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Penicillin-Binding Protein 2 Inactivation in *Escherichia coli* Results in Cell Division Inhibition, Which Is Relieved by FtsZ Overexpression

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Aminoacyl-tRNA synthetase mutants of *Escherichia coli* are resistant to amdinocillin (mecillinam), a β-lactam antibiotic which specifically binds penicillin-binding protein 2 (PBP2) and prevents cell wall elongation with concomitant cell death. The *leuS*(Ts) strain, in which leucyl-tRNA synthetase is temperature sensitive, was resistant to amdinocillin at 37°C because of an increased guanosine 5′-diphosphate 3′-diphosphate (ppGpp) pool resulting from partial induction of the stringent response, but it was sensitive to amdinocillin at 25°C. We constructed a *leuS*(Ts) Δ(*rodA-pbpA*)::Km^r strain, in which the PBP2 structural gene is deleted. This strain grew as spherical cells at 37°C but was not viable at 25°C. After a shift from 37 to 25°C, the ppGpp pool decreased and cell division was inhibited; the cells slowly carried out a single division, increased considerably in volume, and gradually lost viability. The cell division inhibition was reversible when the ppGpp pool increased at high temperature, but reversion required de novo protein synthesis, possibly of septation proteins. The multicopy plasmid pZAQ, overproducing the septation proteins FtsZ, FtsA, and FtsQ, conferred amdinocillin resistance on a wild-type strain and suppressed the cell division inhibition in the *leuS*(Ts) Δ(*rodA-pbpA*)::Km^r strain at 25°C. The plasmid pAQ, in which the *ftsZ* gene is inactivated, did not confer amdinocillin resistance. These results lead us to hypothesize that the nucleotide ppGpp activates *ftsZ* expression and thus couples cell division to protein synthesis.

The enterobacterium *Escherichia coli* owes the rigidity of its rod shape to its peptidoglycan layer, a single macromolecule surrounding the cytoplasmic membrane. The final steps of peptidoglycan synthesis are catalyzed by penicillin-binding proteins (PBPs), enzymes which are located in the cytoplasmic membrane and covalently bind penicillin and its derivatives (35, 59). Peptidoglycan synthesis is necessary not only for the maintenance of cell morphology but also for survival; in the presence of penicillin, cells lyse (20) because they cannot synthesize peptidoglycan (35, 59). Some PBPs seem redundant, whereas others are essential. In particular, PBP3 has been shown to be essential for septation (43). PBP2, coded for by the *pbpA* gene, is required for lateral elongation and for the maintenance of rod shape (43). The RodA protein, coded for by the *rodA* gene in the same transcriptional unit as *pbpA* (32), is required for full PBP2 activity (22). The study of PBP2 function has been facilitated by the availability of a particular penicillin derivative, amdinocillin (mecillinam) (27), which binds specifically to PBP2 (44, 45). In the presence of this antibiotic, cells lose their rod shape, become spherical, and fail to form colonies (24, 31). *E. coli* can become spherical without loss of viability; this occurs in *lpp ompA* double mutants (42), in some *pbpA* mutants (36), and when PBP5 is overexpressed (29). Thus, it is surprising that a wild-type strain cannot form colonies on rich media containing amdinocillin. Since lateral elongation does not seem to be essential, we concluded that PBP2 inactivation resulted in the failure of some vital cellular process (7, 36).

Genetic studies of PBP2 function have been greatly facilitated by the analysis of amdinocillin-resistant mutants. A first class of mutants are spherical even in the absence of amdinocillin and have mutations located at 71 min, in the *mre* locus (53–55). A second class of amdinocillin-resistant mutants maintain their rod shape in the absence of amdinocillin and grow as spherical cells in the presence of the antibiotic. It has been reported that *cya* and *crp* mutants, lacking a functional cyclic AMP-catabolite gene activator protein complex, have this phenotype (3, 23); the amdinocillin resistance of these mutants, however, seems to depend on the genetic background (reference 11 and unpublished results). Aminoacyl-tRNA synthetase mutants also belong to this class (6, 52). These enzymes charge amino acids on their respective tRNAs. A decrease in their activity causes a deficit in aminoacyl-tRNA and induces the stringent response characterized, essentially, by a decrease in the synthesis of stable RNAs and proteins involved in translation. The stringent response thus adjusts the ribosome concentration and translation capacity to aminoacyl-tRNA availability. The effector of the stringent response is a special nucleotide, ppGpp (guanosine 5′-diphosphate 3′-diphosphate), synthesized in wild-type strains when an uncharged cognate tRNA occupies the A site of an active ribosome. The enzyme responsible for the increase in ppGpp under these conditions is called the stringent factor and is coded for by the *relA* gene. The primary effect of ppGpp is on transcription, altering the affinity of RNA polymerase for a large set of promoters (10). We have shown that a *relA* mutation, which abolishes the stringent response, suppresses the amdinocillin resistance of aminoacyl-tRNA mutants and that an increase in the ppGpp level confers amdinocillin resistance on a wild-type strain during normal growth, explaining the amdinocillin resistance of aminoacyl-tRNA synthetase mutants (52).

In the presence of amdinocillin, cells enlarge before death, suggesting a failure of cell division (3, 24, 36, 52). However, it

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TABLE 1. Bacterial strains used for this study

Strain	Genotype	Source or reference
KL231	*leuS31*(Ts) *thyA6 rpsL120*	26
GC3742	*zbe-280*::Tn*10 thyA6 rpsL120*	M. Springer
GC3744	As KL231, Δ(*rodA-pbpA*)::Km^r	This work
GC3747	As KL231, *relA1*	This work
GC3782	Prototroph	Laboratory collection
GC3784	As GC3782, *relA1*	Laboratory collection
GC7393	*argH his* Δ*cya* Δ(*rodA-pbpA*)::Km^r	This work

has proved difficult to quantify the residual division after PBP2 inactivation, either because of complications due to concomitant morphological changes or because of partial cell lysis. We have constructed a conditional system allowing us to study the effects of PBP2 inactivation on cell division without interference from morphological changes or lysis. Using it, we show that PBP2 inactivation results in cell division inhibition, which is relieved by an increased ppGpp pool.

The most documented effect of ppGpp is transcription modulation during the stringent response (10). However, the nucleotide may also be involved in cell division: a Δ*relA* Δ*spoT* double mutant which has no detectable ppGpp forms filaments (60), suggesting that ppGpp is a positive effector of cell division. This led us to hypothesize that PBP2 inactivation results in a deficiency in some cell division protein which can be overcome by an increased ppGpp pool. The FtsZ protein plays a key role in cell division, acting at an early step, possibly initiation (56). FtsZ is distributed randomly in the cytoplasm between successive divisions but condenses to form a ring around the cell center at the onset of septation (4). The relative rate of *ftsZ* expression is inversely correlated with growth rate: when the growth rate decreases, *ftsZ* expression increases (2, 17, 38). The ppGpp pool size is also inversely correlated with growth rate (10), so the FtsZ concentration and the ppGpp pool increase together when the growth rate decreases. It has been claimed that excess RelA activity partially suppresses the *ftsZ84*(Ts) mutation, presumably by increasing the ppGpp pool (as described in reference 19). These observations suggested to us, first, that ppGpp may be a positive regulator of *ftsZ* expression and, second, that an increased ppGpp pool may allow cells to survive without PBP2 because of increased *ftsZ* expression. We show here that overproduction of FtsZ suppresses the cell division inhibition observed after PBP2 inactivation and confers resistance to amdinocillin.

MATERIALS AND METHODS

Bacterial strains and phages. The strains used in this work are all *E. coli* K-12 derivatives, and they are described in Table 1. The *leuS*(Ts) mutant KL231 and its *leuS*$^+$ derivative SK2257 were kindly provided by M. Springer. The *leuS*(Ts) *relA* strain GC3747 is a *cysC*$^+$ transductant of a *leuS*(Ts) *relA cysC*::Tn*10* transductant of KL231. The Δ(*rodA-pbpA*)::Km^r deletion, constructed in our laboratory by T. Ogura like the Δ(*pbpA*)::Km^r deletion (36), was introduced by transformation onto the chromosome of a C600 *recD1009* strain containing the plasmid pAR1(*rodA*$^+$ *pbpA*$^+$) and subsequently transferred by P1 transduction to the Δ*cya* strain PP7860 (36) to make strain GC7393. Plating efficiencies were calculated from overnight cultures; we did not observe differences with exponential cultures. P1 *vir* transductions were carried out as described previously (33).

Media and growth conditions. The medium used was LB broth (33) supplemented with thymine (2.5 g/liter) except for measurements of the ppGpp concentration (see below). The presence of the *relA1* mutation was checked on solid M63 minimal medium (33) supplemented with glucose (0.4%), thiamine (10 μg/ml), thymine (100 μg/ml), and serine, glycine, and methionine (100 μg/ml each); *relA* mutants cannot grow on this medium unless isoleucine and valine (100 μg/ml each) are added (49, 50). Solid media contained 1.5% agar. Antibiotics, when required, were added at the following concentrations: chloramphenicol, 20 μg/ml; kanamycin, 40 μg/ml; amdinocillin, 1 or 10 μg/ml; and tetracycline, 10 μg/ml.

DNA techniques. Plasmid DNA was extracted, and transformation was carried out as described by Sambrook et al. (41). Plasmid pAX242 (36) was kindly provided by T. Ogura and the pBR322 derivatives pZAQ (58) and pAQ were kindly provided by J. Lutkenhaus. pAQ carries a Tn*5* insertion into the *ftsZ* gene (61). We constructed the plasmid pΔ by deletion of the 2.3-kbp *Eco*RI-*Eco*RI fragment of pZAQ (see Fig. 2).

Measurement of ppGpp concentration. To measure the ppGpp concentration by ^{32}P labeling, bacteria were grown in phosphate-depleted LB medium prepared as described by Bukhari and Ljungquist (8). In this medium, we estimated the residual phosphate concentration to be 5×10^{-4} to 1×10^{-3} M.

To an exponential culture of the *leuS*(Ts) Δ(*rodA-pbpA*)::Km^r strain, [^{32}P]phosphate was added at a final activity of 70 μCi/ml. Incubation was continued for 30 to 40 min, and then 80-μl samples were removed periodically and added to 120 μl of cold formic acid (1.2 N final concentration). After 30 min on ice, samples were centrifuged and 5- to 20-μl aliquots of the supernatants were loaded on PEI cellulose (PEI Baker-flex; J. T. Baker, Inc., Phillipsburg, N.J.) and chromatograms were made in Na_2HPO_4-NaH_2PO_4, pH 3.4, at 20°C for 5 h (60). The chromatograms were dried and analyzed with a PhosphorImager (Molecular Dynamics) after 3 to 5 h of exposure; experimental precision was within approximately 10%.

Miscellaneous techniques. Optical density (OD) was measured at 600 nm in a PM6 spectrophotometer with a 1-cm optical path. Morphological observations were made with a Jenamed phase-contrast microscope (Zeiss, Jena, Germany). The volume distribution of cells was determined for samples diluted 20- to 100-fold in filtered saline with a Coulter Counter (model ZB; Coultronics, Margency, France) equipped with a 30-μm orifice and a C1000 Coulter Channelyzer. The particle concentration was evaluated either directly with the Coulter Counter or indirectly as the ratio of the OD_{600} to the average cell volume, normalized to the direct count at time zero.

RESULTS AND DISCUSSION

Construction and characterization of a *leuS*(Ts) Δ(*rodA-pbpA*)::Km^r strain. To design a system in which PBP2 activity is essential in one condition but not in another, we took advantage of the properties of the *leuS*(Ts) strain KL231. This mutant has a thermosensitive leucyl-tRNA synthetase (26). It cannot grow at 44°C because the enzyme, essential to the cells, is completely inactivated. At 37°C, the strain grows with a lower growth rate than its *leuS*$^+$ derivative (GC3742) because of partial inactivation of leucyl-tRNA synthetase, which causes a decrease in the charge of $tRNA^{Leu}$. At 25°C, there was no difference between the two strains, showing that the synthetase is fully active at this temperature. We measured the plating

TABLE 2. Amdinocillin resistance of the *leuS*(Ts) mutant

Strain	Plating efficiency on LB				Acceptance[a] of Δ(*rodA-pbpA*)::Km^r	
	Without amdinocillin		With amdinocillin[b]			
	25°C	37°C	25°C	37°C	25°C	37°C
GC3742 (*leuS*$^+$)	1.0	≡1	2.1×10^{-5}	7.3×10^{-5}	no	no
KL231 [*leuS*(Ts)]	1.2	≡1	1.3×10^{-5}	0.62	no	yes
GC3747 [*leuS*(Ts) *relA1*]	≡1	5.4×10^{-3}	4.5×10^{-7}	4.5×10^{-6}	no	no
GC3744 [*leuS*(Ts) Δ(*rodA-pbpA*)::Km^r]	1.3×10^{-4}	≡1	7.0×10^{-5}	0.91		

[a] Ability to give Km^r transductants when transduced with P1 grown on strain GC7393.
[b] 10 μg/ml.

efficiency of the *leuS*(Ts) and *leuS*$^+$ strains in the presence of amdinocillin (10 μg/ml) at 25 and 37°C. At 25°C, both strains were sensitive to amdinocillin; however, at 37°C, although the *leuS*$^+$ strain remained sensitive, the *leuS*(Ts) strain was resistant (Table 2).

We next verified that the amdinocillin resistance of strain KL231 was due to the *leuS*(Ts) mutation. We transduced the strain to Tc^r with a P1 stock made on a strain carrying a Tn*10* transposon near the *leuS*$^+$ gene; amdinocillin sensitivity at 37°C was 69% cotransducible with tetracycline resistance (22 of 32 transductants). We then chose as the donor strain a Tc^r transductant which had remained amdinocillin resistant at 37°C and transduced the *leuS*$^+$ strain GC3742 to Tc^r; among Tc^r transductants 66% (21 of 32) were amdinocillin resistant at 37°C and sensitive at 25°C. All amdinocillin-resistant cotransductants were thermosensitive at 44°C, a phenotype of the *leuS*(Ts) mutant. Thus, the amdinocillin resistance of strain KL231 at 37°C is tightly linked or identical to the *leuS*(Ts) mutation.

A deletion of the *pbpA* and *rodA* genes, Δ(*rodA-pbpA*)::Km^r, was constructed in vitro (see Materials and Methods). Like the previously described Δ*pbpA*::Km^r deletion (36), it could only be introduced into strains (i) diploid for this region, (ii) that were amdinocillin resistant because of a mutation in an aminoacyl-tRNA synthetase gene, or (iii) carrying a *cya* mutation. We tried to introduce the deletion into the *leuS*(Ts) and parental strains at 25 and 37°C. Km^r transductants were obtained only with the *leuS*(Ts) strain at 37°C; none appeared with the *leuS*(Ts) strain at 25°C or with the parental strain at either temperature (Table 2). The transductants were spherical, as expected when PBP2 is inactivated. One transductant, GC3744, of genotype *leuS*(Ts) Δ(*rodA-pbpA*)::Km^r (see below), was cultivated at 37°C for further analysis. Its plating efficiency at 25°C was about 10^{-4}, similar to the frequency of amdinocillin-resistant mutants in the *leuS*(Ts) strain at 25°C and in the parental strain at 25 or 37°C (Table 2). This transductant was unable to form colonies at 44°C, like the *leuS*(Ts) strain and unlike the isogenic *leuS*$^+$ strain GC3742.

We wished to verify conclusively that the *leuS*(Ts) mutation was present in GC3744, since the *leuS*$^+$ gene is near the *rodA-pbpA* operon and could have been introduced by cotransduction with Δ(*rodA-pbpA*)::Km^r; in this case, another unidentified mutation could allow the strain to tolerate the deletion. We therefore carried out two additional tests on the Km^r transductant GC3744. First, we transduced the *leuS*$^+$ strain GC3742 to Km^r at 25 or 37°C, using P1 stocks made either on the supposed *leuS*(Ts) Δ(*rodA-pbpA*)::Km^r transductant GC3744 or on a *leuS*$^+$ Δ(*rodA-pbpA*)::Km strain Δ*cya* (GC7393). We obtained Km^r transductants only with P1 made on GC3744 and only at 37°C (>300 clones), showing that this strain carries a mutation linked to the *rodA-pbpA* operon which permits it to tolerate the deletion at 37°C. Second, we transformed the transductant GC3744 with plasmid pAX242 carrying the wild-type *pbpA* and *rodA* genes but not the *leuS* gene. Cm^r transformants selected at 37°C all recovered their rod shape and could grow at 25 but not at 44°C. The transformants were resistant to amdinocillin at 37°C and sensitive at 25°C, like the *leuS*(Ts) strain. These results confirm that the *leuS*(Ts) mutation is still present in the transductant GC3744 and is required for the tolerance of the Δ(*rodA-pbpA*)::Km^r deletion.

Although amdinocillin binds preferentially to PBP2 (43), it could have secondary targets, especially at 10 μg/ml, compared with a reported MIC of about 0.03 μg/ml (48). The *leuS*(Ts) Δ(*rodA-pbpA*)::Km^r strain GC3744 had the same plating efficiency at 37°C with or without 10-μg/ml amdinocillin (Table 2), indicating that no vital secondary targets are inactivated at this concentration. Furthermore, addition of 10 μg of amdinocillin per ml to a culture of the *leuS*(Ts) Δ(*rodA-pbpA*)::Km^r strain at 37°C had no effect on the growth rate in mass or cell count. The same experiment with the *leuS*(Ts) strain showed that amdinocillin addition did not affect mass growth but temporarily inhibited cell division during the transition from rod shape to spheres of larger volume (data not shown). We conclude that the only effect of amdinocillin at 10 μg/ml is the inactivation of PBP2; a strain carrying the Δ(*rodA-pbpA*)::Km^r deletion behaves like a *rodA*$^+$ *pbpA*$^+$ strain in the presence of amdinocillin.

Lethality of PBP2 inactivation and ppGpp concentration. We have previously shown that aminoacyl-tRNA synthetase mutants, like wild-type strains with increased RelA activity, owe their amdinocillin resistance, as well as their ability to accept the Δ(*rodA-pbpA*)::Km^r deletion, to a high ppGpp pool synthesized by the RelA protein in response to partial aminoacyl-tRNA starvation (52). To see whether the same was true of the *leuS*(Ts) Δ(*rodA-pbpA*)::Km^r strain GC3744, we tried to introduce the *relA1* mutation into the strain by cotransduction with a *cysC*::Tn*10* insertion. Among 128 Tc^r clones obtained at 37°C, no RelA$^-$ cotransductants were obtained, suggesting that the *relA1* mutation was lethal in the strain. Transduction of the *leuS*(Ts) strain gave 20% cotransduction of *relA* with tetracycline resistance at 25°C. Surprisingly, the *leuS*(Ts) *relA* transductant had decreased plating efficiency at 37°C (Table 2). Amdinocillin decreased this efficiency 1,000-fold, suggesting that the *leuS*(Ts) mutant, like other aminoacyl-tRNA synthetase mutants, owes its amdinocillin resistance to the induction of the stringent response. This is consistent with our previous report, showing that amdinocillin resistance does not result simply from slow growth but rather from increased ppGpp levels (52).

We measured the ppGpp concentration in the *leuS*(Ts) Δ(*rodA-pbpA*)::Km^r strain GC3744 during steady-state growth in phosphate-depleted LB medium at 37°C and after transfer of the culture from 37°C to the nonpermissive temperature, 25°C. The ppGpp concentration dropped threefold within 7

min after the temperature shift and then remained constant for 80 min of further incubation. This result is consistent with our conclusion that the *leuS*(Ts) strain, like other aminoacyl-tRNA synthetase mutants, is amdinocillin resistant because of an increased ppGpp pool. Experiments in progress suggest that the ppGpp concentration has to exceed a critical threshold to permit amdinocillin resistance; preliminary results indicate that a doubling in the ppGpp concentration is sufficient to confer amdinocillin resistance (24a).

Effect of PBP2 inactivation on cell growth and division. In the presence of amdinocillin, wild-type cells become spherical and increase in volume before dying, suggesting that cell division has been inhibited; the morphological change and partial lysis, however, do not allow accurate quantification of the extent of cell division inhibition (3, 24, 36). Using the Δ(*rodA-pbpA*)::Km^r deletion, we constructed a strain which allows us to study the effect of PBP2 inactivation without interference from lysis or the morphological transition induced by amdinocillin; this is the *leuS*(Ts) Δ(*rodA-pbpA*)::Km^r strain GC3744 described above, which is viable and spherical when cultivated at 37°C but unable to form colonies at 25°C. We cultivated the *leuS*(Ts) Δ(*rodA-pbpA*)::Km^r strain at 37°C and then transferred the culture to 25°C and observed the effect on cell growth and division. The control was the *leuS*(Ts) strain KL231. While the *leuS*(Ts) strain rapidly recovered equilibrated growth of mass as well as of cell numbers at 25°C, the *leuS*(Ts) Δ(*rodA-pbpA*)::Km^r strain did not (Fig. 1A and B). Cell concentration increased slowly for 120 min at 25°C, remaining constant after a single doubling. Plating at 37°C revealed a slow loss in viability beginning about 120 min after the shift to 25°C, and after 3 h of incubation at 25°C, about 40% of the cells remained viable (data not shown). For both strains, the mean cell volume began to increase immediately after the temperature shift. It stabilized after 150 min in the *leuS*(Ts) culture but continued to increase in the *leuS*(Ts) Δ(*rodA-pbpA*)::Km^r strain (Fig. 1C), and ultimately these cells began to form ghosts devoid of cytoplasm. The modest volume increase in the *leuS*(Ts) strain at 25°C presumably reflects the release from partial leucyl-tRNA starvation, analogous to the volume increase associated with nutritional shift-ups (25).

The above results show that cell division is inhibited when the ppGpp pool is decreased in the *leuS*(Ts) Δ(*rodA-pbpA*)::Km^r strain. Under these conditions, the cells carry out a single division, enlarge, and gradually lose viability.

FtsZ overproduction confers amdinocillin resistance. We have shown that PBP2 inactivation in wild-type cells results in the inhibition of cell division and that a high ppGpp level allows cells with inactivated PBP2 to divide and survive. Since cells with no ppGpp also exhibit a division defect (60), albeit nonlethal, we speculated that ppGpp activates the transcription of one or more cell division genes. The FtsZ protein, essential for septation initiation, is a key control point of the cell cycle in *E. coli* (28), and a *relA* plasmid can partially suppress the *ftsZ84*(Ts) mutation (described in reference 19), presumably by increasing the ppGpp pool. An attractive hypothesis would then be that a high ppGpp level overcomes the division problem caused by PBP2 inactivation simply by increasing *ftsZ* expression. If division inhibition is the only lethal aspect of PBP2 inactivation, then FtsZ overproduction would be expected to confer amdinocillin resistance and the capacity to support the Δ(*rodA-pbpA*)::Km^r deletion.

To test whether FtsZ overproduction confers amdinocillin resistance, we transformed an amdinocillin-sensitive strain, GC3782, with plasmid pZAQ carrying the *ftsZ*, *ftsA*, and *ftsQ* genes; as controls, we used plasmids pAQ lacking *ftsZ* and pΔ with no intact *fts* genes (see Materials and Methods). The

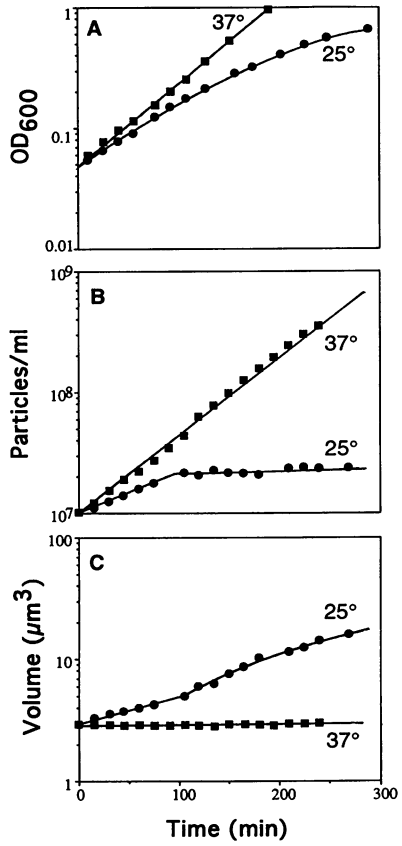

FIG. 1. Division inhibition in the *leuS*(Ts) Δ(*rodA-pbpA*)::Km^r strain at 25°C. Strain GC3744, growing exponentially in LB at 37°C, was divided in two at time zero, and one part was transferred to 25°C. Samples were removed at 15-min intervals and analyzed for OD_{600}, particle count, and average cell volume. (A) OD_{600}. (B) Particle count, determined as the ratio of the OD_{600} to the average cell volume normalized to the direct count at time zero. (C) Mean cell volume, determined from the volume distribution evaluated with a C1000 Coulter Channelyzer.

plasmid pZAQ caused minicell formation, as previously observed when the FtsZ protein was overproduced (58). The size of pAQ transformants varied, including normal size cells, elongated cells, and filaments, denoting a cell division problem; the effects of pAQ are discussed below. None of the effects of pZAQ and pAQ were observed with pΔ. The plasmid pZAQ conferred nearly complete resistance to amdinocillin at 1 μg/ml and partial resistance at 10 μg/ml, whereas pAQ and pΔ transformants remained completely sensitive (Table 3).

Plasmid pZAQ could confer amdinocillin resistance by inducing the stringent response. To test this admittedly unlikely hypothesis, we repeated the above experiments with a *relA1* strain (GC3784) in which stringent response induction cannot occur. Transformants of this strain carrying pZAQ, pAQ, and pΔ had the same morphological characteristics as the *relA*$^+$ (GC3782) transformants except that in the *relA1*/pAQ strain filamentation was more pronounced. Again, pZAQ conferred amdinocillin resistance (Table 3). We conclude that overproduction of FtsZ, the only protein expressed by pZAQ but not by pAQ, confers amdinocillin resistance independent of the stringent response.

We next wished to see whether the amdinocillin resistance conferred by pZAQ permits cells to tolerate the Δ(*rodA-*

TABLE 3. pZAQ-conferred amdinocillin resistance

Strain and plasmid	Plating efficiency[a] at 37°C on LB[b] with amdinocillin at:	
	1 μg/ml	10 μg/ml
GC3782 (*relA*$^+$)		
pZAQ	0.25	4.2×10^{-3}
pAQ	2.2×10^{-5}	3.1×10^{-6}
pΔ	6.0×10^{-5}	1.5×10^{-5}
GC3784 (*relA*)		
pZAQ	0.35	2.3×10^{-3}
pAQ	3.7×10^{-6}	$\leq 2 \times 10^{-6}$
pΔ	8.3×10^{-6}	2.2×10^{-6}

[a] Ratio of colony titer on LB containing amdinocillin to that on LB.
[b] Tetracycline was present in all plates to maintain the plasmids.

pbpA)::Km^r deletion, an essential experiment to conclude that the plasmid allows cells with inactivated PBP2 to survive. We transformed the *leuS*(Ts) and *leuS*(Ts) Δ(*rodA-pbpA*)::Km^r strains with the same plasmids at 37°C; cells of all strains remained spherical. Plasmid pZAQ conferred amdinocillin resistance on the *leuS*(Ts) strain at 25°C and permitted growth of the *leuS*(Ts) Δ(*rodA-pbpA*)::Km^r strain at 25°C (Table 4), showing that pZAQ suppresses the cell division inhibition caused by complete inactivation of PBP2.

The amdinocillin resistance conferred by pZAQ clearly requires FtsZ overproduction, since pAQ is ineffective, but we cannot say whether FtsA or FtsQ overproduction is needed as well. The plasmid pAQ causes filamentation and, surprisingly, the *leuS*(Ts)/pAQ transformant was amdinocillin sensitive at both 37 and 25°C. Furthermore, the pAQ plasmid could not be introduced into the *leuS*(Ts) Δ(*rodA-pbpA*)::Km^r strain at any temperature, again suggesting that overproduction of FtsA and FtsQ without FtsZ is lethal in a strain with inactivated PBP2. In a different genetic background (strain GC3782), the *leuS* (Ts) mutation, introduced by transduction, still conferred amdinocillin resistance at 37°C (but not at 25°C) and the introduction of pAQ again abolished this resistance, indicating that the effect of pAQ does not depend on some cryptic mutation in strain KL231. Since a proper balance between FtsZ and FtsA is necessary to maintain septation activity (13, 16), the effects of pAQ may reflect the lower FtsZ/FtsA ratio, formally resembling a reduction in the FtsZ level. In any case, our results show that overproduction of septation proteins confers amdinocillin resistance.

TABLE 4. Acceptance of Δ(*rodA-pbpA*)::Km^r permitted by pZAQ

Strain and plasmid	Plating efficiency on LB[a]			
	25°C		37°C	
	−	+	−	+
KL231 [*leuS*(Ts)]				
pZAQ	0.85	0.70	≡1	0.70
pAQ	1.3	6.8×10^{-6}	≡1	4.2×10^{-6}
pΔ	0.90	7.4×10^{-6}	≡1	0.45
GC3744 [*leuS*(Ts) Δ(*rodA-pbpA*)::Km^r]				
pZAQ	0.72	0.75	≡1	0.95
pΔ	1.2×10^{-4}	1.5×10^{-4}	≡1	1.1

[a] Tetracycline was present in all plates to maintain the plasmids. −, Incubation without amdinocillin; +, incubation with amdinocillin (10 μg/ml).

Does ppGpp promote the synthesis of a division protein? A high ppGpp level or the presence of the plasmid pZAQ confers amdinocillin resistance on *E. coli*, which is compatible with the idea that the level of FtsZ activity is positively controlled by ppGpp. This could be at the transcriptional level, analogous to the positive regulation by ppGpp of the *his* operon (46) and others (10). It has recently been shown that FtsZ protein has an essential GTPase activity which requires activation in vitro by any of several nucleotides (14, 34). We have suggested that the in vivo activator of FtsZ may be ppGpp (51), implying that elevated ppGpp pool levels increase FtsZ activity directly. To test this hypothesis, we studied the ability of division-inhibited cells to resume cell division when the ppGpp pool is increased, with or without concomitant protein synthesis. We shifted a culture of the *leuS*(Ts) Δ(*rodA-pbpA*)::Km^r strain (GC3744) to 25°C for 3 h and then transferred the culture back to 37°C to increase the ppGpp pool again. Cell division resumed 30 min after the return to 37°C (data not shown). To test whether protein synthesis was required for the recovery of cell division after incubation of the *leuS*(Ts) Δ(*rodA-pbpA*)::Km^r strain at 25°C, we carried out the same experiment but after 3 h of incubation at 25°C transferred the culture to 42 instead of 37°C. At 42°C, nearly complete inactivation of the leucyl-tRNA synthetase severely decreases protein synthesis, with concomitant induction of the stringent response. The particle count increased 10% in 60 min and then remained constant. The shift to 42°C induced slight lysis: the OD decreased 18% in 50 min and then increased very slowly. Cells did not exhibit constrictions or decreased volume, suggesting that the particle count increase reflected partial lysis, not cell division. Thus, an increased ppGpp pool alone could not restore cell division. We obtained similar results by transferring the culture to the permissive temperature of 37°C in the presence of 200 μg of chloramphenicol per ml after 3 h of incubation at 25°C (data not shown); under these conditions, protein synthesis is inhibited without induction of the stringent response (9).

These results show that the recovery of cell division in the *leuS*(Ts) Δ(*rodA-pbpA*)::Km^r strain after division inhibition at 25°C requires de novo protein synthesis in addition to a large ppGpp pool. It is thus unlikely that ppGpp stimulates division by interacting directly with FtsZ.

This conclusion is supported by the observation that the *leuS*(Ts) Δ(*rodA-pbpA*)::Km^r strain at 37°C is hypersensitive to low concentrations (2 to 5 μg/ml) of the RNA polymerase inhibitor rifampin (data not shown), suggesting that amdinocillin resistance due to increased ppGpp levels results from a modulation of transcriptional activity, as previously noted (52). Although other interpretations cannot be ruled out, the simplest explanation of our results is that high ppGpp levels stimulate the synthesis of FtsZ. We are currently testing the effect of ppGpp on the activity of the different *ftsZ* promoters.

The *ftsZ* gene is located at 2 min on the *E. coli* genetic map, in a cluster rich in genes essential for cell division. The structure of the *ftsQAZ* genes (Fig. 2) shows that all the genes can be transcribed from two promoters located in the *ddlB* gene, and *ftsZ* has four additional promoters within the *ftsA* gene (2, 39, 40). The expression of *ftsZ* is inversely correlated with growth rate (2, 17, 38). The P_{Q1} promoter contains a so-called "gearbox" sequence, which is found in other promoters inversely regulated by growth rate (1) and, in one case, has been shown to be essential for this regulation (2). The P_{Z4}-P_{Z3} promoters, studied together in a gene fusion, also exhibit inverse growth rate regulation (17, 38). Since the ppGpp concentration varies in the same way as *ftsZ* expression with respect to growth rate (10), the P_{Q1} and P_{Z4}-P_{Z3} promoters are attractive candidates for ppGpp activation. However, other

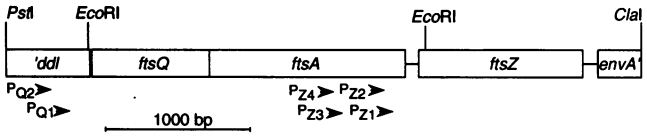

FIG. 2. Structure of the *fts* insert of plasmid pZAQ (58). Arrows indicate the promoters from which *ftsZ* is expressed. Plasmid pAQ carries a Tn*5* insertion within the *ftsZ* gene (61), and pΔ was constructed by deletion of the internal 2.3-kbp *Eco*RI-*Eco*RI fragment.

regulations have been reported and the ppGpp effect could be indirect. The product of the *sdiA* gene has been recently identified as a positive regulator of *ftsZ* expression via modulation of the P_{Q2} promoter (57); the P_{Z4}-P_{Z3} promoters may be regulated by DnaA or by replication initiation (30); and finally, *ftsA* has been reported to regulate P_{Z4}-P_{Z3} expression (17), although this observation has been contested (38).

The FtsZ protein is a key control point in the *E. coli* cell cycle. It is the target of endogenous cell division inhibitors SulA, SfiC, MinC-MinD, and MinC-DicB (28) and is therefore involved in coupling cell division to DNA replication (21) and to correct positioning of the septum (15). FtsZ is essential for an early stage of septation and has been reported to be limiting for this process: overproduction of FtsZ increases the frequency of septation via minicell production (58), and decreases in FtsZ concentration cause a division block (12), although the precise threshold required for viability depends on the experimental system (47). FtsZ is distributed randomly in the cytoplasm between successive divisions but condenses to form a ring around the cell center at the onset of septation (4). Recently, FtsZ has been shown to possess GTPase activity, with a GTP binding site similar to those of eukaryotic tubulins (14, 34, 37). This observation, together with the capacity of FtsZ to aggregate, suggests that the protein plays a structural role in cell division, unlike the enzymatic function of PBP3, for example. FtsZ could polymerize (like eukaryotic tubulin) and form a ring at the cell center, and depolymerization of the ring could cause constriction. The control of cell division could then take place at the level of FtsZ localization (5) or polymerization (51).

What is the mechanism involved in cell division inhibition when PBP2 is inactivated? Spherical cells resulting from PBP2 inactivation have a diameter similar to the length of the same cells with active PBP2 and consequently have a larger volume (18). For example, our *leuS*(Ts) Δ(*rodA-pbpA*)::Km^r strain cultivated at 37°C in LB has about a threefold larger volume than the *leuS*(Ts) strain cultivated in the same conditions. If FtsZ forms a continuous ring around the cells, large spherical cells clearly need more monomers than rod-shaped cells to make the ring. The cell division inhibition observed when the *leuS*(Ts) Δ(*rodA-pbpA*)::Km^r strain is transferred from 37 to 25°C could be due to the inability of the cells to synthesize enough FtsZ to form a ring because *ftsZ* expression, regulated by ppGpp, is decreased. The rescue by increased ppGpp or by the plasmid pZAQ would then result from restoration of a level of *ftsZ* expression sufficient to permit ring formation in large spherical cells.

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