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Murein Synthesis and Beta-Lactam Antibiotic Susceptibility During Rod-to-Sphere Transition in a *pbpA*(Ts) Mutant of *Escherichia coli*

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The conditional morphology mutant of *Escherichia coli* SP45 grows as a rod at 30°C and assumes a spherical shape after 90 min of incubation at 42°C. The rod-to-sphere morphological transition has been found to be associated with the disappearance of penicillin-binding protein 2 (PBP-2), the progressive reduction (as much as 50%) of murein synthesis, as measured both in intact cells and ether-permeabilized bacteria, and alterations in the structure of the cell envelope, including detachment of the outer membrane from the underlying structures. The detachment was initially localized at the poles of the cells and then spread over the entire surface. Shape transition was also linked to increased susceptibility to beta-lactam antibiotics which preferentially bound to PBP-1A (cephalothin, cephaloridine) or to PBP-3 (furazlocillin, piperacillin). Treatment with beta-lactams possessing a high affinity for PBP-1A, although inducing a low degree of peptidoglycan synthesis inhibition (5 to 10%), was associated with a marked loss of cell viability and massive lysis. On the other hand, the simultaneous absence of PBP-2 and inhibition of PBP-3 caused a significant reduction of peptidoglycan synthesis, yet only slightly affected cell viability. Whereas PBP-1A inhibition during shape transition had no effect on morphology, addition of antibiotics binding to PBP-3 30 min after the temperature shift-up caused formation of elongated cells with a centrally located bulge, not observed in similarly treated cells grown at 30°C. Inhibition of PBP-3 in round cells 90 min after temperature shift caused formation of giant cells, indicating complete loss of elongation ability. The different effects of the simultaneous inhibition of two PBPs, combining mutational loss with specific binding in vivo of another PBP by beta-lactams, provide new insight into the role of these proteins and the killing mechanisms of this class of antibiotics.

Different beta-lactam antibiotics induce different effects on *Escherichia coli* (rapid lysis, elongation, formation of round cells) which are linked to the affinity of the antibiotic for the penicillin-binding proteins (PBPs) located in the inner membrane of both gram-positive and gram-negative bacteria (15, 20–22, 24). Mutants of *E. coli* lacking each individual PBP have been described (20), but except for PBP-1Bs-deficient mutants very little is known about the susceptibility of these mutants to different beta-lactam antibiotics and the biochemistry of their murein (21). Purification of some of the functionally major PBPs has recently been reported (22).

The availability of a spontaneous mutant of *E. coli*, SP45 (17), which grows as a normal rod at 30°C and assumes a round shape after incubation at 42°C, prompted us to investigate the effects of several beta-lactams on the morphogenetic ability of *E. coli*. This mutant has been characterized as *pBpA*(Ts) and possesses a thermolabile PBP-2. The mutation is contransducible with *lip* at high frequency and maps at 14 min on the genetic map (7, 18).

In this study we report the effects of antibiotics selectively binding to PBP-1A and -3, as determined by an in vivo assay, on murein biosynthesis and morphology during the rod-to-sphere morphological transition.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* SP45 *pbpA*(Ts) (kindly provided by B. G. Spratt) was selected for mecillinam resistance and temperature-sensitive growth without treatment with mutagens (18) from *E. coli* KN126 *tryE9829*(Am) *tyr*(Am) *ilv sup-126*. The strains were routinely grown in antibiotic medium no. 3 (Pennassay broth [Difco]) at 30°C or at 42°C with vigorous shaking.

Effects of beta-lactam antibiotics on growth and viability during shape transition. The strains

were grown for two generations at 30°C, and then part of the culture was transferred to 42°C. At 30 and 90 min after the temperature shift-up, selected concentrations of cephaloridine, cephalothin, furazlocillin, and piperacillin were added to the cultures. Antibiotic concentrations binding exclusively to their primary target PBP when added to intact growing cells were selected (see below) and used in all of the experiments. Cell viability and absorbance (measured at 650 nm in a Beckman BD spectrophotometer) of the cultures were determined after 60 min of treatment with the antibiotics.

Murein synthesis evaluation. Murein synthesis during shape transition in the presence or absence of antibiotics was determined by incorporation of DL-*meso*-2,6-diamino[1,7-^{14}C]pimelic acid ([^{14}C]DAP) into lysozyme-digested murein and by incorporation of externally added murein precursors to ether-permeabilized cells (23). *E. coli* SP45 and KN126 were grown as described above at 30 or 42°C with or without antibiotics. Cells (50 ml) were harvested by centrifugation 30 and 90 min after the temperature shift-up and suspended in 10 ml of prewarmed incorporation medium [50 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.6), 0.2% glucose, 10 mM $MgCl_2$, 5 mM KH_2PO_4, 0.4 mM glutamic acid (neutralized), 2 mM L-lysine, 1 mM L-alanine, and 200 μg of chloramphenicol per ml] which contained 0.35 μCi of [^{14}C]DAP. Incubation was carried out for 10 min at the same growth temperature, and the incorporation of the radioactive tracer was linear. After washing, the pellet was heated at 100°C for 10 min and suspended in ammonium acetate buffer (50 mM) containing 1 mg of lysozyme per ml (Sigma). After overnight incubation at 37°C, radioactivity was measured in a liquid scintillation system {1 liter of toluene, 500 ml of Triton X-100, 100 mg of PPO [2,5-diphenyloxazole], and 0.4 mg of dimethyl POPOP [1,4-bis-(5-phenyloxazolyl)benzene]; LS 7000 scintillation counter, Beckman}. The remaining supernatant was freeze-dried and then suspended in 20 μl of distilled water, and murein fragments were separated by descending chromatography. Radioactive spots were localized after autoradiography, cut, and counted. The number of counts per minute was related to the amount of protein (Bio-Rad Protein Reagent).

Preparation of ether-treated cells was carried out as described (10, 21). Unlabeled uridine diphosphate-*N*-acetylmuramyl pentapeptide was prepared by treatment (40 min) of *Bacillus cereus* T cultures with vancomycin (30 μg/ml) and purified as reported (8).

Reaction mixtures contained in a final volume of 200 μl, ether-permeabilized cells (1 mg of protein), 50 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.3), 20 mM $MgCl_2$, 10 mM adenosine triphosphate, 100 nmol of uridine diphosphate-*N*-acetylmuramyl pentapeptide, and 90,000 cpm of uridine diphosphate-*N*-[^{14}C]acetylglucosamine. Mixtures were incubated at 30 or 42°C with or without antibiotic for 60 min, and the incorporation of *N*-[^{14}C]acetylglucosamine into trichloroacetic acid-precipitable or sodium dodecyl sulfate (SDS)-insoluble material was determined as described (10).

Assay of murein hydrolase activity in whole cells. Hydrolase activity was measured in cells grown at 30 or 42°C with or without antibiotics, according to the procedure previously reported (4). Cells were labeled in the incorporation medium described above.

Envelope preparation and PBP detection by fluorography. Antibiotic concentrations at which preferential binding to a single PBP occurred were determined as follows. Cultures growing at 30 or 42°C (1 liter) were sampled (80 ml each) and treated for 20 min with drug concentrations ranging from 0.05 to 100 μg/ml. To stop the reaction, the cells were immediately washed in cold phosphate buffer (50 mM, pH 7.0), and the cell envelopes were prepared by differential centrifugation of sonicated cells (19). The washed envelopes were resuspended in the same buffer and incubated for 15 min at the same growth temperature in the presence of [^{14}C]benzylpenicillin (30 μg/ml, final concentration).

Separation of the PBPs by SDS-slab gel electrophoresis and their detection by fluorography were performed as described (16).

Films were scanned with a scanning densitometer (Transydine General Co., Ann Arbor, Mich.), and the concentration range in which the antibiotics bound to only one PBP in vivo was determined.

Phase-contrast and electron microscopy. Techniques for phase-contrast microscopy have been previously described (12). For electron microscopic observations, samples of the cultures growing at 30 or 42°C were harvested by centrifugation and suspended in 2% glutaraldehyde dissolved in 50 mM phosphate buffer (pH 7.4). After 2 h at 4°C the cells were washed with four changes of cold buffer and postfixed for 30 min in cold 1% OsO_4 in the same buffer. Postfixation was followed by dehydration in graded acetone and by embedding in Durcopam ACM epoxy resin. Thin sections were cut with a Reichert OM U3 ultramicrotome, mounted on grids, and double stained with saturated aqueous uranyl-acetate and Reynolds lead citrate. The specimens were examined with a Zeiss EM9 electron microscope.

Chemicals. [^{14}C]benzylpenicillin (51 mCi/mmol), [^{14}C]DAP (40 to 60 mCi/mmol), and uridine diphosphate-*N*-D-[*U*-^{14}C]acetylglucosamine (>200 mCi/mmol) were from the Radiochemical Centre, Amersham, U.K. All the reagents were of the best grade available. Cephaloridine, cephalothin, and piperacillin were commercially available products. Furazlocillin was a gift from James T. Park.

RESULTS

Disappearance of PBP-2 in cells growing at 42°C. Thermolability of PBP-2 in strain SP45 has previously been determined by preincubation of envelope at 42°C for short periods (17). Since these conditions are likely to differ from an in vivo situation, the kinetics of disappearance of PBP-2 in intact cells growing at the nonpermissive conditions was first determined. As shown in Fig. 1, the amount of PBP-2 still available for binding to [^{14}C]benzylpenicillin was reduced 50% after 20 min of growth at 42°C, and 90% of the original amount was no longer detectable after 40 min.

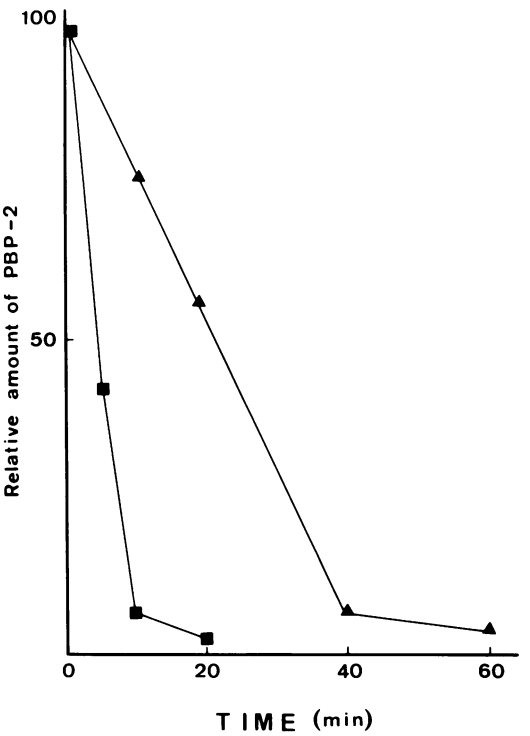

FIG. 1. *Kinetics of the disappearance of PBP-2 in intact cells (▲) growing at 42°C and in envelopes (■) prepared from cells grown at 30°C and incubated at 42°C for short time intervals.*

These results greatly differ from those obtained in experiments carried out with isolated envelopes (Fig. 1; 17). In the latter condition, 10 min of incubation at 42°C was sufficient to almost completely inactivate the ability of PBP-2 to bind the radiolabeled penicillin G.

No changes in the intensity of the other bands were detectable in association with the disappearance of PBP-2.

Biochemical and morphological analysis of rod-to-sphere transition. The kinetics of morphological changes after temperature shift-up was similar to that described by others (6). After 30 min, cells were ovoid in shape, and upon further incubation (90 min) all cells appeared as cocci (see Fig. 3 and 5). The rate of viable count increase was very close to that of the control culture growing at 30°C. Coulter units increased at a higher rate at 42°C (data not shown), probably due to an increase in the average cell size associated with shape change. Absorbance of the culture at 42°C also increased at a higher rate, most probably for the same reason (Table 1). Murein synthesis, measured both in intact cells and in ether-permeabilized bacteria, showed significant modifications. After 30 min of incubation at 42°C it was slightly reduced (Table 2); by the time of complete morphological transition, it was 50% reduced. In ether-treated cells a similar pattern was observed (Table 2) when radioactivity was measured in trichloroacetic acid-precipitable material. However, the amount of externally added precursors incorporated into SDS-insoluble murein progressively increased. At 30°C, 50% of the newly synthesized murein was inserted in the sacculi (SDS insoluble), whereas after 90 min of incubation at 42°C, 75% of the labeled murein was recovered from SDS-treated samples. These differences may well be related to a different rate of insertion of the nascent peptidoglycan in the sacculi.

Murein labeled with [^{14}C]DAP was digested with lysozyme, and the resulting fragments were separated by paper chromatography. The degree of cross-linking during the various phases of shape transition was found to be the same (Table 2).

Associated with shape change was an increase in autolytic activity measured in whole cells labeled with [^{14}C]DAP as a substrate (Table 3). After 30 min at 42°C, autolytic activity was close to that observed at 30°C, but after 90 min it had significantly increased.

Electron microscope observations consistently demonstrated changes in the organization of the envelope layers which were closely associated with the kinetics of shape transition. During the early stages of morphological transition, the outer membrane appeared to be detached from the peptidoglycan layer in limited areas corresponding to the old poles of the cells (Fig. 2). On the lateral surface only slight damage could be observed. When transition was complete, the outer membrane appeared to be detached from the entire surface with few remaining sites of contact (Fig. 3). No alterations were evident in cells grown at 30°C (Fig. 4).

Effect of antibiotic treatment during rod-to-sphere transition. The effect of simultaneous inhibition of different PBPs can be investigated by using multiple mutants or by combining the mutational loss of a PBP with inhibition of another by a beta-lactam antibiotic with specific affinity. Among the functionally major PBPs, PBP-1A, -1Bs, -2, and -3 are included (20–22). In our previous investigations (2) it was not possible to find, among the several antibiotics tested, one showing an exclusive affinity in vivo for PBP-1Bs. The effects of antibiotics showing exclusive affinity for PBP-1A (cephaloridine and cephalothin) and for PBP-3 (furazlocillin and piperacillin) in intact cells over a certain concentration range were tested. Binding affinity was checked in intact cells by pulsing the cultures with different concentrations of the

TABLE 1. *Susceptibility of E. coli SP45 to different beta-lactam antibiotics during rod-to-sphere transition*[a]

Antibiotic concn (60-min pulse)	CFU			OD		
	30°C at 90 min	42°C at:		30°C at 90 min	42°C at:	
		30 min	90 min		30 min	90 min
Control	7×10^8	3×10^8	$6.8 \pm \times 10^8$	0.90	0.35	1.10
Cephaloridine						
1/2 MIC	5×10^8	1.8×10^8	2.3×10^7	0.75	0.28	0.55
MIC	2×10^8	3×10^7	6×10^6	0.50	0.10	0.25
Cephalothin						
1/2 MIC	5×10^8	2.4×10^8	4×10^7	0.75	0.30	0.60
MIC	2.6×10^8	4×10^7	7.2×10^6	0.50	0.15	0.25
Furazlocillin						
1/2 MIC	6×10^8	2×10^8	1.2×10^8	0.90	0.35	0.95
MIC	2.4×10^8	9×10^7	7×10^7	0.80	0.28	0.80
Piperacillin						
1/2 MIC	6×10^8	2.4×10^8	3×10^8	0.90	0.35	1.00
MIC	5×10^8	2×10^8	1×10^8	0.85	0.35	0.92

[a] Antibiotic was added to exponentially growing cultures of strain SP45 at 30°C (at 90 min) and at 42°C (30 and 90 min after the temperature shift-up) for 60 min. Survivors were determined as colony-forming units (CFU) by plating suitable dilutions of the culture. Turbidity (optical density, OD) was measured at 650 nm (BD spectrophotometer, Beckman).

TABLE 2. *Effect of beta-lactam antibiotics on murein synthesis during rod-to-sphere transition in E. coli SP45*

Growth conditions			[^{14}C]DAP incorporated in intact cells (%)	Cross-linking[a]	[^{14}C]UDP-GlcNAc[b] incorporated in ether-treated cells (%)	
Temp (°C)	Time after temp shift (min)	Drug[c]			TCA precipitate	SDS insoluble[d]
30		Control	100	0.98	100	46
		Cephaloridine	95	0.94	98	51
		Furazlocillin	82	0.98	85	40
42	30	Control	92	0.94	88	65
		Cephaloridine	90	1.00	90	65
		Furazlocillin	71	1.10	75	52
42	90	Control	56	1.00	52	75
		Cephaloridine	48	0.98	50	75
		Furazlocillin	36	0.98	31	50

[a] The degree of peptide cross-linkage in the peptidoglycan was determined from analysis of the labeled fragments separated by paper chromatography (isobutyric acid-ammonia, 5:3, vol/vol) after digestion with lysozyme. The labeled spots were located after autoradiography, cut, and counted.

[b] GlcNAc, *N*-Acetylglucosamide.

[c] MICs were used.

[d] Expressed as percent of radioactivity recovered from trichloroacetic acid (TCA)-treated samples.

selected antibiotics and then preparing the envelopes as described (15). Concentrations binding only to the primary target were used for subsequent experiments. Concentrations of cephaloridine and cephalothin binding exclusively to PBP-1A ranged from 0.1 to 1 μg/ml; the concentrations of piperacillin and furazlocillin binding only to PBP-3 ranged from 0.05 to 40 μg/ml, respectively. The 50% saturating concentrations of these antibiotics for their primary targets in relation to the minimal inhibitory concentrations (MIC) are presented in Table 4. It is remarkable that, although strain SP45 was more sensitive to PBP-1A inhibition when

grown at 42°C, no significant difference was observed in the binding affinities at the different growth temperatures.

When PBP-1A was inhibited by a 60-min pulse with cephaloridine or cephalothin concentrations equal to the MIC or one-half of the MIC at 30 and 90 min after the temperature shift-up, no significant morphological changes were evident (Fig. 5). When treated after a 30-min incubation at 42°C, the cells appeared to elongate slightly, but any morphogenetic process was difficult to detect because cells were extremely sensitive to the simultaneous inhibition of PBP-2 and -1A, and massive lysis occurred. At the concentrations used neither cephaloridine nor cephalothin significantly inhibited murein synthesis (Table 2) regardless of the time at which it was added to the culture. Cells treated with these antibiotics were found to autolyse more extensively when lysis was checked in buffer after activation in the cold with 1 M NaCl (Table 3). The effect of the simultaneous inhibition of PBP-3 and -2 was markedly different. When furazlocillin or piperacillin was added after a 30-min incubation, cells were able to elongate, though only to a limited extent (Fig. 5). A short filament was formed with a centrally located bulge. Although this shape closely resembles the one that can be obtained with high concentrations of penicillin for gram-negative rods, it is

TABLE 3. *Murein hydrolase activity in intact cells during rod-to-sphere transition with or without antibiotics*

Strain	Enzyme activity[a] at growth temp:		
	30°C	42°C	
		30 min	90 min
SP45	42	47	71
KN126	40	40	48
SP45 + bound PBP-1A	58	72	86
SP45 + bound PBP-3	48	51	70

[a] Activity is reported as percent of murein liberated to trichloroacetic acid-nonprecipitable material, based on the ratio between that and total amount of radioactivity in the whole cells. Autolysins were activated by treatment with 1 M NaCl in the cold for 10 min. Incubation was carried out at the same growth temperature for 60 min in tris(hydroxymethyl)aminomethane-maleate buffer (pH 6.8) (4).

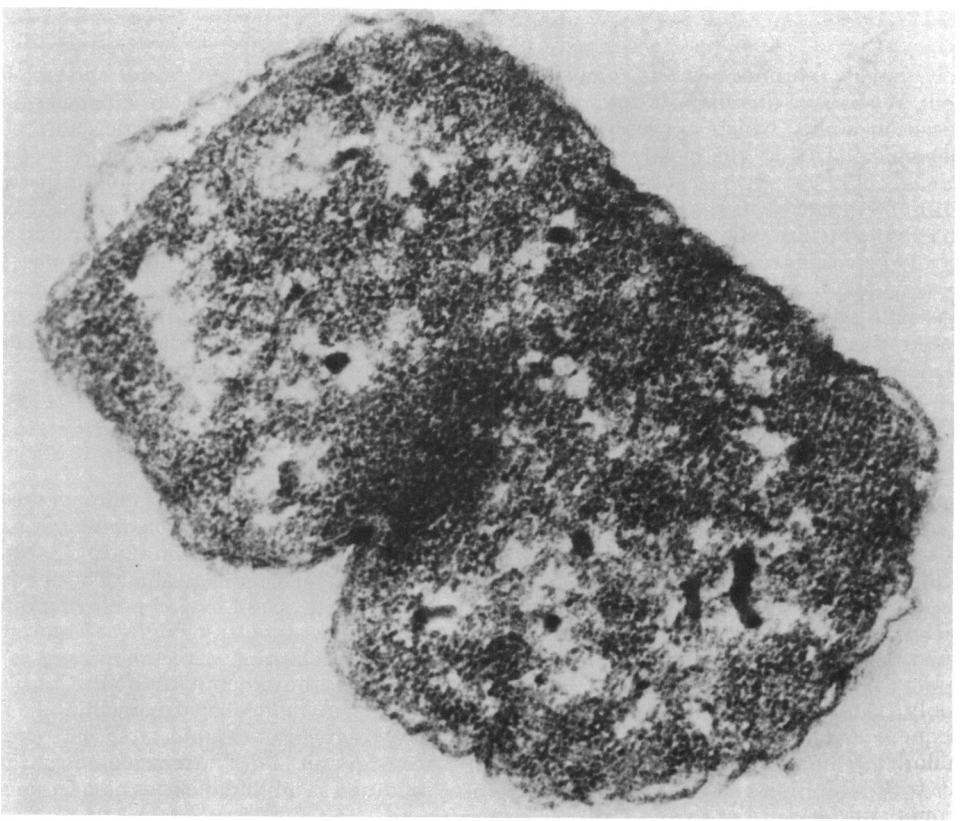

FIG. 2. *Transmission electron micrograph of strain SP45 after 30 min of incubation at 42°C (×15,000).*

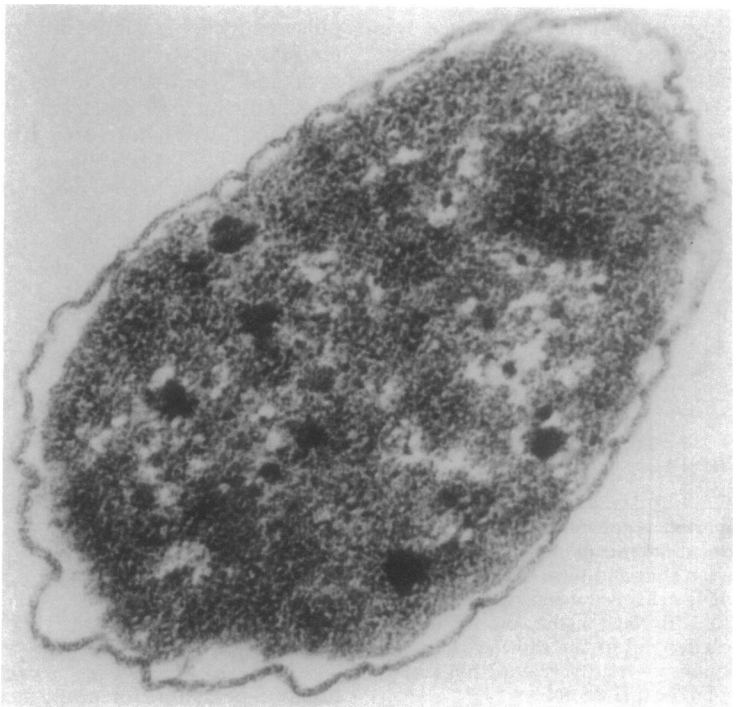

FIG. 3. *Transmission electron micrograph of strain SP45 90 min after temperature shift-up (×15,000).*

likely that its morphogenesis is completely different. We believe this shape is due to a residual elongation ability which is preserved in some biochemical way at the polar regions of the rounded cells.

After 90 min at 42°C the elongation potential was completely lost, and only larger, round cells were detected after the inhibition of cell septation (Fig. 5).

Loss of viability was less marked in cells treated with furazlocillin or piperacillin than in cells treated with cephaloridine or cephalothin, and absorbance decreased to a limited extent after a 20-min pulse (Table 1). When autolysis was checked, a degree of autolytic activity similar to that of the untreated cells was observed (Table 3). However, inhibition of murein synthesis was greater than in cells in which PBP-2 and -1A were simultaneously inhibited. Both piperacillin and furazlocillin caused a progressive increase in the inhibition of [^{14}C]DAP incorporation during shape transition (Table 2). A 20-min pulse with furazlocillin after 30 min from the temperature shift-up caused a 30% inhibition, whereas similar treatment after 90 min of incubation at the nonpermissive temperature inhibited murein synthesis up to 35%. A similar degree of inhibition was obtained with 5 μg of piperacillin per ml (data not shown). Identical results were obtained in ether-permeabilized bacteria both in trichloroacetic acid-precipitable material and SDS-insoluble murein (Table 2).

The lack of a strict correlation between the degree of murein inhibition and cell lysis suggests that, in this system, the increased autolytic activity is not due to an imbalance between murein synthesis and degradation but to the fact that murein synthesized during the simultaneous inhibition of PBP-2 and -1A is qualitatively more prone to undergo autolysis.

DISCUSSION

Conditional morphology mutants are of great interest in the investigation of the mechanism of bacterial shape regulation (2, 10, 12, 13).

Results reported here allow a better definition of the sequence of events occurring during the rod-to-sphere transition in *E. coli* morphological mutants. The first detectable event is the decrease in the amount of PBP-2 available for binding to radiolabeled penicillin G. The 50% loss observed at 20 min after the temperature shift-up still allows a residual elongation when septation is inhibited and is associated with hy-

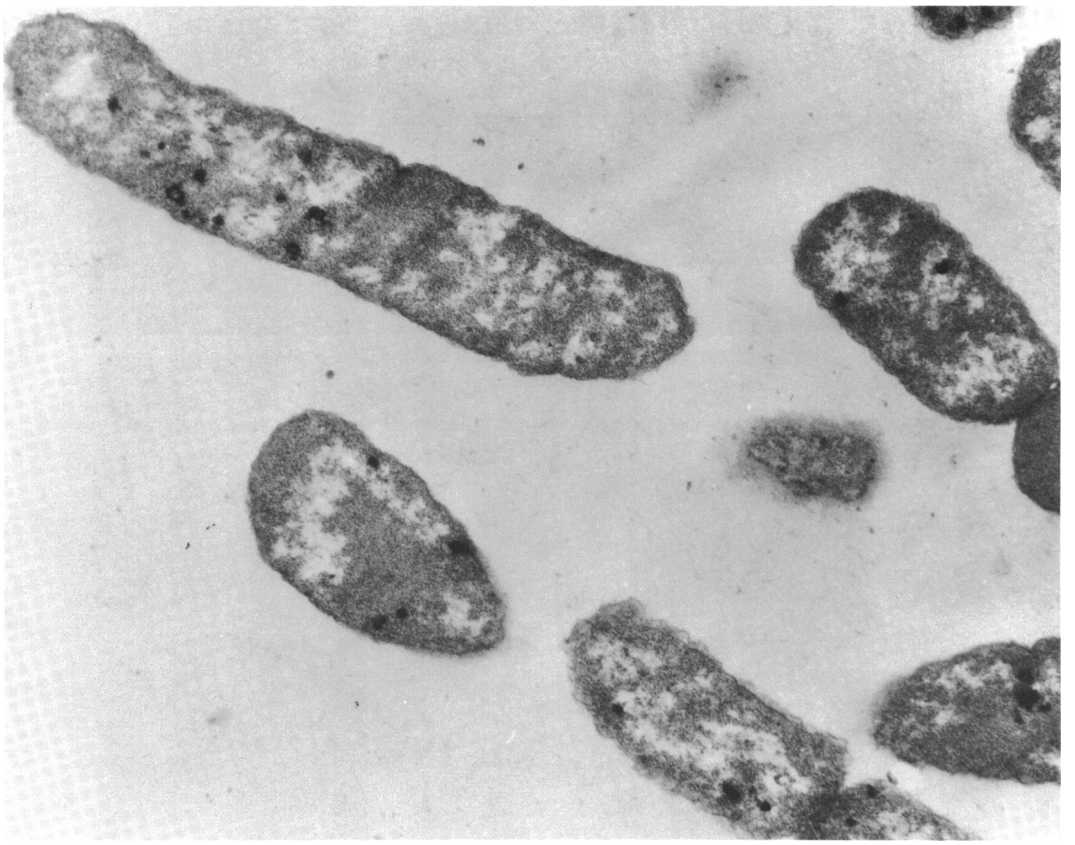

FIG. 4. *Transmission electron micrograph of strain SP45 grown at 30°C (×9,800).*

TABLE 4. *Relation between MICs and the 50% saturating concentrations of beta-lactam antibiotics in E. coli SP45*

Antibiotic	MIC (μg/ml) at:		50% saturating concn (μg/ml) in intact cells
	30°C	42°C	
Cephaloridine	1.6	0.4	0.25[a]
Cephalothin	1.8	1	0.4[a]
Furazlocillin	2	2	0.05[b]
Piperacillin	6	6	0.05[b]

[a, b] Values refer to (*a*) PBP-1A and (*b*) to PBP-3; 50% saturating concentrations of the antibiotics were identical in cells growing at 30 or 42°C.

persensitivity to the simultaneous inhibition of PBP-1A.

A 50% reduction in murein synthesis, similar to that observed after mecillinam treatment, is associated with decrease and eventual disappearance of PBP-2 (11). The murein synthesized seems to possess a normal structure, in agreement with what has been observed by others in a *rod* mutant (5) or in round-shaped cells after addition of mecillinam (11). Although these cells seem to possess a normal murein, they are more susceptible to autolysis. It is possible that the activation of all or some of the autolytic enzymes plays a role in the morphological transition, or that this increased autolytic capacity simply reflects an imbalance between the rates of murein synthesis and degradation. It would be worthwhile to investigate in more detail the activity of different autolytic enzymes and the mechanism of their triggering by beta-lactams (9) during shape transition. In fact, murein hydrolases have been proposed to play a role in cell elongation and septation (14), and enzymes acting on murein have been shown to increase in activity around the time of septum formation (1, 3). It is possible that during sphere transition regulation of the hydrolases differs from that of a normally rod-shaped cell.

Another interesting feature of the rod-to-sphere transition is represented by the peculiar changes observed in the organization of the outer layers of the envelope. The pattern of

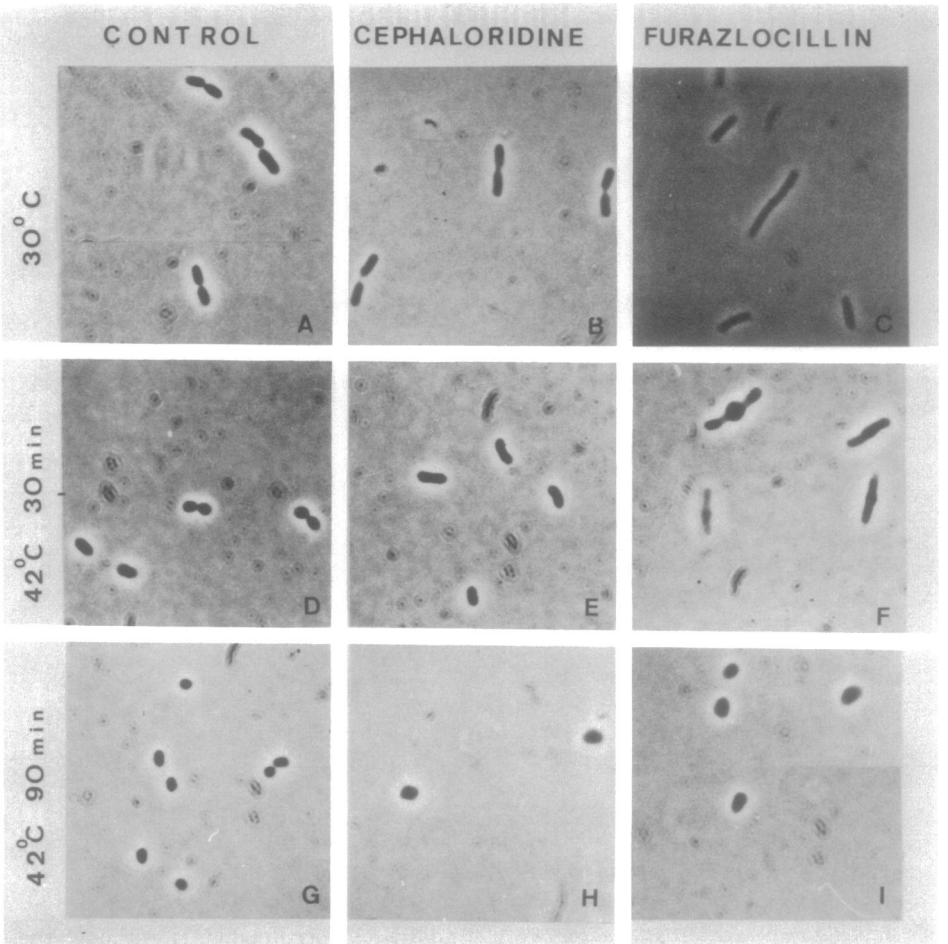

FIG. 5. *Phase-contrast photomicrographs of strain SP45 at 30°C (A) and after 30 min and 90 min of incubation (D and G respectively) at 42°C. The effects of a 60-min treatment with cephaloridine before and after shift-up are shown in (B), (E), and (H). The morphological effects of furazlocillin (1 μg/ml) added to the cultures growing at 30°C and at 30 or 90 min after the shift to the nonpermissive temperature are shown in (C), (F), and (I).*

detachment of the outer membrane from the underlying peptidoglycan might well reflect a deep disarray in the relative rates of synthesis of the two structures or an alteration in the function of insertion of the lipoprotein, considered to be important in the anchorage of the outer membrane to the peptidoglycan layer.

Availability of antibiotics with exclusive affinity for one PBP in intact growing cells is extremely useful for understanding the role of each PBP in the morphogenetic events. The necessity to correlate the effects induced by a beta-lactam antibiotic with its binding properties in vivo has been generally overlooked in studies on murein synthesis. Moreover, binding studies have been generally performed using isolated envelopes, an experimental situation very likely to differ from what occurs in vivo.

The first point of interest in the results reported here is the reduced MIC of the antibiotics binding to PBP-1A for strain SP45 when grown at 42°C. The reduction in the MIC does not correlate with a change in the affinity of the antibiotics for their target. This increase in susceptibility has not been detected for the antibiotics binding to PBP-3.

As reported, treatment with cephaloridine induces cell lysis before any effect on shape is

evident (9, 17). Our results indicate that in the presence of a reduced amount of PBP-2 this effect can be obtained with concentrations which would not be effective if the strain were grown at conditions permissive for PBP-2 synthesis. The massive lysis occurring during the simultaneous inhibition of PBP-1A and -2 in the absence of a comparable effect on murein synthesis inhibition suggests that PBP-1A is involved in the final stages of murein synthesis in some important yet subtle way. Murein, although not grossly altered, is more prone to digestion by autolytic attack. An explanation of these effects is presently very difficult. Mutants lacking PBP-1A did not show any significant alteration in murein structure, but mutants lacking PBP-1Bs and -1A are not viable (20–22). One mutant lacking PBP-1A and -2 was found to grow very poorly (19; G. A. Botta and P. Canepari, unpublished data).

Inhibition of PBP-3, which has been demonstrated to be involved in septation (2, 15, 20), clearly shows that PBP-2 is involved in the initiation of cell elongation. In the presence of a reduced amount of PBP-2 (30 min after the temperature shift), cells maintain the ability, albeit reduced, to elongate. Complete absence of PBP-2 and simultaneous inhibition of septation cause the formation of giant round cells.

The greater inhibition of murein synthesis obtained with furazlocillin or piperacillin at 42°C can be explained on the basis of the recently proposed model for regulation of cell shape in *E. coli* (12). It has been suggested that in rod cells two sites for murein synethesis exist: one responsible for septal murein synthesis and the other responsible for lateral wall formation. Cocci would result from a prevalent activity of the site for septation over the site for elongation. One of the components of the site for septal murein synthesis is PBP-3, and we proved that binding to this protein in filamentous cells does not inhibit murein synthesis, whereas inhibition is maximal at the time of synchronous septation (2). These results, taken together, strongly suggest that during rod-to-sphere transition septal murein synthesis becomes prevalent and the inhibition obtained by PBP-3 inhibition becomes greater.

The approach used here, simultaneous inhibition of different PBPs by combining mutational loss with antibiotic treatment, could represent a model system to test the efficiency of beta-lactam combinations for clinical use.

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