JOURNAL OF BACTERIOLOGY, Sept. 1997, p. 5551–5559 Vol. 179, No. 17
0021-9193/97/$04.00+0

Analysis of the Interaction of FtsZ with Itself, GTP, and FtsA

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Received 30 January 1997/Accepted 23 June 1997

The interaction of FtsZ with itself, GTP, and FtsA was examined by analyzing the sensitivity of FtsZ to proteolysis and by using the yeast two-hybrid system. The N-terminal conserved domain consisting of 320 amino acids bound GTP, and a central region of FtsZ, encompassing slightly more than half of the protein, was cross-linked to GTP. Site-directed mutagenesis revealed that none of six highly conserved aspartic acid and asparagine residues were required for GTP binding. These results indicate that the specificity determinants for GTP binding are different than those for the GTPase superfamily. The N-terminal conserved domain of FtsZ contained a site for self-interaction that is conserved between FtsZ proteins from distantly related bacterial species. $FtsZ_{320}$, which was truncated at the end of the conserved domain, was a potent inhibitor of division although it expressed normal GTPase activity and could polymerize. FtsZ was also found to interact directly with FtsA, and this interaction could also be observed between these proteins from distantly related bacterial species.

FtsZ is an essential cell division protein in *Escherichia coli* (11) and *Bacillus subtilis* (6) and is widely conserved among prokaryotes (4, 29, 40). It functions during cell division by forming a ring, designated the Z ring, at the division site that directs cell division (1, 8; reviewed in reference 25). The dynamics of Z-ring formation during the cell division cycle led to the suggestion that the Z ring is a cytoskeletal element that is formed through a nucleation event and self-assembly of FtsZ (8, 24). In vitro, FtsZ has GTPase activity (14, 30, 34) and can assemble into filaments consistent with its suggested cytoskeletal function (16, 31). The cytoskeletal structures observed by using immunofluorescence (1, 2) and by utilizing an FtsZ-GFP tag (27) are also consistent with the model.

During cell division, FtsZ is likely to interact with many proteins, including itself. Assembly of FtsZ into filaments and two-dimensional (2-D) sheets indicates that there must be several sites for self-interaction (16, 31). At least one interaction must occur between subunits along one axis in a protofilament, and another must occur between subunits in two adjacent protofilaments in 2-D sheets. In addition, FtsZ may interact with other proteins to recruit them to the division site. One of these is FtsA, which has been shown to localize to the division site in an FtsZ-dependent manner (3, 27). Another is ZipA, which has been shown to localize to the division site and interact directly with FtsZ (20). It's possible that other division proteins may also be recruited to the division site through interaction with FtsZ.

ftsZ genes from a variety of organisms have been sequenced, and the lengths of the deduced proteins vary from just over 320 amino acids to over 500 amino acids. This heterogeneity is due largely to differences at the carboxyl terminus (5, 28). FtsZ can be divided into two segments: a large N-terminal segment of approximately 320 residues that is highly conserved and a C-terminal segment that is of quite variable length and less well conserved. Between the *E. coli* and *B. subtilis* FtsZs, which are of similar lengths, there is no homology in the C-terminal 60 amino acids except at the extreme carboxyl terminus (5). This homology is not conserved in archaebacterial FtsZs (4, 29, 40).

FtsZ contains a sequence motif, GGGTGTG, very similar to the tubulin signature motif, GGGTGSG. This observation led to the discovery of its GTPase activity (14, 30). Although involvement of the tubulin signature motif in the GTPase activity of tubulin is speculative, there is evidence that the corresponding sequence in FtsZ plays an important role. A mutation altering this sequence in FtsZ to GGGAGTG results in loss of function in vivo and a loss of GTPase activity in vitro (30), whereas a mutation changing this sequence to SGGTGTG results in temperature sensitivity in vivo and reduced GTPase activity (14, 34) but increased ATPase activity in vitro (35). Similar mutations have not been reported for tubulin.

In tubulin, the GTP binding site is unknown, due to a lack of structural information; however, attempts to identify the sequences that compose the site have been made by sequence comparisons and cross-linking studies involving GTP and its analogs. The latter studies have led to the identification of three different peptides from amino acids 3 to 19, 63 to 77, and 155 to 174 that can be cross-linked to the nucleotides (21, 23, 36). Sequence comparisons, especially with members of the GTPase superfamily, have led to the suggestion that regions from amino acids 103 to 109, 295 to 298, and 203 to 206 may be analogous to the conserved motifs involved in GTP binding and GTPase activity among GTPase superfamily members (37). However, direct testing of this suggestion by mutation did not confirm this possibility (17).

FtsZ and tubulin show additional sequence similarities beyond the tubulin signature motif (24). These similarities include two pairs of closely spaced and highly conserved aspartic acid and asparagine residues. The spacing between these two conserved pairs and the tubulin signature motif is very similar in FtsZ and tubulin. Interestingly, in the GTPase superfamily it is an aspartic acid residue, closely linked to an asparagine residue, that determines the nucleotide specificity through hydrogen bonding to the guanine ring (reviewed in reference 10).

In this study, we have used proteolysis and the yeast two-hybrid system (18) to try to delineate the region of FtsZ involved in interaction with guanine nucleotides and with itself. Having located the GTP/GDP binding site to a central region,

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TABLE 1. Plasmids used in the yeast two-hybrid system

Plasmid	Vector	Construct on plasmid (amino acids)
pGAD424BSZ	pGAD424	$FtsZ_{Bs}$ (1-382)
pCX101	pGAD424	$FtsZ_{Bs}$ (1–326)
pCX102	pGAD424	$FtsZ_{Bs}$ (1–255)
pCX103	pGAD424	$FtsZ_{Bs}$ (1–213)
pCX104	pGAD424	$FtsZ_{Bs}$ (1–121)
pCX105	pGAD424	$FtsZ_{Bs}$ (100–382)
pCX106	pGAD424	$FtsZ_{Bs}$ (200–382)
pCX107	pGAD424	$FtsZ_{Bs}$ (300–382)
pCX116	pGAD424	$FtsZ_{Bs}$ (29–392)
pCX117	pGAD424	$FtsZ_{Bs}$ (60–382)
pGBT9BSZ	pGBT9	$FtsZ_{Bs}$ (1–382)
pCX108	pGBT9	$FtsZ_{Bs}$ (1–326)
pCX109	pGBT9	$FtsZ_{Bs}$ (1–255)
pCX110	pGBT9	$FtsZ_{Bs}$ (1–213)
pCX111	pGBT9	$FtsZ_{Bs}$ (1–121)
pCX112	pGBT9	$FtsZ_{Bs}$ (100–382)
pCX113	pGBT9	$FtsZ_{Bs}$ (200–382)
pCX114	pGBT9	$FtsZ_{Bs}$ (300–382)
pCX120	pGBT9	$FtsZ_{Bs}$ (29–392)
pCX121	pGBT9	$FtsZ_{Bs}$ (60–382)
pJC65	pGAD424	$FtsA_{Bs}$ (1–440)
pJC66	pGBT9	$FtsA_{Bs}$ (1–440)
pCX118	pGBT9	$FtsA_{Bs}$ (21–440)
pCX119	pGBT9	$FtsA_{Bs}$ (1–350)

we have further explored the interaction by examining several mutations that specifically alter the conserved residues. We have also detected an interaction between FtsZ and FtsA.

MATERIALS AND METHODS

Bacterial strains and plasmids. W3110, XL-1-blue, and DH5α were routinely used as strains for cloning and protein expression. JFL101 [*ftsZ84*(Ts)] and JKD7-2(pKD3) were used for complementation (7, 11). The latter strain carries *ftsZ::kan* with *ftsZ* supplied by the temperature-sensitive replicon pKD3.

pCXZ was used for expression of *B. subtilis ftsZ* ($ftsZ_{Bs}$) as described previously (38). pJF118HE was used as an expression vector for various *E. coli ftsZ* derivatives (19). pJC72 contained the first 320 codons of *ftsZ* cloned into pJF118HE. pBEFΔ was derived from pBEF0 by deleting the *Hin*dIII fragments (9). Two-hybrid system plasmids pGBT9 and pGAD424 were utilized (Clontech) with various derivatives constructed by PCR. Primers 5′-ATAGAATTCATG TTGGAGTTCGAAAC and 5′-CATGGATCCATTAGCCGCGTTTATTAC were utilized for $ftsZ_{Bs}$ with pCXZ as a template. The resulting plasmids were designated pGAD424BSZ and pGBT9BSZ, respectively. The primer design ensured that $ftsZ_{Bs}$ was in frame with both the GAL4 binding domain (BD) and the activating domain (AD) in these two plasmids. PCRs were performed by utilizing *Taq* polymerase in a COY Tempcycler II for 30 cycles with each cycle consisting of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C. Other plasmids, containing various portions of $ftsZ_{Bs}$, were constructed with the same approach. These plasmids are listed in Table 1.

PCR was also used to construct derivatives of pGBT9 and pGAD424 containing $ftsA_{Bs}$ with pUCA1 as a template (5). The primers for full-length $ftsA_{Bs}$ construction were 5′-TAAGGATCCAAATGAACAACAATGAAC for the 5′ end and 5′-AATCTGCAGCTATTCCCAAAACATGC for the 3′ end. Other $ftsA_{Bs}$ plasmids used in the yeast two-hybrid system are listed in Table 1 and were constructed by using appropriate primers.

Protein purification and sequencing. *E. coli* FtsZ ($FtsZ_{Ec}$) was expressed and purified as previously described (31, 38). $FtsZ_{320}$ was expressed from plasmid pJC72 and was purified essentially by following the procedures for the wild-type protein. $FtsZ_{Bs}$ was expressed and purified by a similar method except that a Mono-Q anion ion-exchange column (Pharmacia) was used instead of DEAE-cellulose. The sodium chloride gradient for elution was from 0 to 1 M. Protein concentrations were determined by using the Bio-Rad protein assay reagent.

The N-terminal sequences of various fragments were determined with an Applied Biosystems 491 protein sequencer. The fragments were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to polyvinylidene difluoride membranes in the following buffer: 1 mM CAPS (3-[cyclohexylaminol]-1-propanesulfonic acid)–10% methanol, pH 11.0. The membranes were wetted in 100% methanol (high-performance liquid chromatography grade) and equilibrated in transfer buffer immediately before use. The appropriate bands were cut out and sequenced.

GTP blotting and GTPase assay. After protease treatment, the digestion products were separated by SDS-PAGE and transferred onto nitrocellulose membranes as previously described (31, 38). Guanine nucleotide blotting and GTPase assays were done as described previously (31, 38). The GTPase assays were done at the protein concentrations indicated below. The reactions were carried out at 30°C in a 50-μl reaction mixture containing 50 mM HEPES (pH 7.2), 10 mM $MgCl_2$, 200 mM KCl, and 0.5 mM [γ-^{32}P]GTP.

Protease digestion and UV cross-linking. FtsZ (5 μg) was incubated with or without nucleotides at 37°C for 10 min in reaction buffer (50 mM HEPES [pH 7.0], 1 mM $MgCl_2$). Trypsin (Sigma) or chymotrypsin (Sigma) was added at various concentrations, and incubation was continued for another 15 min. The reaction was stopped either by placing the mixture on ice or by adding 1 volume of 2× SDS loading buffer to the mixture and boiling it for 5 min. The reaction mixtures were run on SDS–12.5% PAGE gels.

To assess GTP cross-linking to proteolytic fragments of FtsZ, 5 μg of FtsZ was incubated with [α-^{32}P]GTP at different concentrations and specific activities at 37°C for 15 min under nonhydrolyzing conditions. Then trypsin was added, and the incubation was continued for another 15 min. The reaction was stopped by placing the mixture on ice, and 1 μg of bovine serum albumin (BSA) was added to each reaction mixture. The samples were exposed to UV light for 20 min, and the proteins were precipitated by the addition of an equal volume of 10% trichloroacetic acid. The pellet was washed once with 5% trichloroacetic acid, resuspended in SDS sample buffer, and boiled for 5 min. These samples were subjected to SDS–12.5% PAGE. Subsequently, the gel was stained, dried, and exposed to X-ray film.

Yeast methods. *Saccharomyces cerevisiae* SFY526 and HF7c (Clontech) were transformed with plasmids as previously described (22). β-Galactosidase activity was assessed as described by the manufacturer.

Site-directed mutagenesis of *ftsZ* and analysis of mutations. The *E. coli ftsZ* gene was introduced into M13mp18 on a *Hin*dIII fragment from plasmid pZAQ (26). Site-directed mutagenesis of $ftsZ_{Ec}$ was carried out with a Muta · gene kit obtained from Bio-Rad Laboratories. After screening for the desired mutation, the allele was subcloned into vectors for assessment of complementation and for overexpression. For complementation, each of the alleles was cloned into a derivative of pBEF0 in which the *Hin*dIII fragments had been deleted. Cloning *ftsZ* alleles into this vector provides upstream sequences (to the *Bam*HI site in *ftsQ*) for expression. The plasmids were then introduced into JFL101 to test for complementation or tested to determine if they could replace pKD3 in JKD7-2 by selecting for Spc^r and screening for ampicillin sensitivity. For overexpression of the mutant proteins, the alleles were cloned into pJF118HE, which contains the *tac* promoter. The overexpressed mutant proteins were purified as described previously for the wild-type protein (30).

Polymerization. The polymerization of FtsZ was carried out as follows. FtsZ was added to 50 mM MES (morpholineethanesulfonic acid)-NaOH (pH 6.5), 10 mM $MgCl_2$, and 1 mM GTP to a final concentration of 250 μg/ml, and the mixture was incubated at 30°C. After 1 h, a 10-μl aliquot of the reaction mixture was placed on a carbon-coated copper grid for 2 min, blotted dry with Whatman 1 MM paper, stained briefly with 1% uranyl acetate, and blotted dry again. The grids were then viewed in a JEOL transmission electron microscope (model 100CXII). Polymerization of $FtsZ_{320}$ was done in the same way except that the protein concentration was 500 μg/ml. A more extensive examination of the polymerization conditions will be published elsewhere (32).

RESULTS

Analysis of the interaction of FtsZ with GTP by proteolysis. Sensitivity to protease digestion was used to explore the interaction between FtsZ and nucleotides. Initially, both $FtsZ_{Ec}$ and $FtsZ_{Bs}$ were examined; however, it was determined that $FtsZ_{Bs}$ was more readily protected from proteolytic digestion by nucleotides, presumably because of tighter nucleotide binding (data not shown). Therefore, $FtsZ_{Bs}$ was utilized in subsequent studies, and a diagram of this protein is shown in Fig. 1. Upon SDS-PAGE, the $FtsZ_{Bs}$ migrates with an apparent molecular mass of 45 kDa even though its calculated molecular mass is close to 40 kDa (5). In the absence of nucleotide, most of the $FtsZ_{Bs}$ was readily degraded by trypsin into small fragments of less than 15 kDa, although a band of approximately 40 kDa showed some resistance (Fig. 2). In the presence of GTP, trypsin-resistant fragments of 40 and 32 kDa were observed. Identical results were obtained with GDP, but other nucleotides, including ATP, CTP, UTP, and GMP, provided no protection from proteolysis (data not shown). This result is consistent with nucleotide binding studies done with $FtsZ_{Ec}$

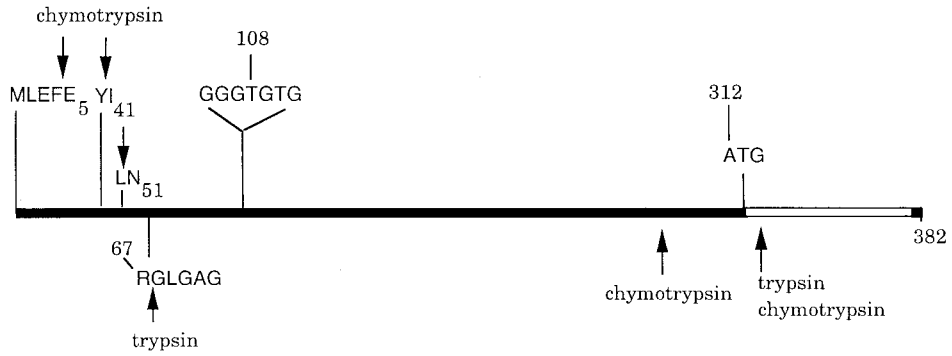

FIG. 1. Map of the FtsZ protein from *B. subtilis*, indicating the relevant features. The positions of proteolytic cleavage sites are indicated. The cleavages at the carboxyl end are only approximate, and not all chymotrypsin cleavage sites are indicated. The conserved regions of FtsZ (filled rectangles) and the nonconserved region (open rectangle) are shown. Another feature is the tubulin signature motif (GGGTGTG).

which demonstrated that it bound GTP and GDP and that this binding could not be competed by other nucleotides (30).

N-terminal sequencing of the 40-kDa fragment revealed that it had an intact N terminus, indicating that it must have arisen by a cleavage near the carboxyl terminus removing 5 kDa of the variable segment. The 32-kDa fragment had an N terminus of GLGAGAN, indicating that it arose from the 40-kDa fragment by cleavage after arginine 67 (Fig. 1). The appearance and relative stability of these proteolytic products in the presence of GTP or GDP suggest that they bind the nucleotide. To test this directly, FtsZ was treated with trypsin in the presence of GTP and the resultant fragments were separated by SDS-PAGE and blotted onto nitrocellulose to test for GTP binding. The 40-kDa fragment bound GTP, whereas the 32-kDa fragment did not (Fig. 3).

One possible reason that the 32-kDa fragment did not bind GTP was that it could not renature on the membrane. To test this possibility, radioactive GTP of various concentrations and specific activities was mixed with FtsZ and then incubated with trypsin. Following incubation with trypsin, the sample was UV-irradiated to cross-link any bound [α-^{32}P]GTP, and the FtsZ was analyzed by SDS-PAGE and autoradiography. The results show that [α-^{32}P]GTP was cross-linked to fragments with molecular masses of 40 and 32 kDa (Fig. 4). The ability of the 32-kDa fragment to be cross-linked to GTP suggests that either this fragment alone binds GTP or the binding of GTP to the intact protein is not sufficiently disrupted by trypsin digestion to eliminate cross-linking. For instance, the N-terminal 7-kDa fragment could remain bound after trypsin digestion and play a role in the binding of GTP.

Additional protease sensitivity experiments were done with chymotrypsin. Incubation of FtsZ with chymotrypsin led to degradation of most of the FtsZ (Fig. 5). The addition of GTP resulted in protection but a more complex pattern than observed with trypsin. Bands that are present in the lane without GTP are enhanced in the presence of GTP. Also, some bands, including bands 4 and 5, appear unique to the lanes with GTP. These results, along with those obtained with trypsin, demonstrate that the FtsZ conformation in the absence of GTP is fairly similar to the nucleotide-bound form; however, the nucleotide-bound form is more resistant to proteolysis. Also, nucleotide binding must cause some conformational change giving rise to bands 4 and 5 with chymotrypsin and the 32-kDa band with trypsin.

Sequence analysis of bands 1 to 5 allowed their origin to be determined. Band 1 is a mixture of two fragments, one having an intact N terminus and a second starting from amino acid 5. Both of these fragments must have arisen by cleavage of a 5-kDa fragment from the carboxyl end, similar to the 40-kDa fragment seen with trypsin. Bands 2 and 3 arose from band 1 by an additional cleavage after amino acids 41 and 51, respectively. Band 4 has an intact N terminus and could arise from band 1 by a second C-terminal cleavage removing a total of 13 kDa from the carboxyl end of the full-length protein. Band 5 is

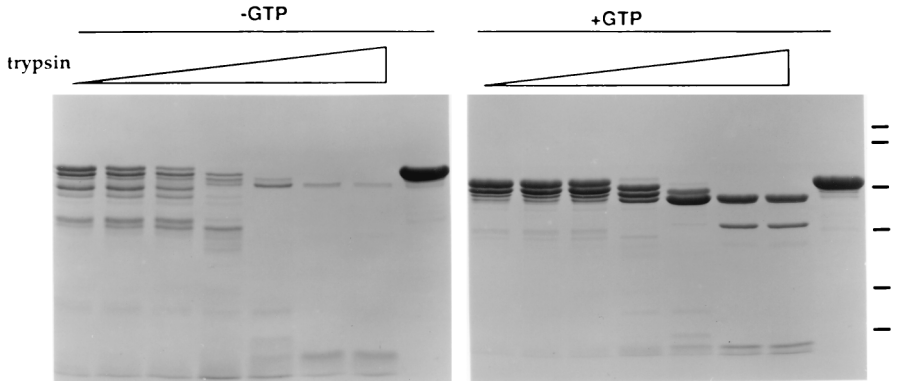

FIG. 2. FtsZ is protected from proteolytic digestion by GTP. FtsZ (5 μg per 10-μl reaction mixture) was incubated with increasing amounts of trypsin (range, 1 to 200 ng per 10-μl reaction mixture) in the absence or presence of 1 mM GTP. The reactions were stopped after 15 min and analyzed by SDS-PAGE. The molecular mass markers (indicated on the right) are as follows (from the top): phosphorylase *b* (97.4 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

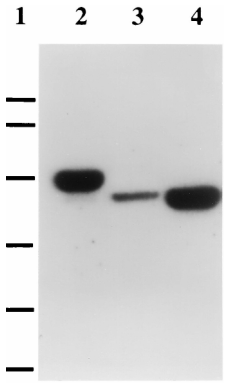

FIG. 3. GTP binding by trypsin fragments of FtsZ. FtsZ was digested with trypsin in the presence of 1 mM GTP (5 μg of FtsZ and 0.15 μg of trypsin in a reaction volume of 10 μl). The reaction was stopped, and the proteolytic fragments were separated by SDS-PAGE. The fragments were transferred to nitrocellulose, incubated with [α-^{32}P]GTP, and autoradiographed. Lane 1, molecular mass markers (the same as in Fig. 2); lane 2, FtsZ without trypsin; lane 3, FtsZ treated with trypsin in the absence of GTP; and lane 4, FtsZ treated with trypsin in the presence of GTP.

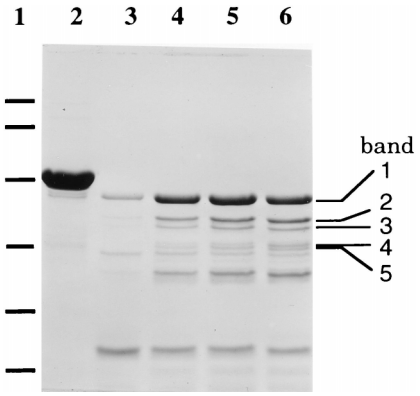

FIG. 5. GTP partially protects FtsZ from digestion with chymotrypsin. Purified FtsZ (5 μg/10 μl) was incubated with chymotrypsin (1.5 μg/10 μl) in the presence or absence of GTP, and the digestion was monitored by SDS-PAGE and Coomassie staining. Lane 1, molecular mass markers (same as in Fig. 2); lane 2, FtsZ; lanes 3 to 6, FtsZ plus chymotrypsin. The GTP concentrations were as follow: lane 3, 0; lane 4, 1 mM; lane 5, 0.1 mM; and lane 6, 0.01 mM.

a mixture of two fragments with the same N termini as those in band 1, but it must be missing a few more amino acids from the carboxyl end than band 4. To determine if any of these proteolytic fragments could bind GTP, they were blotted onto nitrocellulose and tested for binding. The results in Fig. 6 show that the fragments in bands 1 and 5 bind GTP, indicating that the carboxyl-terminal 14 kDa of FtsZ is not required for GTP binding. The GTP binding to band 5 (but not band 4) was confirmed by running a longer gel that clearly separated these fragments (data not shown). These results indicate that the GTP binding site is within the first 26 to 31 kDa.

Mutagenesis of conserved aspartic acid and asparagine residues. The results of the proteolytic digestion experiments suggest that the GTP binding domain of FtsZ is located within a central core region that has limited sequence homology to eukaryotic tubulins. In addition to the glycine-rich tubulin signature motif, there are two aspartate and two asparagine residues that are highly conserved among FtsZs and tubulins, with similar spacing from the tubulin signature motif (Fig. 7) (24). These conserved residues include D158, N165, N207, and D209 in *E. coli* FtsZ and D197, N204, N247, and D249 in yeast β-tubulin (33). To assess the importance of these residues to FtsZ function, we introduced mutations into the *E. coli ftsZ* gene contained on M13. Subsequently, the mutated gene was introduced into a low-copy-number plasmid to test for complementation and also introduced into an expression vector so that the mutant protein could be overproduced and isolated.

Low-copy-number plasmids carrying either the D158-to-N (D158N) or the D158A mutation complemented the *ftsZ84*(Ts) mutation and were able to displace a plasmid carrying the wild-type *ftsZ* gene in a strain with the chromosomal copy of the *ftsZ* gene disrupted (Table 2). These results indicate that this aspartic acid residue is not essential for *ftsZ* function. Also, the FtsZD158A mutant protein displayed a level of GTPase activity higher than that of the wild type (Table 2).

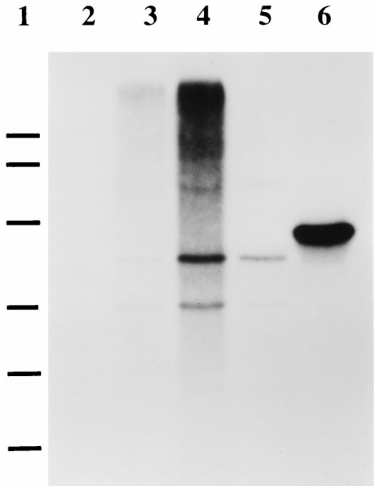

FIG. 4. Cross-linking of GTP to trypsin fragments of FtsZ. FtsZ (5 μg/10 μl) was incubated with trypsin (0.15 μg/10 μl) in the presence of GTP of various concentrations and specific activities. Reactions were stopped by placing the mixture in the cold, and the mixture was exposed to UV light for cross-linking. Samples were subjected to SDS-PAGE and autoradiographed. Lane 1, molecular mass markers (same as in Fig. 2); lane 2, FtsZ and trypsin, with no other additions; lane 3, FtsZ, trypsin, and 1 mM [α-^{32}P]GTP (0.5 Ci/mmol); lane 4, FtsZ, trypsin, and 0.1 mM [α-^{32}P]GTP (5 Ci/mmol); lane 5, FtsZ, trypsin, and [α-^{32}P]GTP (3,000 Ci/mmol); lane 6, FtsZ and [α-^{32}P]GTP (3,000 Ci/mmol).

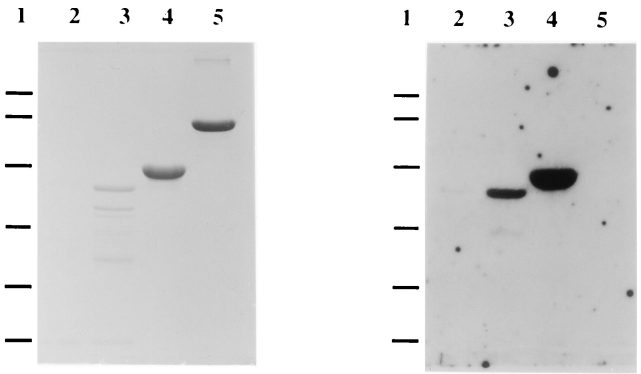

FIG. 6. GTP binding by chymotrypsin fragments of FtsZ, assessed by treating FtsZ with chymotrypsin in the presence of GTP. Proteolytic fragments were separated by SDS-PAGE and blotted onto nitrocellulose, which was incubated with labelled GTP and autoradiographed. Lanes 1, molecular mass markers (same as in Fig. 2); lanes 2, FtsZ and chymotrypsin incubated without GTP; lanes 3, FtsZ and chymotrypsin incubated in the presence of 1 mM GTP; lanes 4, FtsZ alone; lanes 5, BSA.

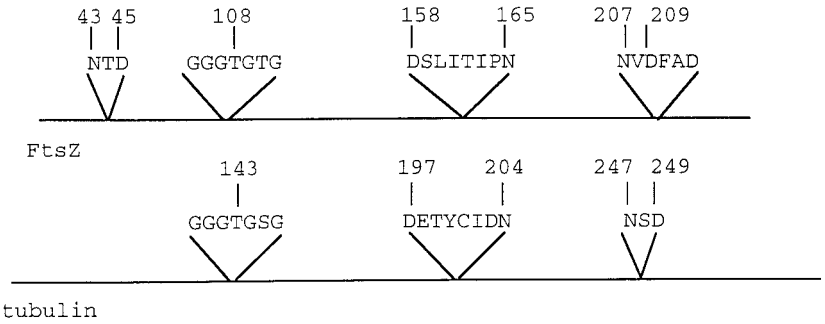

FIG. 7. Diagram indicating the location of the conserved aspartic acid and asparagine residues in FtsZ and the corresponding residues in tubulin. The numbering of the residues is from *E. coli* FtsZ and yeast β-tubulin.

The other three residues all were essential for *ftsZ* function (Table 2). Plasmids carrying *ftsZ*N165Y and *ftsZ*N165D did not display *ftsZ* function in either of the complementation tests. The mutant proteins carrying these substitutions for asparagine 165 retained the ability to bind GTP; however, they displayed markedly reduced GTPase activity. Likewise, *ftsZ*N207D and *ftsZ*D209N were unable to complement *ftsZ*-deficient cells. The FtsZN207D and FtsZD209N proteins both bound GTP as well as the wild type, suggesting that the alterations did not dramatically affect overall conformation; however, they had little GTPase activity (Table 2).

Highly conserved asparagine and aspartic acid residues are present in the N-terminal 67 amino acids of FtsZ. Mutagenesis revealed that they are essential, since both *fts*ZD45N and *ftsZ*N43D failed to complement the *ftsZ* mutants (Table 2). The corresponding mutant proteins bound GTP, indicating that these residues are not necessary for this activity (Table 2). FtsZN43D bound GTP more effectively than the wild type, indicating that this protein either refolded more efficiently on the membrane or had a higher affinity for GTP. However, these two mutant proteins displayed reduced GTPase activity, with that of FtsZD45N more dramatically reduced (Table 2). These mutant proteins, as well as the others, were also examined for ATPase activity, but none displayed a significant amount (data not shown).

TABLE 2. Analysis of the *ftsZ* mutations

Mutation	Complementation[a]		GTP binding[b]	GTPase activity (%)[c]
	ftsZ84(Ts)	Conditional null		
None (wild type)	+	+	+	100
N43D	−	−	++	31
D45N	−	−	+	<5
D158A	+	+	+	155
D158N	+	+	+	ND
N165Y	−	−	+	18
N165D	−	−	+/−	16
N207D	−	−	+	<5
D209N	−	−	+	ND

[a] Complementation tests were done at 42°C with the indicated mutations carried on the low-copy-number plasmid pGB2. The strains used were JFL100 [*ftsZ84*(Ts)] and JKD7-2 (*ftsZ*::*kan*)(pKD3). +, complementation; −, no complementation.

[b] GTP binding determined after transfer of the proteins to nitrocellulose and incubation with [α-^{32}P]GTP. +, binding equivalent to that of the wild type; ++, enhanced binding relative to that of the wild type; +/−, weak binding.

[c] The GTPase activity was measured over 120 min at a protein concentration of 250 μg/ml. The lag for the GTPase activity was variable, so the activity was assessed between 60 and 90 min, during which time the rate of hydrolysis was linear for all mutants. The wild-type activity was set at 100%. ND, not determined.

Interaction of FtsZ with itself. FtsZ probably interacts with itself to form the Z ring. To try to determine in more detail potential interacting sites, we exploited the yeast two-hybrid system. Previously, it was shown that $FtsZ_{Ec}$ has an activation domain, so it cannot be used as bait in the two-hybrid system (22). In contrast, $FtsZ_{Bs}$ does not have an activation domain, as revealed by its failure to turn the appropriate indicator strain blue on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) when fused to the BD of GAL4 (data not shown). Therefore, we used $FtsZ_{Bs}$ fused to the BD of GAL4 in combination with constructs containing $FtsZ_{Bs}$ or $FtsZ_{Ec}$ fused to the AD of GAL4 (22). Full-length constructs yielded blue colonies on X-Gal, indicating that an interaction between FtsZ molecules can be detected in this system (Fig. 8). It also demonstrates that this interaction is conserved between the *E. coli* and *B. subtilis* FtsZs despite the divergence in sequence between these genes. In fact, quantitative determination of the β-galactosidase units indicates that the interaction between the heterologous FtsZs is slightly stronger. To try to further delineate the site of self-interaction, deletions from both the 5′ and the 3′ ends of the *B. subtilis ftsZ* gene were made, and the truncated genes were fused to the BD of GAL4 and tested with a construct expressing either the AD-$FtsZ_{Bs}$ or the AD-$FtsZ_{Ec}$ fusion. The results of a series of deletions are summarized in Fig. 8. Removal of 56 amino acids from the carboxyl terminus of $FtsZ_{Bs}$, which removes the nonconserved region of FtsZ, had no effect on the interaction with either of the FtsZs on the AD. However, with removal of an additional 71 amino acids, no interaction was detected. Removal of 28 amino acids from the N terminus of $FtsZ_{Bs}$ resulted in a decrease in the interaction with $FtsZ_{Ec}$, and the interaction with $FtsZ_{Bs}$ was no longer detectable. Removal of up to 99 amino acids from the N terminus did not have any additional effect on the interaction with $FtsZ_{Ec}$; however, with removal of 200 amino acids from the N terminus, no interaction could be detected. From these results, a site for interaction between FtsZs can be defined in the N-terminal conserved domain, probably occurring between amino acids 100 and 326.

Analysis of the activity of truncated derivatives of FtsZ. The above analysis of FtsZ indicated that the carboxyl-terminal 57 amino acids are not required for FtsZ-FtsZ interaction. In addition, the nucleotide binding studies indicated that the extreme carboxyl segments of FtsZ were not required for GTP binding. To examine this further, several truncated *ftsZ* genes with stop codons at positions 321, 277, 253, and 220 were constructed by PCR and cloned into the expression vector

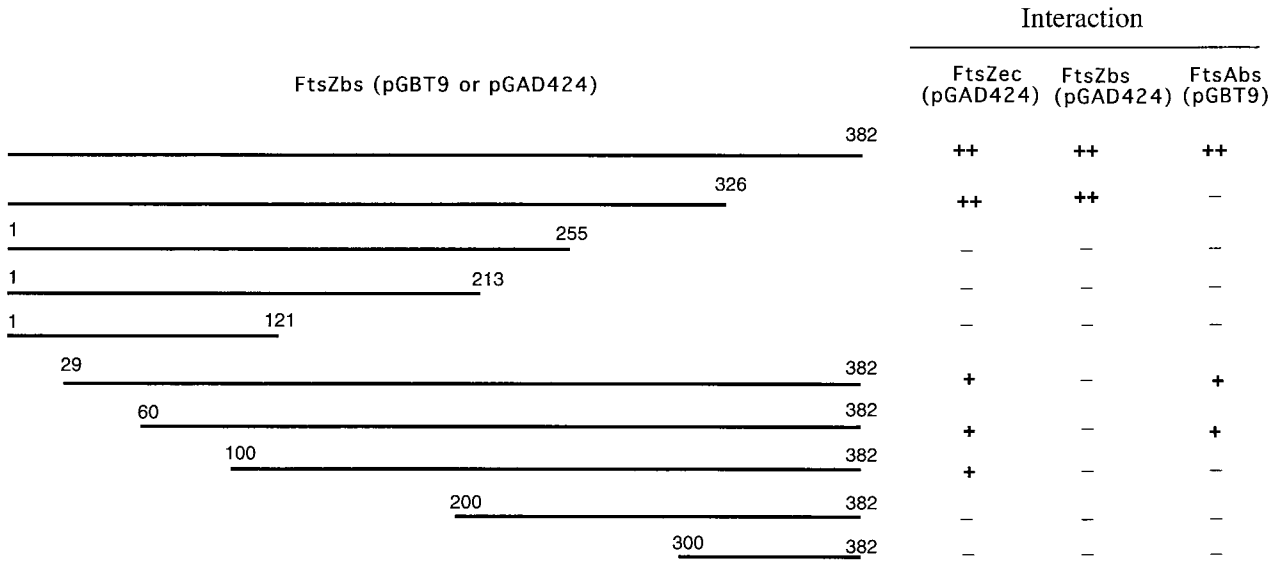

FIG. 8. Diagram showing the inserts that were utilized in the yeast two-hybrid system to map the regions of FtsZ responsible for the interaction of FtsZ with itself and with FtsA. Interactions are indicated on the right, based on the results of color development on indicator plates. ++, color development within 1 h; +, color development within 3 h; −, no color development in 24 h. Quantitation of the β-galactosidase activity gave the following values for the full-length proteins: $FtsZ_{Bs}$-$FtsZ_{Bs}$, 8.5 U, $FtsZ_{Bs}$-$FtsZ_{Ec}$, 12 U; $FtsA_{Bs}$-$FtsZ_{Bs}$, 14 U; and $FtsA_{Bs}$-$FtsZ_{Ec}$, 10 U. These interactions are sufficient to yield a blue color in 30 to 60 min.

pJF118HE to give pJC72, pJC74, pJC71, and pJC73, respectively. The truncations were made with $ftsZ_{Ec}$ so that the resultant alleles could be tested for complementation. Initial attempts at cloning the PCR fragments were unsuccessful, raising the possibility that the basal level of expression was deleterious. In an attempt to overcome this possible problem, pBS58, which contains *ftsQ*, *ftsA*, and *ftsZ* and results in a four- to fivefold increase in FtsZ, was introduced into the cloning strain. Previous work has shown that raising the level of FtsZ can suppress inhibition of cell division and lethality caused by expression of an *ftsZ* gene missing the first 37 codons (41). Using pBS58 allowed us to isolate all four clones. Subsequent retransformation and selection for just the expression vector allowed plasmids containing the three shorter truncations to be separated from pBS58. However, pBS58 was always present with pJC72, even though it was not selected, suggesting that $FtsZ_{320}$ was particularly toxic. Induction of the truncated $FtsZ_{320}$, even in the presence of pBS58, led to filamentation and cell death, indicating that this truncated FtsZ interfered with division (data not shown). Thus, the variable carboxyl segment is essential for FtsZ activity, and a truncated protein lacking this segment is a potent inhibitor of division.

To further analyze the truncated proteins and to assess their biochemical activity, they were overproduced. Overproduction of the three shorter FtsZs led to aggregation, as indicated by their appearance in the low-speed pellet. In contrast, $FtsZ_{320}$ behaved like the wild-type FtsZ throughout purification and was readily purified. Further analysis showed that $FtsZ_{320}$ bound GTP as well as the full-length protein (data not shown) and had a similar level of GTPase activity (Fig. 9A). The GTPase displayed a lag similar to that of the wild-type protein, and the lag decreased with increasing protein concentration, as previously reported for the wild-type protein (Fig. 9B) (14, 30). The ability of $FtsZ_{320}$ to polymerize was examined by utilizing conditions that have been found to promote the polymerization of FtsZ in the absence of promoting agents such as DEAE dextran (to be published elsewhere) (32). This polymerization is GTP dependent and does not occur with GDP. $FtsZ_{320}$ was found to undergo GTP-dependent polymerization at pH 6.5, also in the absence of DEAE dextran (Fig. 10B). The filaments showed a greater tendency to align along their long axis to generate larger structures somewhat similar to those formed by the full-length protein in the presence of DEAE dextran (31). In the absence of DEAE dextran, the full-length protein assembles into filaments with less extensive bundling under these polymerization conditions (Fig. 10A).

Interaction of FtsZ with FtsA. It has been suggested that FtsA interacts with FtsZ (12, 15, 27). To test this possibility, the yeast two-hybrid system was used. We used $FtsA_{Bs}$ fused to the BD of GAL4 and $FtsZ_{Bs}$ fused to the AD of GAL4. Introduction of plasmids carrying these fusions into the appropriate tester strain revealed an interaction as indicated by blue color on X-Gal (Fig. 8). The blue color was dependent upon the presence of both plasmids, since either plasmid alone or either plasmid in the presence of a cognate empty vector yielded no color (data not shown). Interestingly, we observed that $FtsA_{Bs}$ also interacted with *E. coli* FtsZ, indicating that the interaction between FtsZ and FtsA occurs in evolutionarily conserved regions (data not shown). To try to delineate the interacting site, the truncated FtsZs used above were moved into the AD plasmid and tested with BD-$FtsA_{Bs}$. We observed that deletion of the first 28 amino acids of $FtsZ_{Bs}$ resulted in a decrease in the interaction but did not eliminate it. Removal of the first 59 amino acids had the same effect, whereas no interaction was detected following removal of the first 99 amino acids. Removal of the largely nonconserved 57 amino acids from the carboxyl terminus resulted in an elimination of the interaction.

DISCUSSION

In this study, we have examined the interaction of FtsZ with itself, GTP, and FtsA. By exploiting sensitivity to proteases, we found that GTP binds to the N-terminal two-thirds of FtsZ and can be cross-linked to a core region consisting of residues 67 to approximately 250. Using the yeast two-hybrid system, we have

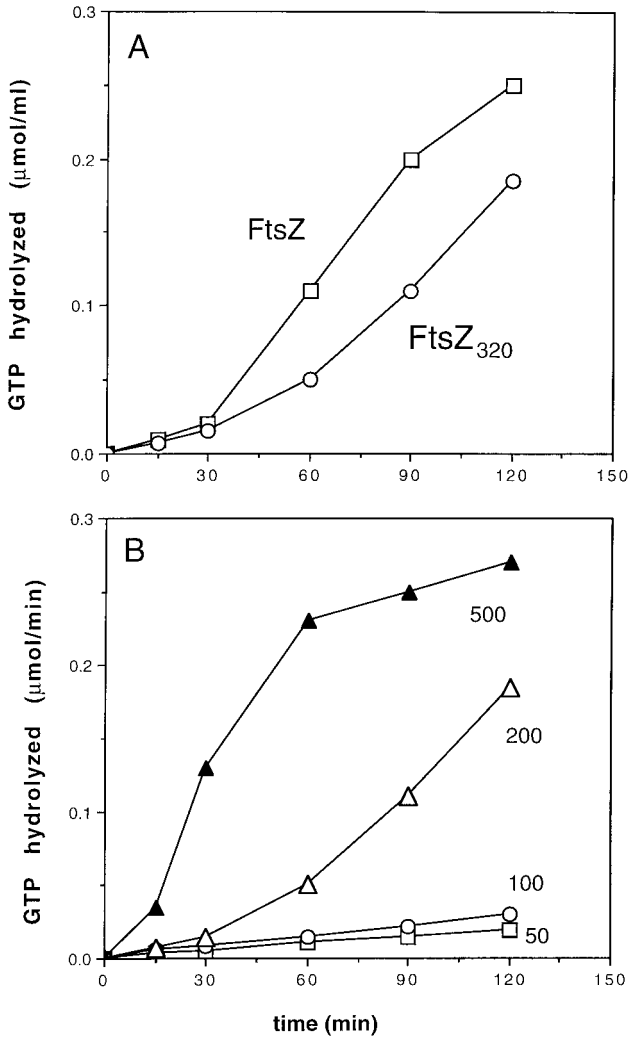

FIG. 9. GTPase activity of $FtsZ_{320}$. (A) The GTPase activity was measured at a protein concentration of 200 μg/ml. FtsZ and $FtsZ_{320}$ were incubated at 30°C in a 50-μl reaction mixture containing 50 mM HEPES (pH 7.2), 10 mM $MgCl_2$, 200 mM KCl, and 0.5 mM [γ-^{32}P]GTP. (B) The effect of the concentration of $FtsZ_{320}$ on the rate of GTP hydrolysis was determined by incubating $FtsZ_{320}$ at the indicated concentrations (in micrograms per milliliter).

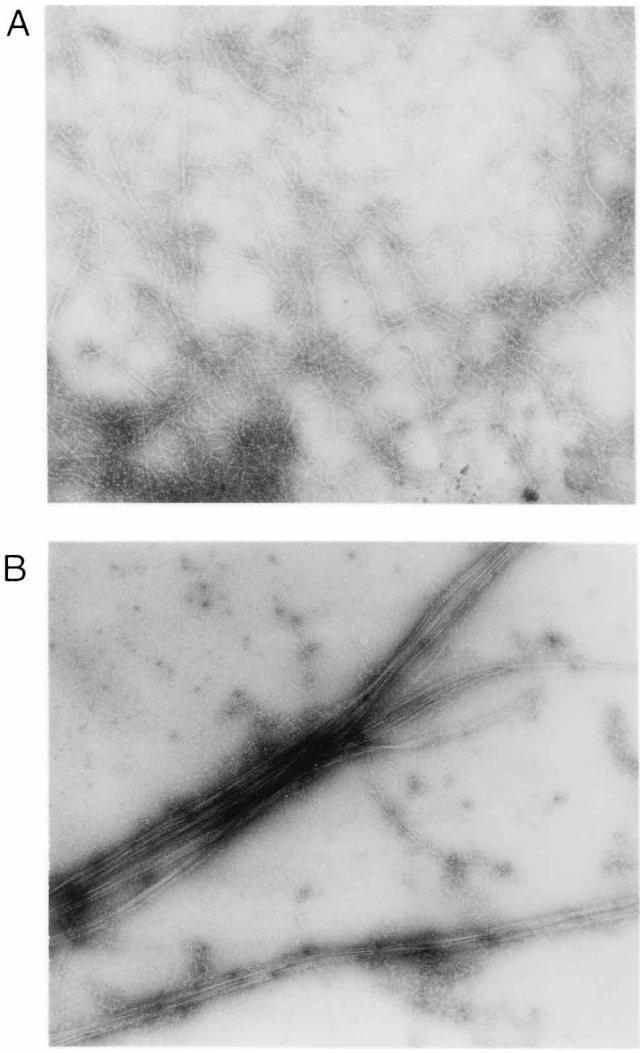

FIG. 10. Polymerization of $FtsZ_{320}$. FtsZ at 250 μg/ml (A) and $FtsZ_{320}$ at 500 μg/ml (B) were incubated in polymerization buffer for 30 min and viewed by negative-stain electron microscopy.

detected an FtsZ-FtsZ self-interaction that is within the N-terminal conserved domain. Furthermore, interaction between FtsZ and FtsA was also detected by using the yeast two-hybrid system. Interestingly, the interaction of FtsZ with itself and with FtsA could also be detected between proteins from the evolutionarily distant species *E. coli* and *B. subtilis*, arguing that it is specified by conserved residues.

In the GTPase superfamily, the nucleotide specificity is determined largely by the sequence NKXD, in which the aspartate residue hydrogen bonds to the guanine ring (10). The FtsZ family contains three pairs of highly conserved aspartate and asparagine residues, two of which can be aligned with conserved residues in tubulin. To explore the interaction of FtsZ with GTP, we first looked at nucleotide protection from proteolysis. The presence of GTP or GDP protected a large, N-terminal, 40-kDa fragment of the *B. subtilis* FtsZ from proteolytic digestion. The carboxyl-terminal segment, which is quite variable among evolutionarily distant bacteria, is rapidly removed by proteolysis and is not required for GTP binding. We have observed this proteolytic sensitivity with several proteases and several different bacterial FtsZs (data not shown), suggesting that the carboxyl tail is quite exposed, even in the presence of bound nucleotide. Furthermore, a truncated FtsZ lacking this variable sequence, $FtsZ_{320}$, bound GTP and had GTPase activity similar to that of the full-length protein. Thus, the variable carboxyl tail is not needed for GTPase activity. The chymotrypsin experiments extend the carboxyl region that is dispensable for GTP binding to 13 kDa. The observation that GTP could be cross-linked to a 32-kDa fragment that lacked approximately 50 amino acids from the carboxyl end and 67 amino acids from the N terminus suggests that at least part of the binding site is within this fragment.

To examine the importance of the three ND pairs in GTP binding and FtsZ function, they were altered by site-directed mutagenesis. The four residues that are in the same relative positions in FtsZ and tubulin (Fig. 7) were not required for GTP binding. D158 was changed to aspartic acid and to alanine. Both of these mutant alleles complemented strains deficient in *ftsZ*, and the D158A protein had enhanced GTPase activity. These studies rule out a critical role for this residue in

FtsZ function. In contrast, the other three residues are critical for function. The N165Y and N165D mutations inactivated the gene, and the corresponding proteins had reduced GTPase activity. Likewise, the N207D and D209N mutant proteins lost *ftsZ* activity in vivo and had no GTPase activity. However, all of these mutant proteins bound GTP (binding by N165D was weak, whereas N165Y binding was similar to that of the wild type), suggesting that their conformation was largely intact and that these aspartic acid and asparagine residues are not playing a direct role in determining nucleotide specificity. The same is true for another aspartic acid residue conserved among FtsZs, D212, which is changed to a G by the *ftsZ2* mutation (7). That mutant protein also binds GTP (13). An additional ND pair that is located nearer the N terminus and is also conserved among FtsZs was found to be essential for growth but not required for GTP binding. D45N and N43D mutant proteins both bound GTP but had reduced GTPase activity. Together, these results indicate that the determinants for the specificity of GTP binding by FtsZ have to be quite different than for the GTPase superfamily.

In vitro, FtsZ is able to polymerize in the presence of GTP to form protofilaments (16, 31). Since these protofilaments can align into a 2-D crystalline array, this suggests that FtsZ has to have several sites, both lateral and longitudinal, for it to interact with itself. By using the yeast two-hybrid system, we can detect at least one of these interactions in the $FtsZ_{Bs}$ protein. This interaction is also seen between the *E. coli* and *B. subtilis* FtsZs, and analysis of truncated derivatives indicates that the site can be defined between residues 100 and 326.

We also used the yeast two-hybrid system to demonstrate an interaction between FtsA and FtsZ. Interaction between these proteins would explain how FtsA is recruited to the Z ring during cell division (3, 27). This interaction did not require the first 59 amino acids of FtsZ but did require the nonconserved carboxyl tail. The latter finding is surprising, since the FtsA-FtsZ interaction was also observed between $FtsA_{Bs}$ and $FtsZ_{Ec}$, indicating that the interaction was between conserved residues. The only conserved residue in the carboxyl tail is at the extreme carboxyl end, which must play some role in the interaction (Fig. 1).

Evolutionarily distant FtsZs cause morphological abnormalities when introduced into *E. coli*. In most cases, this is a block to division (5, 28, 39, 40). The same appears to be true for FtsA, since *B. subtilis ftsA* is toxic to *E. coli* (28). From the results presented here, it is clear that the *B. subtilis* FtsZ and FtsA proteins are able to interact with $FtsZ_{Ec}$. Therefore, the *B. subtilis* proteins interact with the division machinery of the *E. coli* host but must be missing some function. The heterologous FtsZ may be equivalent to $FtsZ_{320}$, which is a potent inhibitor of division. Recently, Ma et al. (27) have shown that introduction of a *Rhizobium meliloti* FtsZ-GFP fusion protein into *E. coli* results in the formation of cytoskeleton-like structures that suggest colocalization with the host FtsZ into nonfunctional structures.

Biochemical analysis of $FtsZ_{320}$ complements the other studies reported here. It confirms that the variable C-terminal region is not required for GTP binding and revealed that it is not required for GTPase activity or polymerization. On the other hand, genetic studies demonstrate that this region cannot substitute for full-length FtsZ and that $FtsZ_{320}$ is a potent inhibitor of cell division. This inhibitory activity is likely to arise from $FtsZ_{320}$ copolymerizing with full-length FtsZ but failing in some subsequent step. Ma et al. (27) found that a similarly truncated FtsZ fused to GFP resulted in the appearance of unusual cytoskeletal structures, including spirals and thick sheets. The latter is consistent with our in vitro observations of the polymers formed by $FtsZ_{320}$. Ma et al. also found that this fusion protein was more inhibitory to division than the full-length fusion protein. This suggests that copolymers formed between FtsZ and $FtsZ_{320}$ are not capable of functioning in septation. Also, the two-hybrid system indicates that the C-terminally truncated FtsZ does not interact with FtsA. Together, these results indicate that the nonconserved carboxyl tail is on the surface of the filaments, where it would be available to interact with other proteins such as FtsA.

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