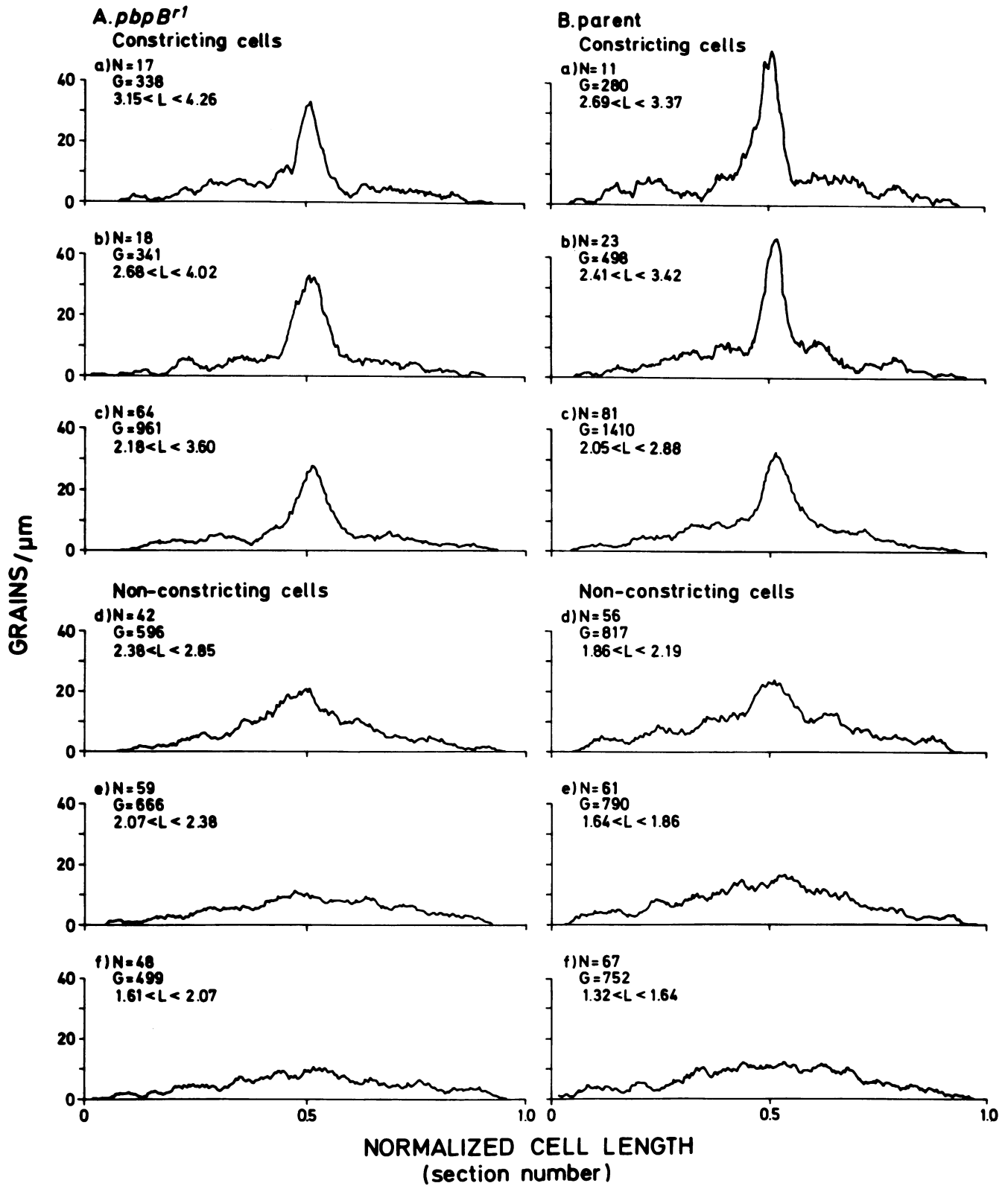


FIG. 5. Distributions of silver grains over whole cells of the *pbpB*^{r1} mutant LMC560 (A) and its parent LMC500 (B). In each panel, silver grain distributions for deeply constricted (a), moderately constricted (b), and slightly constricted (c) cells, and for three length classes of nondividing cells (d, e, and f) are shown. The longest cell half of constricting cells has been positioned to the left. The total number of cells (N), the total number of silver grains (G), and the range of cell length (L) are indicated. Distributions can be compared, since the data were obtained from the same preparations.

between these characteristics (Table 2). This apparent contradiction can be understood when we take a closer look at the two different methods used to obtain the mutants. The *pbpB*^{r1} mutant and its derivatives were isolated by selection for resistance to cephalosporins. Resistance to cephalosporins is, in the most ideal case, caused by a change in the active site of PBP3 which reduces its affinity for cephalosporins without affecting its affinity for its normal substrate or its thermostability. A mutant with such a mutation has never been found. Probably the affinity of PBP3 for its normal substrate is impaired in the mutants with the pointed polar caps. The same mutation which causes the reduction in substrate affinity may decrease the temperature at which denaturation occurs, as seems to be the case with the *pbpB*^{r1} mutation.

Other conditional *pbpB* mutants have been isolated by screening for temperature-sensitive cell division (1, 20, 25). In some of these mutants (SP63, LMC579, and JE10730), the denaturation temperature of PBP3 seems to be decreased without significant influence on its substrate affinity at the permissive temperature. The shape of the polar caps was not changed in these mutants. In the other temperature-sensitive mutants (LMC510 and SP33), the affinity of PBP3 for cephalosporins is slightly decreased and the shape of the polar caps is also somewhat altered (Table 2). Thus, our results suggest that the shape of the polar caps might be an indicator for the activity of PBP3.

It has been proposed that the peptidoglycan layer in rod-shaped bacteria is synthesized by two different systems (8, 23, 24). One of these systems synthesizes the lateral wall, and the other takes care of polar cap formation. Results obtained with specific β -lactam antibiotics, like mecillinam and furazlocillin, as well as with *rodA*, *pbpA*, and *pbpB* mutants, suggested that RodA and the mecillinam-sensitive PBP2 were involved in the synthesis of the lateral wall and that PBP3 was involved in polar cap formation. These results, however, do not exclude the existence of a single enzyme complex capable of functioning in the synthesis of both the lateral wall and the polar caps. According to the surface stress theory (16), a local increase in the activity of these complexes could result in a switch from lateral wall synthesis to polar cap formation. For geometric reasons, it could be envisaged that lateral wall synthesis requires only peptidoglycan synthesis in a longitudinal direction, whereas polar cap formation requires synthesis in both longitudinal and transverse directions. In this case, the locally increased activity represents the additional synthesis in the transverse direction. The ratio between transverse and longitudinal synthesis during the constriction process would then determine the correct formation of cell poles.

If the cell is not able to increase the activity of any of the complexes, no constriction will be initiated and a just initiated constriction might persist as it was formed. This was observed in *ftsZ* and *ftsQ* mutants (28). If the cell is able to activate the complexes only partially, blunt constrictions may be formed as observed in *pbpB* and *ftsA* mutants (28). Similar constrictions were observed in the *sefA* mutant (21) and in cells overproducing a PBP3 in which the active site Ser-307 had been changed to Cys (Fig. 1A; also unpublished results). In addition, cells treated with the β -lactam antibiotics furazlocillin, piperacillin, cephalothin, and cephalosporins had blunt constrictions (5, 12, 22). However, pointed polar caps were not observed previously in cells treated with these antibiotics, probably because concentrations which completely inhibit cell division were used. We have found that partial inhibition of PBP3 activity by subinhibitory concen-

trations of β -lactam antibiotics, like cephalosporins, furazlocillin, and piperacillin, can delay the constriction process, resulting in the formation of blunt or threadlike constrictions which can be finished to form slightly pointed polar caps. Further studies will have to show whether pointed cell poles can be formed by temperature-sensitive *pbpB* mutants grown at intermediary temperatures. Probably because the amount of transverse surface synthesis is relatively small in comparison with that of longitudinal surface synthesis, a slight inhibition of the rate of transverse surface synthesis could not be found by measurement of the DAP incorporation. On the other hand, the kinetics of DAP incorporation (Fig. 4) might not be affected by a decreased transpeptidase activity, when existing peptidoglycan chains are elongated, because the incorporation of labeled precursors by transglycosylase activity of PBP1A, PBP1Bs, PBP2, and PBP3 in vitro seemed not significantly affected by β -lactam antibiotics (13–15, 19).

The topography of the peptidoglycan synthesis, as determined by autoradiography, was not altered markedly in the pointed polar cap mutant (Fig. 5). In other words, the synthesis of pointed polar caps probably does not change the redistribution of surface synthesis. We therefore reach the conclusion that small changes in the transpeptidase activity of PBP3 already result in major changes in cell shape.

Bulge formation, as found in the pointed polar cap mutant after a temperature shift to 42°C in TY broth (Fig. 1F), has previously been observed as the result of treatment with low concentrations of ampicillin or penicillin (5, 12, 23) and with mecillinam and cephalosporins simultaneously (24) and in the cell shape-cell division double mutants of Begg and Donachie (3). Recently, Ishino and co-workers (15) have shown that the in vitro peptidoglycan synthesis by PBP2 requires the presence of RodA, which functions just like PBP2 in the maintenance of cell shape (27). However, RodA is not required for cell division, because deletion of the *rodA* gene is not lethal (26). Since PBP3 and RodA probably interact with each other (4), the bulge formation in the pointed polar cap mutant could be due to an impaired interaction between PBP3 and RodA, which indirectly affects PBP2. The interaction between these proteins, which were thought to function exclusively in constriction formation or cell elongation, respectively, could point to a peptidoglycan-synthesizing enzyme complex which is activated in different manners for constriction formation and cell elongation.

ACKNOWLEDGMENTS

This work was supported by the Foundation for Fundamental Biological Research, which is subsidized by the Netherlands Organization for the Advancement of Pure Research, The Hague, The Netherlands.

We thank J. R. Walker and R. Geerse for providing plasmids; J. Leutscher for drawings and photographs; N. Nanninga and A. Koch for critically reading the manuscript; E. Pas for technical assistance; and P. Huls for help with the computer.

LITERATURE CITED

- Allen, J. S., C. C. Philip, R. A. Gustafson, R. G. Allen, and J. R. Walker. 1974. Regulation of bacterial cell division: genetic and phenotypical analysis of temperature-sensitive, multinucleate, filament-forming mutants of *Escherichia coli*. *J. Bacteriol.* **117**: 978–986.
- Barrett, A. N., and I. D. J. Burdett. 1981. A three-dimensional model reconstruction of pole assembly in *Bacillus subtilis*. *J. Theor. Biol.* **92**:127–139.
- Begg, K. J., and W. D. Donachie. 1985. Cell shape and division in *Escherichia coli*: experiments with shape and division mu-

- tants. *J. Bacteriol.* **163**:615–622.
4. Begg, K. J., B. G. Spratt, and W. D. Donachie. 1986. Interaction between membrane proteins PBP3 and RodA is required for normal cell shape and division in *Escherichia coli*. *J. Bacteriol.* **167**:1004–1008.
 5. Burdett, I. D. J., and R. G. E. Murray. 1974. Septum formation in *Escherichia coli*: characterization of septal structure and the effects of antibiotics on cell division. *J. Bacteriol.* **119**:303–324.
 6. Cohen, S. N., A. C. Y. Chang, and L. Hsu. 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R factor DNA. *Proc. Natl. Acad. Sci. USA* **69**:2110–2114.
 7. Fletcher, G., C. A. Irwin, J. M. Henson, C. Fillingim, M. M. Malone, and J. R. Walker. 1978. Identification of the *Escherichia coli* cell division gene *sep* and organization of the cell division-cell envelope genes in the *sep-mur-fisA-envA* cluster as determined with specialized transducing lambda bacteriophages. *J. Bacteriol.* **133**:91–100.
 8. Hartmann, R., J.-V. Höltje, and U. Schwarz. 1972. Targets of penicillin action in *Escherichia coli*. *Nature (London)* **235**:426–429.
 9. Hedge, P. J., and B. G. Spratt. 1985. Amino acid substitutions that reduce the affinity of penicillin-binding protein 3 of *Escherichia coli* for cephalixin. *Eur. J. Biochem.* **151**:111–121.
 10. Hedge, P. J., and B. G. Spratt. 1985. Resistance to β -lactam antibiotics by re-modelling the active site of an *E. coli* penicillin-binding protein. *Nature (London)* **318**:478–480.
 11. Higgins, M. L. 1976. Three-dimensional reconstruction of whole cells of *Streptococcus faecalis* from thin sections of cells. *J. Bacteriol.* **127**:1337–1345.
 12. Iida, K., S. Hirata, S. Nakamura, and M. Koike. 1978. Inhibition of cell division of *Escherichia coli* by a new synthetic penicillin, piperacillin. *Antimicrob. Agents Chemother.* **14**:257–266.
 13. Ishino, F., and M. Matsuhashi. 1981. Peptidoglycan synthetic enzyme activities of highly purified penicillin-binding protein 3 in *Escherichia coli*: a septum-forming reaction sequence. *Biochem. Biophys. Res. Commun.* **101**:905–911.
 14. Ishino, F., K. Mitsui, S. Tamaki, and M. Matsuhashi. 1980. Dual enzyme activities of cell wall synthesis, peptidoglycan transglycosylase and penicillin-sensitive transpeptidase, in purified preparations of *Escherichia coli* penicillin-binding protein 1A. *Biochem. Biophys. Res. Commun.* **97**:287–293.
 15. Ishino, F., W. Park, S. Tomioka, S. Tamaki, I. Takase, K. Kunugita, H. Matsuzawa, S. Asoh, T. Ohta, B. G. Spratt, and M. Matsuhashi. 1986. Peptidoglycan synthetic activities in membranes of *Escherichia coli* caused by overproduction of penicillin-binding protein 2 and RodA protein. *J. Biol. Chem.* **261**:7024–7031.
 16. Koch, A. L. 1983. The surface stress theory of microbial morphogenesis. *Adv. Microb. Physiol.* **24**:301–366.
 17. Lodder, J., and N. J. W. Kreger-Van Rij. 1952. The yeasts: a taxonomic study. North-Holland Publishing Company, Amsterdam.
 18. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 19. Nakagawa, J., S. Tamaki, S. Tomioka, and M. Matsuhashi. 1984. Functional biosynthesis of cell wall peptidoglycan by polymorphic bifunctional polypeptides. Penicillin-binding protein 1Bs of *Escherichia coli* with activities of transglycosylase and transpeptidase. *J. Biol. Chem.* **259**:13937–13946.
 20. Nishimura, Y., Y. Takeda, A. Nishimura, H. Suzuki, M. Inouye, and Y. Hirota. 1977. Synthetic ColE1 plasmids carrying genes for cell division in *Escherichia coli*. *Plasmid* **1**:67–77.
 21. Normark, S., L. Norlander, T. Grundström, G. D. Bloom, P. Boquet, and G. Frelat. 1976. Septum formation-defective mutant of *Escherichia coli*. *J. Bacteriol.* **128**:401–412.
 22. Olijhoek, A. J. M., S. Klencke, E. Pas, N. Nanninga, and U. Schwarz. 1982. Volume, growth, murein synthesis, and murein cross-linkage during the division cycle of *Escherichia coli* PA3092. *J. Bacteriol.* **152**:1248–1254.
 23. Schwarz, U., A. Asmus, and H. Frank. 1969. Autolytic enzymes and cell division of *Escherichia coli*. *J. Mol. Biol.* **41**:419–429.
 24. Spratt, B. G. 1975. Distinct penicillin-binding proteins involved in the division, elongation, and shape of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **72**:2999–3003.
 25. Spratt, B. G. 1977. Temperature-sensitive cell division mutants of *Escherichia coli* with thermolabile penicillin-binding proteins. *J. Bacteriol.* **131**:293–305.
 26. Spratt, B. G. 1980. Deletion of the penicillin-binding protein 5 gene of *Escherichia coli*. *J. Bacteriol.* **144**:1190–1192.
 27. Spratt, B. G., A. Boyd, and N. Stoker. 1980. Defective and plaque-forming lambda transducing bacteriophage carrying penicillin-binding protein-cell shape genes: genetic and physical mapping and identification of gene products from the *lip-dacA-rodA-phpA-leuS* region of the *Escherichia coli* chromosome. *J. Bacteriol.* **143**:569–581.
 28. Taschner, P. E. M., P. G. Huls, E. Pas, and C. L. Woldringh. 1988. Division behavior and cell shape of isogenic *ftsZ*, *ftsQ*, *ftsA*, *phpB*, and *ftsE* cell division mutants of *Escherichia coli* during temperature shift experiments. *J. Bacteriol.* **170**:1533–1540.
 29. Taschner, P. E. M., J. G. J. Verest, and C. L. Woldringh. 1987. Genetic and morphological characterization of *ftsB* and *nrdB* mutants of *Escherichia coli*. *J. Bacteriol.* **169**:19–25.
 30. Trueba, F. J., and C. L. Woldringh. 1980. Changes in cell diameter during the division cycle of *Escherichia coli*. *J. Bacteriol.* **142**:869–878.
 31. Weidel, W., and H. Pelzer. 1964. Bag-shaped molecules—a new outlook on bacterial cell walls. *Adv. Enzymol.* **26**:193–232.
 32. Wientjes, F. B., E. Pas, P. E. M. Taschner, and C. L. Woldringh. 1985. Kinetics of uptake and incorporation of meso-diaminopimelic acid in different *Escherichia coli* strains. *J. Bacteriol.* **164**:331–337.
 33. Woldringh, C. L., M. A. de Jong, W. van den Berg, and L. J. H. Koppes. 1977. Morphological analysis of the cell division cycle of two *Escherichia coli* substrains during slow growth. *J. Bacteriol.* **131**:270–279.
 34. Woldringh, C. L., P. Huls, E. Pas, G. J. Brakenhoff, and N. Nanninga. 1987. Topography of peptidoglycan synthesis during elongation and polar cap formation in a cell division mutant of *Escherichia coli* MC4100. *J. Gen. Microbiol.* **133**:575–586.
 35. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains. Nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**:103–119.