Cell cycle regulation and cell type-specific localization of the FtsZ division initiation protein in Caulobacter

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ABSTRACT Many genes involved in cell division and DNA replication and their protein products have been identified in bacteria; however, little is known about the cell cycle regulation of the intracellular concentration of these proteins. It has been shown that the level of the tubulin-like GTPase FtsZ is critical for the initiation of cell division in bacteria. We show that the concentration of FtsZ varies dramatically during the cell cycle of Caulobacter crescentus. Caulobacter produce two different cell types at each cell division: (i) a sessile stalked cell that can initiate DNA replication immediately after cell division and (ii) a motile swarmer cell in which DNA replication is blocked. After cell division, only the stalked cell contains FtsZ. FtsZ is synthesized slightly before the swarmer cells differentiate into stalked cells and the intracellular concentration of FtsZ is maximal at the beginning of cell division. Late in the cell cycle, after the completion of chromosome replication, the level of FtsZ decreases dramatically. This decrease is probably mostly due to the degradation of FtsZ in the swarmer compartment of the predivisional cell. Thus, the variation of FtsZ concentration parallels the pattern of DNA synthesis. Constitutive expression of FtsZ leads to defects in stalk biosynthesis suggesting a role for FtsZ in this developmental process in addition to its role in cell division.

The cell division cycle and the DNA replication cycle must be well-coordinated for cell proliferation to proceed normally and for each progeny cell to inherit its complement of genetic material. In bacteria with no obvious differentiation, such as Escherichia coli during exponential growth, the task of coordinating the expression and function of a multitude of cell division and DNA replication genes is a formidable one (reviewed in ref. 1). Another layer of complexity is added in differentiating bacteria that coordinate specific developmental stages with cell division and DNA replication.

In the dimorphic alpha-purine bacterium Caulobacter crescentus, compartmentalization of the predivisional cell is coincident with the establishment of distinct programs of gene expression in the incipient swarmer and stalked cells (for reviews, see refs. 2 and 3). In this organism, cell division always produces two different cell types: (i) a swarmer cell and (ii) a stalked cell. The swarmer cell is a slow-growing, motile, and chemotactically competent cell that is incapable of DNA replication. After approximately 25–33% of the cell cycle, the swarmer cell differentiates into a stalked cell: it sheds its flagellum, initiates DNA replication, and synthesizes a stalk (a thin cylindrical extension of the cell surface layer) at the pole previously occupied by the flagellum. Cell growth eventually leads to the formation of a predivisional cell in which a flagellum is synthesized de novo at the pole opposite the stalk. After cell division, the progeny stalked cell is immediately capable of initiating a new round of DNA replication and cell growth.

Analysis of the effects of cell division mutations and of penicillin on C. crescentus has determined that the completion of early cell division steps is required for developmental events such as the activation of the flagellum, stalk formation, and for the correct assembly of surface structures at the nascent swarmer pole of the cell, even though DNA replication still proceeds normally (4). The coupling between cell division and differentiation events is also exemplified by the fact that mutants blocked early in flagellum synthesis or assembly (class II mutants) exhibit a delay in the timing of cell division, resulting in the formation of filamentous cells (2, 3). This indicates that a developmental checkpoint links early flagellum morphogenesis to cell division. Expression of the early class II flagellar genes is required for the expression of late flagellar genes. Because late flagellar genes have been shown to be transcribed specifically in the swarmer compartment of the predivisional cell after a cell division barrier has been formed (5, 6), this checkpoint would ensure that late flagellar gene expression occurs after initiation of cell division. Conversely, the gyrB gene, encoding the gyrase B subunit, and an early flagellar gene, flIF, are transcribed only in the stalked compartment of the predivisional cell where they are required immediately after division (7, 8).

In bacteria, the initiation of cell division requires the tubulin-like GTPase FtsZ (reviewed in refs. 9 and 10). FtsZ is a highly conserved protein (11, 12) that polymerizes into a ring structure associated with the cytoplasmic membrane at the site of cell division (13, 14). The assembly of this ring is thought to select the site of cell division, to recruit other cell division proteins, and/or to constrict and provide mechanical force for division (9, 10, 15). To investigate the coupling of cell division and cell differentiation in C. crescentus, we have initiated a study of the ftsZ gene. Here we report the cloning and characterization of ftsZ. We show that ftsZ is essential and that the intracellular concentration of FtsZ is highly regulated during the cell cycle. FtsZ is first detected at approximately the swarmer-to-stalked cell differentiation stage and its level is maximal during the early stages of cell division. After cell division, FtsZ is only present in stalked cells, presumably as a result of its degradation in swarmer cells. This indicates a coupling between the cell cycle and developmental regulation of this important cell division protein.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Media, and Growth Conditions. E. coli DH11S F' (16) was used as a host for cloning and S17–1 (17) was used for conjugal transfer of plasmids to C. crescentus. C. crescentus strains were all derivatives of strain NA1000 (18). All strains were grown as described (19). T46 is a cosmid containing ~30 kb of C. crescentus NA1000 DNA. It was identified as containing ftsZ by screening the C. crescentus

Abbreviation: IPTG, isopropyl β-D-thiogalactoside.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U40273).

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comsid library described (20). pHB2.0 and pHB2.0rev are pSKII- and pSKII+ (Stratagene) derivatives, respectively, containing the complete ftsZ gene on a 2-kb BamHI–HindIII fragment oriented such that ftsZ is in the same orientation as lacZ in pHB2.0 and in the opposite orientation in pHB2.0rev. Plasmids pΔN, pΔC, and pΔNC were created by subcloning a 1.4-kb PvuII (codon 64)–HindIII (downstream of the terminator) fragment, a 690-bp BamHI–PstI fragment (containing the ftsZ promoter through codon 158), and a 1-kb PstI (from codon 158 to 483) fragment from pHB2.0 into appropriately digested pBGST18 [a derivative of pBGST18 (21)], obtained from M. R. K. Alley (Imperial College, London), that contains a RK2 oriT fragment into the two DraI sites, respectively. DptacZ is the BamHI–HindIII ftsZ fragment cloned into pGLO10 (obtained from D. Helsinki, University of California, San Diego) generating pGLO10ftsZ, with an upstream 2-kb EcoRI fragment containing pac and lacFΔ (from plasmid pLctac7; obtained from M. R. K. Alley).

DNA Manipulations, Genetic Techniques, Immunoblotting, and Microscopy. General cloning, DNA sequencing and analysis, and genetic procedures were as described previously (19). For immunoblotting, total cell lysates were resolved on a SDS/PAGE gel and transferred to nitrocellulose (Schleicher & Schuell). FtsZ was detected using the primary antibody (anti-E. coli FtsZ; a gift from J. Luktenhaus, University of Kansas Medical Center) at a 1:5,000 dilution and the secondary antibody [goat anti-rabbit IgG (H + L)–horseradish peroxidase (HRP)] conjugate ( Gibco/BRL) preabsorbed with acetone powders (22) from E. coli DH115SF and C. crescentus NA1000 at a 1:20,000 dilution in blocking buffer TTBS containing 0.05% (vol/vol) Tween 20 with 10% (wt/vol) Carnation nonfat dry milk. Chemiluminescent detection of the IgG-HRP conjugate was done as described in the Enhanced Chemiluminescence kit (Amersham). For cell cycle studies, swarmer cells from a late log phase culture of C. crescentus NA1000 were isolated and processed as described (23). Light photomicroscopy was performed on a Zeiss Axioplan light microscope with a 100X Plan Neofluor oil objective using Nomarski differential interference contrast optics. Transmission electron microscopy was done as described (19) using a JEOL model JEM-1010 electron microscope at 60 kV.

RESULTS
Cloning and Mapping of a Caulobacter Homolog of the ftsZ Gene. A C. crescentus homolog of ftsZ was cloned from a cosmid library (20) using a PstI fragment encoding the N-terminal portion of the Rhizobium meliloti ftsZ1 gene (24) as a probe as previously described for rpoN (19). From the identified cosmid (T46), a 2.0-kb BamHI–HindIII fragment that hybridized to the R. meliloti probe was subcloned into pSKII- and sequenced on both strands. One ORF with a high G+C bias at the third position was identified, measuring 1523 bp with the potential to code for a 508 amino acid protein with a predicted molecular weight of 54 kDa.

The predicted protein product of this ORF has a high degree of sequence similarity with all the predicted products of ftsZ genes sequenced thus far (Fig. 1) and will henceforth be referred to as FtsZ. After the highly conserved N-terminal region (the first ~330 amino acids), the only other region of sequence similarity is a highly conserved extreme C-terminal sequence found in the vast majority of FtsZ sequences. The C-terminal domain of similarity has the consensus sequence L(D,E)(I,V)PX(F,Y)(L,I)(R,K) and is found only a few amino acids before the end of the FtsZ proteins. The region between the N-terminal domain of similarity and the C-terminal domain of similarity varies extensively, both in length and in sequence. Even between the closely related Streptomyces griseus and Streptomyces coelicolor, this spacer region has only 56% sequence identity, whereas the rest of the protein has only one nonconserved amino acid out of 319. A striking feature of C. crescentus FtsZ also found in R. meliloti FtsZ1 is the length of the spacer region: 178 and 261 amino acids, respectively, compared with an average of 63 amino acids for the other FtsZ proteins. The spacer region of C. crescentus FtsZ contains an unusually high number of proline (16%) and charged residues (8% arginines and 12% glutamates) as compared with the rest of the protein, as is the case in R. meliloti FtsZ1 (24). The spacer region of C. crescentus FtsZ is predicted by the PROTEINSTRUCTURE program to be highly flexible and to have a high probability to be located at the surface of the protein. We hypothesize that this region acts as a flexible spacer between the N-terminal domain of similarity and the C-terminal domain of similarity and could also serve as a target for proteolysis (see below).

The position of the ftsZ gene on the physical map of C. crescentus was determined by Southern blot hybridization as described previously (19). The ftsZ probe hybridized to the 223-kb Spel fragment, the 405-kb AseI fragment, and the 133-kb DraI fragment at approximately map position 3150 kb (data not shown).

The ftsZ Gene Is Essential for Viability in C. crescentus. We used homologous recombination of plasmids containing different portions of ftsZ to determine whether ftsZ is essential in C. crescentus. Because the conjugative plasmid pBGST18 cannot replicate in C. crescentus, selection for kanamycin-resistant transconjugants selects for integration of the plasmid by homologous recombination within a chromosomal locus if the plasmid contains DNA from the same locus. We constructed three plasmids. As a control for homologous recombination at the ftsZ locus, we found that integration of pΔN and pΔC occurred readily (2 × 10^-6) kanamycin-resistant transconjugants per C. crescentus cell. Integration of these plasmids recreates a complete copy of ftsZ in addition to generating a truncated copy of ftsZ. In contrast, integration of plasmid pΔNC, containing a 1-kb PstI fragment coding for an internal portion of ftsZ (from codon 158 to codon 483), occurred at a 1000-fold lower frequency (7 × 10^-6) kanamycin-resistant transconjugants per C. crescentus cell). Matings carried out under the same conditions using pBGST18 containing no C. crescentus DNA gave a comparable number of kanamycin-resistant colonies. Because the pΔNC plasmid is missing both the N- and C-terminal coding regions, its integration by homologous recombination results in a gene disruption in ftsZ. When a second copy of ftsZ was provided on cosmids T46, integration of pΔNC occurred readily with a frequency equivalent to that of the integration of pΔN or pΔC into NA1000 or NA1000/T46 (3 × 10^-6) kanamycin-resistant transconjugants per C. crescentus cell). We conclude from these experiments that the ftsZ gene is essential in C. crescentus.

Identification of the C. crescentus FtsZ Protein. We have used an antibody raised against E. coli FtsZ to detect the C. crescentus FtsZ protein. This antibody cross-reacts specifically with a major immunoreactive protein in a wide array of Gram-negative and Gram-positive bacteria (11). Fig. 2A shows that this is also the case in C. crescentus, where the cross-reactive protein has an apparent molecular mass of 65 kDa. This is larger than the expected molecular mass of 54 kDa predicted from the primary sequence of ftsZ. To confirm that this cross-reacting protein was indeed FtsZ, we introduced a plasmid containing C. crescentus ftsZ under the control of the lac promoter into E. coli DH11S. Induction of the lac promoter by addition of isopropyl β-D-thiogalactoside (IPTG) induces the expression of a cross-reacting protein with the same apparent molecular weight as the putative C. crescentus FtsZ. This protein is undetectable in E. coli in the absence of pH2.0 and is barely detectable in E. coli/pHB2.0 in which the lac promoter is not induced. The aberrant migration of FtsZ is similar to that of other acidic proteins (the pI of C. crescentus FtsZ is 4.5) and is also seen with R. meliloti FtsZ1 (24).
Cell Cycle Variation of FtsZ Level. To determine if the concentration of FtsZ varies during the cell cycle of *C. crescentus*, swarmer cells were isolated and were allowed to proceed synchronously through the cell cycle. At 15-min intervals, aliquots from the synchronous culture were removed and used to prepare cell lysates that were analyzed by SDS/PAGE. Proteins were transferred to nitrocellulose paper and immunoblotted using an anti-*E. coli* FtsZ antibody. The immunoblot in Fig. 2B shows that the level of FtsZ varies dramatically during the cell cycle. No FtsZ is detectable in swarmer cells early in the cell cycle (0 and 15 min). Under these conditions, the McpA protein is detected in swarmer cells, indicating that our failure to detect FtsZ in swarmer cells is not due to problems in cell lysis (data not shown). FtsZ was detected for the first time at 30 min, just before the differentiation of swarmer cells into stalked cells, which occurred at 45 min. The level of FtsZ then increased until 0.6 cell division units when the cells had begun to divide. From this point until the time of cell separation (150 min or 1 cell division unit), the level of FtsZ decreased dramatically with a half-life of ~30 min. Pulse-chase immunoprecipitation experiments with mixed cultures indicate that the half-life of FtsZ is 30–35 min (unpublished results). The level of FtsZ increased again as the stalked cells initiated a new cell cycle (150 to 195 min). We investigated the fate of FtsZ after cell division by separating swarmer and stalked cells from the 150 min culture and subjecting them to immunoblot analysis. All the FtsZ was found in the stalked cell fraction and none was detected in swarmer cells (Fig. 2B).

Expression of the *C. crescentus* ftsZ Gene in *E. coli* Affects the Frequency of Cell Division. To determine if *C. crescentus* FtsZ has biological activity in *E. coli*, we examined the *E. coli* cells containing either pHB2.0 or pHB2.0rev under various conditions. Cell division is inhibited almost immediately when ftsZ is expressed at a relatively high level under the control of the fully induced lac promoter (Fig. 3D). Cell elongation is obvious only 1 hr after addition of IPTG and subsequently leads to the formation of long, smooth filaments with no sign of constriction, indicating that division has been inhibited at the initiation step. Even without IPTG to induce the lac promoter, cells are filamentous when they reach stationary phase (data not shown). This may be due to the background level of lac promoter activity transcribing ftsZ or to the activity of the native ftsZ promoter. Indeed, overexposure of immunoblots indicate that there is a low level of FtsZ expression under these conditions (data not shown). The inhibition of *E. coli* cell division caused by overexpression of *C. crescentus* FtsZ is consistent with the inhibition of cell division caused by an
overproduction of E. coli FtsZ to a level 5-fold higher than normal (25). The presence of pHB2.0rev in E. coli causes a broadening of the cell-length distribution in exponential phase compared to a culture of DH11S without plasmid or DH11S containing pSK11* (data not shown). This leads to a higher level of cells that measure 50–70% of the mean cell length of a culture of DH11S/pHB2.0rev grown in the presence of IPTG (Fig. 3 B and C). The accumulation of smaller cells is even more dramatic in early stationary phase. Because the growth rate of the two strains is not significantly different (data not shown), this suggests that expression of C. crescentus ftsZ leads to erratic placement of the cell division site in E. coli. This is also the case with expression of R. meliloti FtsZ1 gene (24) and for a 2- to 3-fold overexpression of E. coli FtsZ (25). The expression of ftsZ in pH2B.0rev probably comes from a C. crescentus promoter or a cryptic promoter within the 200-bp upstream of the translation start site of ftsZ that can be recognized in E. coli. Indeed, we have found that the native ftsZ promoter is in this region (A. J. Kelly and Y.V.B., unpublished work). The fact that induction of the lac promoter by addition of IPTG to DH11S/pHB2.0rev restores cell-length distribution to normal (Fig. 3C) suggests that strong transcription coming from the lac promoter inhibits transcription of ftsZ or produces anti-sense ftsZ RNA, thereby eliminating the low level of FtsZ responsible for increased division in DH11S/pHB2.0rev grown without IPTG.

We attempted, without success, to complement the E. coli ftsZ84 Ts mutation with both pHB2.0 and pHB2.0rev in the presence of various concentrations of IPTG. Thus, even if C. crescentus FtsZ is able to interact with the E. coli cell division machinery, it is unable to completely substitute for E. coli ftsZ.

**Constitutive Expression of ftsZ Affects Stalk Biosynthesis.** To address the significance of the temporal regulation of FtsZ concentration, we used plasmid ptcZ in which ftsZ is transcribed from its own promoter and from the IPTG inducible ptc promoter. Cultures of C. crescentus NA1000 containing ptcZ were grown in the presence of different concentrations of IPTG and examined by transmission electron microscopy. In the absence of IPTG, cells have a normal appearance (data not shown, but identical to Fig. 4A). This was also the case for NA1000 containing pGL10 (without ftsZ) in the presence of 10 mM IPTG (Fig. 4A). When NA1000/ptcZ was grown in the presence of 1 or 10 mM IPTG, the majority of the cells had bifurcated stalks with a flagellum still attached to one of the stalk branches (Fig. 4B–D). Cells with flagellated or bifurcated stalks occur very rarely in wild-type populations (0.3%: see ref. 26). At 10 mM IPTG, cells became filamentous (data not shown), suggesting that high expression of ftsZ inhibits cell division in C. crescentus as it does in E. coli.

**DISCUSSION**

Although many bacterial genes involved in DNA replication and cell division have been identified, it is not yet known whether the intracellular concentrations of their protein products vary during the cell cycle. We initiated this study for two reasons: (i) C. crescentus provides an excellent system in which to address the cell cycle control of genes due to the ease with which cell populations can be synchronized and (ii) considerable evidence indicates that the processes of cell division and...
cell differentiation are tightly linked in *C. crescentus*. Cell division events affect the developmental program and vice versa. This lead to the proposal that the site of cell division may serve as an organizational center at the new poles (27-29). Because *ftsZ* is the earliest acting cell division gene known in bacteria and it is involved in the positioning of the division site, it is likely to be involved in the coupling of cell differentiation and the cell division cycle. We report an example of cell cycle variation in the abundance of a bacterial cell division protein, the FtsZ protein of *C. crescentus*. In addition, we show that this important cell division initiation protein is developmentally regulated and is only present in the stalked cell after cell division.

The most striking finding from this study is the dramatic cell cycle variation in the level of FtsZ. The appearance of FtsZ during the cell cycle coincides roughly with the time of initiation of DNA replication and the differentiation of swarmer cells into stalked cells: FtsZ is absent from swarmer cells (which do not replicate DNA) and it starts accumulating coincident with the initiation of DNA replication. Following a period (coincident with the bulk of DNA replication) where FtsZ is at its highest, there is a dramatic drop in the concentration of FtsZ ~150 min into the cell cycle (Fig. 2B). Interestingly, this coincides with the post-synthetic gap (G2) in DNA synthesis (30, 31). Thus, the variation in FtsZ concentration parallels the pattern of DNA synthesis. This regulation of FtsZ concentration in parallel with DNA replication is particularly interesting in light of recent experiments suggesting that cell division is directly coupled to the initiation of DNA replication in *E. coli* (32). The fact that FtsZ is absent from swarmer cells and is present in stalked cells after cell division implies that the drop in FtsZ level seen in G2 mostly reflects its degradation in swarmer cells or in the swarmer compartment of the predivisional cell where DNA replication is blocked. Furthermore, the half-life of FtsZ is much less than one generation time, at least during the G2 period, as illustrated by the drastic decrease in FtsZ concentration between 90 and 135 min into the cell cycle (Fig. 2B). Our estimate from this and other cell cycle immunoblots and from pulse-chase immunoprecipitations is that the half-life of FtsZ is less than 0.2 cell division unit. This is in contrast to the situation in *E. coli* where the half-life of FtsZ is more than one generation (33). This may reflect the fact that in *E. coli* both progeny cells are essentially equivalent and initiate a new round of growth and division, whereas cell division in *C. crescentus* gives rise to cells that differ in their ability to replicate DNA. This dramatic regulation of FtsZ concentration in *C. crescentus* is a clear example of developmental regulation of a cell division protein. In *S. griseus*, the level of both the *ftsZ* mRNA and of the FtsZ protein have been found to be essentially the same during both vegetative growth and sporulation (34), and in *Bacillus subtilis*, the level of FtsZ decreases 2- to 3-fold during the first 2 hr of sporulation, but then remains constant (35). In nutritional shift-up experiments in *E. coli*, the level of *ftsZ* mRNA has been shown to reach a maximum at about the time DNA replication initiates (33). It is not known to what extent the level of FtsZ varies during the cell cycle in *E. coli*. However, the fact that FtsZ is rate-limiting for cell division, that an overproduction of FtsZ leads to the formation of minicells, and that a higher overproduction of FtsZ inhibits cell division has been interpreted as meaning that the level of FtsZ is tightly controlled and that FtsZ needs to accumulate to a critical level for cell division to be initiated (9, 33).

FtsZ is rapidly degraded late in cell division. This leads to the cell type-specific localization of FtsZ upon cell separation. We hypothesize that this is due to either a differential degradation or a differential synthesis of FtsZ in the swarmer and stalked compartments, or a combination of both. A site for FtsZ proteolysis may be the proline-rich region between the N-terminal domain of similarity and the C-terminal domain of similarity. Proline-rich regions have been shown to be exposed to solvent and to exhibit substantial conformational flexibility (36) because they are unable to fold into a compact structure (37). Other cases of developmental regulation by proteolysis are known in *C. crescentus*. The chemoreceptor protein McPα is present in swarmer cells and is degraded during the swarmer-to-stalked cell differentiation stage (23). McPα degradation requires a short 14 amino acid sequence located at its C terminus. The DNA methyltransferase CcrM is also subject to proteolysis as cells approach division (38) and constitutive expression of CcrM causes developmental abnormalities (39).

Developmentally regulated proteolysis presumably serves to prevent the presence of certain proteins at the wrong place or at the wrong time during cell differentiation. In the case of FtsZ, its constitutive expression causes the synthesis of bifurcated stalks with a flagellum still attached to one of the stalk branches. This phenotype could be caused indirectly by FtsZ binding to the flagellar pole and interfering with cell division or with polar differentiation events, or it might be indicative of a more direct role of FtsZ in stalk synthesis. Because FtsZ is synthesized just before the time of stalk biosynthesis, we speculate that FtsZ plays a direct role in stalk biosynthesis. Its expression early in swarmer cells when it is transcribed by the induced *tac* promoter might lead to premature initiation of stalk biosynthesis, thus trapping the flagellum at the tip of the stalk. FtsZ could be involved in the topological reorientation of peptidoglycan biosynthesis required for stalk synthesis in a manner analogous to its role in cell division.

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