JOURNAL OF BACTERIOLOGY, Oct. 1990, p. 5863–5870
0021-9193/90/105863-08$02.00/0

Vol. 172, No. 10

Differential Effect of Mutational Impairment of Penicillin-Binding Proteins 1A and 1B on *Escherichia coli* Strains Harboring Thermosensitive Mutations in the Cell Division Genes *ftsA*, *ftsQ*, *ftsZ*, and *pbpB*

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Received 28 February 1990/Accepted 23 July 1990

To study the functional differences between penicillin-binding proteins (PBPs) 1A and 1B, as well as their recently postulated involvement in the septation process (F. García del Portillo, M. A. de Pedro, D. Joseleau-Petit, and R. D'Ari, J. Bacteriol. 171:4217–4221, 1989), a series of isogenic strains with mutations in the genes coding for PBP 1A (*ponA*) or PBP 1B (*ponB*) or in the cell division-specific genes *ftsA*, *ftsQ*, *pbpB*, and *ftsZ* was constructed and used as the start point to produce double mutants combining the *ponA* or *ponB* characters with mutations in cell division genes. PBP 1A seemed to be unable to preserve cell integrity by itself, requiring the additional activities of PBP 2, PBP 3, and FtsQ. PBP 1B was apparently endowed with a more versatile biosynthetic potential that permitted a substantial enlargement of PBP 1A-deficient cells when PBP 2 or 3 was inhibited or when FtsQ was inactive. β-Lactams binding to PBP 2 (mecillinam) or 3 (furazlocillin) caused rapid lysis in a *ponB* background. The lytic effect of furazlocillin to *ponB* cell division double mutants was suppressed at the restrictive temperature irrespective of the identity of the mutated cell division gene. These results indicate that PBPs 1A and 1B play distinct roles in cell wall synthesis and support the idea of a relevant involvement of PBP 1B in peptidoglycan synthesis at the time of septation.

Growth of the bacillar eubacterium *Escherichia coli* is characterized by the alternation of elongation and division periods. Synthesis of the transverse septum is subjected to complex regulatory mechanisms ensuring proper timing and location at the center of the cell and is followed by the actual separation of the daughter cells. The covalently closed nature of the peptidoglycan layer (sacculus) of the cell wall makes growth and division of the cell depend strictly on the metabolism of this particular component (9, 17).

The final steps in the synthesis of the sacculus are mediated by penicillin-binding proteins (PBPs), a set of membrane bound proteins characterized by their ability to interact with β-lactam antibiotics specifically at the active center forming covalent, enzymatically inactive complexes (9, 20, 29).

In *E. coli* seven genetically independent PBPs have been identified (18–22). These proteins, highly redundant enzymatically, are supposed to play specific functions in peptidoglycan metabolism and therefore in cell growth. According to the functional scheme generally accepted, in exponentially growing cells, PBPs 1A and 1B (PBPs 1) insert new precursors necessary for cell wall elongation; PBP 2 is involved in maintenance of cell shape and initiation of cell wall growth; PBP 3 mediates septum formation at cell division; PBP 4 participates in postinsertional modifications of the sacculus; and PBPs 5 and 6 regulate the proportion of pentapeptide side chains (9, 20, 22). However, there is still considerable incertainty about the precise physiological role of the individual PBPs with the exception of PBP 3, whose specific involvement in septation seems firmly established (3, 11, 18, 20–22, 24).

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In this context, growing evidence indicates that physiology of PBPs 1 might be more complex than suspected. Although PBPs 1 were initially considered equivalent proteins, later evidence indicates that they might play specific functions in peptidoglycan biosynthesis (12, 16, 18, 20, 23, 31). Furthermore, the activity of PBPs 1 might be essential for survival only at a defined stage of the cell cycle coincident with the septation period (5, 7, 8). This statement is based on the observation that, at least under certain conditions, PBP 2 seems able to synthesize by itself the peptidoglycan necessary for cell enlargement throughout the elongation period, up to a point when a septation related event makes the activity of PBPs 1 indispensable for normal division (7).

The hypothesis of a period of action for PBPs 1 coordinated with the septation process immediately suggests the possibility of interactions between PBPs 1 and the products of cell division genes. Furthermore, it questions whether both PBPs, 1A and 1B, are involved in the presumptive interactions with cell division gene products or whether their activities are subjected to independent regulatory pathways.

A possible way to learn more about these questions is by investigation of the phenotypic alterations induced in strains deficient in PBPs 1 as a result of mutations affecting cell division genes. With this idea in mind, we have obtained families of double mutants with deletions either in of the *ponA* (*mrcA*) and *ponB* (*mcrB*) genes, coding for PBP 1A and 1B, respectively (22, 31), and a thermosensitive mutation in one of the cell division-specific genes *ftsA*, *ftsQ*, *ftsZ*, and *pbpB* (1, 2, 6, 24, 26). The results of our study indicate that the two PBPs 1 might differ substantially in their functionality and favor the idea of an involvement of PBPs 1 in septation.

TABLE 1. Genotypic properties of *E. coli* K-12 strains

Strain	Genotype	Source or reference
MC6RP1	F⁻ *thrA leuA proA dra drm lysA*	15[a]
SP1026	F⁻ *his lac tsx supF rpsL ponB*::Spc^r	31
SP1027	F⁻ *his lac tsx supF rpsL ponA*::Km^r	31
D3	F⁻ *his ilv trp*(Am) *thyA ara*(Am) *galU42 galE lac*-125(Am) *tsx*(Am) *tyrT* [*supFA81*(Ts)] *ftsA3*(Ts)	26
TOE1	F⁻ *thyA leu proA his thi argE lacY galK xyl mtl ara tsx rpsL supE ftsQ*1(Ts)	2
TOE0	TOE1 *leu*$^+$	This work[b]
AX655	F⁻ *thr leu thi arg proA his pbpB2158*(Ts)	28
AX6550	AX655 *leu*$^+$	This work[b]
LMC509	F⁻ *araD139* (*argF-lac*)*U169 rpsL150 flbB5301 ptsF25 deoC1 rbsR relA1 lysA1 ftsZ84*(Ts)	24
QCA2	MC6RP1 *ponA*::Km^r	P1(SP1027) × MC6RP1
QCB1	MC6RP1 *ponB*::Spc^r	P1(SP1026) × MC6RP1
RP11	MC6RP1 *leu*$^+$ *ftsA3*(Ts)	P1(D3) × MC6RP1
RP31	MC6RP1 *leu*$^+$ *ftsQ*(Ts)	P1(TOE0) × MC6RP1
RP41	MC6RP1 *leu*$^+$ *pbpB2158*(Ts)	P1(AX6550) × MC6RP1
RP61	MC6RP1 *leu*$^+$ *ftsZ84*(Ts)	P1(LMC509) × MC6RP1
ARP110	RP11 *ponA*::Km^r	P1(SP1027) × RP11
ARP310	RP31 *ponA*::Km^r	P1(SP1027) × RP31
ARP410	RP41 *ponA*::Km^r	P1(SP1027) × RP41
ARP610	RP61 *ponA*::Km^r	P1(SP1027) × RP61
BRP110	RP11 *ponB*::Spc^r	P1(SP1026) × RP11
BRP310	RP31 *ponB*::Spc^r	P1(SP1026) × RP31
BRP410	RP41 *ponB*::Spc^r	P1(SP1026) × RP41
BRP610	RP61 *ponB*::Spc^r	P1(SP1026) × RP61

[a] Intermediate strain in the construction of MC6RP3.
[b] Spontaneous *leu*$^+$ revertant.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used are listed in Table 1. Cells were routinely grown in LB broth (13) supplemented with thymine (50 mg/liter) at 30°C under vigorous aeration. Strains harboring resistance determinants were grown in the presence of kanamycin (30 μg/ml) or spectinomycin (40 μg/ml). Growth was monitored by periodically measuring the optical density at 550 nm (OD_{550}) of the cultures. Particle number was measured in a Coulter Counter ZM electronic particle analyzer (Coulter Electronics, Luton, United Kingdom).

Antibiotics. Ampicillin, mecillinam, piperacillin, azthreonam, and furazlocillin were generous gifts from Laboratorios Abelló S.A. (Madrid, Spain), Leo Pharmaceutical Products (Ballerup, Denmark), Lederle Piperacillin, Inc. (Carolina, Puerto Rico), E. R. Squibb & Sons, Inc. (Princeton, N.J.), and Bayer (Wuppertal, Federal Republic of Germany), respectively.

Transduction of genetic characters. Bacteriophage P1 *virA*-mediated transduction was performed as described previously (14). Given the thermosensitive nature of the genetic characters transduced, incubations were performed at 30°C. *leuA*$^+$ transductants were selected on mineral-citrate agar plates (27) supplemented according to the nutritional requirements of the strain in use. Thermosensitivity was tested on LB agar plates containing 0.1% (wt/vol) NaCl.

Microscopic observation. Cells were fixed in 0.2% (vol/vol) Formalin–0.9% (wt/vol) NaCl, layered on top of agar-covered slides, and observed, or photographed, in a Nikon type 104 microscope (Nikon, Nippon Kogaku, Tokyo, Japan) with phase-contrast optics.

Treatment of exponentially growing cells with β-lactam antibiotics. Cultures of the strains to test were kept in exponential growth for at least 10 generations at 30°C. At an OD_{550} of 0.2, the cultures were divided into a number of subcultures. Half of them were transferred to 42°C, and 5 min later β-lactams were added to pairs (30 and 42°C) of subcultures. Samples were periodically withdrawn to monitor the effects of the treatments. As a positive control for the induction of cell lysis in the different experiments, a subculture of each series was treated with ampicillin at 100 μg/ml.

RESULTS

Effects of β-lactams on growth and division of isogenic single mutants affected in genes coding for PBPs 1 or for cell division-specific proteins. A series of isogenic strains harboring mutations in genes *ponA* (coding for PBP 1A), *ponB* (coding for PBP 1B), *ftsA*, *ftsQ*, *ftsZ* (*sulB*, *sfiB*), and *pbpB* (coding for PBP 3) was constructed by transferring the mutated alleles from pertinent strains into *E. coli* MC6RP1 by phage P1-mediated transduction (Table 1).

The response of the mutants against β-lactams preferentially binding to PBPs 2 (mecillinam, 10 μg/ml) and 3 (furazlocillin, 1 μg/ml) was studied to find out whether the indicated mutations could alter the response of wild-type cells against the indicated β-lactams characteristically leading to spherical morphology and filamentation, respectively. The specificity of binding of the β-lactams was confirmed by means of competition experiments performed as described by Spratt (18, 19). At the concentrations used, mecillinam and furazlocillin bound preferentially to PBPs 2 and 3, respectively (data not shown). Because of the thermosensitive nature of most mutants, all observations were systematically performed at 30 and 42°C.

The effects of β-lactams on the growth and morphology of strains QCA2 (*ponA*) and QCB1 (*ponB*) are illustrated in Fig. 1. Whereas the *ponA* strain QCA2 behaved exactly as the wild-type MC6RP1 in all instances, impairment of PBP 1B provoked a radical change in the reaction of QCB1 cells against mecillinam and furazlocillin. Neither mecillinam nor furazlocillin was lytic to the wild-type strain, but both were so to QCB1 cells, which lysed promptly after addition of

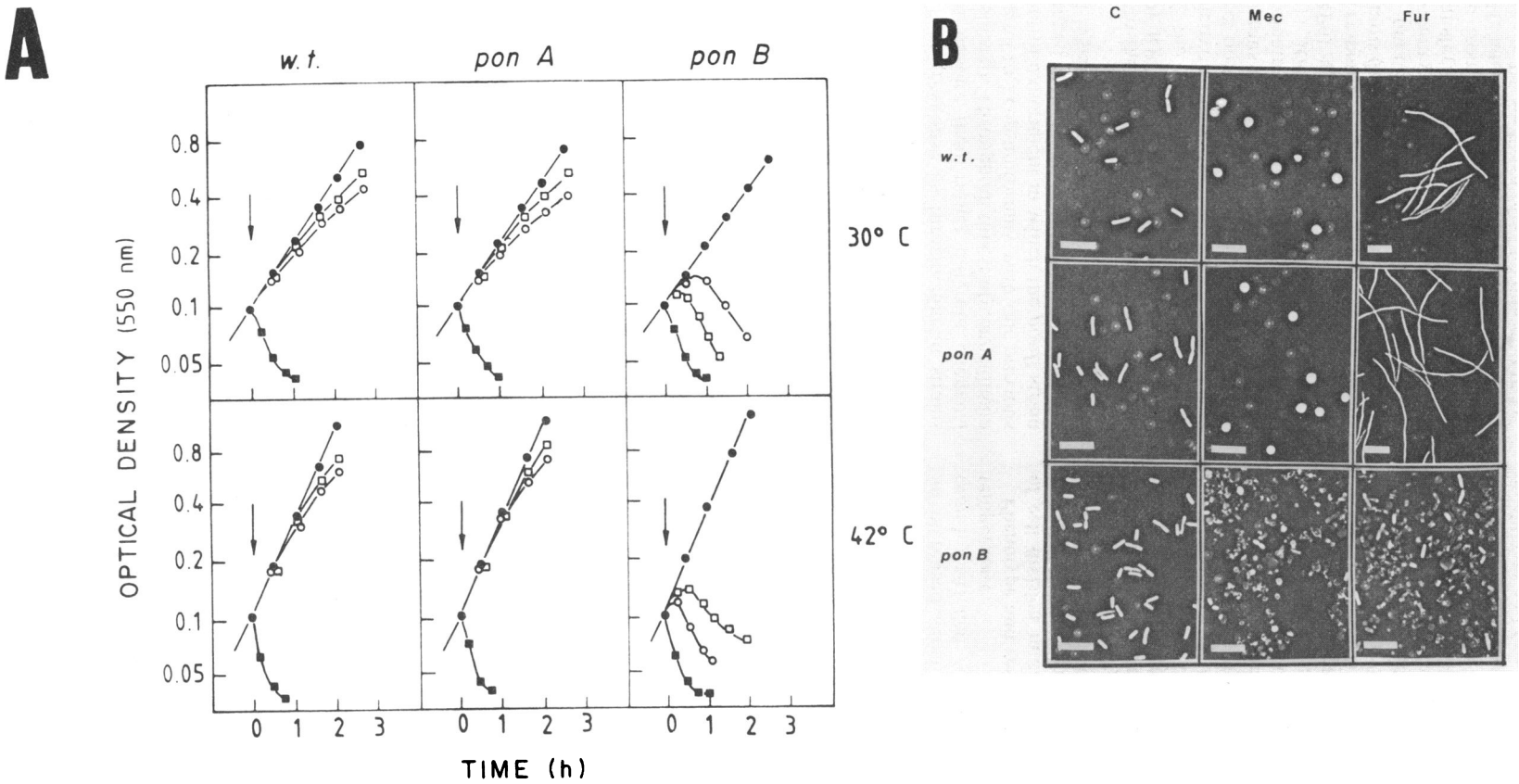


FIG. 1. Effects of β-lactams on growth and morphology of *ponA* and *ponB* mutants. (A) Cultures of *E. coli* MC6RP1 (wild type [w.t.]), QCA2 (*ponA*), and QCB1 (*ponB*) growing exponentially at 30 and 42°C were divided into a number of subcultures at an OD_{550} of 0.1, which were treated with different β-lactams (arrows) and monitored by periodically measuring OD_{550}. Symbols: (●) untreated control; (○) mecillinam (10 μg/ml); (□) furazlocillin (1 μg/ml); (■) ampicillin (100 μg/ml). (B) At 90 min after addition of β-lactams, samples withdrawn from the subcultures growing at 30°C were fixed in 0.4% Formalin–0.9% NaCl, spread on agar-covered slides, and observed by phase-contrast microscopy. C, Untreated subcultures; Mec, mecillinam-treated subcultures; Fur, furazlocillin-treated subcultures. Bars, 10 μm.

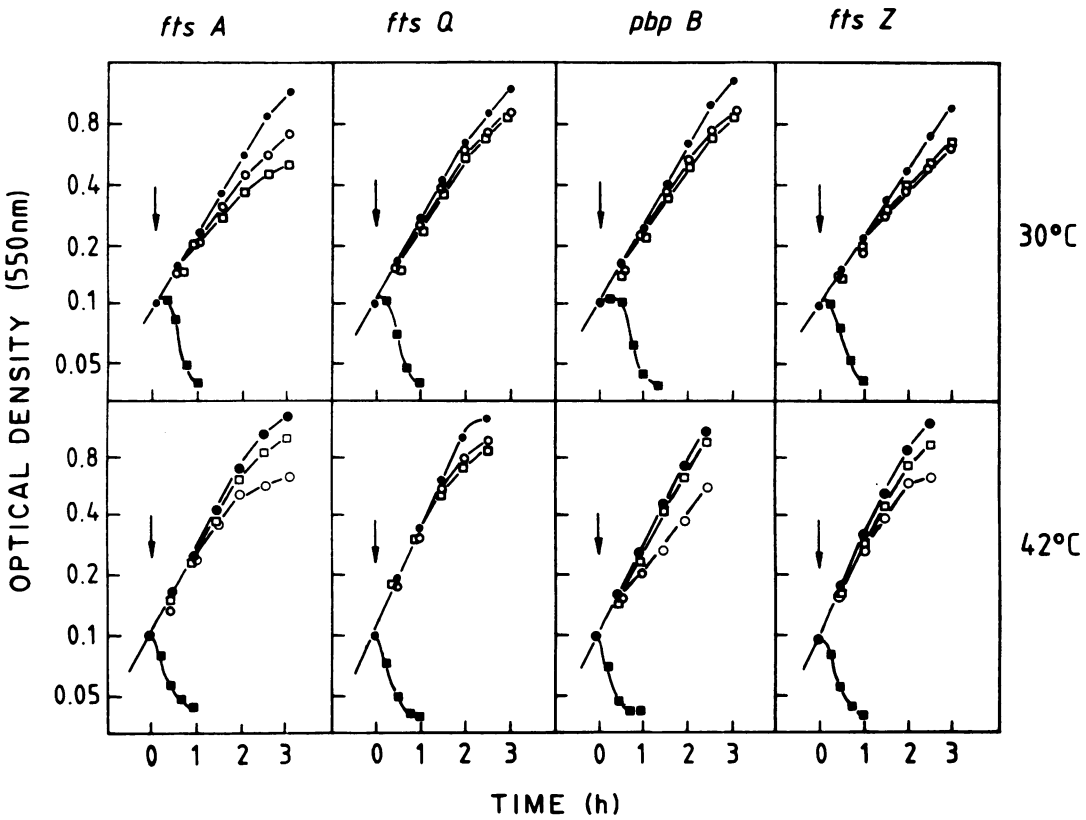


FIG. 2. Effects of β-lactams on the growth of isogenic cell division mutants. Cultures of *E. coli* RP11 (*ftsA*), RP31 (*ftsQ*), RP41 (*pbpB*), and RP61 (*ftsZ*) growing exponentially at 30°C were divided into two series of subcultures at an OD_{550} of 0.1. One series was transferred to a 42°C water bath, and 5 min later parallel subcultures of both series were treated with different β-lactams (arrows) and monitored by periodically measuring OD_{550}. Symbols: (●) untreated control; (○) mecillinam (10 μg/ml); (□) furazlocillin (1 μg/ml); (■) ampicillin (100 μg/ml).

either drug. Piperacillin (6 μg/ml) and azthreonam (0.5 μg/ml), two other β-lactams with high affinity to PBP 3 (4, 10), elicited the same response as furazlocillin in strains QCA2 and QCB1 despite their rather different chemical natures (data not shown). Throughout this study, mecillinam was used at 10 μg/ml to keep uniformity with our previous work (5, 7). A control experiment in which the *ponB* strain QCB1 was challenged with mecillinam at lower concentrations showed that lysis was readily induced at a concentration of 0.1 μg/ml.

The responses to the selected β-lactams of strains RP11 (*ftsA*), RP31 (*ftsQ*), RP41 (*pbpB*), and RP61 (*ftsZ*) are shown in Fig. 2. At 30°C, the mutant strains behaved like the wild type. At the restrictive temperature (42°C), furazlocillin had no noticeable effect on the formation of filaments by the *fts* mutants. Simultaneous inhibition of PBP 2 and cell division resulted in the formation of partially constricted filaments with bulges in RP11, RP31, and RP41 or oblated ellipsoids in the *ftsZ* mutant RP61, as expected (1).

Phenotypes and responses to β-lactams of an isogenic family of cell division mutants defective for PBP 1A. A set of isogenic strains harboring mutations in gene *ponA* (PBP 1A) and in one of the cell division genes *ftsA*, *ftsQ*, *pbpB*, and *ftsZ* was constructed by transferring the thermosensitive allele of the cell division gene from the corresponding single mutant, RP11, RP31, RP41, and RP61, respectively, into the *ponA* strain QCA2 by phage P1-mediated transduction (Table 1).

The phenotypes and responses against β-lactams of the double mutants (Fig. 3) were identical to those of the corresponding single cell division mutants (Fig. 2) except for an increased sensitivity to mecillinam in strains ARP110 (*ponA ftsA*) and ARP410 (*ponA pbpB*) at the restrictive temperature.

Phenotype and response to β-lactams of an isogenic family of cell division mutants defective for PBP 1B. A series of isogenic strains with mutations in *ponB* and in one of the cell division genes under study was constructed as described above but using the *ponB* strain QCB1 as the recipient in the transduction experiments (Table 1).

The four different *ponB* cell division mutants, strains BRP110 (*ponB ftsA*), BRP310 (*ponB ftsQ*), BRP410 (*ponB pbpB*), and BRP610 (*ponB ftsZ*), grew well at 30°C with a normal bacillar morphology. At 42°C, the mutants in *ftsA*, *pbpB*, and *ftsZ* behaved like the single mutants affected in the corresponding cell division gene, producing very long unseptated filaments. However, the *ftsQ* strain BRP310 had only a reduced capacity of growth at 42°C. After cell mass increased two- to threefold, lysis was observed (Fig. 4). The behavior of strain BRP410 (*ponB pbpB*) was unexpected. This strain was predicted to lyse at 42°C on the basis of the lytic response of the *ponB* single mutant QCB1 against the PBP 3-specific inhibitor furazlocillin (Fig. 1); however, it was in fact able to elongate as much as RP41 (*pbpB*) at 42°C.

Most interesting was the reaction of the double mutants to mecillinam and furazlocillin. At the permissive temperature (30°C), the effect of the β-lactams on the double mutants was

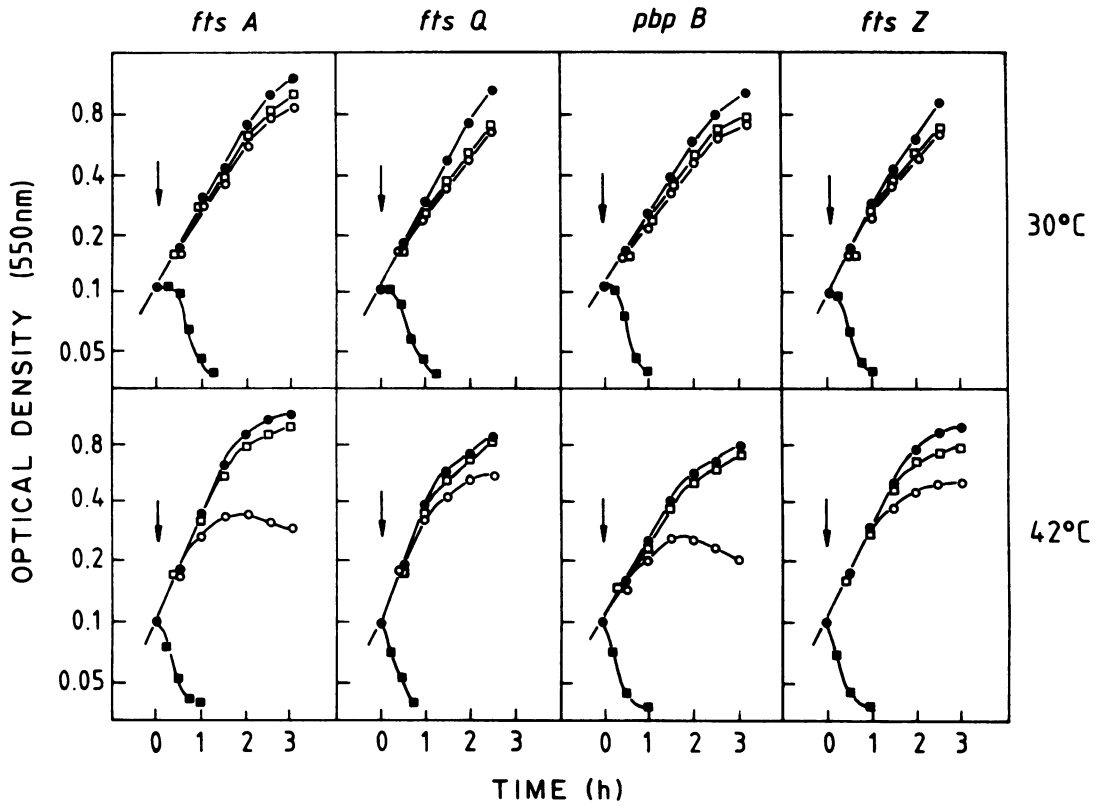


FIG. 3. Effects of β-lactams on the growth of isogenic *ponA* cell division mutants. Cultures of *E. coli* ARP110 (*ponA ftsA* [*ftsA*]), ARP310 (*ponA ftsQ* [*ftsQ*]), ARP410 (*ponA pbpB* [*pbpB*]), and ARP610 (*ponA ftsZ* [*ftsZ*]) growing exponentially at 30°C were divided into two series of subcultures at an OD_{550} of 0.1. One series was transferred to a 42°C water bath, and 5 min later parallel subcultures of both series were treated with different β-lactams (arrows) and monitored by periodically measuring OD_{550}. Symbols: (●) untreated control subcultures; (○) mecillinam (10 μg/ml); (□) furazlocillin (1 μg/ml); (■) ampicillin (100 μg/ml).

similar to that on QCB1 (*ponB*) (compare Fig. 4 and 1). Although OD measurements suggested that furazlocillin had very poor, if any, lytic action against strain BRP310 (*ftsQ ponB*), microscopical observation clearly revealed rather extensive cell lysis in the corresponding cultures (Fig. 4).

At the restrictive temperature (42°C), mecillinam was lytic to all four *ponB* cell division double mutants, as it was to QCB1 (Fig. 1). However, furazlocillin was unable to trigger lysis in any of the double mutants, in contrast to its lytic action against QCB1 (*ponB*) and the double mutants at 30°C (Fig. 4). Microscopic observation of the β-lactam-treated cultures confirmed that furazlocillin had no discernible effects either in the morphology or in the integrity of the filaments formed when the double mutants were grown at 42°C (Fig. 4B). These results indicate that blockade of septation by mutation of any of the cell division genes tested suppresses the lytic effect of furazlocillin in *ponB* strains.

DISCUSSION

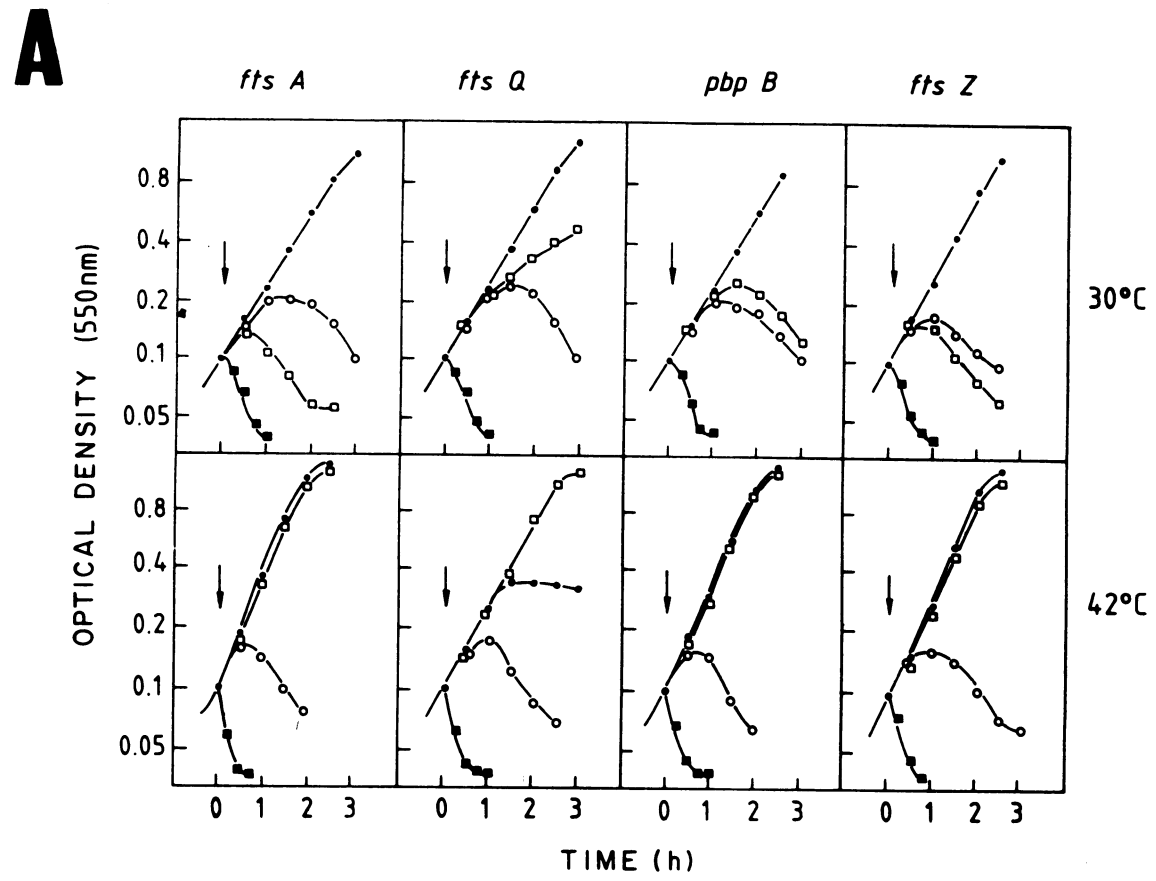
Earlier investigations of the characteristics of the bacteriolytic response of *E. coli* cells to β-lactams specifically binding to PBPs 1 led us to consider the likelihood of a regulatory relationship between the activity of PBPs 1 and the initiation of septation. The main reason was the observation that under a number of different growth conditions, lysis subsequent to inhibition of PBPs 1 would happen at a time coincident with septation but independently of the actual formation of the septum (5, 7, 8).

To further explore this hypothesis, we investigated the phenotypic properties of series of isogenic strains with deletions in genes *ponA* or *ponB*, coding for PBPs 1A and 1B, respectively, alone and in combination with thermosensitive mutations in *ftsA*, *ftsQ*, *ftsZ*, and *pbpB*, the best-characterized cell division genes (1, 6, 24).

Analysis of the single mutants QCA2 and QCB1 (*ponA* and *ponB*, respectively) revealed drastic differences in their responses to inhibitors of PBP 2 or PBP 3, in congruence with the results of a similar study reported some years ago by Schmidt et al. (16). β-Lactams binding specifically to these proteins induced similar morphological alterations in QCA2 (*ponA*) and MC6RP1 (wild type) but were lytic to QCB1 (*ponB*) cells.

The morphology and growth of the *ponA fts* double mutant strains at 30 and 42°C, as well as their responses to β-lactams at both temperatures, were identical to those of the corresponding cell division single mutants except for the increased sensitivity to mecillinam of ARP110 (*ponA ftsA*) and ARP410 (*ponA pbpB*) at 42°C. The similarity in behavior of the *pbpB* and *ftsA* double mutants might reflect the proposed direct regulatory action of FtsA protein on PBP 3 (25). These results suggest that lack of functional PBP 1A had no serious effects on cell division mutants except when PBPs 2 and 3 were simultaneously blocked, a situation that apparently made the cell more susceptible to further alterations to the peptidoglycan-synthesizing enzymes.

Investigation of the *ponB fts* double mutants revealed


A
fts A
fts Q
pbp B
fts Z
OPTICAL DENSITY (550nm)
0.8
0.4
0.2
0.1
0.05
0 1 2 3
TIME (h)
30°C
42°C

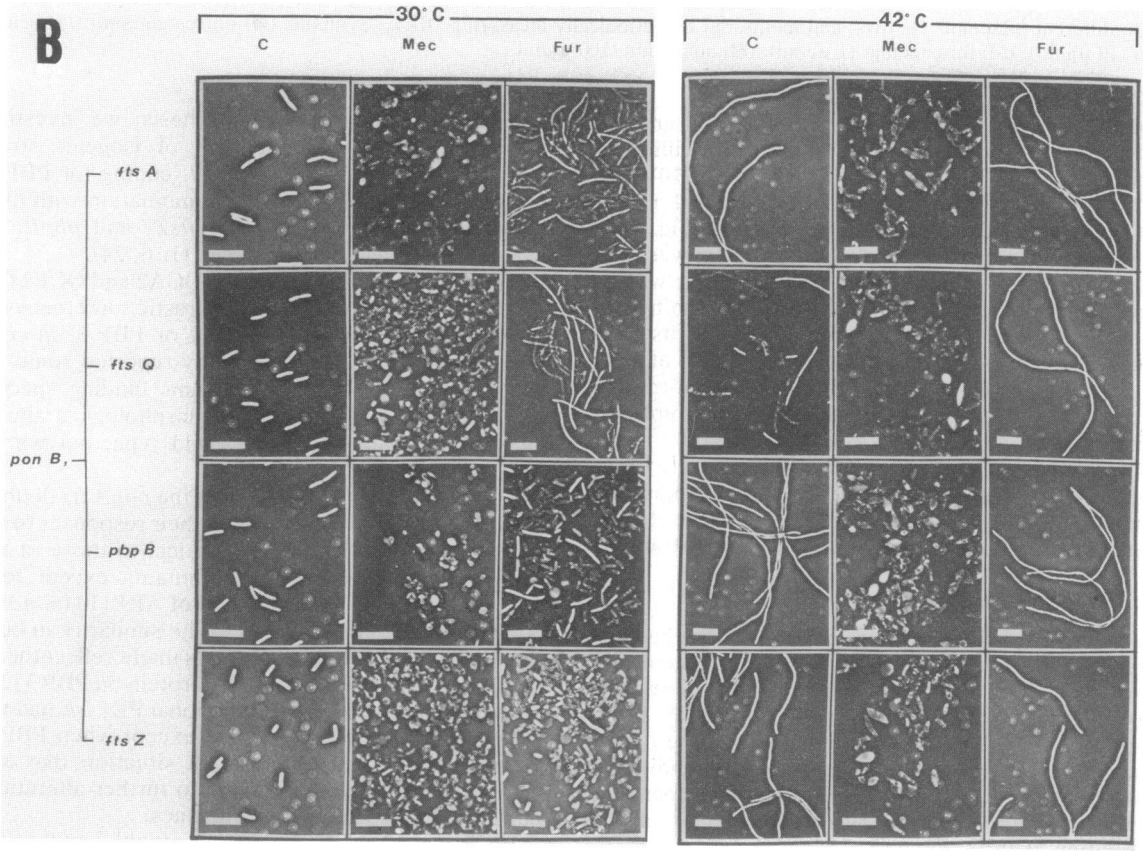

B
30° C
42° C
C
Mec
Fur
fts A
fts Q
pon B,
pbp B
fts Z

FIG. 4. Effects of β-lactams on growth and morphology of isogenic *ponB* cell division mutants. (A) Cultures of *E. coli* BRP110 (*ponB ftsA* [*ftsA*]), BRP310 (*ponB ftsQ* [*ftsQ*]), BRP410 (*ponB pbpB* [*pbpB*]), and BRP610 (*ponB ftsZ* [*ftsZ*]) growing exponentially at 30°C were divided into two series of subcultures at an OD_{550} of 0.1. One series was transferred to a 42°C water bath, and 5 min later parallel subcultures of both series were treated with different β-lactams (arrows) and monitored by periodically measuring OD_{550}. Symbols: (●) untreated control subcultures; (○) mecillinam (10 μg/ml); (□) furazlocillin (1 μg/ml); (■) ampicillin (100 μg/ml). (B) After addition of antibiotics, samples were withdrawn at 90 min (control [C] 30°C, mecillinam [Mec], 30 and 42°C, furazlocillin [Fur] 30°C) or 180 min (control 42°C, furazlocillin 42°C), fixed in 0.4% Formalin–0.9% NaCl, spread on agar-covered slides, and observed by phase-contrast microscopy. Bars, 10 μm.

quite unexpected phenotypic characteristics at the restrictive temperature (42°C), although at 30°C all of them behaved like the parental *ponB* single mutant QCB1. The first surprising aspect was the ability of BRP410 (*ponB pbpB*) to form long filaments when incubated at 42°C, whereas according to the observed effects of furazlocillin on QCB1 (*ponB*) and in BRP410 itself at 30°C, it was expected to lyse. This apparent discrepancy suggested that the outcome of inhibiting the septum-synthesizing activity of PBP 3 might be strongly dependent on the nature of the inhibitory action. For instance, mutational inhibition could prevent activation of the protein at the time of septation (3), bringing an extended elongation period, whereas a β-lactam would inhibit an already activated protein, disturbing the interplay between synthetic and hydrolytic activities required for cell wall growth leading to cell lysis.

The growth potential of BRP310 (*ponB ftsQ*) at 42°C was apparently limited. After increasing in mass two- to threefold, a relatively slow lytic process took place. However, the single *ftsQ* mutant did not show any particular tendency to lyse. This finding suggests that impairment of *ftsQ* function puts some extra demand on the peptidoglycan biosynthetic machinery that can be accomplished by PBP 1B but not PBP 1A.

The most intriguing observation was the suppression of the lytic action of furazlocillin in all four *ponB* cell division double mutants at the restrictive temperature. As expected from its action on QCB1, furazlocillin was lytic to the four strains at 30°C; however, at 42°C the β-lactam lost its lytic effect, and cells generated long filaments, indicating that under these conditions PBPs 1A and 2 suffice to permit continuous elongation of the cell. The behavior of BRP310, the *ponB ftsQ* double mutant, was particularly striking; addition of furazlocillin at 42°C prevented the lytic process from taking place at the restrictive temperature as discussed above.

In theory, the lytic effect of furazlocillin on *ponB* strains could be triggered by a slight inhibitory action of the β-lactam on PBP 1A, undetectable in competition assays. However, suppression of lysis at 42°C in *ponB fts* double mutants strongly argues against this hypothesis. In fact, simultaneous inhibition of the two PBPs 1 in *fts* mutants leads to cells lysis irrespective of growth temperature (8).

The behavior of the *ponB fts* double mutants could be explained by assuming that activation of PBP 3 at the time of septation generates a change in peptidoglycan biosynthesis that can be withstood or bypassed by PBP 1B, but not PBP 1A, in conjunction with PBP 2 if PBP 3 is inhibited by a β-lactam before termination of the septum. Blockade of septation in cell division mutants at the restrictive temperature would prevent activation of PBP 3 and therefore would allow indefinite enlargement of the cell by the joint action of PBPs 1A and 2 in the presence of furazlocillin, which would be ineffective because its target enzyme would remain physiologically inactive. The behavior of BRP410 (*ponB pbpB*) could also be explained in this context by assuming that the thermosensitive mutation in *pbpB* yields a form of PBP 3 that cannot be activated at 42°C.

The observations discussed above indicate that in the absence of a functional PBP 1B, PBP 1A is unable to preserve cell integrity by itself, requiring the additional activities of PBPs 2 and 3 to avoid cell lysis. In contrast, PBP 1B is apparently endowed with a higher or more versatile biosynthetic potential, permitting a substantial enlargement of PBP 1A-deficient cells even when PBP 2 or 3 is inhibited. However, simultaneous inhibition of PBPs 2 and 3 in ARP410 (*ponA pbpB*) treated with mecillinam allowed only a moderate cell enlargement (two- to threefold increase in mass), indicating that PBP 1B also requires the concerted action of PBPs 2 and 3 for prolonged peptidoglycan synthesis. Of course, for normal division, the activity of both PBPs 2 and 3 is required.

From our perspective, these results indicate that the two PBPs 1 play distinct roles in cell wall synthesis and support the idea of a relevant involvement of PBP 1B in peptidoglycan synthesis at the time of septation, probably as a PBP 3 helper protein, a concept recently put forward by Wientjes and Nanninga (30).

ACKNOWLEDGMENTS

We thank B. G. Spratt and M. Vicente for sending us bacterial strains. The excellent technical assistance of J. de la Rosa is gratefully acknowledged.

F.G.P. was supported by a fellowship from Fondo de Investigaciones Sanitarias. This work was supported by grant BIO88-0251-C03-03 of the C.I.C.Y.T. Plan Nacional de Biotecnología and an institutional grant from Fundación Ramón Areces.

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