

Temperature-Sensitive Cell Division Mutants of *Escherichia coli* with Thermolabile Penicillin-Binding Proteins

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The thermostability of the penicillin-binding proteins (PBPs) of 31 temperature-sensitive cell division mutants of *Escherichia coli* has been examined. Two independent cell division mutants have been found that have highly thermolabile PBP3. Binding of [¹⁴C]benzylpenicillin to PBP3 (measured in envelopes prepared from cells grown at the permissive temperature) was about 30% of the normal level at 30°C, and the ability to bind [¹⁴C]benzylpenicillin was rapidly lost on incubation at 42°C. The other PBPs were normal in both mutants. At 30°C both mutants were slightly longer than their parents and on shifting to 42°C they ceased dividing, but cell mass and deoxyribonucleic acid synthesis continued and long filaments were formed. At 42°C division slowly recommenced, but at 44°C this did not occur. The inhibition of division at 42°C was suppressed by 0.35 M sucrose, and in one of the mutants it was partially suppressed by 10 mM MgCl₂. PBP3 was not stabilized in vitro at 42°C by these concentrations of sucrose or MgCl₂. Revertants that grew as normal rods at 42°C regained both the normal level and the normal thermostability of PBP3. The results provide extremely strong evidence that the inactivation of PBP3 at 42°C in the mutants is the cause of the inhibition of cell division at this temperature and identify PBP3 as an essential component of the process of cell division in *E. coli*. It is the inactivation of this protein by penicillins and cephalosporins that results in the inhibition of division characteristic of low concentrations of many of these antibiotics.

The process of bacterial cell division is poorly understood (17). Physiological studies with *Escherichia coli* have shown that over a wide range of growth rates division follows the termination of a round of deoxyribonucleic acid (DNA) replication by a fixed interval (the D period) of about 25 min at 37°C (7). Soon after the termination of a round of DNA replication, cell division becomes independent of further ribonucleic acid and protein synthesis (3, 5). Presumably, at this time all the enzymes and structural or regulatory proteins required for the assembly of the membrane and peptidoglycan layers of the septa have been synthesized. The first visible sign of division is the ingrowth of the inner membrane and a double layer of peptidoglycan, commencing about 15 min before division and being completed only 1 to 2 min before division, resulting in the physiological separation of the cell into two compartments (3, 13, 24). The final assembly of the outer membrane between the double layer of peptidoglycan and the physical separation of the cells occupies the last 1 or 2 min of the division cycle (3, 13, 24).

Biochemical and genetic approaches to the process of cell division are severely limited by the inability to study division at the subcellular level. Genetic analysis has identified at least eight genes in *E. coli* that are specifically required for cell division (8, 14, 15, 17); temperature-sensitive mutants at these loci (*ftsA-H*) cease division abruptly and grow into long filaments when transferred from the permissive to the restrictive temperature. The usefulness of these mutants is restricted, since the identification of the defective gene products has not been achieved.

One approach to the biochemistry of cell division is to study specific inhibitors of the process. In theory, it should be possible to identify the target with which the inhibitor interacts and thereby to identify a specific component of the division process. Although many chemicals result in the filamentation of bacterial cells, the only ones known to act specifically on division are the penicillins and cephalosporins (2, 4, 16, 18). Low concentrations of most of these β -lactam antibiotics inhibit cell division in *E. coli* without influencing DNA synthesis or cell

mass increase and result in the formation of long filaments. At higher concentrations these antibiotics inhibit cell elongation, resulting in cell lysis and the production of spheroplasts (16, 18). β -Lactam antibiotics inhibit several enzymes involved in the terminal stages of peptidoglycan metabolism (2), and it has been proposed that their differential effects on cell division and cell elongation are due to the involvement of separate enzyme systems in the two types of peptidoglycan growth (6, 12, 16, 18, 23). Inactivation of a penicillin-sensitive enzyme (PSE) specifically required for cell division will result in the growth of the cell as long filaments, and therefore mutants that produce a highly thermolabile form of this enzyme should be temperature-sensitive cell division mutants. The screening of cell division mutants for alterations in the thermostability of one of the several PSEs is impracticable, but fortunately these enzymes can be conveniently detected and studied as those proteins that bind [^{14} C]benzylpenicillin (2, 19, 22). Six penicillin-binding proteins (PBPs) are consistently detected in *E. coli* envelopes, and two additional PBPs are less consistently detected (19). Previous studies on the properties of the *E. coli* PBPs have implicated PBP3 as the target at which penicillins and cephalosporins interact to inhibit cell division (18-20). We have examined the thermostability of the individual PBPs in 31 temperature-sensitive cell division mutants of *E. coli* and have identified two independent mutants that have highly thermolabile PBP3. One of these mutants has been briefly described previously (18). The properties of these mutants and their revertants provide strong evidence that PBP3 is specifically required for the divi-

sion of *E. coli* and is the target at which β -lactam antibiotics interact to inhibit cell division and cause filamentation.

MATERIALS AND METHODS

Bacterial strains. Table 1 lists the *E. coli* strains used in this work. LEB18 and its derivatives were B/r strains, whereas all other strains were K-12 derivatives. Temperature-sensitive mutants of KN126 and LEB18 were isolated after mutagenesis for 30 min at 37°C with 100 μ g of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml in 50 mM tris(hydroxymethyl)aminomethane-maleate buffer, pH 6.0 (1). Cells were washed free of mutagen, grown overnight in broth at 30°C to allow mutations to be expressed, and plated on nutrient agar at 30°C. Colonies were replica plated to the same agar at 42°C, and temperature-sensitive mutants were purified and grown in broth at 30 and 42°C. Those mutants that were normal rods at 30°C, but which filamented when shifted to 42°C, were kept for further study. In the mutant selection in which SP63 was obtained, the mutagenized cells were subjected to a primary selection at 30°C with cephalixin (5 μ g/ml), cephalixin-resistant colonies were replica plated to 42°C, and temperature-sensitive mutants were screened as above (18).

Media and conditions of growth. Cells were grown in Penassay broth (Difco antibiotic medium no. 3) in all experiments unless otherwise stated. The minimal medium used in some experiments was M9 supplemented with 0.2% glucose and required L-amino acids (20 μ g/ml). Cultures were initiated by taking a loop of bacterial growth from a stock agar plate into about 20 ml of medium and growing the cells at the appropriate temperature overnight. In the morning, the cultures were diluted and grown with vigorous aeration for at least a further five cell doublings (maintaining the absorbance below 0.4 by periodic dilution) before experiments were started.

Measurement of cell growth. Growth of the cultures was followed by measuring the absorbance at

TABLE 1. *Bacterial strains*

Strain	Relevant genotype or properties	Source
KN126	<i>tryE</i> (Am)9829 <i>tyr</i> (Am) <i>ilv sup-126</i>	T. Nagata (11)
SP63	Temperature-sensitive cell division mutant derived from KN126 after nitrosoguanidine mutagenesis	This paper and reference 18 (strain 6-30)
SP631 and SP632	Spontaneous temperature-resistant revertants of SP63	This paper
LEB18	<i>lacZ rpsL</i>	P. Meacock
SP25	Temperature-sensitive mutant derived from LEB18 after nitrosoguanidine mutagenesis	This paper
SP258	Spontaneous partial revertant of SP25	This paper
SP2581 and SP2582	Spontaneous temperature-resistant revertants of SP258	This paper
T84	<i>ftsA</i>	J. L. Strominger (15)
MFT84	<i>ftsB</i>	F. Jacob (15)
MFT108	<i>ftsC</i>	J. L. Strominger (15)
MFT103	<i>ftsD</i>	J. L. Strominger (15)
MFT99	<i>ftsE</i>	F. Jacob (15)
MFT1141	<i>ftsF</i>	F. Jacob (15)
ASH501	<i>ftsH</i>	I. B. Holland (8)

550 nm (A_{550}) (for broth cultures) or A_{450} (for minimal medium cultures) in a Gilford model 300 automatic sampling spectrophotometer using a 1-cm-path length cuvette. Total cell numbers were measured using a model B Coulter counter. Cells were diluted into ice-cold membrane-filtered saline containing 0.37% formaldehyde and were counted with a 30- μ m probe. To measure cell size, exponentially growing cultures were sampled in triplicate, and the absorbances and total cell numbers were measured. Average cell size was expressed as A_{550} per cell number. DNA synthesis was measured by incorporation of [^{3} H]thymidine in the presence of 1.5 mM uridine, as described previously (21).

Preparation of envelopes and measurement of PBPs. For all experiments, cell envelopes (consisting of inner membrane, outer membrane, and peptidoglycan) were prepared as described previously (19) from cells that had been grown in Penassay broth at 30°C. For the standard assay of PBPs, 200 μ l of envelopes (5 to 10 mg/ml in 50 mM sodium phosphate buffer, pH 7.0) were incubated for 10 min at 30°C with 20 μ l of a 50- μ Ci/ml amount of [^{14} C]benzylpenicillin. The final concentration of [^{14} C]benzylpenicillin for all samples in all experiments was 31.3 μ g/ml. The binding was terminated, and the inner membrane proteins were solubilized by the addition of 10 μ l of 20% (wt/vol) Sarkosyl NL-97 and 5 μ l of a 120-mg/ml amount of nonradioactive benzylpenicillin (19). After 20 min at room temperature, the Sarkosyl-insoluble outer membrane and peptidoglycan were pelleted by centrifugation at 40,000 $\times g$ for 60 min at 10°C in the SM-24 rotor of a Sorvall RC2B centrifuge. The Sarkosyl-soluble inner membrane proteins were fractionated, according to apparent molecular weight, on a 10% sodium dodecyl sulfate-polyacrylamide slab gel, and the PBPs were detected by fluorography on prefogged (9) Kodak RP Royal X-ray film at -80°C as described previously (19).

The in vitro thermostability of the PBPs in temperature-sensitive mutants was examined by preparing envelopes from cells that had been grown at the permissive temperature (30°C) and measuring their ability to bind [^{14} C]benzylpenicillin at 30°C (as above) and at 42°C, after various periods of preincubation at this temperature.

Quantitation of the levels of [^{14} C]benzylpenicillin bound to each PBP was measured by densitometry of the X-ray films, using a Joyce-Loebl MK111c microdensitometer.

Measurement of minimal inhibitory concentrations. The minimal inhibitory concentrations of β -lactam antibiotics were measured by serial dilution. Tubes containing 1 ml of Penassay broth and twofold serial dilutions of the antibiotic were inoculated with 0.1 ml of a 10^{-4} dilution of an overnight culture of bacteria. After incubation for 24 h at 30°C, the minimal inhibitory concentrations were read as the lowest concentration that inhibited turbid growth.

Chemicals. [^{14} C]benzylpenicillin (specific activity, 53 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, England. Unlabeled benzylpenicillin, cephalixin, and cephaloridine

were kindly provided by Glaxo Laboratories, Greenford, England. Sarkosyl NL-97 was obtained from Geigy Industrial Chemicals, New York.

RESULTS

Isolation of mutants. Twenty-four temperature-sensitive cell division mutants were isolated after mutagenesis of strains KN126 and LEB18 as described above. The temperature sensitivity of the binding of [^{14} C]benzylpenicillin to the individual PBPs was measured in these mutants and in seven previously described cell division mutants. There were no defects in the PBPs of any of the *fts* mutants we examined (listed in Table 1), but two of the newly isolated mutants had thermolabile PBP3. One of these mutants (SP25) was isolated from LEB18, and the other (SP63) was isolated from KN126. Both mutants retained the nutritional markers of their respective parents and had not gained any additional requirements during mutagenesis.

SP63 formed colonies on nutrient agar at 42°C with a frequency of 2.1×10^{-5} . This was reduced to 1.3×10^{-7} on nutrient agar at 44°C. Many of these colonies were not true revertants, since they grew as mixtures of filaments and normal cells at 42°C. Two true revertants, which grew as normal rods at 42°C, were, however, isolated and designated SP631 and SP632. These revertants had the same nutritional requirements as their parent.

SP25 formed short filaments at 42°C and then lysed, with the production of spheroplasts. No revertants of SP25 that form colonies on nutrient agar at 42°C (reversion rate, $>1:10^9$) have been obtained, and this strain is almost certainly a double mutant, with one defect that results in filamentation and another that results in lysis at the restrictive temperature. In support of this, we have been able to isolate derivatives of SP25 that form long filaments but do not lyse at 42°C by plating cells on nutrient agar at 37°C. Under these conditions approximately $1:10^7$ cells formed colonies above a background lawn of growth, and 8 of 13 of these partial revertants filamented without lysis at 42°C. One of these latter strains was chosen for further study and was designated SP258. It grew at the same rate as SP25 and no lysis has been observed, at either 42 or 44°C. SP258 produced revertants on nutrient agar at 42°C, and two of these, which grew as normal rods, were designated SP2581 and SP2582.

Properties of the PBPs of mutants SP25 and SP63. Envelopes of mutant SP25 and its parent (LEB18) were prepared from cells grown

at the permissive temperature, and the level of [^{14} C]benzylpenicillin bound to each of the PBPs was measured at 30 and 42°C. The mobilities of the PBPs on the gels in these B/r strains were identical to those in *E. coli* K-12 (Fig. 1A and J), although a PBP with an apparent molecular weight of 32,000 (PBP7) was consistently detected in the B/r strains, but less consistently, and in lesser amounts, in K-12 strains (19). The level of [^{14} C]benzylpenicillin binding to PBP3 of mutant SP25 at 30°C was about 30% of that in the parent LEB18 (compare the ratio of PBP2/PBP3 in Fig. 1A and D). The low level of PBP3 in mutant SP25 at 30°C was not due to a failure to saturate the protein with [^{14} C]benzylpenicillin, since raising the concentration of the latter from 31 to 116 μ g/ml did not increase the level of binding. As PBP3 in this mutant was highly thermolabile (see below), it was possible that it was being partially inactivated during envelope preparation or during the assay at 30°C. Attempts to increase the level of PBP3 by taking additional precautions to prevent loss of activity during envelope preparation (e.g., replacing the standard 10 mM sodium phosphate buffer with 50 mM phosphate buffer containing 10% glycerol and only washing the membranes once) or during the PBP assay (e.g., by reducing the assay temperature to 20°C and including 10% glycerol in the assay mix) had no effect.

Binding of [^{14} C]benzylpenicillin to the PBPs of LEB18 was not reduced by preincubation of the envelopes for 8 min at 42°C; i.e., the PBPs were thermostable for at least 8 min at 42°C. Envelopes of the mutant SP25 rapidly lost the ability to bind [^{14} C]benzylpenicillin to PBP3 when incubated at 42°C, although the other PBPs were thermostable (Fig. 1E through G). The inactivation of PBP3 was irreversible under these conditions, since little recovery of binding activity was observed after the envelopes had been returned from 42 to 30°C (Fig. 1H and I).

The second mutant with a thermolabile PBP3 has been described briefly previously (18), where it was called 6-30, and it had penicillin-binding properties very similar to those of SP25. Envelopes prepared from cells of SP63 grown at the permissive temperature bound a low level of [^{14} C]benzylpenicillin to PBP3 at 30°C, and binding was thermolabile at 42°C (18). The inactivation of PBP3 at 42°C was irreversible, since after 10 min of preincubation at 42°C no binding of [^{14} C]benzylpenicillin to PBP3 was detected either at 42°C (Fig. 2B) or for at least 30 min after returning the envelopes to 30°C (Fig. 2C through F). As with mutant SP25, the level of binding to PBP3 of SP63 at 30°C

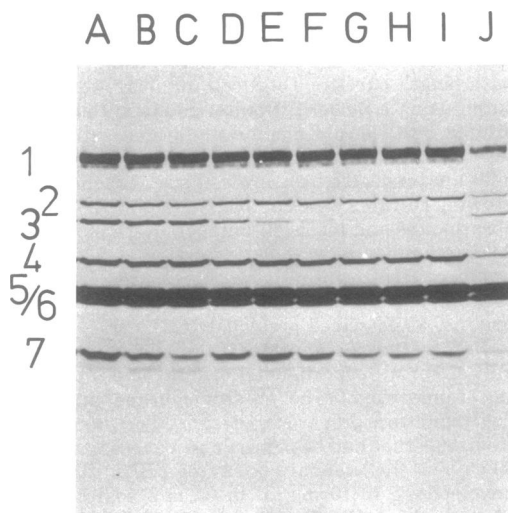


FIG. 1. Binding of [^{14} C]benzylpenicillin to envelopes of LEB18 and SP25. [^{14} C]benzylpenicillin was bound to envelopes of LEB18 for 10 min at 30°C (A). Envelopes of LEB18 were incubated at 42°C, and after 4 min (B) or 8 min (C) [^{14} C]benzylpenicillin was added for a further 2 min at this temperature. [^{14} C]benzylpenicillin was added to envelopes of SP25 for 10 min at 30°C (D). Envelopes of SP25 were incubated at 42°C, and after 0 min (E), 4 min (F), or 8 min (G), [^{14} C]benzylpenicillin was added for a further 2 min at this temperature. A portion of envelopes of SP25 was incubated at 42°C for 10 min and then transferred back to 30°C, and after 1 min (H) or 7 min (I) [^{14} C]benzylpenicillin was added for a further 10 min at this temperature. The PBPs of an *E. coli* K-12 strain (KN126) are included for comparison (J). The binding was terminated, the samples were loaded on a 10% sodium dodecyl sulfate-polyacrylamide slab gel, and a fluorograph was prepared by exposure of the gel to X-ray film for 66 days as described in the text.

could not be increased by raising the concentration of [^{14} C]benzylpenicillin or by taking additional precautions to prevent inactivation during envelope preparation and PBP assay.

Properties of the PBPs of revertants of SP25 and SP63. Figures 3 and 4 show the thermostability of the PBPs in revertants of SP25. The partial revertant SP258, which filamented at 42°C but did not lyse, was identical to SP25 in the level and thermolability of PBP3 (Fig. 3). The full revertants SP2581 and SP2582, which grew as normal rods at 42°C, had regained both the normal level and the normal thermostability of PBP3 (Fig. 4). Revertants of SP63 that grew as normal rods at 42°C also regained the normal level and thermostability of PBP3 (Fig. 5). In this figure PBP1 is split into two bands. Recent experiments using a differ-

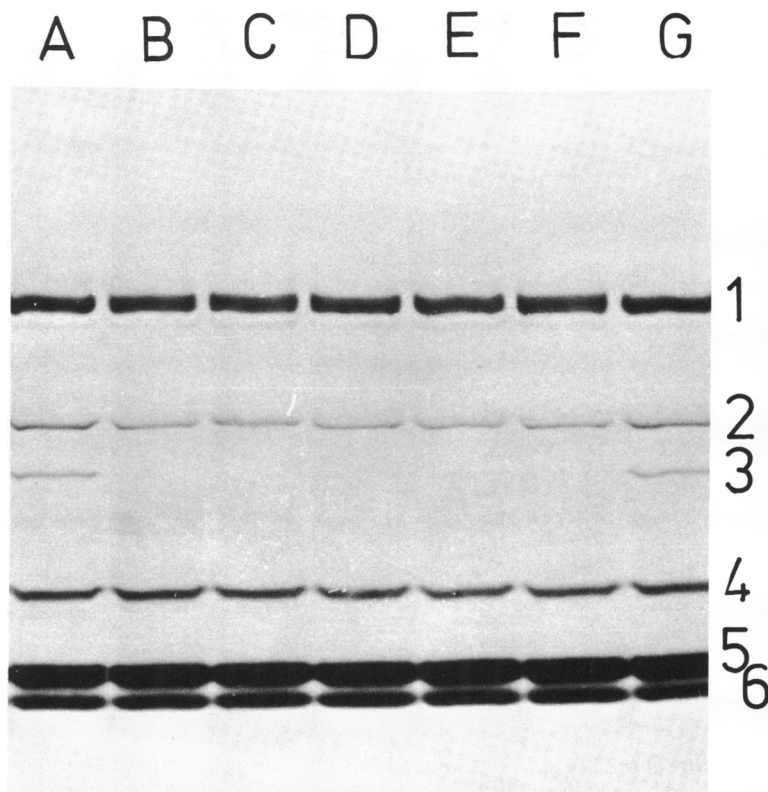


FIG. 2. Binding of [^{14}C]benzylpenicillin to envelopes of SP63. [^{14}C]benzylpenicillin was added to envelopes of SP63 for 10 min at 30°C (A, G). Envelopes of SP63 were incubated at 42°C for 10 min, and [^{14}C]benzylpenicillin was added to one portion for a further 5 min at 42°C (B), while the remainder was transferred back to 30°C and [^{14}C]benzylpenicillin was added after 0 min (C), 10 min (D), 20 min (E), or 30 min (F) for a further 10 min at this temperature. Samples were fractionated, and a fluorograph was prepared by exposure of the slab gel to X-ray film for 49 days.

ent polyacrylamide gel system have shown that PBP1 is at least two proteins (U. Schwarz and B. G. Spratt, unpublished experiments), as previously suspected (19). On this gel PBP7 fails to bind [^{14}C]benzylpenicillin at 42°C. However, PBP7 of the parent and other derivatives was also thermolabile in many experiments and is presumably a rather unstable protein.

Growth and division of SP258 and SP63. Mutants SP258 and SP63 grew rapidly in broth at 30°C with mean generation times that were indistinguishable from those of their parent strains (32 to 34 min for SP258 and 34 to 36 min for SP63). At 30°C the size (A_{550} per cell) of SP258 was 22% greater than that of LEB18, and SP63 was 29% greater than KN126. The increased size of the mutants was due to an increased average cell length.

After a shift from 30 to 42°C, both mutants accelerated their rate of DNA synthesis and cell mass increase, but cell division ceased after a residual increment of 20 to 25%. After about 50

min at 42°C, cell division slowly recommenced and a doubling in cell number, over that at the time of the temperature shift, occurred by 80 min (Fig. 6 and 7). At this time cell mass had increased 9-fold in SP63 and 11-fold in SP258, and long filaments were observed under the phase-contrast microscope (Fig. 8). The slow recovery of cell division at 42°C was found both in minimal medium and in broth; it occurred whether the cell number at the time of the temperature shift was low (e.g., 3×10^6 cells/ml) or relatively high (e.g., 3×10^7 cells/ml). When 44°C was used as the restrictive temperature, the residual increment in cell number of SP258 and SP63 was slightly smaller (10 to 20%) than that at 42°C, and recommencement of division during continued incubation at 44°C did not occur. Figure 9 shows the division of SP63 and KN126 at 44°C in broth. After 130 min at 44°C, cell number had only increased by 20% whereas cell mass had increased by 20-fold, resulting in the production of extremely long

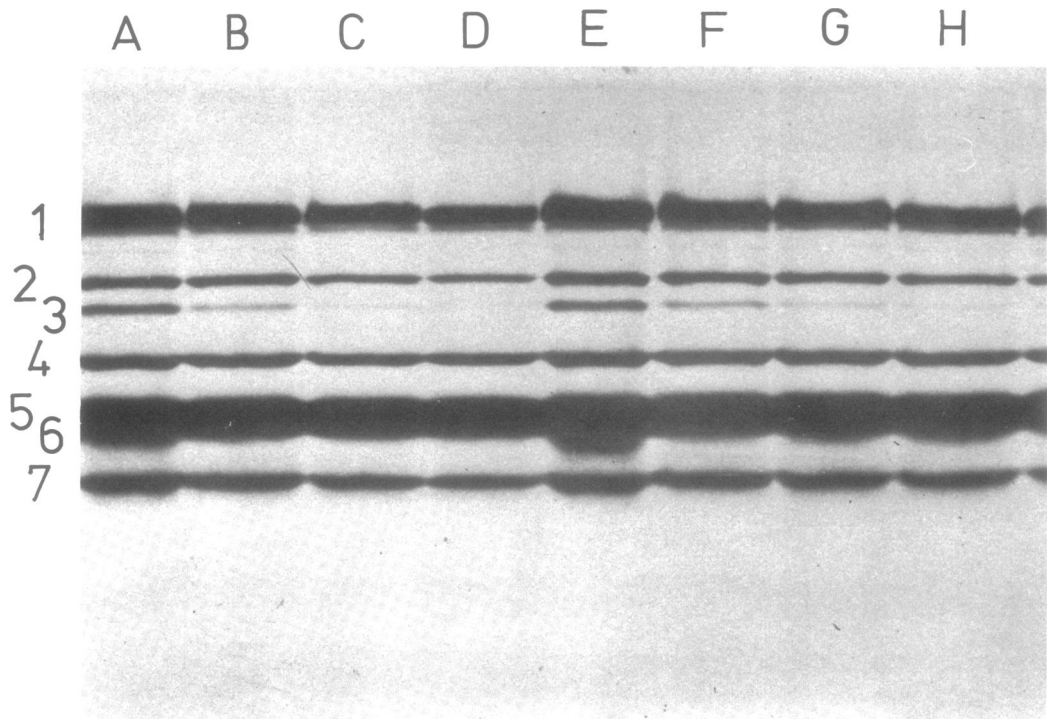


FIG. 3. Thermostability of the PBPs in SP25 and SP258. [^{14}C]benzylpenicillin was bound to envelopes of SP25 for 10 min at 30°C (A). Envelopes of SP25 were incubated at 42°C, and after 0 min (B), 4 min (C), or 8 min (D) [^{14}C]benzylpenicillin was added for a further 2 min at this temperature. [^{14}C]benzylpenicillin was bound to envelopes of SP258 for 10 min at 30°C (E). Envelopes of SP258 were incubated at 42°C, and after 0 min (F), 4 min (G), or 8 min (H) [^{14}C]benzylpenicillin was added for a further 2 min at this temperature. Samples were fractionated, and a fluorograph was prepared by exposure of the slab gel to X-ray film for 55 days.

filaments (Fig. 8). Shifting SP63 back to 30°C, after 40 min at 44°C, resulted in a burst of division after a further 25 min. Filaments of SP63 and SP258, as well as those formed by their parents in the presence of low concentrations of β -lactam antibiotics, often showed a single, centrally positioned incomplete septum when observed under phase-contrast or Nomarski optics.

The inhibition of cell division at 42°C was overcome by the addition of 0.35 M sucrose to broth in strain SP63 and was partially overcome in strain SP258. MgCl_2 (10 mM) also partially reversed the inhibition of division in strain SP63 but had no effect on SP258. DL-Diaminopimelic acid (40 $\mu\text{g}/\text{ml}$), D-alanine (20 $\mu\text{g}/\text{ml}$), NaCl (0.34 M), and cyclic adenosine 5'-monophosphate (1 mM) failed to stimulate division at 42°C in either mutant. The suppression of the defect in cell division at 42°C by sucrose or MgCl_2 could be the result of the stabilization of PBP3 under these conditions. We compared

the thermolability of [^{14}C]benzylpenicillin binding to PBP3 in the presence or absence of 0.35 M sucrose or 10 mM MgCl_2 . Neither compound stabilized PBP3 in the mutants.

Additional properties of mutants SP258 and SP63. Table 2 shows the minimal inhibitory concentrations of the mutants and their parents for three β -lactam antibiotics.

SP63 was isolated in a strain that contains a temperature-sensitive amber suppressor (*sup-126*; 11), and the lesion in the gene coding for PBP3 could have been an amber mutation. If this had been the case, revertants of the temperature-sensitive suppressor should form colonies at 42°C and at the same time should lose their requirements for tryptophan and tyrosine (which are also due to amber mutations). However, although the amber suppressor reverted to thermostability with a frequency of 6×10^{-6} (measured as the reversion rate of KN126 to tryptophan and tyrosine independence at 42°C), we did not obtain colonies of SP63 that grew at

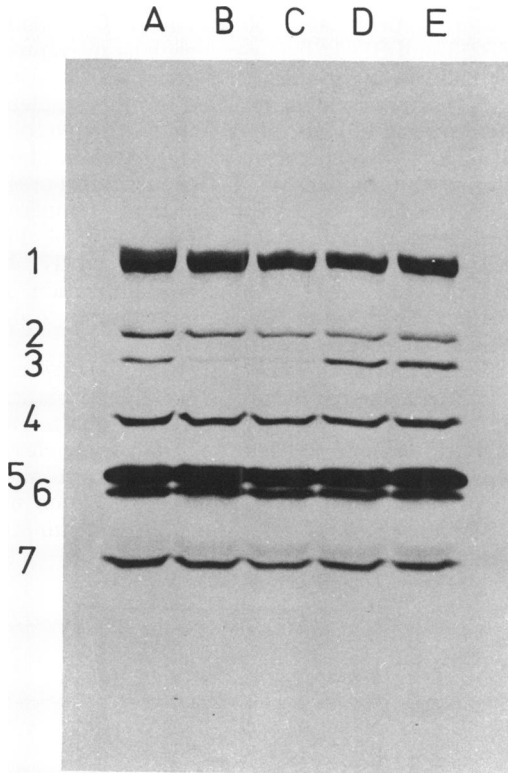


FIG. 4. Thermostability of the PBP3 in revertants of SP258. [^{14}C]benzylpenicillin was bound to envelopes of SP25 for 10 min at 30°C (A). Envelopes of SP25 (B), SP258 (C), SP2581 (D), and SP2582 (E) were incubated at 42°C for 8 min, and [^{14}C]benzylpenicillin was added for a further 2 min at this temperature. Samples were fractionated, and a fluorograph was prepared by exposure of the slab gel to X-ray film for 48 days.

42°C in the absence of tryptophan and tyrosine. The lesion in the gene coding for PBP3 of SP63 was therefore not an amber mutation.

DISCUSSION

The identification of the defective gene products in temperature-sensitive cell division mutants is difficult. Since most of these mutants have been isolated after mutagenesis, particular care is needed to show that any difference between the mutant and its parent is the cause of the defect in division. Loss of a protein or changes in its rate of synthesis on shifting a cell division mutant from the permissive to the restrictive temperature are not sufficient evidence for a role of that protein in the temperature-sensitive phenotype. Two criteria should be satisfied: first, the protein should be shown

to be demonstrably altered in its enzymatic or physicochemical properties, and, second, revertants that divide normally at the restrictive temperature should regain the normal properties of the protein.

The knowledge that penicillins and cephalosporins inhibit cell division by inactivating an enzyme involved in peptidoglycan metabolism and the availability of a convenient method for screening mutants for an alteration in this protein have resulted in the identification of two mutants, SP258 and SP63, in which the above criteria have been satisfied.

Envelopes of SP258 and SP63 prepared from cells grown at the permissive temperature possessed a PBP3 that was active when assayed at 30°C but was rapidly inactivated on incubation in vitro at 42°C. The level of binding of [^{14}C]benzylpenicillin (and the rate of binding [unpublished experiments]) to PBP3 was lower in the mutants at 30°C than in their parents. The lower activity of PBP3 at the permissive temperature correlated well with the increased length of both mutants, suggesting that division even at this temperature was to some extent impaired by the alteration of PBP3. The reason for the lower level of PBP3 is unclear; it could be due to a decreased synthesis, to a decreased ability of the altered protein to be correctly inserted into the cytoplasmic membrane, or to a loss of activity during envelope

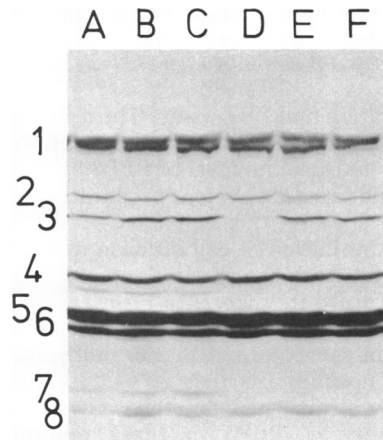


FIG. 5. Thermostability of PBP3 in SP63 and its revertants. [^{14}C]benzylpenicillin was bound to envelopes of (A) SP63, (B) SP631, and (C) SP632 for 10 min at 30°C. Envelopes of (D) SP63, (E) SP631, and (F) SP632 were incubated at 42°C for 5 min, and [^{14}C]benzylpenicillin was added for a further 5 min at this temperature. Samples were fractionated, and a fluorograph was prepared by exposure of the slab gel to X-ray film for 31 days.

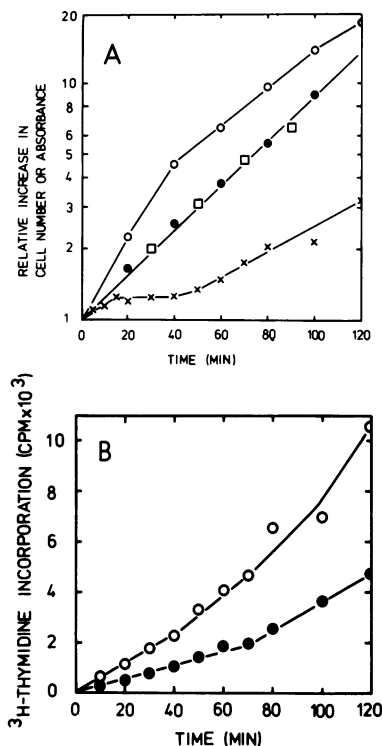


FIG. 6. Growth of SP258 at 30 and 42°C. (A) SP258 was grown in broth at 30°C and shifted to 42°C at zero time. Symbols: (A) Division at 30°C (□) and at 42°C (×); absorbance at 30°C (●) and at 42°C (○). (B) DNA synthesis at 30°C (●) and at 42°C (○). The initial cell numbers and absorbance at zero time were 9.1×10^6 and 0.045, respectively.

preparation and PBP assay. The latter possibility was made less likely by the failure of attempts to raise the level of PBP3 by using gentler conditions of envelope preparation and PBP assay.

The inhibition of cell division at 42°C correlated well with the inactivation of PBP3 at this temperature *in vitro*. The recovery of the normal level and the normal thermostability of PBP3 in revertants of SP258 and SP63, which divide normally as rods at 42°C, provides extremely strong evidence that it was the thermostability of PBP3 in these mutants that caused the inhibition of cell division at 42°C. PBP3 is therefore the first example of a protein that has been shown to be an essential component of the process of cell division in *E. coli*. It is the inactivation of this protein by β -lactam antibiotics that causes the inhibition of division characteristic of the majority of these compounds (18).

Some recovery from the inhibition of cell di-

vision occurred after 50 min at 42°C in both SP258 and SP63. This recovery was prevented by using a slightly higher restrictive temperature and presumably resulted from incomplete inactivation of PBP3 at 42°C under *in vivo* conditions. The suppression of the defect in division at 42°C by sucrose, or the partial suppression by $MgCl_2$, is a feature of many temperature-sensitive cell division mutants (8, 14, 15, 17), although its basis is not understood (17). It was probably not due to the stabilization of PBP3 by sucrose or $MgCl_2$, as attempts to do this *in vitro* were unsuccessful.

SP258 and SP63 were slightly more resistant than their parents to three β -lactam antibiotics at 30°C. SP63 was selected as resistant to a low level of cephalixin, but it is far from clear whether the resistance in this mutant and SP258 has any relevance to the alteration in PBP3 since several *fts* mutants show a similar resistance to benzylpenicillin (15).

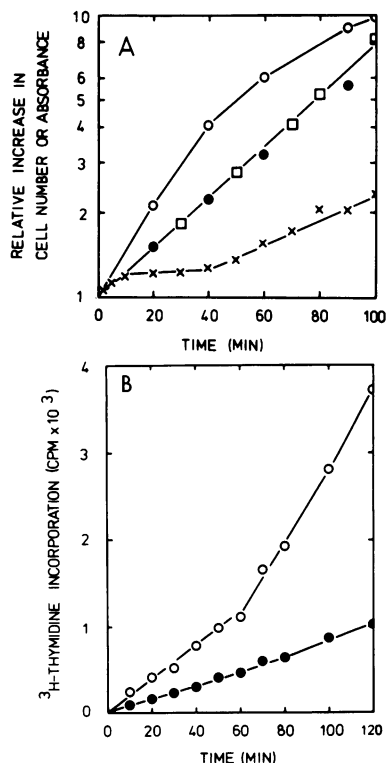


FIG. 7. Growth of SP63 at 30 and 42°C. (A) SP63 was grown in broth at 30°C and shifted to 42°C at zero time. Symbols: (A) Division at 30°C (□) and at 42°C (×); absorbance at 30°C (●) and at 42°C (○). (B) DNA synthesis at 30°C (●) and at 42°C (○). The initial cell numbers and absorbance at zero time were 1.36×10^7 and 0.081, respectively.

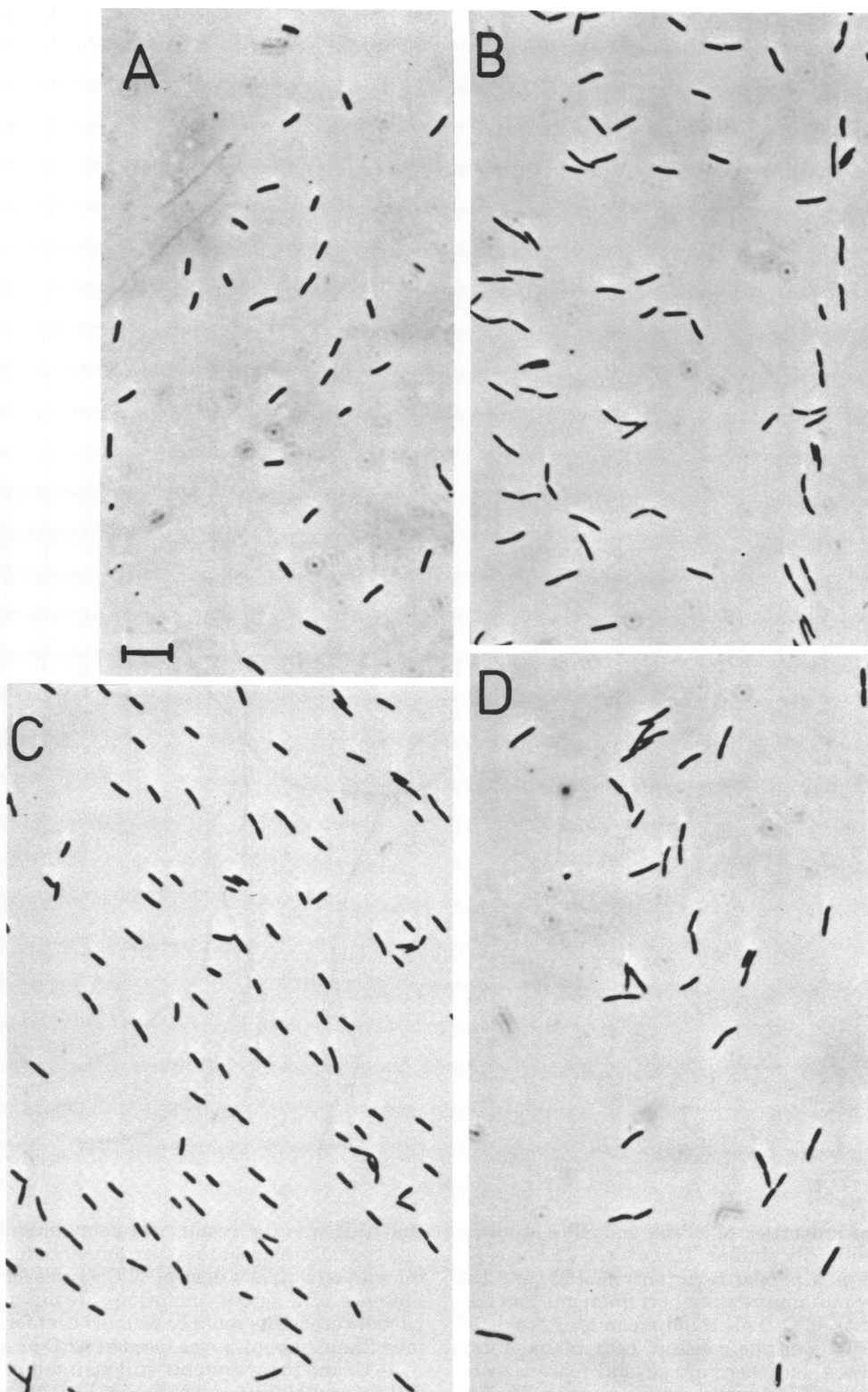
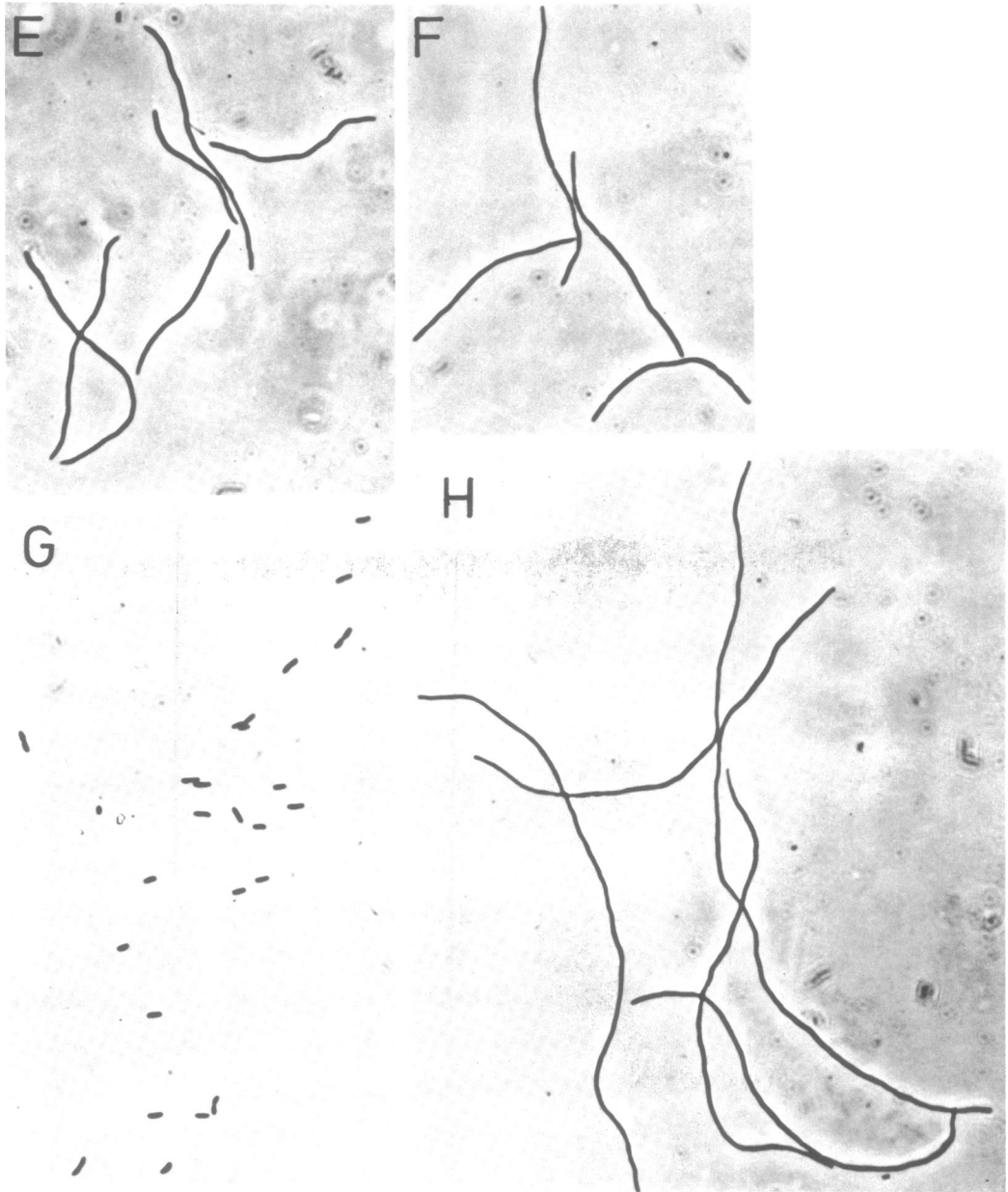
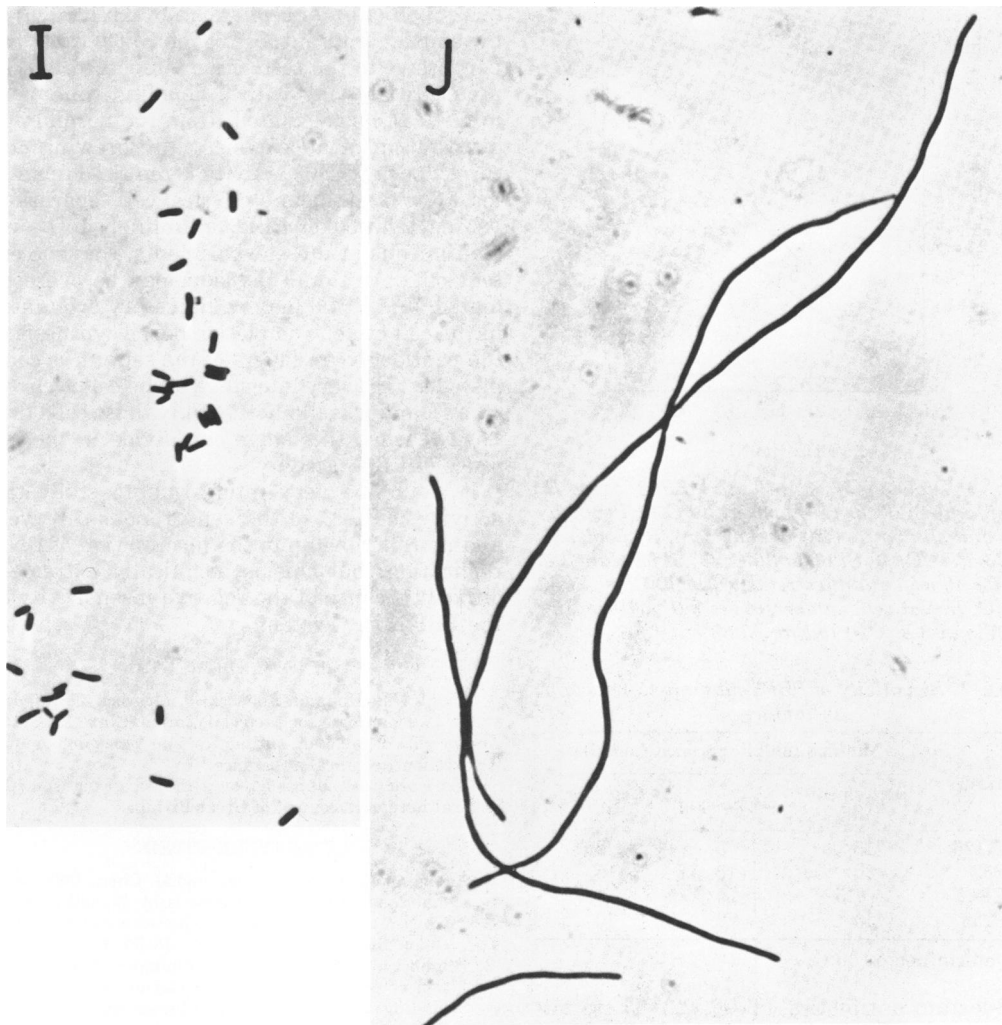


FIG. 8. (A) LEB18, (B) SP258, (C) KN126, and (D) SP63 grown in broth at 30°C; (E) SP258 and (F) SP63 shifted to 42°C for 80 min; (G) LEB18, (H) SP258, (I) KN126, and (J) SP63 shifted to 44°C for 130 min. The bar in (A) corresponds to 10 μ m.



The properties of SP258 and SP63 were extraordinarily similar. However, SP258 was isolated as a partial revertant of SP25, and this latter mutant grew as short filaments and then lysed at 42°C. This could mean that the defect in PBP3 was the cause of both filamentation and lysis, but there are several reasons to believe that SP25 was a double mutant. The fact that PBP3 was thermolabile in SP25 suggests

that this was the result of a point mutation, and the failure to obtain revertants that formed colonies on nutrient agar at 42°C argues for the presence of a second mutation. Furthermore, partial revertants could be isolated that formed long filaments but never lysed at 42°C or even at 44°C, and these mutants still maintained the full thermolability of PBP3. Finally, SP63 had a thermolabile PBP3 and yet never lysed at



42°C. The cause of lysis in SP25 was presumably due to a defect in cell wall synthesis, but it was not due to the defect in PBP3 and none of the other PBPs were thermolabile.

SP258 and SP63 are considered to be mutations in the structural gene for PBP3, and I propose to call this gene *pbpB* (penicillin-binding protein). *pbpA* is the structural gene for PBP2 defined by temperature-sensitive cell shape mutants that have thermolabile PBP2 (B. G. Spratt, manuscript in preparation). The *pbpB* mutations of SP258 and SP63 have not been mapped, but they appear to be distinct from those of the *fts* mutants that we examined (Table 1), as these latter mutants had normal PBP3.

PBP3 has an apparent molecular weight of

60,000, and a single rapidly growing *E. coli* cell has been estimated to contain about 50 molecules of this protein (19). PBP3 is presumed to correspond to a minor PSE involved in peptidoglycan metabolism specific to cross-wall formation at division. From its abundance, molecular weight, and penicillin-binding properties, it is clearly distinct from the two major PSEs that have been purified from *E. coli* (6, 12, 23). Purely on the basis of a difference in the sensitivities of the two PSEs to β -lactam antibiotics, it has been suggested that one is involved in division and one in cell elongation (6, 12, 23). More recent work has shown that these assignments were premature, and it is now believed that it is minor PSEs, corresponding to minor PBPs (e.g., PBP3), that are the killing targets

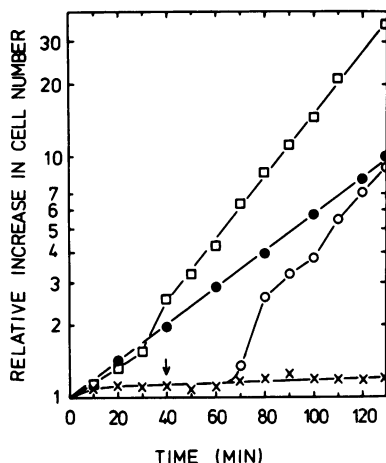


FIG. 9. Division of KN126 and SP63 at 44°C. KN126 and SP63 were grown at 30°C in Penassay broth and shifted to 44°C at time zero. Symbols: □, KN126 at 44°C; ●, SP63 at 30°C; ○, SP63 shifted to 44°C for 40 min and then returned to 30°C; ×, SP63 at 44°C. Initial cell numbers were 9.9×10^6 /ml for KN126 and 8.8×10^6 /ml for SP63.

TABLE 2. Sensitivity of PBP3 mutants to β -lactam antibiotics

Strain	Minimal inhibitory concn (μ g/ml) ^a		
	Benzylpenicillin	Cephalexin	Cephalexidine
KN126	15	5	2.5
SP63	30	10	5
LEB18	7.5	10	2.5
SP258	15	20	10

^a Determined at 30°C.

for β -lactam antibiotics (18–20, 22). These minor PBPs have not been purified yet. The function of the major PSEs in cell growth remains obscure.

PBP3 has been shown to be the target at which β -lactams bind to inhibit cell division and cause filamentation, but its precise physiological function is unknown. It is attractive to believe that PBP3 is synthesized, or activated, at the termination of a round of DNA replication and results in the production of a special type of peptidoglycan necessary for division. Protein synthesis is no longer necessary for division from about 15 min before division (3, 5), and presumably PBP3 has been synthesized by this time, but its activity is probably required during most of the D period and until very close to the moment of physical cell separation, as only 10 to 20% of a culture of *E. coli* growing in broth divide when PBP3 is inactivated (by the addition of low concentrations of

benzylpenicillin or cephalexin to KN126 [20] or by shifting mutant SP258 of SP63 from the permissive to the restrictive temperature). For a culture growing with a doubling time of 23 min it can be calculated (from the normal age distribution of an exponentially growing culture, 10) that PBP3 activity is required up to at least 3 to 6 min before physical cell separation. As inactivation of PBP3 is unlikely to be instantaneous in these experiments, the true extent of the residual division may be overestimated, and PBP3 may be necessary even later in the cell cycle. Completion of the synthesis of the peptidoglycan layers of the septum is completed at this very late stage of the cell cycle (3, 13, 24), and it is likely that only those cells that have reached this stage can divide in the absence of PBP3 activity.

It would be very interesting to study the activity of PBP3 in the synchronous cell cycle and its relationship to the termination of DNA replication, but this is technically extremely difficult because of the relative insensitivity of the PBP assay system.

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