Temperature Shift Experiments with an \textit{ftsZ84}(Ts) Strain Reveal Rapid Dynamics of FtsZ Localization and Indicate that the Z Ring Is Required throughout Septation and Cannot Reoccupy Division Sites Once Constriction Has Initiated

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FtsZ is an essential division protein in bacteria that functions by forming a ring at midcell that mediates septation. To further study the function of the Z ring the effect of a temperature-sensitive mutation, \textit{ftsZ84}(Ts), on ring dynamics and septal progression was examined. Shifting a strain carrying an \textit{ftsZ84}(Ts) mutation to the nonpermissive temperature led to loss of Z rings within 1 min. Septal ingrowth was immediately inhibited, and sharply demarcated septa, present at the time of the shift, were gradually replaced by blunted septa. These results indicate that the Z ring is required throughout septation. Shifting filaments to permissive temperature led to a rapid localization of FtsZ84 at regular intervals. Included in these localization events were complete and partial rings as well as spots, although some of these eventually aborted. These results reveal the rapid dynamics of FtsZ localization and indicate that nucleation sites are formed in the absence of FtsZ function. Interestingly, Z rings could not reform at division sites that were constricted although they could reform at sites that had not begun constriction.

An early event in bacterial cell division is the formation of a Z ring at the future division site (1, 6). Once formed, the Z ring remains at the leading edge of the invaginating septum throughout septation. The functions of the Z ring may be to provide energy for invagination of the septum (4) and to recruit FtsA and possibly other proteins to the division site (2, 3, 13). The recruitment of FtsA has been documented by immunostaining of fixed cells (1, 4, 6) and by using FtsA tagged by green fluorescent protein (17). ZipA binds to FtsZ, and its appearance at the division site coincides with that of FtsZ (13). FtsN recruitment has also been documented by immunostaining, and its appearance is delayed significantly after that of FtsZ, ZipA, and FtsA (2).

Determination of the structure of the Z ring and of how the Z ring is positioned at the division site are two important questions. It has been suggested that the Z ring is positioned by a nucleation site whose function is cell cycle regulated (6, 15). In this model the nucleation site positions the Z ring by functioning to initiate polymerization of FtsZ into the Z ring. In vitro work with FtsZ has shown that it can undergo GTP-dependent polymerization to form protofilaments which further assemble into sheets or tube-like structures (12, 18). The most likely structure of the Z ring is a flat sheet of protofilaments that run perpendicular to the long axis of the cell (12).

The study of two temperature-sensitive \textit{ftsZ} mutations, \textit{ftsZ84(Ts)} (16, 22) and \textit{ftsZ26(Ts)} (7), has provided useful information about FtsZ localization. The failure of strains carrying either of these mutations to divide under nonpermissive conditions coincides with a failure of the mutant proteins to localize (1). At the permissive temperature FtsZ26 assembles into spirals which lead to spiral-shaped septa, providing strong evidence that the FtsZ cytoskeletal element determines septal morphology (4). The fact that in small cells these spirals are at midcell suggests that FtsZ26 still recognizes the nucleation site but that the protofilaments either are structurally altered or have an altered interaction with membrane components such as the nucleation site to produce the spirals. Spirals have also been seen using FtsZ that is tagged with GFP (17). These spirals do not appear to lead to division, which is likely due either to the inhibitory effect of the fusion protein or to an imbalance in the division proteins.

Previously (1), it was shown that shifting cultures of strains carrying either the \textit{ftsZ84(Ts)} or the \textit{ftsZ26(Ts)} mutation to the nonpermissive temperature leads to a loss of FtsZ localization as long filaments lacking Z rings are formed. We wondered if Z rings fell apart upon the temperature shift or if Z rings already formed were able to complete septation. Previously, investigators have reported some residual division after a temperature shift, suggesting that at least some Z rings that exist at the permissive temperature are able to complete division or that FtsZ is not required throughout septation (22, 23, 25). We also wondered how quickly Z rings form upon shifting filaments from nonpermissive to permissive temperatures and about their position in the filament, since potential division sites (i.e., nucleation sites) could be formed during the period at the nonpermissive temperature. In addition, if Z rings fall apart at the nonpermissive temperature, it is of interest to determine if they can reform at these sites.

**MATERIALS AND METHODS**

**Bacterial strain and medium.** The \textit{Escherichia coli} K-12 strain MCZ84 \textit{[lecT::Tn10 ftsZ84(Ts)]} was used in this study and has been described previously (1, 9). LB broth was used as a rich medium (6).

**Immunofluorescence microscopy.** Cells were processed for immunofluorescence microscopy essentially as described previously (1). Samples of cultures growing exponentially at 30 or 42°C were added to test tubes containing a fixative solution (final concentrations: 2.6% [vol/vol] glutaraldehyde, 0.04% [vol/vol]...
paraformaldehyde, and 32.25 mM Na3PO4). After incubation for 10 min at room temperature and for 50 min on ice, the cells were collected and washed three times with phosphate-buffered saline (PBS) (10 mM Na2HPO4 [pH 7.4], 150 mM NaCl, 15 mM KCl). Washed cells were resuspended in GTE (50 mM glucose, 10 mM Tris [pH 7.5], and 1 mM each of MgCl2 and MnCl2) in which 20% ethanol (vol/vol) was added. The final concentration of lysozyme depended upon the period of time cells were stored in GTE (up to 1 week at 4°C) but was in the range of 4 to 20 μg/ml. The cells were immediately transferred to the wells of a multwell fluorescence microscope slide (ICN Pharmaceuticals) that was pretreated with poly-L-lysine (0.1%, wt/vol). After 2 min the liquid was aspirated, and each well was washed twice with PBS and allowed to air dry. The cells were rehydrated with PBS for 4 min after which a blocking solution (2% [wt/vol] bovine serum albumin in PBS) was added, and incubation was continued for 10 to 15 min. The blocking solution was replaced with a 1:250 dilution (in the blocking solution) of an affinity-purified rabbit polyclonal antibody to E. coli FtsZ or FtsA (3, 6). Incubation was continued at room temperature for several hours or overnight at 4°C. Each well was washed 10 times with PBS before a secondary antibody (10 μl of a 1:100 dilution of a Cy3-conjugated anti-rabbit immunoglobulin G antibody [Jackson Immunoresearch]) was added. Incubation was continued for 1 to 3 h, and then the cells were washed 10 times with PBS and once with SlowFade equilibration buffer (Molecular Probes) for 5 min before being mounted in SlowFade (Molecular Probes). The slides were stored at −20°C until microscopic observation. Cells were photomicrographed with a Nikon Optiphot fluorescence microscope equipped with a 100× objective and a Contax 167MT camera with Kodak Ektachrome 400 film. Fluorescence was observed by using a 510- to 560-nm excitation filter and a 590-nm barrier filter (G-2A; Nikon). Images were scanned from slides with a Nikon LS-3510 AF slide scanner and imported into Adobe Photoshop software.

Fate of Z rings and septa in MCZ84 at the nonpermissive temperature. A culture of MCZ84 growing exponentially at 30°C in L broth was shifted to 42°C. The temperature shift was carried out by diluting the culture at 30°C 1:4 into prewarmed broth at 42°C to ensure rapid temperature equilibration. Samples were taken at various times after the shift and fixed and processed for immunofluorescence microscopy to visualize the location of FtsZ as described previously (1). Figure 1 compares the position of Z rings in MCZ84 cells at the nonpermissive temperature, although FtsZ was not present at the cell quarters. In such cells, it appears that a Z84 ring at midcell aborted or failed to form, resulting in larger cells. Since these cells contained a central constriction this suggests that the ring aborted. Thus, one can conclude that the FtsZ84 protein is properly positioned to the division site at permissive temperature, although it appears that a Z84 ring at midcell aborted or failed to form, and then decreased about 20% over the next 10 to 15 min before any cell division had occurred (data not shown). This result indicates that 20% of these early localization events were aborted.

Some cells examined 2 min after the shift to 42°C contained sharply demarcated septa, as indicated in the photomicrographs in Fig. 2A. Such septa appeared identical to septa in control cultures, indicating that the temperature shift had little effect on the morphology of nascent septa (data not shown). In contrast, cells that had been shifted to 42°C for longer times (10 min) lacked sharply demarcated septa, as shown in the examples in Fig. 2B. Instead, many such cells contained blunt indentations at midcell, suggesting that they arose from the well-demarcated septa seen at early times. Some blunt constrictions were quite deep and must have arisen from cells that were in the latter stages of septation at the time of the shift, confirming that even cells late in septation were blocked. Thus, the continued invagination of septa present at the time of the temperature shift is blocked, and septa gradually become less demarcated as cells continue to grow. These results indicate that the Z ring is required throughout septation.

Recovery of Z rings in MCZ84 filaments shifted to the permissive temperature. The position of Z rings in MCZ84 cells at permissive temperature was determined (Fig. 3A). Most cells fell within a twofold range of cell lengths and contained a Z ring at midcell. Some cells in the population, however, were longer and contained one or two rings. Interestingly, these rings were usually positioned at the cell quarters. In such cells, it appears that a Z84 ring at midcell aborted or failed to form, resulting in larger cells. Since these cells contained a central constriction this suggests that the ring aborted. Thus, one can conclude that the FtsZ84 protein is properly positioned to the division site at permissive temperature, although it appears that Z84 rings are not as stable as wild-type Z rings and abort at a detectable frequency.

Having determined that FtsZ84 is positioned normally at the permissive temperature, we examined the consequences of shifting MCZ84 to permissive temperature after 30 min at nonpermissive temperature (Fig. 4). Examples of cells sampled 2 min after the return to permissive temperature are shown in Fig. 5. Ninety percent of the cells had at least one localization event. Three things can be observed from this figure: (i) FtsZ84 is localized to multiple locations within the filaments within 2 min, (ii) the position of FtsZ84 in the filaments is rather regular, and (iii) complete rings, partial rings (Fig. 5B), and some spots are evident (Fig. 5D). The pattern of fluorescence indicated in Fig. 5B, two diametric dots, was previously observed in Rod- mutants of E. coli where altering the plane of focus revealed an arc connecting the two dots (4). The distance of all localized fluorescence (complete and partial rings or spots) to one of the cell poles was plotted as a function of cell length (Fig. 3B). Lines were added to indicate the theoretical positions of division sites. Inspection of the graph indicates that FtsZ84 is not located randomly in these short filaments but is mostly positioned either at midcell, the cell’s quarters, or the cell’s eightths. Cells were observed that had FtsZ84 localized at one to seven sites per cell with an average of 2.8 rings or partial rings per cell (Fig. 6). Quantitation of the total number of FtsZ84 localization events after the shift back to the permissive temperature revealed that localization events peaked at 1 to 2 min and then decreased about 20% over the next 10 to 15 min before any cell division had occurred (data not shown). This result indicates that 20% of these early localization events were aborted.

The results obtained with a culture shifted to 42°C for 45 min (Fig. 4) and back to permissive temperature for 2 min are similar to those for a culture shifted for 30 min. The number of localization events increased to an average of 3.7 rings, partial rings, and spots per cell. Again, FtsZ84 is positioned at regular intervals, indicating it is localized at potential division sites
FIG. 1. Z rings rapidly disappear upon shift of MCZ84 to nonpermissive temperature. A culture of MCZ84 growing exponentially at 30°C was shifted to 42°C. Samples were taken before the shift and 2 min after the shift and processed for immunofluorescence microscopy to determine the location of FtsZ as previously described (1). The FtsZ was visualized by staining with antibodies to FtsZ and a secondary antibody conjugated to the fluorophore Cy3. (A and B) Phase-contrast and immunofluorescence photomicrographs of cells from the 30°C culture. (C and D) Phase-contrast and immunofluorescence photomicrographs of cells 2 min after shift to 42°C.
FIG. 2. Morphological appearance of septa upon shift of MCZ84 to the nonpermissive temperature. Cells from the cultures in the experiment depicted in Fig. 1 were observed by scanning electron microscopy (4). (A) Examples of cells 2 min after the shift (note the sharply demarcated septa indicated by the arrowheads). (B) Examples of cells 10 min after the shift (note the blunt constrictions at midcell indicated by the arrowheads).
Many of these localization events fall on the line nearest the x axis, which indicates the 1/16 position. This position is occupied in many of these longer cells. It is also occupied in a few of the longer cells from the 30-min shift but not in the shorter cells. These results suggest that, upon return to permissive temperature, FtsZ84 is localized to division sites that were formed at the nonpermissive temperature. Since greater than 80% of the cells had a Z84 ring at midcell before the temperature shift, our finding of many Z84 rings at midcell after a shift back from nonpermissive temperature (in about 40% of the cells shifted back after 30 min at 42°C) suggests that FtsZ84 can return to at least some of these vacated sites. Further analysis of this phenomenon (see below) revealed that not all midcell sites can be reoccupied.

To further document these FtsZ84 localization events we examined the localization of FtsA. Previous results have shown that FtsA is localized to the septum and that it is dependent upon prior localization of FtsZ (3, 17). These prior results also showed that FtsA localization is coincident with that of FtsZ. Therefore, cells from the same temperature shift experiment (30 min at 42°C and 2 min at 30°C) were processed to determine the localization of FtsA. Figure 6 compares the number of localization events per cell for these two proteins. The distributions are quite similar with the number of FtsA localization events per cell closely mimicking that of FtsZ84. The total numbers of localization events are also quite similar but with FtsZ84 events exceeding those of FtsA. We have noticed this before (3) and do not know if it is due to lower sensitivity for FtsA detection or to the FtsA localization rate lagging slightly behind that of FtsZ. Nonetheless, it suggests that the FtsZ84 localization events are normal events in that they recruit FtsA.

**FIG. 3.** Location of Z rings in MCZ84 at permissive temperature. (A) Cells of MCZ84 grown at the permissive temperature (experiment depicted in Fig. 1) were analyzed for the location of the Z ring. The distance of the Z ring to the nearest cell pole was plotted against cell length. The three lines indicate 25, 50, and 75% of the cell length and are the theoretical positions of septa (24). (B) Location of Z rings and partial rings in recovering filaments of MCZ84. The distance of Z rings from the nearest pole in recovering filaments of MCZ84 was determined and plotted against the cell length. In cells with multiple bands of fluorescence all distances were measured to only one of the poles. The pole chosen contained the nearest band of fluorescence. Data from cells shifted to 42°C for 30 min and back to 30°C for 2 min were indicated by the open triangles and data from cells shifted to 42°C for 45 min and back to 30°C for 2 min were indicated by the closed triangles. The lines drawn represent the positions of predicted division sites. Each line is at a 1/8 position except the (dashed) line nearest the x axis, which is at a 1/16 position. Note that only longer cells have a ring at this position.
cells had a Z84 ring at midcell (60%), confirming that FtsZ84 could reoccupy at least some of the vacated sites (Fig. 8). This must be the case since this value approached the value in the preshift culture (60 versus 85%). Close inspection of cells with rings at midcell revealed that they did not contain a central constriction. Instead, cells with a central constriction contained a ring at either one or both of the quarter positions, indicating that some new sites for FtsZ84 localization had formed during the period of the temperature shifts (as seen with the longer periods at 42°C) and that FtsZ84 could not again localize to vacated midcell sites that were in the process of constriction prior to the temperature shifts.

**DISCUSSION**

The results of this study reveal the rapid dynamics of FtsZ localization within bacterial cells. A shift of MCZ84 to the nonpermissive temperature results in a loss of FtsZ84 localization within 1 min and an immediate block to septation, indicating that the Z ring is required throughout septation. Earlier temperature shift experiments done with ftsZ84(Ts) were consistent with FtsZ being required for initiation of septation but did not address the requirement for FtsZ throughout septation (5, 22, 23, 25). The present results clearly address this issue and are consistent with immunocytochemical data demonstrating that FtsZ is present at the leading edge of the septum throughout septation (1, 6). The nascent septa that are sharply demarcated at the time of the temperature shift gradually become blunt with continued cell growth, and many are eventually difficult to observe. This gradual disappearance of these demarcated septa and the failure to form any new septa are consistent with the “smooth” morphology normally associated with filaments formed as result of a lack of ftsZ function (5, 23). The gradual disappearance of the septa is most likely due to the loss of polarized growth that is observed during septation. Through much of the cell cycle, growth of the cell envelope is diffuse and is polarized only during septation when most envelope growth occurs.
centripetally at midcell to form the septum (26, 27). The disappearance of the Z ring must result in a return to diffuse growth, which gradually smoothes out the aborted septum.

Shifting MCZ84 to the permissive temperature after a period at the nonpermissive temperature led to rapid localization of FtsZ84 at regularly spaced intervals. This regular spacing suggests that FtsZ84 was localizing to division sites (nucleation sites) and therefore suggests that division sites are formed at the nonpermissive temperature in the absence of FtsZ function. Cook and Rothfield (8) have provided evidence that plasmolysis bays, thought to be markers for periseptal annuli, are regularly spaced in the absence of FtsZ function. Thus, FtsZ84 could be localizing to these annuli. We found that the capacity of MCZ84 to accumulate potential for ring formation was retained for at least 45 min at the nonpermissive temperature.

The localization events following the shift back to the permissive temperature included complete and partial rings as well as some spots of fluorescence. With wild-type cells partial rings are difficult to detect, also indicating that FtsZ ring formation is very rapid. Previously, partial rings were observed in Rod" mutants; presumably their larger circumference allowed intermediate stages to be observed (4). Here, we may have observed intermediate stages of ring formation due to the additional time required for FtsZ84 to renature and/or to the fact that many rings are attempting to form simultaneously. Nonetheless, we observe that FtsA is recruited in a similar pattern to FtsZ, suggesting that these events are behaving as expected.

Interestingly, the experiment designed to test if FtsZ84 could return to sites vacated by a temperature shift yielded mixed, but clear, results. FtsZ84 returned to sites that had not yet initiated constriction; however, it did not return to sites that had a constriction. This result suggests that once constriction is initiated the localizing signal for FtsZ is lost or masked. One possibility is that the Min system (11), which blocks old sites (the cell poles) from being reused, comes into play as the constriction process is initiated. With this model the initiation of constriction would convert a nascent division site into an old division site by the action of min.

It appears that some FtsZ84 rings are aborted at the permissive temperature, giving rise to longer cells. The failure of Z rings to always complete division has also been inferred from the study of other cell division mutants growing at the permissive temperature, notably ftsQ1(Ts) and ftsI23(Ts) but also ftsA12(Ts) and ftsW(Ts) (1, 14). Thus, the presence of mutationally altered division proteins, including FtsZ itself, results in a readily detectable frequency at which Z rings fail to complete division. The addition of cephalaxin, which blocks PBP3(FtsI), also results in the dissolution of Z rings that are in the process of constriction (19). Thus, constricting Z rings appear to be more fragile than Z rings that have not initiated constriction. In the present experiments the failure of localization events is perhaps due to a mixture of FtsZ84 molecules, some of which might not be completely renatured, as well as to competition between different rings.
These results as well as those obtained by Pogliano et al. (19) suggest that the Z ring has two distinct states. One state precedes constriction and appears more stable and another occurs during constriction and appears more fragile, as evidenced by a tendency to abort in various situations that are expected to prevent septal progression. In the first state the Z ring is joined by FtsA and ZipA but not by FtsN and probably not by the other known division proteins that are thought to act later, such as FtsI (2, 3, 13). In the constricting state the Z ring is joined by FtsN and presumably these other later acting proteins. Inhibition of invagination of the septum causes the constricting Z ring to abort. This is seen very clearly with the addition of cephalin (19) but also occurs with many temperature-sensitive cell division mutants at a readily detectable frequency at the permissive temperature (1).

It is not clear what biochemical activity of FtsZ84 is affected upon shift to the nonpermissive temperature. In vitro, FtsZ84 has reduced GTPase activity but it is not temperature dependent (10, 20). In addition, it has increased ATPase activity (21). It is likely, however, that FtsZ84’s GTPase is temperature dependent in vivo. One possibility is that shifting to the nonpermissive temperature only blocks assembly of FtsZ84. If that is the case, the rapid disassembly of FtsZ84 rings would indicate that Z rings are not static but are rapidly turning over.

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