Non-sporulating *ftsZ* mutants in *Streptomyces coelicolor* reveal amino acid residues critical for FtsZ polymerization dynamics

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During sporulation of *Streptomyces coelicolor*, the cytokinin protein FtsZ is assembled into dozens of regularly spaced Z rings, which orchestrate the division of aerial hyphae into spores. We have previously found that a missense allele of *ftsZ*, *ftsZ17*(Spo), primarily affects sporulation septation rather than formation of cross-walls in vegetative mycelium. To clarify what aspect of FtsZ function is compromised in such non-sporulating mutants, we here use a genetic strategy to identify new *ftsZ*(Spo) alleles and describe how some of the mutations affect the biochemical properties of FtsZ. We have established a system for purification of recombinant untagged *S. coelicolor* FtsZ, and shown that it assembles dynamically into single protofilaments, displays a critical concentration indicative of cooperative assembly and has a rate of GTP hydrolysis that is substantially higher than that of the closely related *Mycobacterium tuberculosis* FtsZ. Of the nine isolated *ftsZ*(Spo) mutations, four affect the interface between the two main subdomains of FtsZ that is implicated in the assembly-induced conformational changes thought to mediate the GTP/GDP-driven cooperative assembly of FtsZ. We find that all these four mutations affect the polymerization behaviour of FtsZ *in vitro*. In addition, at least one *ftsZ*(Spo) mutation at the longitudinal contact surface between subunits in protofilaments strongly affects formation of polymers *in vitro*. We conclude that the assembly of Z rings during sporulation of *S. coelicolor* is highly sensitive to disturbances of FtsZ polymerization and therefore constitutes an excellent system for analysis of the elusive properties of FtsZ that mediate its characteristic polymerization dynamics.

INTRODUCTION

Bacterial cell division is orchestrated by the tubulin homologue FtsZ, which assembles on the inner surface of the cytoplasmic membrane to form a cytoskeletal structure known as the Z ring. This ring establishes the site of cytokinesis, acts as a scaffold for the other proteins involved in cell division and contributes force for constriction of the cell. *In vitro*, FtsZ assembles into protofilaments in the presence of GTP. The incoming subunit contributes a catalytically important residue to the GTPase active site on the neighbouring FtsZ molecule, and therefore the polymeric form hydrolyses GTP and becomes highly dynamic (for recent reviews, see Aylett *et al.*, 2011; Erickson *et al.*, 2010; Lutkenhaus *et al.*, 2012). Even under conditions when FtsZ assembles into single protofilaments, the polymerization is cooperative, and both polymer formation and GTPase activity display a critical concentration. The basis for this cooperativity has been enigmatic (Oliva *et al.*, 2007), but has been proposed to involve an assembly-induced conformational change of GTP-bound FtsZ that allows further subunits to bind to protofilaments with high-affinity (Chen *et al.*, 2005; Chen & Erickson, 2011; Martin-Galiano *et al.*, 2010). Recent structural data on *Staphylococcus aureus* FtsZ in complex with the inhibitor molecule PC190723 provide support for such an assembly-induced conformational switch, involving movement of the two main subdomains of FtsZ in relation to each other (Elsen *et al.*, 2012). These two independently folding subdomains – the N-terminal GTPase domain and the C-terminal activation domain – are separated by the central helix H7 and make up the core of the FtsZ molecule (Løwe & Amos, 1998; Oliva *et al.*, 2004). *In vivo*, FtsZ assembly is also modulated by a number of proteins. In

**Abbreviation:** Zap, Z-associated protein.

Three supplementary figures and a supplementary table are available with the online version of this paper.
Escherichia coli, FtsA and ZipA act as membrane tethers for the Z ring, and additional Z-associated proteins (Zap) also contribute to the stabilization of the Z ring (Galil & Gerdes, 2010; Hale & de Boer, 1997; Pichoff & Lutkenhaus, 2005). Other mechanisms involved in spatial or temporal control of FtsZ assembly include the Min system that prevents division at cell poles, nucleoid occlusion systems that prevent division over unsegregated nucleoids, and cell cycle checkpoint mechanisms like the SOS system of Escherichia coli. These systems include proteins that directly interact with FtsZ and interfere with its assembly (reviewed by Adams & Errington, 2009; Erickson et al., 2010; Lutkenhaus et al., 2012). Overall, assembly of the Z ring is determined both by the polymerization dynamics of FtsZ itself and by proteins that regulate FtsZ behaviour, ensuring the assembly of Z rings at the right time and place. After the Z ring has formed, it matures into a divisome by recruitment of a number of additional proteins, in E. coli including the essential division proteins FtsK, FtsQ, FtsB, FtsL, FtsW, FtsI and FtsN (reviewed by de Boer, 2010; Lutkenhaus et al., 2012). The divisome is a set of division machinery that can invaginate the cytoplasmic membrane, synthesize septal peptidoglycan and eventually split the septum to separate the daughter cells.

Although the FtsZ-based cell division machinery is broadly conserved, there is substantial variation in how assembly of the Z ring is controlled in different bacterial groups. A striking example is the Actinobacteria, which is a large phylum of Gram-positive organisms comprising medically interesting genera like Corynebacterium and Mycobacterium (Del Sol et al., 2006; Ploclinski et al., 2011), and direct interaction between the division protein FtsW and FtsZ may also help to stabilize the Z rings (Bennett et al., 2009; Datta et al., 2006; Mistry et al., 2008). However, the exact mechanisms by which cell division is developmentally regulated in Streptomyces remain unclear.

We have previously identified a missense allele, ftsZ17(Spo) that specifically interferes with sporulation septation while allowing essentially normal vegetative growth and hyphal cross-wall formation, thereby resulting in a non-sporulating phenotype in S. coelicolor (Grantcharova et al., 2003). This ftsZ mutation prevents normal assembly of Z rings in sporogenic hyphae, but the mechanistic basis for the differential effect on the two types of cell division in S. coelicolor is unclear. One possibility is that the ftsZ(Spo) mutation interferes with the interaction between FtsZ and sporulation-specific regulator(s) of Z ring assembly like SsgB (Willemse et al., 2011). Alternatively, if sporulation septation is more sensitive to disturbance of FtsZ function than vegetative cross-wall formation, ftsZ mutations that give a more general defect in FtsZ function may have stronger phenotypic effects on sporulation than on vegetative growth (Grantcharova et al., 2003). In order to clarify what aspect of FtsZ function is compromised in such non-sporulating mutants, we have set out to isolate further ftsZ(Spo) mutations and analyse how they affect the biochemical properties of FtsZ. We find that several ftsZ(Spo) mutations affect the intrinsic polymerization activity of FtsZ itself. The results identify critical amino acid residues involved in central but poorly understood aspects of the polymerization dynamics of FtsZ, and they underline the importance of dynamic remodelling of FtsZ polymers during sporulation of Streptomyces hyphae.

**METHODS**

**Bacterial strains and growth conditions.** S. coelicolor strains used in this study were derivatives of strain M145 (Kieser et al., 2000), and included the previously described strains HU133 (ftsZ::aphD1), J2417 (ftsZ::aphD1 attB2C31::pKF32[ftsZ]*) and O17 (ftsZ::aphD1 attB2C31::pO17[ftsZ17(Spo)])(Flärdh et al., 2000; Grantcharova et al., 2003; McCormick et al., 1994). E. coli strain DH5α (Hanahan, 1983) was used for cloning and plasmid propagation. Other E. coli strains are mentioned below. Culture conditions, antibiotic concentrations, genetic manipulations and recombinant DNA work generally followed previously described procedures for E. coli (Sambrook & Russell, 2001) and Streptomyces (Kieser et al., 2000). S. coelicolor strains were cultivated on mannitol soy flour (MS) agar plates or in yeast extract malt extract (YEME) liquid medium (Kieser et al., 2000).
Stratégie for isolation of ftsZ(Spo) mutants. The Diversify PCR random mutagenesis kit (Clontech Laboratories) was used to amplify ftsZ from plasmid pKF29 using standard M13 sequencing primers (Färärd et al., 2000). The PCR product was cloned in pCR2.1-TOPO using the TOPO TA cloning kit (Invitrogen) and then excised using EcoRI and XbaI digestion of plasmid DNA extracted from pools of several thousand transformants. The excised ftsZ gene was used to replace the normal ftsZ in plasmid pKF32, digested with EcoRI and XbaI and introduced into the promising conjugation donor strain E. coli ET12567/pUZ8002 by electroporation (Kieser et al., 2000). Plasmid pKF32 carries ftsZ and its promoter region in a vector that is transferrable from E. coli to S. coelicolor by conjugation, and it integrates into the &phi;C31 attB site on the S. coelicolor chromosome and fully complements the ΔftsZ::aphI mutation (Färärd et al., 2000). Transformant pools were used as donors for conjugation into the S. coelicolor ΔftsZ strain HU133 as described previously (Grancharova et al., 2003). From over 20,000 exconjugants, candidates with normal-sized pale grey or white colonies (among normally growing and sporulating ftsZ strains with dark grey colonies) were isolated and investigated by phase-contrast microscopy to identify those with defects in sporulation septation. The pKF32-derived plasmid was recovered from the putative mutants and transformed to E. coli ET12567/pUZ8002 as described previously (Grancharova et al., 2003). Two transformants from each potential mutant were used for conjugal transfer of the plasmid to the S. coelicolor ΔftsZ strain HU133. In cases where the majority of exconjugants gave rise to colonies with the same plate phenotype as the originally isolated putative mutant, the ftsZ allele on these plasmids was sequenced.

Plasmids for heterologous expression of ftsZ alleles in E. coli. S. coelicolor ftsZ was cloned in the expression plasmid pTBY1 from the IMPACT system (New England Biolabs) to generate a C-terminal fusion to the inin-chitin-binding-domain (FtsZ-intein-CBD). The ftsZ gene was amplified from plasmid pKF32 using primers KF44 and KF268 (see Table S1 available with the online version of this paper), digested with Ndel and Sapl and ligated into pTBY1 digested with the same enzymes. The resulting plasmid was named pKF176.

Phusion High-Fidelity DNA polymerase (Finnzymes) was used for site-directed mutagenesis in order to introduce specific mutations into pKF176. Back-to-back primer pairs with the desired point mutation introduced in one of the primers (Table S1) were phosphorylated at their 5′-end using T4 polynucleotide kinase (New England Biolabs) and used in PCR amplification of the entire pKF176 sequence. Amplified products were purified, treated with Dpnl to cleave any remaining plasmid template, religated and used to transform E. coli strain DH5α. The ftsZ alleles created in this way were verified by DNA sequencing.

Heterologous expression of ftsZ alleles and protein purification. pKF176 and its derivatives were transformed into E. coli strain ER2566 (New England Biolabs) for expression of ftsZ. Cultures were grown in 1 L LB medium (Sambrook & Russell, 2001) with carbenicillin (50 µg mL−1) to an OD600 of 0.6–0.8 at 37 °C, then transferred to 16 °C, induced with 0.5 mM IPTG, incubated overnight and harvested by centrifugation. Each pellet was resuspended in 25 mL cold column buffer (20 mM HEPES–NaOH pH 8.0, 500 mM NaCl, 1 mM EDTA), supplemented with Complete EDTA-free protease inhibitor cocktail (Roche). Each suspension was passed twice through a French press cell at 20,000 p.s.i. (138,000 kPa) and clarified by centrifugation at 20,000 g for 30 min at 4 °C.

Cleared cell lysate was loaded onto columns containing 5 mL chitin bead slurry (New England Biolabs), previously equilibrated with 50 mL cold column buffer. The column was washed with 50 mL wash buffer 1 (20 mM HEPES–NaOH pH 8.0, 500 mM NaCl, 1 mM EDTA, 0.1 % (w/v) Triton X-100), followed by 50 mL wash buffer II [20 mM HEPES–NaOH pH 8.0, 1 M NaCl, 1 mM EDTA, 1 % (w/v) Triton X-100] and three washes with 10 mL Wash buffer III (20 mM HEPES–NaOH pH 8.0, 500 mM NaCl, 1 mM EDTA, 5 mM ATP, 10 mM MgCl2), incubating for 3 min at room temperature between each wash with wash buffer III. ATP and MgCl2 were included in Wash buffer III to remove ATP-dependent chaperones from the column. After washing with 20 mL column buffer to remove the ATP, 15 mL freshly prepared cleavage buffer (20 mM HEPES–NaOH pH 8.0, 500 mM NaCl, 1 mM EDTA, 50 mM dithiothreitol) was allowed to pass through. The column was plugged, incubated overnight at 4 °C and then 15 mL cold GF-buffer (50 mM HEPES–NaOH pH 7.2, 0.1 mM EDTA, 10 % (w/v) glycerol) was added and seven 2 mL fractions were collected. To analyse protein yield and purity, all samples were analysed by SDS–PAGE using a Mini-PROTEAN 3 electrophoresis system (Bio–Rad) and stained with Coomassie brilliant blue. The fractions containing the protein were pooled and dialysed against 100 volumes ice-cold GF-buffer to purify the protein from dithiothreitol and salts using a Spectra/Por molecular porous membrane tubing (molecular mass cut-off of 12,000–14,000; Spectrum Laboratories). The protein concentration of a sample of wild-type FtsZ was determined by quantitative amino acid analysis and used as a reference to validate the in-house concentration determinations measuring A280 with a ND-1000 NanoDrop spectrophotometer. It has not been possible to ascertain how large a fraction of FtsZ molecules in our preparations are active.

GTPase assay. The PiColorlock Gold kit (Innova Biosciences), a malachite-green-based assay of released phosphate (P), was used to monitor GTPase activity of the purified FtsZ proteins. The Pi release was quantified colorimetrically at 620 nm. FtsZ in GF buffer was diluted to the desired concentration in MMK buffer (50 mM MES pH 6.5, 200 mM potassium acetate, 10 mM magnesium acetate). As a precaution both the GF and MMK buffers were treated with PBind resin (Innova Biosciences) to remove any contaminating traces of phosphates. The activity was tested in triplicate at 30 °C. The reactions were started by addition of 50 µM GTP to 3.5 µM FtsZ and samples were taken at 0, 2.5, 5, 10, 20 and 40 min. Then the reaction was stopped by adding an equal volume of 0.6 M perchloric acid. The standard curve was plotted using the absorbance data from an analytically prepared dilution series of potassium phosphate ranging from 2.5 to 100 µM.

Polymerization assay. Right-angle light-scattering assays were conducted in a Shimadzu RF-5301 PC spectrophotometer. The samples were illuminated at 350 nm and the 90° light scattering was measured at 350 nm with a slit width of 1.5 nm for both incoming and outgoing light. FtsZ in Gf buffer was diluted to 3.5 µM with MMK buffer. The reactions were started by adding GTP to 50 µM.

Electron microscopy. 7 µM of FtsZ (all variants) in MMK buffer was allowed to polymerize for 5 min with 500 µM GTP at 30 °C. Samples were diluted 10-fold in MMK-buffer containing 500 µM GTP immediately before 3.5 µl of the samples were spotted on carbon-coated copper grids and incubated for 3 min at 30 °C. The grids were then quickly washed twice in 50 µl drops of distilled H2O followed by one quick immersion in a 50 µl drop of 0.8 % uranyl formate followed by a 30 s immersion in a second drop of uranyl formate. Grids were carefully blotted against Whatman filter paper between each step. Negatively stained samples were air-dried and stored at room temperature before being observed. Images were obtained by a Philips CM120 transmission electron microscope operating at 100 kV and recorded with a MegaView III CCD camera (1376 × 1032 pixels) and analySIS software (Olympus Soft Imaging Solutions).

Homology modelling. Pair-wise alignment of S. coelicolor FtsZ with Mycobacterium tuberculosis FtsZ (gi52695400) was the basis for
creating a homology model with PDB entry 1RQ7 (sequence identity 79%) (Leung et al., 2004) as the template in the program SOD (Kleywegt et al., 2001). The model was adjusted in the program O (Jones et al., 1991), using rotamers that would improve packing in the interior of the protein. The model is arranged as a dimer as in the Mycobacterium tuberculosis FtsZ structure (PDB entry 1RQ7), but we have only used chain A as a basis for any interpretations. The model is available upon request from the authors.

RESULTS AND DISCUSSION

Isolation of new ftsZ(Spo) mutants

To isolate new ftsZ(Spo) mutants, we used error-prone PCR to achieve random mutagenesis of the S. coelicolor ftsZ gene in a plasmid vector and introduced the resulting library of mutant alleles into the ftsZ null mutant strain HU133. Exconjugants were screened for white colony phenotype, indicating that the incoming plasmid has restored vegetative cell division, but not sporulation septation. This screen also selects against severely dysfunctional ftsZ alleles, which do not allow growth on the medium used for conjugative transfer of plasmids (Grantcharova et al., 2003). The ftsZ-carrying plasmid was extracted from selected strains and reintroduced into the ΔftsZ strain HU133 to verify that the phenotype was linked to the ftsZ allele on the plasmid. In this way, nine new ftsZ(Spo) mutant alleles were found and the mutations were identified by DNA sequencing (Table 1).

The plate phenotype for the isolated mutants ranged from completely white colonies, [ftsZ18(Spo), ftsZ22(Spo), ftsZ26(Spo)], to very pale grey [(ftsZ19(Spo), ftsZ20(Spo), ftsZ24(Spo)] or intermediate grey colonies [ftsZ23(Spo), see Fig. 1]. The microscopic examination of aerial hyphae revealed to what extent the ftsZ(Spo) mutants had abolished or reduced formation of normal spores, sporulation septa and aberrantly sized spore-like hyphal fragments (Table 1 and Fig. 2).

Mutations affecting the C-terminal tail of FtsZ

Two of the new mutant alleles [ftsZ19(Spo) and ftsZ23(Spo), see Table 1] had mutations affecting the conserved C-terminal peptide of FtsZ that in other bacteria is known to bind FtsZ-interacting proteins, including FtsA, ZipA and MinC (Din et al., 1998; Haney et al., 2001; Lutkenhaus et al., 2012; Ma & Margolin, 1999; Mosyak et al., 2000; Shen & Lutkenhaus, 2009). Thus, these amino acid residues might be involved in direct interaction of FtsZ with proteins that aid the assembly of proper Z rings in Streptomyces. One candidate partner is FtsW (Mistry et al., 2008), while SsgB has been suggested to interact with S. coelicolor FtsZ (ScFtsZ) via other routes than the C-terminal peptide (Willemse et al., 2011). It is also possible that the C-terminal tail has a role in lateral interactions between protofilaments, but the positively charged residues at the extreme C terminus of Bacillus subtilis FtsZ that have recently been implicated in protofilament bundling are not present in ScFtsZ (Buske & Levin, 2012). Both the ftsZ19(Spo) and ftsZ23(Spo) mutations gave relatively mild phenotypes and have not been further investigated here (Table 1).

Mapping the ftsZ(Spo) mutations on the structural model of the FtsZ core

The residues affected by ftsZ(Spo) mutations have been highlighted on a ribbon cartoon of the homology model of the S. coelicolor FtsZ structure, based on the crystal structure of FtsZ from the closely related Mycobacterium tuberculosis (MtFtsZ; Fig. 3). The positions of mutated residues suggest possible features that may be affected by them. For example, the ftsZ22(Spo) mutation N273I is in helix H10 in a region known from previous studies to be directly involved in longitudinal contact with the next FtsZ molecule within a single protofilament (Oliva et al., 2004). The ftsZ25(Spo) mutant has the A275T mutation in helix H10. This residue is not directly involved in longitudinal contacts, but may disrupt the z helix structure in this region and thereby influence the interaction with the next subunit in the protofilament. However, this mutation appears also to affect the stability of the protein since the A275T mutant was the only one of our isolated mutants that showed a substantially reduced level of FtsZ in Western blot analyses of cell extracts (data not shown). In vivo, the A275T mutation has a very strong effect on vegetative growth, suggesting a strong general cell division defect, and it has therefore been excluded from further detailed analyses. The V10D substitution in ftsZ20(Spo) affects a residue at the interface between the two subunits in the crystallized dimer of MtFtsZ (PDB entry 1RQ7), but it is not clear whether the contact surface between the two monomers in this crystal structure has any resemblance to real lateral interactions between FtsZ protofilaments in vivo (Leung et al., 2004). Interestingly, three of the new mutations (I126F, S189T and L246P), as well as the A249T mutation in the previously described ftsZ17(Spo) allele (Grantcharova et al., 2003), affect residues in the interfaces between the two main core domains of FtsZ and the central helix H7 that separates them. This region has recently been implicated in conformational changes that underlie the putative assembly switch of FtsZ (Chen & Erickson, 2011; Elsen et al., 2012). In order to test the hypothesis that several of the ftsZ(Spo) mutations affect the intrinsic polymerization behaviour of FtsZ, we set out to investigate the effect of selected ftsZ(Spo) mutations on FtsZ activity in vitro (see below). We focus here on the mutations that affect the internal interface between the two main subdomains of FtsZ. Although the D196N substitution also affects this region, it does not confer a sporulation phenotype when separated from the other mutations in ftsZ27(Spo) and has therefore not been further investigated. We aimed to include the V10D variant in further analyses, but this mutant protein was for unknown reasons impossible to produce in E. coli.
| ftsZ(Spo) allele | Nucleotide change | Mutation | Colony size* | Degree of pigmentation† | Sporulation septation‡ | Polymerization§ | GTP hydrolysis (GTP/FtsZ min⁻¹)|||| CC¶ |
|-------------------|------------------|----------|--------------|-------------------------|------------------------|-----------------|---------------------|----------------------|------------------|
| –                 | –                | Wild-type| ++           | +                       | Regular spores         | + + +           | 0.89 ± 0.01         | 0.94                 |
| 17                | G746A            | A249T    | ++           | +                       | No spores.             | + + +           | 0.53 ± 0.02         | 0.36                 |
| 18                | A376T            | I126F    | +            | +                       | Very few and           | – #             | –                   | –                   |
|                   |                  |          |              |                         | irregular spores.      |                 |                     |                      |
|                   |                  |          |              |                         | Mostly hyphal          |                 |                     |                      |
|                   |                  |          |              |                         | fragments.             |                 |                     |                      |
| 19                | A1073T A1187G    | E358V D396G | ++           | + (+)                  | Spores form, but       | ND**            | ND                  | ND                  |
|                   |                  |          |              |                         | irregularly shaped and reduced numbers compared with WT. |                 |                     |                      |
| 20                | T29A             | V10D     | +            | +                       | Very few and           | ND              | ND                  | ND                  |
|                   |                  |          |              |                         | irregular spores.      |                 |                     |                      |
|                   |                  |          |              |                         | Mostly hyphal          |                 |                     |                      |
|                   |                  |          |              |                         | fragments.             |                 |                     |                      |
| 22                | A818T            | N273I    | +            | +                       | Very few and           | +               | 0.54 ± 0.01         | 0.45                 |
|                   |                  |          |              |                         | irregular spores.      |                 |                     |                      |
|                   |                  |          |              |                         | Mostly hyphal          |                 |                     |                      |
|                   |                  |          |              |                         | fragments.             |                 |                     |                      |
| 23                | A1172T           | E391V    | +            | +                       | Spores form, but       | ND              | ND                  | ND                  |
|                   |                  |          |              |                         | irregularly shaped and moderately reduced numbers compared with WT. |                 |                     |                      |
| 24                | T565A            | S189T    | +            | +                       | Very few and           | + +             | 0.58 ± 0.03         | 1.21                 |
|                   |                  |          |              |                         | irregular spores.      |                 |                     |                      |
|                   |                  |          |              |                         | Mostly hyphal          |                 |                     |                      |
|                   |                  |          |              |                         | fragments.             |                 |                     |                      |
| 25                | G823A T142C††    | A275T    | +            | +                       | Poor growth and        | ND              | ND                  | ND                  |
|                   |                  |          |              |                         | aerial mycelium        |                 |                     |                      |
|                   |                  |          |              |                         | formation. No          |                 |                     |                      |
|                   |                  |          |              |                         | spores.               |                 |                     |                      |
| 26‡‡              | T737C            | L246P    | +            | +                       | No spores.             | +               | 0.95 ± 0.04         | 1.44                 |
|                   |                  |          |              |                         | Occasional septa      |                 |                     |                      |
|                   |                  |          |              |                         | divide spore-like      |                 |                     |                      |
|                   |                  |          |              |                         | hyphal fragments.      |                 |                     |                      |
| 27                | A170T C219T G586A| D57V G75C D196N | +            | +                       | No spores.             | ND              | ND                  | ND                  |
|                   |                  |          |              |                         | Occasional septa      |                 |                     |                      |
|                   |                  |          |              |                         | divide spore-like      |                 |                     |                      |
|                   |                  |          |              |                         | hyphal fragments.      |                 |                     |                      |
Table 1. Cont.

*Estimation of colony size in comparison to wild-type colonies was used as indicator of effects on vegetative growth. ++, colony size similar to ftsZ+ strain J2417. +, colonies clearly smaller than ftsZ+ strain.
†Degree of grey pigmentation of colonies (see Fig. 1).
‡Formation of spores and sporulation septa was estimated by phase-contrast microscopy of impression prints from the aerial mycelium of developing colonies (see Fig. 2).
§Based on light scattering and transmission electron microscopy assays.
||GTP hydrolysis rate during the initial 10 min (5 min for L246P) of GTP hydrolysis at 3.5 μM FtsZ, where the reaction proceeds in a linear fashion. For the L246P protein, only the first 5 min were used since the slope thereafter deviated from linearity.
¶Critical concentration (CC) determined by plotting the initial GTP hydrolysis rate against FtsZ concentration (at 1.5, 2.5 and 3.5 μM FtsZ) and extrapolating the linear regression line backwards to where it meets the x-axis (Fig. S2).
#No biochemical activity was detected for the I126F mutant.
**ND, Not determined.
††Silent mutation.
†††The originally isolated mutant had also a mutation in the ftsZ promoter region, but we have here used a reconstructed strain with only the single missense mutation, showing the same phenotype as the original strain.

Purification and biochemical analyses of S. coelicolor FtsZ

First, systems had to be established for heterologous production, purification and biochemical analyses of S. coelicolor FtsZ. Attempts to produce hexahistidine-tagged S. coelicolor FtsZ in E. coli have previously been unsuccessful due to toxicity and insolubility of the product (K. Flärnhed, unpublished observations). However, such problems were avoided by fusing the C terminus of FtsZ to the large intein–chitin binding domain of the IMPACT system (New England Biolabs), which allowed efficient production and purification of soluble untagged FtsZ after intein-mediated cleavage of the fusion protein.

Addition of GTP to purified ScFtsZ in the presence of the poly-cationic crowding agent DEAE-dextran induced formation of stable polymers consisting of bundled protofilaments, and subsequent addition of an excess of GDP caused depolymerization (Fig. S1). In the absence of DEAE-dextran, the GTP-induced polymers were shorter and appeared as single protofilaments, as observed by right-angle light scattering assays and electron microscopy of negatively stained samples, respectively (Figs 4a and 5a). Upon depletion of GTP, FtsZ depolymerized rapidly (Fig. 4a). In a malachite-green-based GTPase assay (Fig. 4c), ScFtsZ hydrolysed about one GTP per FtsZ min⁻¹ at our standard reaction conditions (30 °C, 3.5 μM FtsZ, 50 μM GTP). We found the critical FtsZ concentration for GTPase activity to be 0.94 μM for ScFtsZ (Table 1 and Fig. S2), which is similar to E. coli FtsZ (EcFtsZ) and MtFtsZ at about 1 and 2 μM respectively (Chen & Erickson, 2005; Chen et al., 2007).

Overall, ScFtsZ shows polymerization activity similar to typical FtsZs, but does not show any bundling or overt lateral interactions under the tested conditions (except in the presence of DEAE-dextran). The GTP hydrolysis rate (0.89 GTP/FtsZ min⁻¹) was lower than that reported for EcFtsZ (around four GTP/FtsZ min⁻¹) and higher than that of the phylogenetically closely related M. tuberculosis FtsZ (approximately 0.08 GTP/FtsZ min⁻¹ at room temperature) (Chen et al., 2007; White et al., 2000). The slow GTP hydrolysis of MtFtsZ may be related to the very slow growth of M. tuberculosis. While S. coelicolor grows significantly faster than M. tuberculosis, the high GTPase activity of ScFtsZ may also be related to the need for highly dynamic polymerization and remodelling involved in forming the multiple Z rings during S. coelicolor sporulation (Grantcharova et al., 2005).

Three mutations around the internal domain interface of FtsZ affect in vitro polymerization dynamics and result in typical sporulation-defective phenotypes

Mutant FtsZs were purified as described above. We first characterized the original A249T mutant protein (Grantcharova et al., 2003). This protein showed a light-scattering signal of similar magnitude to the wild-type protein, suggesting similar amount or average size of polymers (Fig. 4a), and similar polymers were revealed by electron microscopy (Fig. 5b). However, the GTP hydrolysis rate was approximately half that of the wild-type (Fig. 4c; Table 1). This was reflected in the polymerization assays, since GTP was consumed at a lower rate compared with the wild-type FtsZ, the A249T protein had a longer-lasting steady-state plateau before onset of the overall decay of polymers due to GTP exhaustion (Fig. 4a). Furthermore, once overall depolymerization commenced, the decay rate of the light scattering signal was reproducibly lower than that for the wild-type protein, indicating a slower subunit turnover in the A249T mutant polymers than in the wild-type.

We then focussed on the new mutations affecting the internal domain interface. The ftsZ26(Spo) allele encodes the L246P mutant protein, which upon addition of GTP showed a weaker light scattering signal compared with the wild-type FtsZ. The steady-state period for polymerization of the L246P protein was very short before the amount of polymers decreased again (Fig. 4a). Only short protofilaments were seen...
in the negative staining (Fig. 5c). The L246P protein had an initial GTP hydrolysis rate similar to that of the wild-type protein, but it then decreased around the time when half the GTP had been consumed (Fig. 4c). Based on these results, we hypothesized that this mutant FtsZ has a higher affinity for GDP than the normal FtsZ protein and therefore shows decreased polymerization when the concentration of GDP approaches that of GTP. This was tested by adding a mixture of GTP and GDP to the wild-type and L246P proteins. The L246P protein showed a strong reduction of GTP hydrolysis rate when GTP and GDP are added at equal concentrations, while the wild-type protein hydrolysed GTP at a substantially higher rate than the L246P protein under these conditions (Fig. S3). Thus, initially FtsZ(L246P) hydrolyses GTP with high turnover rates, but it is strongly affected by the presence of GDP, possibly contributing to the smaller size and the lower abundance of the polymers formed by this mutant protein compared with the wild-type (Figs 5c and 4c).

The loop between H9 and S8 that is affected by L246P and A249T is located at the end of the cleft that separates the two main domains of FtsZ. The region between helix H9 and \( \beta \) strand S8 has previously been shown to play a role in FtsZ polymerization. One of the mutations (S272A) introduced into Methanococcus jannaschii FtsZ (MtFtsZ)
by Martín-Galiano et al. (2010) to probe the conformational switch of FtsZ corresponds to position S244 in ScFtsZ. This mutation promoted stability of polymers. Mutating the D254–E255 residues to alanines in Caulobacter crescentus FtsZ (corresponding to E at position 248 in ScFtsZ, i.e. immediately between L246 and A249) led to unstable FtsZ assembly in vivo and cells that could initiate but not complete cell division (Wang et al., 2001). Furthermore, two of the non-lethal ftsZ mutations isolated in B. subtilis based on effects on prespore-specific gene expression are in this region and affect the end of H9 (ftsZ3, V260A) or the beginning of S8 (ftsZ24, I245F) (Feucht & Errington, 2005). In summary, A249T and L246P are likely to affect ScFtsZ polymerization by influencing the intramolecular interactions between the domains, and it should be noted that both mutations influence the critical concentration of FtsZ, which is indicative of the cooperativity of FtsZ protofilament formation (Table 1 and Fig. S2).

The cleft between the domains is also the site of the ftsZ24(Spo) mutation (S189T). S189 is located on the central H7 helix and faces the cleft that separates the GTPase and activation domains of FtsZ (Fig. 3). The S189T mutant FtsZ showed reduced GTP hydrolytic activity compared with the wild-type and L246P (Fig. 4d). The magnitude of the light scattering signal was lower than for the wild-type, but despite the decreased GTP consumption, the duration of the steady state was shorter compared with the wild-type protein (Fig. 4b). Therefore, whether activity of the S189T protein was inhibited by GDP in the same way as the L246P protein was tested, but this was not the case (Fig. S3). Importantly, this cleft corresponds to the taxol binding site on tubulins, and it is also the binding site of the FtsZ inhibitor PC190723 that stabilizes FtsZ polymers from some Gram-positive bacteria (Andreu et al., 2010; Elsen et al., 2012; Haydon et al., 2008; Tan et al., 2012). Residue G193 in ScFtsZ corresponds to G196 in the S. aureus FtsZ that is involved in forming a hydrogen bond between the central

**Fig. 3.** Ribbon cartoon of the homology model of S. coelicolor FtsZ showing the location of the mutations studied in this work. The C-terminal activation domain is shown in blue, the central helix (H7) in cyan, the N-terminal GTPase domain in green and the bound GDP in orange. The amino acid residue mutations described in this study are indicated as sticks in red (mutations for which the gene product has been purified and subjected to biochemical analyses) and yellow (for those mutations that have not been subjected to biochemical analyses). The model is based on chain A of the crystal structure of Mycobacterium tuberculosis FtsZ (PDB entry 1RQ7).
H7 helix and the C-terminal activation domain in the GDP-bound conformation, while being separated from the C-terminal domain by the PC190723 molecule when FtsZ is in the so called ‘high-affinity’ state proposed by (Elsen et al., 2012). The leucine at position 187 of ScFtsZ corresponds to L189 of EcFtsZ, at which an inserted tryptophan revealed a particularly large assembly-induced conformational change (Chen & Erickson, 2011). The S189T mutation is apparently not a dramatic change, but it is likely that S189 plays a specific role in the interaction between the C- and N-terminal domains within one monomer. The change to threonine (S189T) probably interferes with these interactions and therefore leads to the observed alteration of the polymerization dynamics of FtsZ causing the defect in sporulation septation.

**Mutants with stronger general defects in FtsZ function**

I126, which is mutated to a phenylalanine in ftsZ18(Spo), is an internal residue in the conserved β strand S5 on the GTPase domain. I126 faces the back side of helix H7 relative to the region corresponding to the taxol-binding site discussed above, and also influences the interface with the activation domain and helix H7. The neighbouring residue in strand S5 corresponds to a predicted hinge involved in conformational changes in MjFtsZ (Martin-Galiano et al., 2010). Mutation of this residue in MjFtsZ leads to more unstable polymers with enhanced GTPase activity, and a corresponding mutation confers thermosensitive cell division to E. coli, indicating more unstable Z-ring assembly (Addinall et al., 2005; Martin-Galiano et al., 2010). The effect of I126F on ScFtsZ was the strongest among our investigated ftsZ alleles. The purified protein was essentially inactive in the biochemical assays and no GTPase activity or light scattering was detected (data not shown). The poor activity of the I126F protein was not due to major misfolding since the circular dichroism spectrum of the mutant protein was very similar to that of the wild-type FtsZ (data not shown). Furthermore, some very short protofilaments were seen by electron microscopy, suggesting a weak activity (arrows in Fig. 5e).

Although our preparations of the I126F FtsZ protein did not polymerize well in vitro, this protein still supported some cell division in vivo. Possibly, other proteins may promote FtsZ assembly in vivo and thereby allow this defective FtsZ to show some functionality. The S. coelicolor ftsZ18(Spo) mutant strain grew slowly and formed small colonies (Fig. 1), indicating a strong effect on vegetative growth. The effect of this mutation is also less specific for sporulation septation than the others described above and it allows some degree of sporulation septation in the aerial mycelium (Fig. 2e). The N273I mutation in ftsZ22(Spo) is a substitution in the contact interface between two consecutive FtsZ monomers within a protofilament. This mutation does not allow
formation of sporulation septa, but it is also an example of a mutant with a more general defect in cell division. It forms smaller and thinner colonies than the congenic strain with wild-type ftsZ (Fig. 1), although it grows substantially better than a true ftsZ null mutant. Also the in vitro polymerization is strongly affected, and although detectable protofilaments were formed, both the GTPase activity and light-scattering signal were lower compared with the wild-type protein (Fig. 4b and d). An unusual feature of this mutant protein was a tendency to polymerize slightly also in the absence of added nucleotide. This was seen as a noisy and drifting baseline in the light-scattering assays, but the signal disappeared when GDP was added to the assay (data not shown). The results from N273I and I126F mutations show that also mutants with relatively strong general defects in FtsZ function and cell division can be identified by our screening strategy.

Conclusions

The screen for white-colony ftsZ mutants identified amino acid residues that both affect the in vitro activity of purified FtsZ and the ability to form sporulation septa. Several of these residues are located in regions of the protein predicted to influence the conformational changes that underlie the GTP/GDP-driven polymerization dynamics. Understanding the effect of such mutations is important for elucidating the molecular basis for FtsZ assembly. The findings corroborate S. coelicolor sporulation as a powerful model system for genetic analysis of the polymerization dynamics and the elusive assembly switch of FtsZ.

Our results reveal that Z ring assembly is more sensitive to perturbations in FtsZ polymerization dynamics during sporulation than during vegetative crosswall formation. The latter process appears significantly more robust, as several ftsZ mutations that are severely affected in sporulation septation still supported normal vegetative growth. Likely reasons for this differential effect are that the assembly of FtsZ during sporulation septation (i) takes place in cells with substantially higher FtsZ concentration due to the transcriptional upregulation of ftsZ, (ii) involves formation of large numbers of closely spaced Z rings in these cells and (iii) is subject to multiple developmental control mechanisms in order for division to take place at the correct time and place, and to be coordinated with the position and segregation of chromosomes (Flärdh, 2003; Jakimowicz & van Wezel, 2012; McCormick, 2009). During this process, FtsZ assembly is likely to be modulated by several proteins, acting both negatively and positively on polymer formation and stability. The balance between these factors is critical for the remodelling and coalescence of helical FtsZ filaments into multiple and regularly spaced Z rings in the long sporulating hyphae. This set of new ftsZ(Spo) alleles will provide useful tools in genetic analyses of Streptomyces sporulation and cell division, for example in isolation of suppressor mutations or identification of genes that when overexpressed can rescue the ability of specific ftsZ(Spo) mutants to sporulate.

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REFERENCES


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