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A *lacZ-pbpB* Gene Fusion Coding for an Inducible Hybrid Protein That Recognizes Localized Sites in the Inner Membrane of *Escherichia coli*

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An in-phase gene fusion consisting of the 5′-terminal 1,314 base pairs (bp) of the structural gene for β-galactosidase (*lacZ*) and the 3′-terminal 1,644 bp of the structural gene coding for penicillin-binding protein 3 (*pbpB*) of *Escherichia coli* was constructed and cloned in the plasmid pDIAM64. The product of the fusion gene was a remarkably stable protein with an apparent molecular weight of 110,000 (p110) that retained the ability to covalently interact with β-lactam antibiotics. The fusion protein was found associated with the membrane at low levels of induction, but it accumulated in the cytoplasm of cells induced for a long time as inclusion bodies of high density. Inclusion bodies were localized at defined positions corresponding to septal sites in all of the pDIAM64-containing strains tested except PAT84 and GD113 (which carry the *ftsZ84* mutant allele). These findings indicate a possible role of the FtsZ protein in the integration of Pbp3 into the membrane and in septum localization during the cell division cycle.

Penicillin-binding protein 3 (Pbp3) of *Escherichia coli* is a membrane-bound protein involved in septum formation at the time of cell division (3, 36, 37). Current evidence indicates that Pbp3 is a bifunctional enzyme catalyzing both a β-lactam-sensitive D,D-transpeptidase reaction and a β-lactam-insensitive peptidoglycan transglycosylase reaction (14). It has been repeatedly suggested that the activity of Pbp3 is under temporal and topological control, participating exclusively in biosynthesis of the septal peptidoglycan (3, 41, 45). Recently, Tormo et al. (39) have shown a molecular interaction between Pbp3 and the product of the *ftsA* gene, a protein with a structural (40) and regulatory (9) role in septation. Also, Begg et al. (2) suggested that an interaction between Pbp3 and RodA is required for normal cell shape and division in *E. coli*. The *ftsZ* gene product, another protein of the 2.0-min cluster, controls a key step in cell division, as suggested by genetic evidence (44). Low levels or absence of the protein produces cell filamentation, but overproduction of FtsZ induces minicell formation by increasing additional septations at the cell pole. Investigations of the SOS response (19, 21) assigned to the FtsZ protein the role of target of action of SfiA, which leads to inhibition of cell division.

Pbp3 is coded by the gene *pbpB* (*ftsI*, *sep*) located in a large cluster of genes (min 2.0 of the *E. coli* linkage map) (37), all of them involved in different aspects of cell envelope growth and division. *pbpB* has been cloned and sequenced (27). From the reported nucleotide sequence, as well as from additional genetic and biochemical evidence, it has been proposed that Pbp3 is synthesized as a precursor protein (27). The predicted additional N-terminal peptide consists of an initial segment of 23 amino acids, rich in basic and hydrophobic sequences, followed by a highly hydrophobic region extending from residues 24 to 43. Analysis of the amino acid sequence shows possible sites of processing by signal peptidases I (28, 48) and II (8, 28) at amino acid residues 40 and 29 of the precursor form, respectively. The role of the additional peptide in integration of the mature protein into the membrane remains unclear.

Gene fusion techniques have proved to be a very useful approach to the study of the mechanisms of protein insertion into the membrane (33). By using these techniques, we have constructed a chimeric protein derived from Pbp3 that both removed the hypothetical signal peptide sequence and fused the protein to the N-terminal part of β-galactosidase. The hybrid protein appears in the membrane fraction when induced at a low level but seems to accumulate in the cytoplasm as inclusion bodies with a regular arrangement at a higher level of induction. This regular location of the grains is absent in strains carrying an *ftsZ* mutant allele. Reinitiation of cell division after induction of grain formation occurs at the positions previously defined by the grains, except in the *ftsZ* mutant strains. The implications of these results for both the integration of Pbp3 into the membrane and the function of FtsZ are discussed.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The *E. coli* K-12 strains used throughout this study are listed in Table 1. The plasmid vectors pDIA9204 (18) and pLC26-6 (4) were gifts of J. Perea and Y. Hirota, respectively. Plasmid pUC9 (42) was purchased from Bethesda Research Laboratories GMbH, Federal Republic of Germany. Cells were routinely grown in L broth (17) supplemented with 0.2% (wt/vol) glucose, at 30°C, under vigorous aeration. To induce the expression of genes cloned under the control of the lambda Pr promoter in vectors derived from pDIA9204, cultures grown at 30°C and at an OD_{550} ranging from 0.2 to 0.6 were transferred to 42°C and incubated for the required periods of time.

DNA methods. DNA manipulation (restriction, ligation, and purification) and transformation were done by standard methods (23).

Small-scale DNA purification was carried out by the lysis-by-boiling method (23). DNA fragments were purified from low-melting-point agarose.

Calf intestine alkaline phosphatase, T4 DNA ligase, and

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TABLE 1. Strains used

Strain	Relevant marker	Reference	Source
JM83	Wild type	25	J. Messing
SP33	*pbpB33*(Ts)		B. G. Spratt
SP63	*pbpB63*(Ts)	35	B. G. Spratt
TOE1	*ftsQ1*(Ts)	1	M. Vicente
D3	*ftsA3*(Ts)	40	M. Vicente
PAT84	*ftsZ84*(Ts)	22	M. Vicente
GC2467	*sfiA*::Tn*5*	6	R. D'Ari
GD113	*ftsZ84*(Ts)	7	G. R. Drapeau
AD12	*lon-1 ftsZ84*(Ts)	7	G. R. Drapeau

restriction endonucleases were used according to the instructions provided by the commercial suppliers.

Detection of Pbps. Cells harvested by centrifugation (3,000 × *g*, 10 min, 5°C) of an appropriate volume of culture (100 to 200 ml) were suspended in 1/10 the initial volume of 50 mM phosphate buffer, pH 7.0, and then subjected to ultrasonic disruption (three pulses of 1 min each with interspersed cooling periods) on a Brandsonic MSE sonicator set at 18 μm amplitude. Unbroken cells were discarded by centrifugation (3,000 × *g*, 10 min, 5°C), and the supernatant was further centrifuged (50,000 × *g*, 60 min, 5°C) to obtain a particulate fraction which was suspended in 50 mM phosphate buffer, pH 7.0, at a final concentration of 5 mg of protein per ml, as measured by the method of Lowry et al. (20). Pbps were detected in the particulate fractions as described by Spratt (34) by using the radioactive β-lactams *N*-([2,3-^{3}H]propionyl)ampicillin (^{3}H-ampicillin) (370 MBq/mmol) at a final concentration of 100 μM or *N*-[3-(4-hydroxy-5-[^{125}I]iodophenyl)propionyl]ampicillin (^{125}I-ampicillin) (74 TBq/mmol) at 50 nM final concentration, prepared as described by Schwarz et al. (32). Samples (4 μl) of the particulate suspension were incubated for 10 min at 37°C with clavulanic acid (200 μg/ml) to inhibit the β-lactamase activity present in the cell envelopes before the addition of the radioactive β-lactam. Binding was allowed to proceed for 20 min at 37°C and then stopped by the addition of ampicillin to a final concentration of 500 μg/ml. Subsequently, the sample was boiled for 10 min after the addition of sodium dodecyl sulfate (SDS) to a final concentration of 1% (wt/vol). Solubilized proteins were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) in 8% (wt/vol) acrylamide gels (15). Labeled proteins were detected by autofluorography (16) or autoradiography, depending on the radioactive β-lactam used.

Optical and electron microscopy. Optical microscopy was performed on a Zeiss photomicroscope III equipped with phase-contrast optics. Cells were fixed in 1% Formalin–0.9% NaCl and spread over agar-coated glass slides for observation.

For the examination of bacteria (in thin sections) by electron microscopy, cells from 1 ml of culture were harvested by centrifugation (13,000 × *g*, 1 min), suspended in an equal volume of 50 mM phosphate buffer, pH 7.0, at 4°C, sedimented under the same conditions, and fixed with 2% (wt/vol) glutaraldehyde and 4% (wt/vol) tannic acid in 50 mM phosphate buffer, pH 7.0. After 1 h at room temperature, the samples were washed four times with 50 mM phosphate buffer, pH 7.0, and postfixed with 1% (wt/vol) OsO_4 at 4°C for 30 min. Cells were then stained with 2% (wt/vol) uranyl acetate for 60 min and washed twice with 50 mM phosphate buffer, pH 7.0, and after gradual dehydration in increasing concentrations of acetone, the samples were embedded in Vestopal W (Serva, Heidelberg, Federal Republic of Germany). After polymerization at 65°C for 24 h, thin sections were obtained in a Sorval MT2-B ultramicrotome, put on collodion-covered grids, and stained with Reynolds lead citrate for 5 min.

For the study of negatively stained bacteria, a drop of a growing culture was fixed with 1% Formalin–0.9% NaCl and prepared for observation under the electron microscope by the agar filtration technique (47). Staining of the specimens was done by floating the grids on a drop of 2% (wt/vol) phosphotungstate, pH 7.0, for 2 min, followed by three washes on drops of distilled water.

All specimens were observed in a JEOL 100-B electron microscope operating at 80 kV.

Granule position measurements. Cell length and granule-to-pole distances were measured in magnified prints from negatives obtained with a Zeiss photomicroscope III by using the fixing and photographic procedures described by Donachie et al. (10). Distance measurements were recorded with the help of a Hewlett Packard 9111A graphics tablet coupled to an HP-85 computer. The software program MLENG was based on the 9111A-HP-85 system tutorial, which includes a continuous digitizing option. Graphic representation of data was done with the program DPLOT, supplied by Hewlett Packard in the HP-85 Standard Pac, and printouts from the computer were redrawn for reproduction.

Fractionation of the fusion protein. A culture of the appropriate strain was grown in L broth medium at 30°C to an OD_{550} of 0.3. A sample was left at 30°C, and the rest was transferred to 42°C. At defined time intervals, 40 ml was taken from the culture and centrifuged (10,000 × *g*, 5 min, 5°C). The cells were resuspended in the same volume of phosphate buffer, 50 mM, pH 6.8. After sonication of the cells (three pulses of 30 s each at 18 μm amplitude with interspersed periods of cooling), the pellet from a low-speed centrifugation (3,000 × *g*, 5 min, 5°C) was obtained. The supernatant was centrifuged again at high speed (40,000 × *g*, 30 min, 5°C), and the pellet was collected. Both pellets were suspended in equal volumes of sample buffer (30.5 mM Tris hydrochloride, 1% SDS, 5% β-mercaptoethanol) and boiled for 5 min. Solubilized proteins were fractionated by SDS-PAGE in 8% acrylamide gels by the method of Laemmli and Favre (15). Proteins were detected by Coomassie brilliant blue R staining of the gel. The intensity of the bands was estimated by densitometric analysis of the stained gel with the aid of a Joyce Loebl Chromoscan 3 integrating densitometer.

RESULTS

Construction of the *lacZ-pbpB* gene fusion. The plasmid vector pDIA9204 (18), a derivative of pBR322, was used in the construction of the *lacZ-pbpB* gene fusion. pDIA9204 carries a translational *cro-lacZ* fusion downstream from the lambda Pr promoter and the lambda *cro* ribosome-binding site and also carries the structural gene *c*I857, coding for a thermosensitive form of the lambda *c*I repressor. When the temperature is shifted up to 42°C, the *c*I857 repressor is inactivated, allowing expression of genes downstream from Pr, leading to high-level overproduction of the corresponding proteins (50). pDIA9204 has three target sites for the restriction endonuclease *Mlu*I, all of them within the β-galactosidase structural gene. pDIAM14, a derivative of pDIA9204 with a unique *Mlu*I restriction site at the nucleotide position corresponding to amino acids 438 and 439 of β-galactosidase, was constructed by in vitro digestion of pDIA9204 with *Mlu*I, followed by religation with T4 DNA

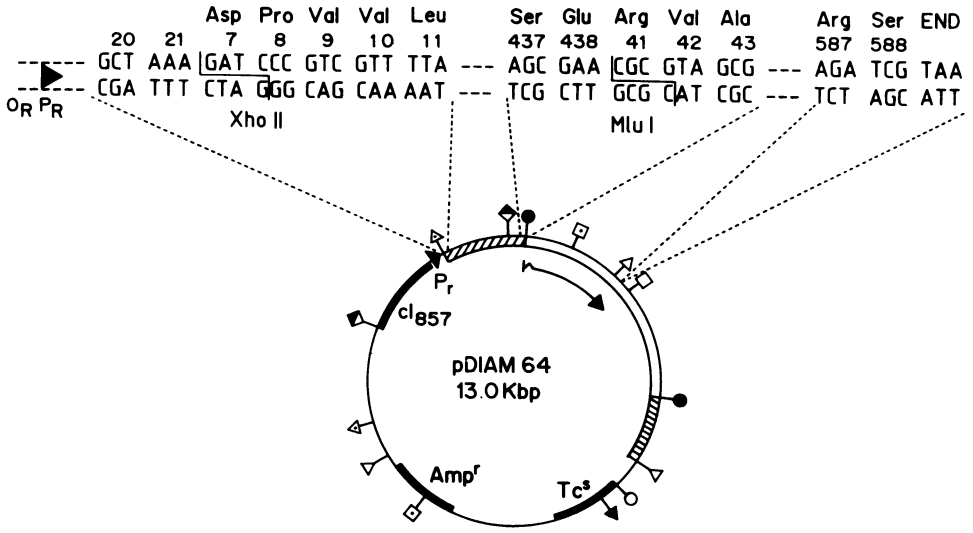

FIG. 1. Genetic organization of the *lacZ-pbpB* gene fusion in pDIAM64. pDIAM14 was obtained from pDIA9204 by *Mlu*I digestion and religation. The *pbpB* structural gene was obtained as a 7.2-kbp *Eco*RI-*Hin*dIII fragment from the Clarke and Carbon plasmid pLC26-6 (4) and was inserted into the multiple cloning region of pUC9 (42). A 3.5-kbp *Mlu*I fragment containing part of the *pbpB* gene was inserted into the single *Mlu*I site of pDIAM14. The resulting plasmid (pDIAM64) is drawn showing the sequences across the *cro-lacZ* and *lacZ-pbpB* junctions. The positions of sites for endonucleases *Pst*I (⊡), *Hin*dIII (○), *Sal*I (▲), *Hgi*AI (□), *Eco*RI (▽), *Kpn*I (△), *Mlu*I (●), *Cla*I (◇), and *Xho*II (◬) are shown. Amino acids 9 to 438 were from the *lacZ* gene, and residues 41 to 588 were from the *pbpB* gene, as deduced from the published sequences for *pbpB* (27) and *lacZ* (12). Amino acids 20 and 21 and 7 and 8 correspond to the *cro* gene and polylinker region, respectively.

ligase. pDIAM14 differs from pDIA9204 in the elimination of a 1,205-bp *Mlu*I internal fragment of the *lacZ* gene, which abolishes the enzymatic activity of β-galactosidase. *pbpB*, the gene coding for Pbp3, contains an *Mlu*I restriction site located in a position corresponding to amino acids 40 and 41 of Pbp3, which was used to construct an in-phase fusion with the defective *lacZ* gene cloned in pDIAM14 (Fig. 1).

The coding region of the *pbpB* gene was obtained by cloning a 7.2-kbp *Hin*dIII-*Eco*RI fragment from pLC26-6 (from the collection of Clarke and Carbon [4]) between the *Hin*dIII and *Eco*RI target sites in the polylinker region of pUC9 to produce an intermediate plasmid (pHE5).

A 3.5-kpb *Mlu*I fragment, encoding the 3′-terminal 1,644 bp of *pbpB*, was obtained from pHE5 and inserted in the *Mlu*I site of pDIAM14 (Fig. 1). After transformation of *E. coli* JM83 with the ligation mixture and subsequent screening, we selected two plasmids containing the fragment of the coding region of *pbpB*, pDIAM62 and pDIAM64, which differed in the orientation of the insert.

Expression of the *lacZ-pbpB* gene fusion cloned in pDIAM62 and pDIAM64. When the temperature is shifted up to 42°C, JM83(pDIAM64) should express at a high level a protein of M_r 110,000, comprising the first 21 N-terminal amino acids of the Cro protein, the 430 N-terminal amino acids of β-galactosidase, and the 548 C-terminal amino acids of Pbp3. This protein should not be expressed under identical conditions by JM83(pDIAM62). Figure 2 shows the results of an experiment in which the total proteins synthesized by both JM83(pDIAM62) and JM83(pDIAM64) strains were analyzed by SDS-PAGE, first at 30°C and then after a shift to 42°C. A very prominent protein with an apparent M_r of 110,000, similar to that predicted for the fusion protein, was found in JM83(pDIAM64) after incubation at 42°C. This protein was not detectable in JM83(pDIAM62) or in any of the strains at 30°C, when the *c*I857 repressor was active. Consequently, we assumed that the protein was the product of the expression of the *lacZ-pbpB* gene fusion. The hybrid protein is referred to as p110.

The level of expression of p110 in cells of JM83(pDIAM64) incubated at 42°C was very high, as expected for a protein under the control of the lambda Pr promoter (50). The amount of fusion protein accumulated after 90 min of incu-

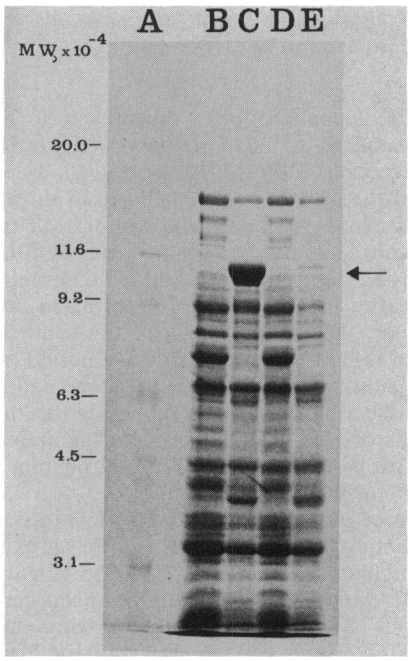

FIG. 2. Detection and identification of the p110 protein. Cells of JM83(pDIAM64) (B, C) and JM83(pDIAM62) (D, E) were grown in L broth at 30°C to OD_{550} of 0.5. The culture for each strain was divided in half, and the temperature of one half (C, E) was shifted to 42°C, as described in the text. After centrifugation, sonication, and boiling in SDS, 80-μl samples were fractionated by SDS-PAGE (8% acrylamide gel) and stained with Coomassie blue. The arrow shows the position of the p110 fusion protein.

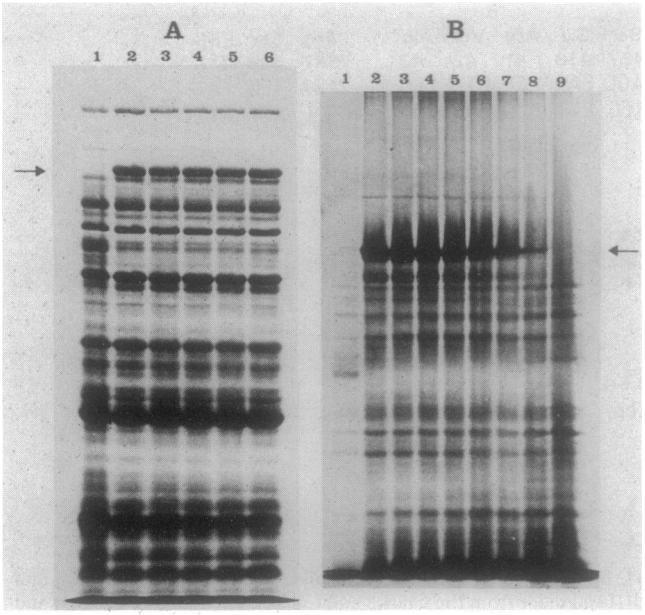

FIG. 3. Stability of p110 in cell lysates and in induced cells after a temperature shiftdown. (A) JM83(pDIAM64) grown in L broth at 30°C and then shifted up to 42°C for 15 min. Cells were treated as described in the text, sonicated, and incubated at 37°C for 0 h (lane 2), 1 h (lane 3), 2 h (lane 4), 3 h (lane 5), or 4 h (lane 6), after which they were boiled in 2% SDS, fractionaed by SDS-PAGE on a 10% acrylamide gel, and stained with Coomassie blue together with a sample of a lysate from uninduced cells (lane 1). (B) JM83 (pDIAM64) grown in L broth at 30°C and shifted to 42°C for 15 min. After sonication and centrifugation (50,000 × *g*, 60 min), the pellets were boiled in 2% SDS, fractionated by SDS-PAGE on an 8% acrylamide gel, and stained with Coomassie blue. Lanes: 1, uninduced control cells; 2, induction for 15 min at 42°C; 3 to 9, induction for 10, 20, 30, 40, 60, or 90 min at 30°C, respectively. The arrows indicate the position of p110.

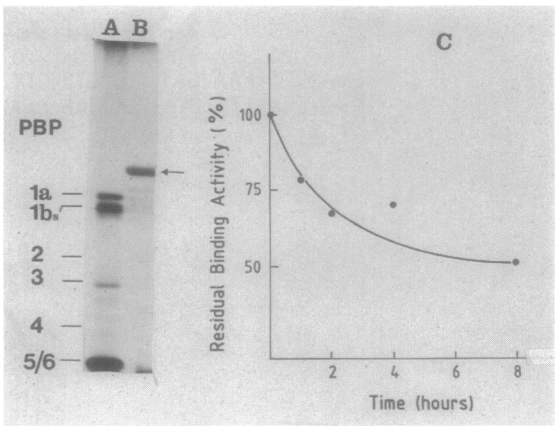

FIG. 4. Stability of the ^{3}H-ampicillin-binding activity of p110. A culture of JM83(pDIAM64) growing exponentially at 30°C in L broth (lane A) was heated to 42°C at an OD_{550} of 0.5 and incubated for 15 min to induce the production of p110 (lane B). Cells were harvested and prepared for detection of Pbps as described in the text. The particulate fraction was transferred to a water bath at 37°C, and 4-μl samples were taken periodically and assayed for the binding of ^{3}H-ampicillin as described in the text. Autofluorograms show the patterns of binding of ^{3}H-ampicillin to particulate fractions from uninduced cells (A) and induced cells (B). (C) Decay of the binding activity of p110 with preincubation time. Quantification was performed by densitometric analysis of the corresponding autofluorograms. The arrow indicates the position of p110.

bation at 42°C accounted for as much as 10 to 12% of total cell protein and 85 to 90% of the protein in the purified particulate fraction of the cell.

Stability of the p110 hybrid protein. p110 showed remarkable stability. In a whole-cell extract of JM83(pDIAM64), obtained by ultrasonic disruption of the cells after an induction period of 15 min at 42°C, the p110 protein remained undegraded after incubation at 37°C for up to 240 min (Fig. 3A). However, when intact cells of the same strain were shifted down to 30°C after an induction period of 15 min at 42°C, the fusion protein was quickly degraded by the cell (Fig. 3B), indicating that it was recognized as an abnormal protein. Cells of JM83(pDIAM64) growing actively at 42°C reached a steady-state level of p110 after 60 min, accounting for 12 to 15% of the total cell proteins.

Interaction of p110 with β-lactam antibiotics. The penicillin-binding activity of p110 was tested in an experiment in which a particulate fraction of induced (15 min at 42°C) cells of JM83(pDIAM64), obtained by high-speed centrifugation (50,000 × *g*, 60 min) of sonically disrupted cells, was incubated in the presence of ^{3}H-ampicillin for 20 min at 37°C, and the radioactively labeled proteins were detected by autofluorography. The results clearly indicated that p110 was able to bind ^{3}H-ampicillin (Fig. 4B). Furthermore, the binding activity of p110 was remarkably stable. Figure 4C shows the results of a similar assay in which the particulate fractions used in the binding assay were incubated at 37°C for increasingly longer periods prior to addition of the radioactive antibiotic. After 8 h of incubation, the residual binding activity of p110 was still more than 50% of the initial level (Fig. 4C). However, the affinity of p110 to ^{3}H-ampicillin was lower than that of Pbp3. The estimated half-saturating concentrations were 25 to 30 μg/ml (60 to 70 μM) and 2.8 μg/ml (7 μM) for p110 and Pbp3, respectively (data not shown). The differences in the intensity of the bands corresponding to Pbp3 (Fig. 4A) and p110 (Fig. 4B) are due to higher expression of p110 and not to an increased affinity of the fusion protein.

Morphological alterations caused by overproduction of p110 in JM83(pDIAM64). Observation of cells of JM83(pDIAM64) incubated at 42°C for 90 min under the phase-contrast microscope indicated that overproduction of p110 drastically modified the morphology of the cells, which appeared as filaments with one or more refringent granules (Fig. 5A). Observation under the electron microscope of negatively stained (Fig. 5C) and thin sections (Fig. 5B) of cells from the same culture showed amorphous intracellular formations at the poles or the center of the cell, positions corresponding to the location of the refringent granules observed by phase-contrast microscopy. The absence of such formations in cells of JM83(pDIAM62) treated in parallel experiments (Fig. 5D) suggests that they represent cytoplasmic aggregates of the fusion protein. During the staining procedures, negatively stained cells of JM83(pDIAM64) lysed, and when observed under the electron microscope (Fig. 5C), they showed the granules as very dense structures tightly attached to the cell envelope.

The morphology (except grain distribution) of induced cells of JM83(pDIAM64) under the phase-contrast microscope was similar to that reported for other protein-overproducing strains (24, 31, 43, 46). However, we did not find any evidence for the accumulation of p110 in the form of membrane-coated inclusions, such as those found in thin sections of ATP synthase-overproducing cells (43), but the granules appeared to be tightly associated with the membrane (Fig. 5B and C).

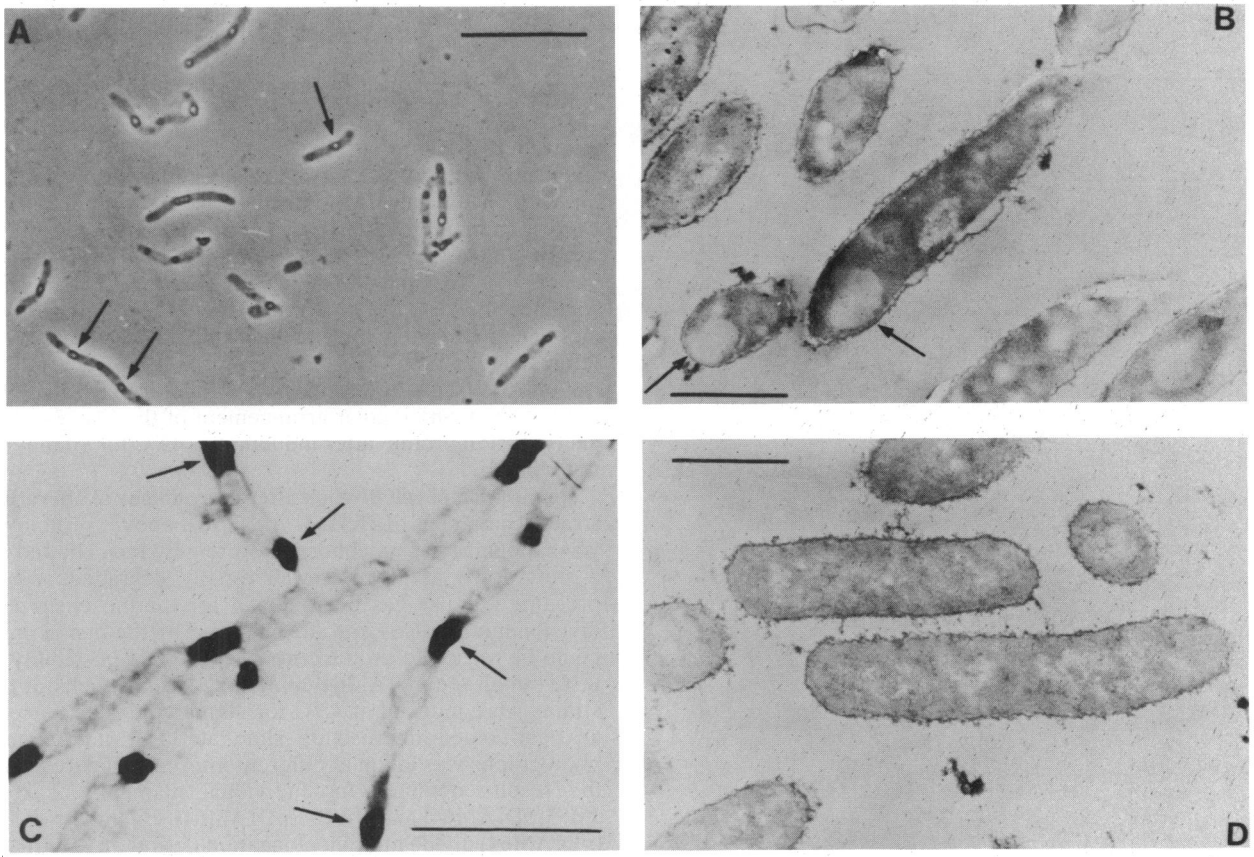

FIG. 5. Morphological alterations caused by the overproduction of p110 in JM83(pDIAM64). Cells of JM83(pDIAM62) and JM83(pDIAM64) growing in L broth at 30°C were induced at 42°C for 90 min. Cells were harvested and prepared for microscopic examination as described in the text. JM83(pDIAM64) phase-constrast microscope (A), thin-section (B) and negative-staining (C), electron microscope. JM83(pDIAM62) thin-section (D), electron microscope. The arrows indicate the location of refringent granules. Bars, 10 μm (A); 1 μm (B, C, D).

Interaction of p110 with the cell envelope. Initial experiments to determine the cellular location of p110 indicated that the fusion protein could be quantitatively recovered in the particulate fraction after centrifugation of 90-min-induced disrupted cells, suggesting that it might be a membrane-associated protein or, alternatively, that p110 was accumulated as fast-sedimenting protein aggregates. To obtain further information on this point, a culture of JM83(pDIAM64) was induced for different time periods, and the cells were disrupted. Pellets from low- and high-speed centrifugations were then analyzed for protein content (Fig. 6). After a short induction time (less than 15 min), the fusion protein was recovered essentially in the cell envelope fraction (40,000 × *g*, 30 min), but after a longer induction time the aggregates had a high density and p110 was found in the particulate fraction (3,000 × *g*, 5 min).

After 15 min of induction at 42°C, production of p110 in JM83(pDIAM64) accounted for about 15% of the maximum level (maximum levels were found in 60-min-induced cells). When the fusion protein was produced at this level, grains were formed and morphological alterations appeared, but p110 was essentially recovered in the cell envelope fraction. Attempts were made to solubilize p110 from this cell envelope fraction. These included the use of 0.5% (wt/vol) Sarkosyl, 0.5% (wt/vol) Triton X-100, 0.5% (wt/vol) Nonidet P-40, 0.5 M NaCl, and 5 mM EDTA. The first three treatments solubilized p110, but the last two did not. All these treatments were unsuccessful when applied to isolated granules purified from induced cells, and in this case only 8 M urea and 2% SDS solubilized the hybrid protein (data not shown).

Expression of plasmid pDIAM64 in *E. coli* carrying thermosensitive alleles of cell division genes. As described above, initial observations of cells of JM83(pDIAM64) under the phase-contrast microscope showed that the overproduction of p110 drastically altered the morphology of the cells, which appeared as filaments with one or more refringent granules (Fig. 5A). A more detailed inspection of the photographs revealed that the aggregates of the protein were localized in precise sites of the cell, corresponding to pre- and postseptal positions (Fig. 7). In order to see whether this regular arrangement was altered in mutants carrying mutations in genes involved in cell division, cells of *E. coli* strains carrying thermosensitive alleles of morphogenes, D3 (*ftsA*), SP63 and SP33 (*ftsI* or *pbpB*), PAT84 and GD113 (*ftsZ*), and TOE1 (*ftsQ*), or related cell division genes, GC2467 (*sfiA*) and AD12 (*lon ftsZ*), were transformed with the plasmid pDIAM64. A culture of a transformant from each strain was induced at 42°C for 90 min and prepared for optical microscopy observation, and a set of photographs was obtained. The position of the granules was measured in at least 400 cells for each strain as described in Materials and Methods. Figure 7 shows the pattern of grain distribution found in strains JM83(pDIAM64) and PAT84(pDIAM64). The grains

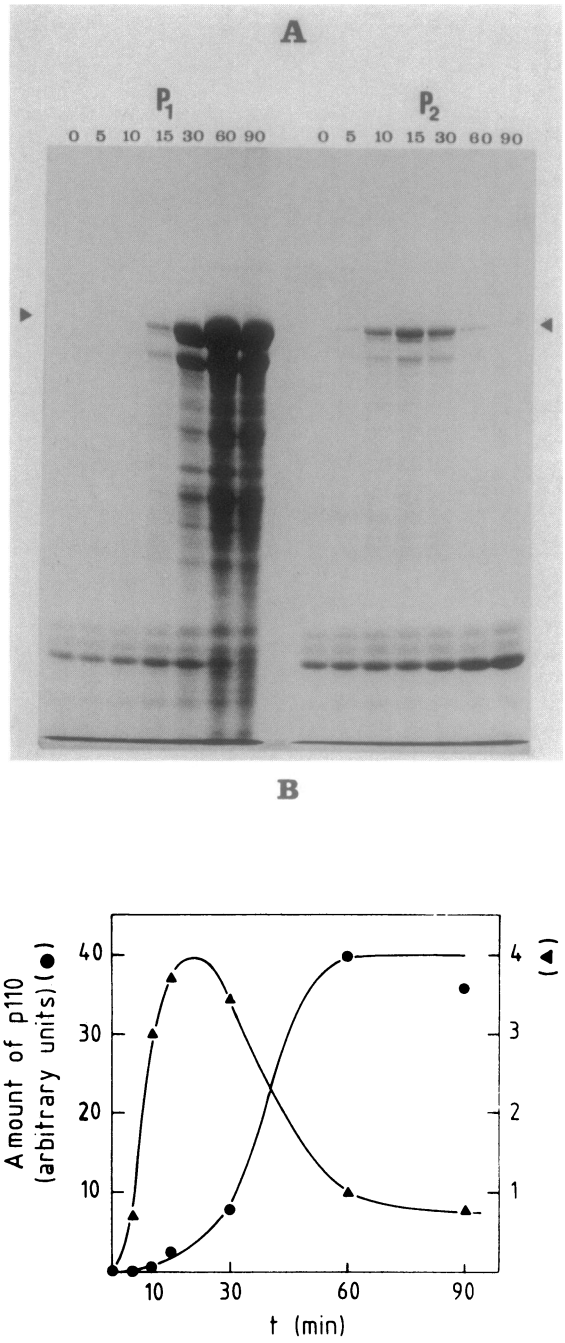

FIG. 6. Kinetics of induction of p110 in particulate and cell envelope fractions of JM83(pDIAM64). (A) A culture of JM83 (pDIAM64) growing in L broth at 30°C was transferred to 42°C at an OD_{550} of 0.3. A 40-ml portion was separated from the culture after 0 (lane 0), 5 (lane 5), 10 (lane 10), 15 (lane 15), 30 (lane 30), 60 (lane 60), and 90 (lane 90) min of incubation. Cells were collected, washed in phosphate buffer, and sonicated as described in Materials and Methods. Sonicated whole-cell extract was centrifuged at low speed (3,000 × *g*, 5 min, 5°C), and a particulate fraction (P1) was obtained. The supernatant was again centrifuged at high speed (40,000 × *g*, 30 min, 5°C), and a second pellet of cell envelopes (P2) was obtained. Both pellets were solubilized in sample buffer and boiled for 5 min. Proteins were separated by SDS-PAGE (8% acrylamide gel) and stained with Coomassie blue. Arrowheads indicate the position of p110. (B) Stained bands were quantified directly from the gel with a Joyce Loebl Chromoscan 3 integrating densitometer. The integrated area (arbitrary units) caused by p110 found in the particulate (●) and cell envelope (▲) fractions was plotted versus induction time.

were localized preferentially in a postseptal position in strain JM83(pDIAM64) (Fig. 7A and C), but some grains were present in the center of the cell corresponding to the actual septum. Strains D3(pDIAM64), SP63(pDIAM64), SP33 (pDIAM64), TOE1(pDIAM64) and GC2467(pDIAM64) showed a similar pattern, with an approximately equal number of grains in septal and postseptal positions (data not shown). In longer cells (>8 μm), two new grain positions corresponding to one-quarter and three-quarters of total cell length appeared. No regular arrangement was found in strains PAT84(pDIAM64) (Fig. 7B and D), GD113 (pDIAM64), or AD12(pDIAM64), and cell division was stopped immediately after the shift up to 42°C in these three strains.

Control strains JM83(pDIA9204) and PAT84(pDIA9204) did not show any regular arrangement of the Cro–β-galactosidase fusion protein after induction at 42°C for 90 min (data not shown).

Reinitiation of cell division after morphological alteration in strains containing pDIAM64. As shown above, protein p110 was recognized as an abnormal protein by the cell, and after a shift down to 30°C, it was rapidly degraded. We can presume that, as has been shown for thermosensitive cell division mutants (11), the site occupied by the fusion protein could be reused to produce new septa. This possibility was tested in all the pDIAM64-containing strains. Cells of these strains were induced at 42°C for 30 min and then placed on an agar-coated microscope slide at 30°C, and a set of photographs was taken at different intervals. Figure 8 shows the results obtained for individual filaments of strains JM83(pDIAM64) and PAT84(pDIAM64). Filaments of JM83 (pDIAM64) divide after an elongation period of 30 min at the predicted sites marked by the granule positions. However, filaments of strain PAT84(pDIAM64) elongated but did not divide after the shift down to 30°C during a time equivalent to three doubling times, and after this period, septa appeared at the ends of the elongated filaments where no grains had formed during induction.

DISCUSSION

We have constructed a fusion hybrid gene, under the control of the lambda Pr promoter, coding for a protein (p110) consisting of the 430 N-terminal amino acids of β-galactosidase and the 458 C-terminal amino acids of Pbp3. A similar construction that codes for a small and very unstable fusion protein has been described by Hedge and Spratt (13). Thus, the p110 protein is the first Pbp-derived hybrid protein that retains stable binding ability. When wild-type Pbp3 is solubilized from the membrane, it rapidly loses its β-lactam-binding ability (26). p110, however, retained its ^{3}H-ampicillin-binding ability in the isolated inclusion bodies after being incubated at 37°C for 8 h. This stabilization could then be attributed to interactions among the molecules of p110 in the aggregate, creating a hydrophobic environment resembling that of the cytoplasmic membrane. In contrast, the protein produced by a 15-min period of induction at 42°C was rapidly degraded in vivo after the temperature was shifted down to 30°C.

Removal of the first 40 N-terminal amino acids in Pbp3 presumably prevents (27; L. R. Desviat and J. A. Ayala, manuscript in preparation) both processing of the protein by the *E. coli* signal peptidases and membrane insertion. We then constructed the pDIAM64 plasmid, expecting the fusion protein to be localized in the cytoplasm because it has a hydrophilic portion of a soluble protein (β-galactosidase)

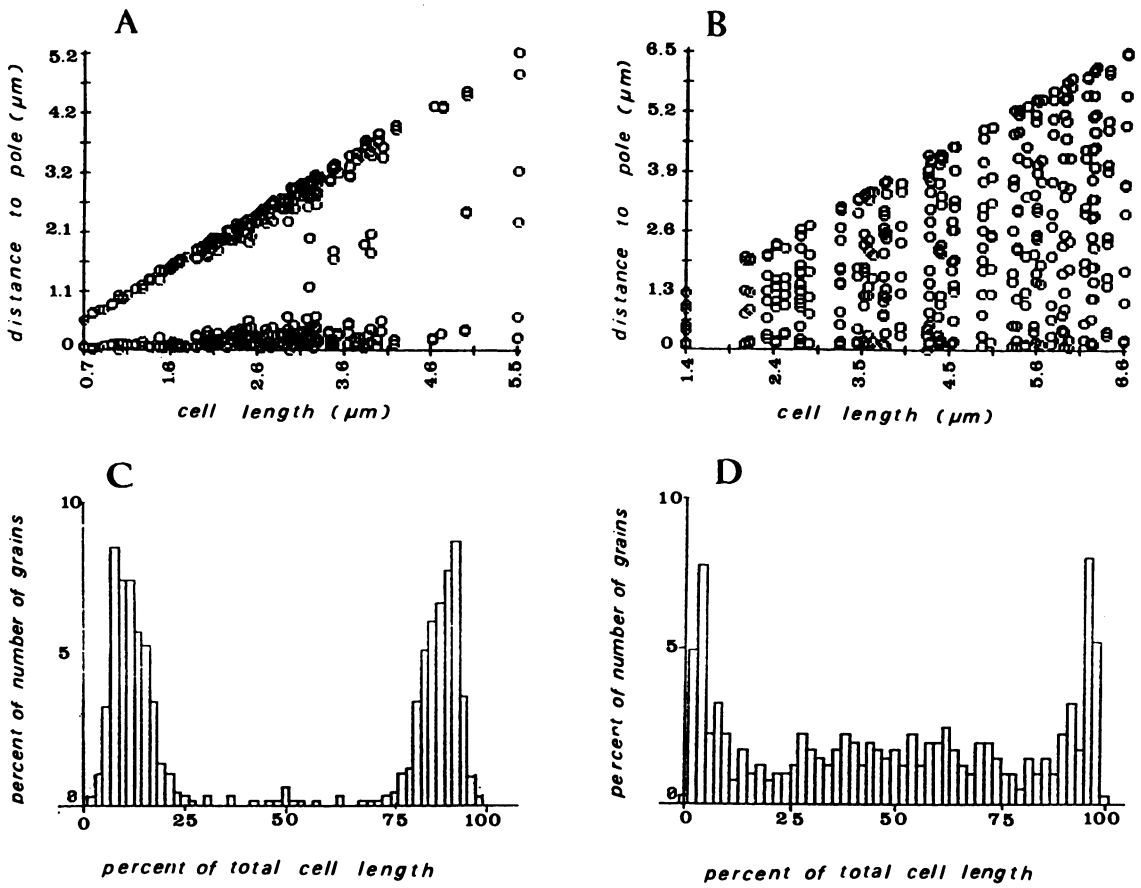

FIG. 7. Patterns of grain distribution of p110. Exponentially growing cultures of JM83(pDIAM64) (A, C) and PAT84(pDIAM64) (B, D) at 30°C were transferred to 42°C for 90 min. Cells were fixed and prepared for optical microscopy observation, and a series of photographs was obtained. Cell length and granule-to-pole distances for each individual cell were measured from positive prints of negatives amplified fivefold, with the help of the devices described in Materials and Methods. An average of 400 cells were measured for each strain. Data are presented as a direct representation of the granule-to-pole versus cell length distances (A, B). The cell length of every individual cell was made uniform, and the distance to pole for every grain was accordingly corrected. The total number of grains found at each relative position in the cell was calculated and is displayed (C, D) as a percentage of the total grains measured for each strain.

fused to a membrane protein (Pbp3) without the signal peptide-like sequence. However, when the fusion protein was produced at levels at which no aggregates of high density were formed (Fig. 6), p110 was recovered in the cell envelope fraction. This result suggests a strong interaction between the protein and the cell envelope. Furthermore, the interaction seems to be hydrophobic, because it was resistant to the action of ionic strength and chelating agents, although the protein could be solubilized by detergents but at lower concentrations than those needed to solubilize inner membrane proteins.

Production of p110 at levels above 3% of total cell protein leds to the accumulation of the protein as dense aggregates, similar to those found for other overproduced proteins (24, 31, 38, 43, 46). These aggregates were tightly bound to the cell envelope and found in localized septal positions. This regular arrangement of the aggregates indicates that the first molecular interaction might take place at defined positions, corresponding to pre- and postseptal sites. The absence of any regular arrangement in the control strain JM83 (pDIA9204) argues in favor of this explanation. The arrangement of granules at septal positions could be explained if the synthesis of the protein occurred at this special location in membrane-attached ribosomes, as has been suggested for outer membrane proteins (30, 49). But this would not be the case, since p110 lacks the signal peptide-like structure. Alternatively, a molecular interaction with some component of the membrane could take place, prefixing a point of aggregation for the next-produced p110 molecules. The absence of regular distribution in the strains carrying an *ftsZ* mutant allele argues in favor of the latter possibility and suggests that FtsZ could play a role in the interaction of p110 with the inner membrane and localization of the hybrid protein in the septal position. In this sense, it was suggested recently (J. Barondess, J. Silverman, and J. Beckwith. Abstr. EMBO Workshop: Molecular Basis of Bacterial Growth and Division, Segovia, Spain, 1987, p. 116) that FtsZ is an integral membrane protein with a single transmembrane domain and the N-terminal domain extending into the cytoplasm. So FtsZ itself might be the membrane component interacting with p110. We presume that this localization mechanism for p110 is brought about by the presence of domains in the hybrid protein (actually present in the wild-type Pbp3) that retain the ability to interact with the membrane components, and consequently the same mechanism of localization for Pbp3 will act in a wild-type strain. The actual interaction between Pbp3 and other proteins involved in cell division is currently under investigation in our labo-

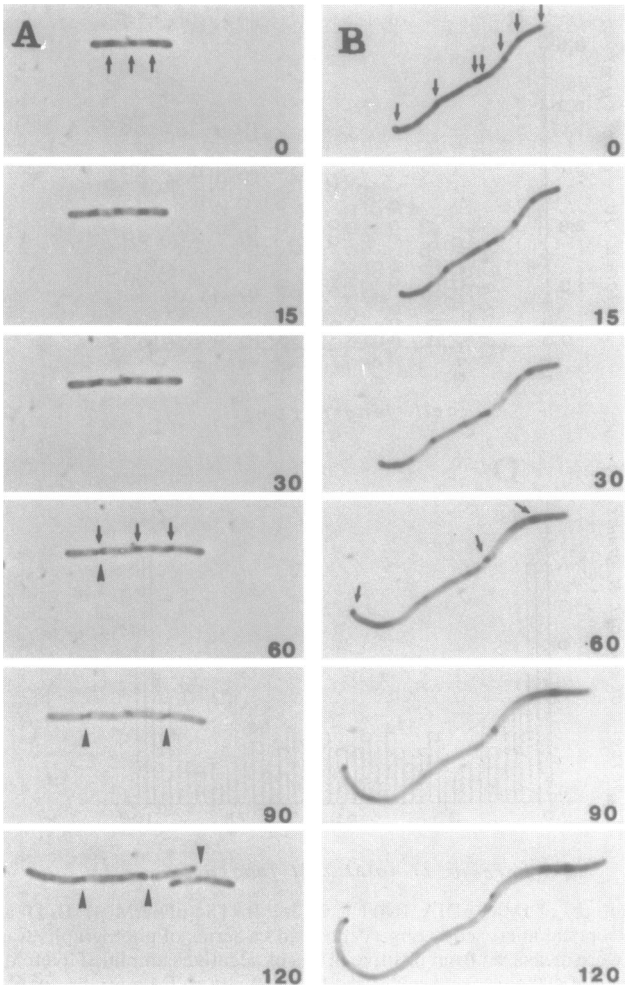

FIG. 8. Reinitiation of cell division after morphological alteration in strains containing pDIAM64. Exponentially growing cultures of JM83(pDIAM64) and PAT84(pDIAM64) (B) were induced at 42°C for 30 min and then placed on agar-coated microscope slides at 30°C. Individual filaments of both strains were analyzed at 0, 15, 30, 60, 90, and 120 min after the temperature shiftdown. The arrows show the positions of granules in the filaments, and the arrowheads show the appearance of septa.

ratory. We are analyzing the environments of Pbp3 in the cell by using bifunctional compounds, i.e., a β-lactam and a photoreactive group separated by arms of different lengths, such as those described by Rodriguez-Tebar et al. (29).

As the synthesis of the hybrid protein occurred after a temperature shift up to 42°C and this increase induced, at the same time, the synthesis of heat shock proteins such as the Lon protease, it could be argued that there are factors impairing grain localization in *ftsZ* mutants other than the presence of a mutant FtsZ protein. The increased level of Lon protease would produce a decrease in the levels of SfiA, which is an inhibitor of FtsZ. Consequently, the number of potential inhibitory SfiA-FtsZ complexes would be greatly reduced. If the altered localization of granules were due to this reduced SfiA-FtsZ interaction, then differences in grain distribution should have been observed between strains GD113(pDIAM64) (*ftsZ*) and AD12(pDIAM64) (*ftsZ lon*). However, an equivalent irregular distribution of grains was found for the last two strains, suggesting that the *ftsZ* mutation is responsible for this failure in grain localization. Moreover, the pattern of grain distribution in strains JM83(pDIAM64) (*sfiA*$^+$) and GC2467(pDIAM64) (*sfiA*::Tn*5*) corresponded to the septal positions, so a decreased level or absence of the SfiA protein does not impair the regular localization of p110 grains.

It has been suggested that the level or activity of FtsZ is rate limiting for cell division (44). This could take place, as suggested by the present study and discussed above, by prefixing some components of the cellular machinery required for cell division (namely Pbp3) to the preseptal membrane sites. If this took place, then the defect in the mutant FtsZ protein could cause a failure to interact or a false interaction with some of these components, impairing correct localization and consequently making this site unfit for cell division. This was also suggested by the finding that while induced cells of JM83(pDIAM64) divided after a shiftdown to 30°C, at the positions defined by the grains (Fig. 8), the induced pDIAM64-containing *ftsZ* mutant did not divide at any site after the shiftdown during a time equivalent to three doubling times. However, thermosensitive defects in the FtsZ84 mutant protein were reversible after the temperature shiftdown, allowing septum formation without de novo protein synthesis in the PAT84 strain (11); then, absence of cell division in the pDIAM64-containing *ftsZ* mutant after the shiftdown to 30°C could be explained in a simple way if the rate of degradation of the fusion protein were reduced in this strain, but this was not the case, because grains disappeared even faster in PAT84 (pDIAM64). Alternatively, we could explain the behavior of this strain by an irreversible false interaction between p110 and the FtsZ84 mutant protein that not only blocks actual septum localization and leaves the potential preseptal site unfit for cell division, but also impairs any new septum localization and cell division. The model proposed by Cook et al. (5) for cell division in gram-negative bacteria suggests that a preexisting site of cell division generates future division sites by a replication-displacement mechanism. Our last conclusion is in accord with this model, and it lends support to the idea that the FtsZ protein could take on an important role in the generation of the division sites.

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