**INTRODUCTION**

A fundamental problem in prokaryotes is the nature of the mechanism of cell division, which is tightly coupled to cell growth, chromosome replication and segregation. Thereby, the cell ensures that each daughter cell is of equal size, and inherits a complete genome. Cytokinesis in bacteria begins with the assembly of the essential GTPase FtsZ into ring-like structures at the nascent division site (reviewed by Errington et al., 2003; Romberg & Levin, 2003). FtsZ homologues can be found in nearly all bacteria, and have also been found in a number of eukaryotic organelles, e.g. mitochondria and chloroplasts (reviewed by Vaughan et al., 2004). The crystal structure of FtsZ closely resembles that of eukaryotic α- and β-tubulin (Lowe & Amos, 1998; Nogales et al., 1998). FtsZ consists of two domains, of which the GTP-binding interactions lie in its N-terminal domain (Lowe & Amos, 1998). The GTPase is split across two monomers, with the N-terminal domain of one monomer providing the GTP-binding site, and the C-terminal domain of the other nucleotide hydrolysis (Lowe & Amos, 1999; Scheffers et al., 2002). Like tubulin, FtsZ undergoes GTP/GDP-dependent polymerization, forming protofilaments, sheets and minirings in vitro (Bramhill & Thompson, 1994; Erickson et al., 1996; Mukherjee & Lutkenhaus, 1994, 1998; Yu & Margolin, 1997). The extreme C-terminus of FtsZ, which is not visible in the crystal structure, has been shown to be required for interaction with the essential division proteins FtsA and ZipA in *Escherichia coli* (Ma & Margolin, 1999; Mosyak et al., 2000). In the rod-shaped bacterium *Bacillus subtilis*, six additional proteins, FtsA, FtsW, DivIB, DivIC, FtsL and PBP2B, have been implicated in cell division, and their localization to the division site is dependent on FtsZ (Beall & Lutkenhaus, 1992; Daniel & Errington, 2000; Daniel et al., 2000; Feucht et al., 2001; Katis et al., 2000). Therefore, the FtsZ ring provides a cytoskeletal scaffold that recruits these other division proteins to form the cytokinetic machinery, and directs the ingrowth of the septum. FtsA, ZapA and the sporulation-specific protein SpoIIIE have been shown to interact directly with FtsZ to stabilize FtsZ ring formation, but so far little is known about the residues that are involved in these interactions (Ben-Yehuda & Losick, 2002; Gueiros-Filho & Losick, 2002; Low et al., 2004; Lucet et al., 2000; Wang et al., 1997).

On starvation, a developmental process called sporulation is initiated, and early in sporulation, the medial FtsZ ring disassembles and spirals out towards the cell poles, where it reassembles into a ring structure near both cell poles (Ben-Yehuda & Losick, 2002). The shift in position of the Z-ring depends both on an increase in FtsZ expression levels, and on the sporulation-specific SpoIIIE protein. SpoIIIE is an integral membrane protein that interacts directly with FtsZ (Lucet et al., 2000), and it has been known for several years that polar FtsZ ring formation and asymmetric division are impaired in spoIE-null mutants (Barak & Youngman, 1996; Feucht et al., 1996; Khvorova et al., 1998). Additionally,
SpoIIE plays a key role in $\sigma^F$ activation (reviewed by Errington, 2003).

So far, three factors that act negatively on FtsZ ring formation have been described in \textit{B. subtilis}: the Min system, the nucleoid and the Z-ring-associated protein EzrA. The Min system consists of three proteins, MinC, MinD and DivIVA, which prevent cell division near the cell pole (Marston & Errington, 1999). Recently, Wu & Errington (2004) identified a protein, Noc, that associates non-specifically with the nucleoid, and is required to inhibit cell division in its vicinity. EzrA is a membrane protein that interacts directly with FtsZ to prevent polymerization and aberrant FtsZ machinery is still not fully understood, and the above survey shows that there are a myriad of potential protein–protein interactions involving FtsZ, of which only a few have been characterized in any detail. The isolation of point mutations in \textit{ftsZ} with subtle effects on phenotype might provide an important tool to analyse FtsZ function. Most previous genetic studies of \textit{ftsZ} have focused on the isolation of mutants with severe division defects (Stricker & Erickson, 1993). One unit of $\beta$-galactosidase catalyses the production of 1 nmol 4-methylumbelliferone min$^{-1}$.

The role of FtsZ in assembly and constriction of the division machinery is still not fully understood, and the above survey shows that there are a myriad of potential protein–protein interactions involving FtsZ, of which only a few have been characterized in any detail. The isolation of point mutations in \textit{ftsZ} with subtle effects on phenotype might provide an important tool to analyse FtsZ function. Most previous genetic studies of \textit{ftsZ} have focused on the isolation of mutants with severe division defects (Stricker & Erickson, 1993). One unit of $\beta$-galactosidase catalyses the production of 1 nmol 4-methylumbelliferone min$^{-1}$.

**RESULTS**

Isolation of non-lethal \textit{ftsZ} mutations

A plasmid carrying the \textit{ftsZ} gene, but lacking the first ATG codon, was randomly mutated by propagation in the \textit{E. coli} mutator strain XL1-Red (Stratagene). The mutated plasmid library was transformed into several different \textit{B. subtilis} strains, with selection for plasmid integration via single crossover into the host chromosome on the basis of chloramphenicol resistance. The resultant clones contained a mutated, but full-length, copy of \textit{ftsZ}, under its natural chromosomal DNA was prepared by a scaled-down method based on the one described by Errington (1984). \textit{B. subtilis} cells were made competent for transformation with DNA using the method of Anagnostopoulou & Spizizen (1961), as modified by Jenkinson (1983). Nutrient agar (NA; Oxoid) was used as a solid medium, and PAB (Oxoid Antibiotic Medium no. 3) or hydrolysed casein medium (CH) as a liquid medium for growing \textit{B. subtilis}. Chloramphenicol (5 mg ml$^{-1}$), tetracycline (10 mg ml$^{-1}$), erythromycin (1 mg ml$^{-1}$), lincomycin (25 mg ml$^{-1}$) and 0-01% X-Gal were added as required. \textit{E. coli} strains were grown in 2 x TY medium (Sambrook \textit{et al}., 1989) or on NA supplemented with ampicillin (100 mg ml$^{-1}$), as required.

**METHODS**

**Strains and plasmids.** These are described in Table 1. Plasmid pSG1928 was constructed by cloning a 1158 bp DNA fragment containing the \textit{ftsZ} gene without the ATG start codon into plasmid pSG1301. The DNA segment was amplified by PCR using 168 wild-type chromosomal DNA with primers 5'-GCCCTAGATTGGAGTTGCAAAAAACAATA-3' and 5'-CCGAATTCCTTTAGCCGCTTTTATACGG-3', which introduced XbaI and EcoRI sites, respectively, and these were then used for cloning. The cloned \textit{ftsZ} fragment was sequenced, and thereby several discrepancies from the published sequence were found (Beall \textit{et al}., 1988): codon 327 (CGT instead of CGC), codon 345 (GAG instead of GAC) and codon 346 (CCA instead of CCA). In several other independent PCR reactions using either 168 or SG8 wild-type chromosomal DNA as a template, we invariably found the same sequences as for the cloned construct. Site-directed mutagenesis (Stratagene) was used to introduce mutations into the \textit{ftsZ} fragment of plasmid pSG1928. The following primers were used: (38-1fw) 5'-GGTTATTAAATGAAAATCCAAAAGATGAGATTGTGG-3', (38-1rev) 5'-GTTGTTATTACGG-3', (38-2fw) 5'-GCTTACTGTGTTCTGCCGCTGAGGTTCCTC-3', (38-2rev) 5'-GCTCTAGA-GAGGAACCTCAGCGGCAGAACACAGTAAGC-3', (38-3fw) 5'-GCTCTAGA-GAGGAACCTCAGCGGCAGAACACAGTAAGC-3', (38-3rev) 5'-GCTCTAGA-GAGGAACCTCAGCGGCAGAACACAGTAAGC-3'. Each mutant plasmid was sequenced before introduction into the \textit{B. subtilis} chromosome. Also, the mutated \textit{ftsZ} gene was sequenced from the chromosome of constructed strains to verify the \textit{ftsZ} mutation. \textit{ftsZ} mutations were crossed back into the parental strain by preparing and then transforming their chromosomal DNA into the parental strain, with selection for chloramphenicol resistance.

**General methods.** DNA manipulations and \textit{E. coli} transformations were carried out using standard methods (Sambrook \textit{et al}., 1989). B. \textit{subtilis} chromosomal DNA was prepared by a scaled-down method based on the one described by Errington (1984). \textit{B. subtilis} cells were made competent for transformation with DNA using the method of Anagnostopoulou & Spizizen (1961), as modified by Jenkinson (1983). Nutrient agar (NA; Oxoid) was used as a solid medium, and PAB (Oxoid Antibiotic Medium no. 3) or hydrolysed casein medium (CH) as a liquid medium for growing \textit{B. subtilis}. Chloramphenicol (5 mg ml$^{-1}$), tetracycline (10 mg ml$^{-1}$), erythromycin (1 mg ml$^{-1}$), lincomycin (25 mg ml$^{-1}$) and 0-01% X-Gal were added as required. \textit{E. coli} strains were grown in 2 x TY medium (Sambrook \textit{et al}., 1989) or on NA supplemented with ampicillin (100 mg ml$^{-1}$), as required.

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The plasmid was mutagenized.

library of mutant first screen was based on use of a strain (1272) bearing a transcriptional control, followed by the integrated plasmid D*Strain generated by the genome function project.

For DNA transformations, the source of the DNA is shown, followed by an arrow, then the recipient strain, with the antibiotic selection in parentheses after "ftsZ". Numbers in parentheses after each entry indicate the strain number and the source of the DNA.

transcriptional control, followed by the integrated plasmid and a second (inactive) copy of ftsZ that lacked promoter, ribosome-binding site and start codon sequences. The library of mutant ftsZ alleles was screened in two ways. The first screen was based on use of a strain (1272) bearing a $\sigma^F$-dependent spoIIQ–lacZ reporter gene. $\sigma^F$ activation during sporulation is dependent on formation of the asymmetric division septum (Feucht et al., 1999; King et al., 1999). This reporter is also inhibited by perturbations in vegetative cell length, which affect polar septation during

Table 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant genotype</th>
<th>Construction, source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B. subtilis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BFS1038*</td>
<td>trpC2 ezrA::pMUTIN2.mcs (ermC)</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>FG356</td>
<td>zapA–yshBA::tet amyE::P,$\sigma_r$zapA (cat) thrC::P,$\omega_c$,yshB (erm)</td>
<td>Guereos-Filho &amp; Losick (2002)</td>
</tr>
<tr>
<td>SG38</td>
<td>trpC2 amyE</td>
<td></td>
</tr>
<tr>
<td>168</td>
<td>trpC2</td>
<td></td>
</tr>
<tr>
<td>279.1</td>
<td>metC3 tal-1 ftsA279</td>
<td></td>
</tr>
<tr>
<td>1272</td>
<td>trpC2 Δ(youV::spoIIQ–lacZ ermC)</td>
<td></td>
</tr>
<tr>
<td>1356</td>
<td>trpC2 zapA–yshBA::tet</td>
<td></td>
</tr>
<tr>
<td>1357</td>
<td>As 1272 $\Omega$(ftsZ::pSG1928 ftsZ3 (V260A) cat)</td>
<td>FG356→168 (Tc)</td>
</tr>
<tr>
<td>1358</td>
<td>As 1272 $\Omega$(ftsZ::pSG1928 ftsZ4 (A285T) cat)</td>
<td>pSG1928→1272 (Cm)</td>
</tr>
<tr>
<td>1359</td>
<td>As 1272 $\Omega$(ftsZ::pSG1928 ftsZ6 (E300K) cat)</td>
<td>pSG1928→1272 (Cm)</td>
</tr>
<tr>
<td>1360</td>
<td>As 1272 $\Omega$(ftsZ::pSG1928 ftsZ11 (S271R) cat)</td>
<td>pSG1928→1272 (Cm)</td>
</tr>
<tr>
<td>1361</td>
<td>As 1272 $\Omega$(ftsZ::pSG1928 ftsZ24 (I245F) cat)</td>
<td>pSG1928→1272 (Cm)</td>
</tr>
<tr>
<td>1362</td>
<td>As 1901 $\Omega$(ftsZ::pSG1928 ftsZ20 (V38A) cat)</td>
<td>pSG1928→1901 (Cm)</td>
</tr>
<tr>
<td>1363</td>
<td>As 1901 $\Omega$(ftsZ::pSG1928 ftsZ5 (D174N) cat)</td>
<td>pSG1928→1901 (Cm)</td>
</tr>
<tr>
<td>1364</td>
<td>As 1901 $\Omega$(ftsZ::pSG1928 ftsZ38 (L302P, Q353R) cat)</td>
<td>pSG1928→1901 (Cm)</td>
</tr>
<tr>
<td>1365</td>
<td>As 2770 $\Omega$(ftsZ::pSG1928 ftsZ8 (S219L) cat)</td>
<td>pSG1928→2770 (Cm)</td>
</tr>
<tr>
<td>1366</td>
<td>As 1272 $\Omega$(ftsZ::pSG1928 ftsZ20 (V38A) cat)</td>
<td>1362→1272 (Cm)</td>
</tr>
<tr>
<td>1367</td>
<td>As 1272 $\Omega$(ftsZ::pSG1928 ftsZ5 (D174N) cat)</td>
<td>1363→1272 (Cm)</td>
</tr>
<tr>
<td>1368</td>
<td>As 1272 $\Omega$(ftsZ::pSG1928 ftsZ3 (L302P, Q353R) cat)</td>
<td>1364→1272 (Cm)</td>
</tr>
<tr>
<td>1369</td>
<td>As 1272 $\Omega$(ftsZ::pSG1928 ftsZ8 (S219L) cat)</td>
<td>1365→1272 (Cm)</td>
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<tr>
<td>1370</td>
<td>As 168 $\Omega$(ftsZ::pSG1928 ftsZ38-1 (L302P) cat)</td>
<td>pSG1929→1272 (Cm)</td>
</tr>
<tr>
<td>1371</td>
<td>As 168 $\Omega$(ftsZ::pSG1928 ftsZ38-2 (Q353R) cat)</td>
<td>pSG1930→1272 (Cm)</td>
</tr>
<tr>
<td>1372</td>
<td>As 1272 $\Omega$(ftsZ::pSG1928 cat)</td>
<td>pSG1928→1272 (Cm)</td>
</tr>
<tr>
<td>1901</td>
<td>trpC2 amyE Δ(minD::ermC)</td>
<td>Marston et al. (1998)</td>
</tr>
<tr>
<td>2023</td>
<td>trpC2 amyE noc::ermC</td>
<td>Sievers et al. (2002)</td>
</tr>
<tr>
<td>2770</td>
<td>trpC2 Δ(minCD::ermC)</td>
<td>A. Leung, University of Oxford (unpublished)</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCI061</td>
<td>F$^-$ hsdR mcrB araD139 Δ(arabABC–leu)7679 galU</td>
<td>Meissner et al. (1987)</td>
</tr>
<tr>
<td>XL1-Red</td>
<td>endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac mutD5</td>
<td>Stratagene</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pSG1301</td>
<td>bla cat</td>
<td>Stevens et al. (1992)</td>
</tr>
<tr>
<td>pSG1928</td>
<td>bla cat ftsZ(4-1146)</td>
<td>This study</td>
</tr>
<tr>
<td>pSG1929</td>
<td>bla cat ftsZ38-1(L302P)</td>
<td>pSG1928 containing the ftsZ38-1 (L302P) mutation</td>
</tr>
<tr>
<td>pSG1930</td>
<td>bla cat ftsZ38-2(Q353R)</td>
<td>pSG1928 containing the ftsZ38-2 (Q353R) mutation</td>
</tr>
</tbody>
</table>

*Strain generated by the B. subtilis genome function project.
†Numbers in parentheses after ftsZ refer to the first and last nucleotides of the ftsZ coding sequence.
‡For DNA transformations, the source of the DNA is shown, followed by an arrow, then the recipient strain, with the antibiotic selection in parentheses.
§The plasmid was mutagenized.
sporulation indirectly. Transformants of this strain were grown at 37 °C, and screened for decreased or increased $\sigma^F$ activation on the basis of β-galactosidase activity on plates containing X-Gal. Of approximately 30 000 transformants screened, five mutants were obtained that formed colonies which were either darker (mutant ftsZ24) or paler blue (mutants ftsZ3, ftsZ4, ftsZ6 and ftsZ11), suggesting an altered activation of $\sigma^F$ compared with the parental strain. The colour changes in strains carrying the ftsZ3 or ftsZ4 mutation were slight. All five mutations were crossed back into the parental strain 1272. Segregation of darker and lighter blue colonies confirmed that the mutations lay within the ftsZ locus.

In parallel, the mutated plasmid library was also transformed into host strains 1901 and 2770 containing deletions of minD and minCD, respectively. Both strains are slightly impaired in sporulation (Levin et al., 1998; Thomaides et al., 2001). Transformants were grown at 37 °C, and screened for altered sporulation frequency on the basis of colony appearance. Around 4500 and 9000 transformants were screened, yielding three mutations (ftsZ5, ftsZ20 and ftsZ38) from the minD mutant, and one (ftsZ8) from the minCD mutant. All of the mutants appeared to be further impaired in sporulation than the parental strain on NA. The mutations were crossed into the minCD+ strain 1272. Segregation of mutations ftsZ5, ftsZ20 and ftsZ38 into colonies with normal and reduced sporulation efficiency showed that these mutations also had an effect in the wild-type background, and lay within the ftsZ locus. Mutation ftsZ8, which produced only a slight sporulation defect in 2770, did not have a detectable phenotype in the wild-type background. Therefore the presence of the mutation was confirmed by sequencing the ftsZ gene of four transformants.

DNA sequencing (Table 2) revealed that only one of the new ftsZ alleles, ftsZ20, would produce a substitution (V38A) in the N-terminal domain of the protein. Allele ftsZ5 (D174N) would affect the beginning of the core helix H7 that connects the N- and C-terminal domains of FtsZ. Six of the other seven alleles had mutations that would affect residues in the less-characterized C-terminal domain of FtsZ. The exception, ftsZ38, had two mutations: L302P, which lay close to the ftsZ6 mutation, and Q353R, which lay in the extreme C-terminus, for which no structural data are available. The substitution V38A (ftsZ20) is in an amino acid position that is highly conserved between other organisms for which genome sequences are available (Vaughan et al., 2004). The changes I245F (ftsZ24), V260A (ftsZ3), E300K (ftsZ6), L302P (ftsZ38) are in less-conserved residues, whereas D174N (ftsZ5), S219L (ftsZ8), S271R (ftsZ11), A285T (ftsZ4) and Q353R (ftsZ38) are not conserved (Vaughan et al., 2004).

### Morphology of the ftsZ mutant strains

To examine the morphological phenotype of the mutants in more detail, cultures of the wild-type and mutant strains were grown in PAB, and exponentially growing cells were analysed by microscopy (Fig. 1). At this point, each of the mutations was examined in a min+ background, independent of the background in which it was isolated. The wild-type strain (1272) is illustrated in Fig. 1(A). Cells producing FtsZS219L (ftsZ8; Fig. 1B, C) had a mild phenotype manifested only in the presence of a small percentage (<1%) of minicells (arrow in Fig. 1B). In the minCD strain background that the mutation was originally isolated from, it formed abnormally long cells (Fig. 1D), which probably accounts for the reduction in sporulation frequency that led to its isolation.

Several mutant proteins [FtsZI245F (ftsZ24), FtsZL302PQ353R (ftsZ38), FtsZV260A (ftsZ3) and FtsZA285T (ftsZ4)]

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**Table 2. Phenotypic effects of ftsZ mutants in the wild-type**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Derivation*</th>
<th>Amino acid change</th>
<th>FtsZ localization pattern in wild-type background</th>
<th>Morphological phenotype in wild-type background†</th>
</tr>
</thead>
<tbody>
<tr>
<td>ftsZ3</td>
<td>IIQ ↓</td>
<td>V260A</td>
<td>Normal, extra protein at cell poles</td>
<td>Spo+; ~10% minicells</td>
</tr>
<tr>
<td>ftsZ4</td>
<td>IIQ ↓</td>
<td>A285T</td>
<td>Normal, extra protein at cell poles</td>
<td>Spo+; ~10% minicells</td>
</tr>
<tr>
<td>ftsZ6</td>
<td>IIQ ↓ ↓</td>
<td>E300K</td>
<td>Broad diffuse bands, aberrant structures</td>
<td>Spo−; normal and filamentous cells; twisted divisions</td>
</tr>
<tr>
<td>ftsZ11</td>
<td>IIQ ↓ ↓</td>
<td>S271R</td>
<td>Long helices</td>
<td>Spo−; normal and filamentous cells</td>
</tr>
<tr>
<td>ftsZ24</td>
<td>IIQ ↑</td>
<td>I245F</td>
<td>Normal</td>
<td>Spo−; ~5% minicells</td>
</tr>
<tr>
<td>ftsZ20</td>
<td>minD</td>
<td>V38A</td>
<td>Helices</td>
<td>Spo−; twisted divisions</td>
</tr>
<tr>
<td>ftsZ5</td>
<td>minD</td>
<td>D174N</td>
<td>Normal, helices, aberrant structures</td>
<td>Spo−; normal and slightly filamentous cells</td>
</tr>
<tr>
<td>ftsZ38</td>
<td>minD</td>
<td>L302P (Q353R)</td>
<td>Normal, short helices</td>
<td>Spo−; ~5% minicells</td>
</tr>
<tr>
<td>ftsZ8</td>
<td>minCD</td>
<td>S219L</td>
<td>Normal, some helices, extra protein at cell poles</td>
<td>Spo−; &lt;1% minicells</td>
</tr>
</tbody>
</table>

*Mutations were identified in a minD background, a minCD background, or a wild-type background, as a colony showing increased (IIQ ↑), decreased (IIQ ↓) or severely decreased (IIQ ↓ ↓) expression of a spoIIQ-lacZ fusion.
†Spo+, sporulation proficient; Spo−/−, weak sporulation defect; Spo−, strong sporulation defect; as judged by colony appearance on NA incubated at 37 °C.
caused the formation of significant numbers of minicells. As seen in Fig. 1(E) and (F), cells producing FtsZI245F protein (ftsZ24) looked slightly shorter than those of the wild-type, and up to about 5% of the cells formed minicells (example arrowed). The majority of minicells were DNA free, but occasionally they contained DNA (data not shown). The FtsZL302P-Q353R protein (ftsZ38) also caused formation of ~5% minicells, but in this case, unusually, the majority

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**Fig. 1.** Morphological phenotype of the mutant *ftsZ* strains. Exponentially growing cells were stained with DAPI or FM5-95 to visualize the nucleoids or membranes, respectively. Phase-contrast (A, B, D, E, G, I, K, M, O, P, R and T) and fluorescence micrographs stained with DAPI (C, F, H, J, L and N) or FM5-95 (Q, S and U) of the same field of cells are shown. (A) Strain 1272 (wild-type), (B, C) strain 1369 (*ftsZ8*; FtsZS219L), (D) strain 1365 (*ΔminCD*, *ftsZ8*; FtsZS219L), (E, F) strain 1361 (*ftsZ24*; FtsZI245F), (G, H) strain 1368 (*ftsZ38*; FtsZL302P-Q353R), (I, J) strain 1357 (*ftsZ3*; FtsZV260A), (K, L) strain 1358 (*ftsZ4*; FtsZA285T), (M, N) strain 1366 (*ftsZ20*; FtsZV38A), (O) strain 1363 (*minD*, *ftsZ5*; FtsZD174N), (P, Q) strain 1387 (*ftsZ5*; FtsZD174N), (R, S) strain 1359 (*ftsZ6*; FtsZE300K), and (T, U) strain 1360 (*ftsZ11*; FtsZS271R). Scale bars, 5 μm.
of them contained DNA (Fig. 1G, H). Otherwise, the cell length was similar to that of the wild-type strain. The \textit{ftsZ38} mutation was isolated in a \textit{minD}\textsuperscript{−} background, and originally large numbers of minicells were formed (data not shown). However, the mutation seemed to be unstable in the 1901 strain background, and derivatives showing improved growth developed within the colonies after a few days of growth on NA containing chloramphenicol. Cells producing FtsZV260A protein (\textit{ftsZ3}) grew normally and produced \~{}10\% minicells, of which about half contained DNA (arrows in Fig. 1I, J). Production of the FtsZA285T protein (\textit{ftsZ4}) generated cells with a similar cell-length distribution to that of the wild-type, though again \~{}10\% minicells were produced (Fig. 1K, L). For this mutant, the vast majority of the minicells contained no DNA [minicells with (arrow) and without (arrowhead) DNA are labelled].

Cells producing FtsZV38A protein (\textit{ftsZ20}) had a slightly increased cell length (Fig. 1M, N). Around 15\% of sister cells appeared to divide in a twisted manner to produce a ‘seagull’ morphology (white arrows). Twisted divisions appeared to occur in both shorter and longer cells. Some of the twisted divisions trapped DNA in the septum (upper white arrow). Many cells had a misshaped pole with a pointed tip or a tilted minicell-like appendage (black arrow).

As shown in Fig. 1(O), the \textit{ftsZ5} mutant (\textit{ftsZD174N}), when first isolated in the \textit{minD}\textsuperscript{+} background, formed long filaments. The phenotype was less severe in the wild-type background, and the cells were only slightly longer, on average, than the wild-type (Fig. 1P). Staining the membranes of the cells with the dye FM9-95 showed that some of the longer cells occasionally formed a septum (Fig. 1Q). Perhaps because of the impairment in division, \textit{ftsZ5} mutant colonies appeared to be unstable in both \textit{minD} and wild-type backgrounds when propagated on NA (with or without chloramphenicol selection).

Exponentially growing cells producing FtsZE300K (\textit{ftsZ6}; Fig. 1R, S) and FtsZS271R (\textit{ftsZ11}; Fig. 1T, U) mutant proteins formed a mixture of short wild-type-like cells and long filaments with few division septa (as seen by staining the membranes with FM9-95; Fig. 1S, U). In addition, production of FtsZE300K (\textit{ftsZ6}) protein in some cells twisted divisions, either within or near the end of the cell filament, again forming tilted minicell-like appendages (arrows). This mutant also appeared to be unstable on NA, whereas the \textit{ftsZ11} mutant strain was stable.

As some of the \textit{ftsZ5} mutant strains were unstable, all cultures were propagated in liquid PAB medium for minimal periods of time. As a further check on the reproducibility of the phenotypes, the mutants were also examined directly from NA, and also immediately after growth on the primary transformation plates, yielding similar findings (data not shown).

It has been shown that lowering or increasing FtsZ expression levels causes filamentation or an increase in minicell formation, respectively (Palacios \textit{et al.}, 1996; Ward & Lutkenhaus, 1985; Weart & Levin, 2003). Therefore, FtsZ protein levels in the various mutants were examined by Western blotting (Fig. 2). All of the mutants appeared to produce normal levels of FtsZ. Suprisingly, the FtsZL302P–Q353R protein (\textit{ftsZ38}) ran at a higher molecular mass position, suggesting that the tertiary structure of this mutant protein might be altered or more resistant to SDS treatment. Sequencing of the \textit{ftsZ38} gene from the chromosome verified the absence of any duplicated sequences.

\textbf{FtsZ ring formation in the mutant \textit{ftsZ} strains}

To determine the effect of the mutations on FtsZ ring formation, we performed IFM on exponentially growing cells, using polyclonal anti-FtsZ antibodies. All mutations were analysed in the \textit{minCD}\textsuperscript{+} strain background (strain 1272). As shown in Fig. 3(A) and (B), FtsZ ring formation in cells producing FtsZI245F protein (\textit{ftsZ24}) was indistinguishable from that of the wild-type strain, in which single bright transverse bands of fluorescence were evident near the midpoints of each cell. The majority of the FtsZI245F (\textit{ftsZ24}) bands were correctly localized at mid-cell, either between separated sister nucleoids, or over bilobed-shaped nucleoids (the bilobed nucleoids have most likely terminated DNA replication, but not segregation; Sharpe \textit{et al.}, 1998) (Fig. 3B, C). A few FtsZI245F (\textit{ftsZ24}) bands localized close...
Novel mutations in \textit{ftsZ}
to one pole of the cell (arrowhead), probably resulting in minicell formation.

The FtsZV260A (ftsZ3) and FtsZA285T (ftsZ4) proteins formed FtsZ rings similar to the wild-type, but, in addition, mutant cells frequently exhibited a strong fluorescent signal at the poles of the cell (Fig. 3D, F). This suggests that some FtsZ is retained there after septation, or that the mutant proteins are resistant to the normal action of the Min system in preventing FtsZ polymerization close to the poles.

A number of mutant proteins [FtsZL302P-Q353R (ftsZ38), FtsZS219L (ftsZ8) and FtsZV38A (ftsZ20)] formed helical FtsZ structures with one to two turns at mid-cell, in addition to normal FtsZ bands. The FtsZL302P-Q353R protein (ftsZ38) generated the weakest phenotype, with few helical FtsZ structures (Fig. 3H). The FtsZS219L protein (ftsZ8) formed helical structures in many cells, but they also usually had abnormal amounts of FtsZ at the poles (Fig. 3I). Interestingly, the gross morphology of this strain was nearly indistinguishable from that of the wild-type strain (apart from a few minicells), suggesting that the helical FtsZ rings usually lead to division. However, in this case the topology of division reflected the shape of the FtsZ structures, and many of the cells formed abnormal, twisted septa (see above).

The FtsZD174N protein (ftsZ5) formed a range of abnormal structures and accumulations of FtsZ (Fig. 3N). As cultures of this mutant included normal but also longer cells, some of the FtsZ structures are presumably non-functional.

Elongated helical FtsZ structures were particularly evident in mutant cells producing FtsZS271R (ftsZ11) mutant protein. The helices often overlapped the area occupied by the DNA (white bars in Fig. 3P, Q). A similar FtsZ localization pattern has been observed in cells with a disruption of the noc gene, which is required for nucleoid occlusion in B. subtilis (Wu & Errington, 2004).

FtsZE300K protein (ftsZ6) formed diffuse broad FtsZ structures, but in contrast to FtsZS271R (ftsZ11), these structures tended to be located between nucleoids (arrowheads in Fig. 3R, S). Most of the FtsZE300K (ftsZ6) structures are probably non-functional, as the strain forms elongated filaments, with only occasional cells of normal length.

**Effects of the ftsZ mutations in various division-mutant backgrounds**

The range of phenotypes exhibited by the collection of ftsZ mutants suggested that they might be affected in different aspects of FtsZ function, or interactions with other components of the division machinery. We reasoned that we might get more information by combining the ftsZ mutations with mutations in other division genes. Therefore, we attempted to introduce each of the new ftsZ mutations into strains bearing mutations in various division genes (ezrA, ftsA279, minC, minD, noc and zapA), none of which seriously affects growth under normal conditions, and look for additional effects on the phenotype.

Most of the ftsZ mutations (ftsZ3, ftsZ4, ftsZ8, ftsZ11, ftsZ20, ftsZ24 and ftsZ38) had either only a weak or no additional phenotype when combined with any of the division mutations tested (data not shown). Two mutations, ftsZ5 and ftsZ6 (producing FtsZD174N and FtsZ300K, respectively), had lethal or near-lethal effects in most or all of the mutant backgrounds. The ftsZ6 mutation had the most severe effect, and double-mutant progeny were recovered at greatly reduced frequency in combination with mutations in ezrA, minD, minC and ftsA279. The mutation was viable in combination with zapA or noc mutation, but the colonies were small. Therefore, production of FtsZ300K (ftsZ6) mutant protein makes cells sensitive to a range of perturbations of the division machinery that are normally well tolerated. FtsZD174N (ftsZ5) mutant protein caused a similar, but less severe, effect. The ftsZ5 double mutants were generally viable, but the cells grew into long filaments, and sporulation was abolished. Absence of EzrA had the strongest effect, and double mutants could not be recovered at all.

**Mutations ftsZ24 and ftsZ38 affect sporulation**

Next we examined whether any of the ftsZ mutations that had approximately normal cell length, and produced FtsZ rings similar to those of the wild-type, had an effect on asymmetric cell division during sporulation, as measured by activation of the sporulation-specific α-factor, αF (Feucht et al., 1999; King et al., 1999). Liquid cultures of various mutant strains were induced to sporulate, and β-galactosidase activity was measured from a lacZ fusion to the αF-dependent reporter gene spoIIQ. The ftsZ3 and ftsZ4 mutant strains (producing FtsZV260A and FtsZA285T, respectively) had a similar level of αF activity as an isogenic ftsZ+ strain (data not shown). As shown in Fig. 4(A), an ftsZ24 mutant (producing FtsZ1245F) exhibited slightly higher β-galactosidase activity than the wild-type. In contrast, the ftsZ38 mutant strain (producing FtsZL302P-Q353R) showed a drastically reduced β-galactosidase activity. These data were in good agreement with the darker and paler colour development seen on X-Gal plates by the ftsZ24 and ftsZ38 mutant strains, respectively. Interestingly, around 60% of cells of strain 1368 (ftsZ38) appeared to have reached or gone beyond the stage of asymmetric septum completion 120 min after induction of sporulation (as judged by the formation of condensed prespore nucleoids; 64% in the equivalent ftsZ+ strain), suggesting that the reduced activation of αF is not due to a block in formation of the asymmetric septum. To determine which of the two
substitutions in the original ftsZ38 mutant was responsible for the impairment of sporulation, each mutation was reconstructed in the vector plasmid (pSG1928) using site-directed mutagenesis, and the mutations were introduced separately into strain 1272. Fig. 4(B) shows that the strain containing the mutation generating the Q353R (ftsZ38-2) substitution sporulated like the wild-type on plates grown at 37°C, whereas the L302P (ftsZ38-1) substitution produced a defect in sporulation like that of the original double mutant. During the course of this work, we also observed that the ftsZ38 mutant strain was temperature sensitive. Colonies grown at room temperature were indistinguishable for sporulation from the wild-type, whereas a defect in sporulation was detected when the strain was grown at 37 or 48°C (Fig. 4B and data not shown). Preliminary results showed that the mean length of vegetative cells was not affected at higher growth temperatures, suggesting that the FtsZL302P-Q353R protein (ftsZ38) is specifically affected in sporulation (Fig. 1G, H and data not shown).

**DISCUSSION**

An important contribution of the present study is the development of a simple strategy to generate and analyse mutations in the essential division gene ftsZ in vivo. The N-terminal domain of FtsZ contains the GTP-binding site, and is involved in monomer–monomer interactions within a single protofilament (Lowe & Amos, 1998, 1999). In contrast, the C-terminal domain of FtsZ is far less conserved, and little is known about its function, except that it often seems to be the site for interaction with other proteins. Interestingly, eight out of the nine mutations described in this study would affect the C-terminus of FtsZ. All of the amino acid substitutions appear to localize in loops, or close to the beginning or end of a helix or sheet structure (Fig. 5), supporting the idea that the C-terminal fold of FtsZ is conserved, and that mutations affecting secondary structural elements are probably lethal, and therefore would not be picked up by this screening approach. All FtsZ mutant proteins, except FtsZS219L (ftsZ8), caused a morphological phenotype in the wild-type strain background, ranging from filaments, to minicells with and without DNA, to twisted cells (Fig. 1), suggesting that the C-terminal domain of FtsZ is important for topological features of cell division, particularly the location and orientation of FtsZ assembly.

Our data suggest that two of the mutant proteins, FtsZD174N (ftsZ5) and FtsZE300K (ftsZ6), might be affected in their ability to assemble, as both mutants grew into normal and filamentous cells, demonstrating that some of the aberrant FtsZ structures seen by IFM are non-functional (Figs 1 and 3). Additionally, combining these ftsZ mutations with mutations in other division genes had lethal or near-lethal effects, suggesting that both mutants require the action of accessory proteins for cell division, perhaps to stabilize FtsZ polymers, to disassemble aberrant structures, and/or to replenish the pool of soluble mutant FtsZ protein. Modelling the structure of the *B. subtilis* FtsZ (Fig. 5) using the solved protein structure of *Methanococcus jannaschii* FtsZ1 (Lowe & Amos, 1998; D. Brown, personal communication) suggests that the ftsZ5 and ftsZ6 mutations lie close together on one surface of the protein, consistent with these mutations having similar effects on the assembly of FtsZ polymers.

Bi & Lutkenhaus (1992) described the isolation of a temperature-sensitive ftsZ26 mutant in *E. coli* that forms spiral...
invaginated septa (Addinall & Lutkenhaus, 1996). The mutant turned out to have an insertion of six additional amino acids between amino acids 38 and 39 of FtsZ. Interestingly, the \textit{ftsZ20} mutant strain, which exhibits a similar phenotype to the \textit{ftsZ26} mutant (Figs 1 and 3), carries a single valine to alanine substitution at position 38. These data strongly support the notion that the shape of the FtsZ ring dictates the pattern of septal ingrowth (Addinall & Lutkenhaus, 1996), and it appears that residue V38 is directly involved in determining the direction of polymerization, and, when altered, leads to the formation of FtsZ spirals, and thus to twisted cells. The fact that several mutant proteins, and especially FtsZS271R (\textit{ftsZ11}), formed FtsZ spirals, but no twisted cells were detected, suggests that the mutant FtsZ spirals seen by IFM differed in their ability to direct the shape of the invaginating septum. Alternatively, the shape of the FtsZ ring might not be enough to determine the shape of the invaginating septum.

Stricker & Erickson (2003) isolated \textit{ftsZ} mutations in \textit{E. coli} that formed extensive spirals, but were not capable of cell division. Production of the FtsZS271R (\textit{ftsZ11}) mutant protein caused a similar phenotype, but here the mutant FtsZ is partially functional, as normal and filamentous cells were observed (Figs 1 and 3). It seems that long spirals extending over the nucleoid are formed in \textit{B. subtilis} under various conditions, e.g. in a \textit{noc} mutant strain, or in cells undergoing sporulation where formation of a spiral-like filament is an intermediate step of the switch from medial to polar FtsZ position (Ben-Yehuda & Losick, 2002; Wu & Errington, 2004).

Interestingly, a number of mutant proteins [FtsZV260A (\textit{ftsZ3}), FtsZA285T (\textit{ftsZ4}) and FtsZI245F (\textit{ftsZ24})] caused the formation of a significant amount of DNA-free minicells (Fig. 1, Table 2). The approximately normal cell length, and the ability to form discrete linear FtsZ bands similar to those of the wild-type (Figs 3), imply that the mutated FtsZs retained their normal assembly and constriction function. The formation of minicells suggests that these mutant proteins are less sensitive to the destabilizing action of MinC. The mutations probably do not abolish binding to MinC completely, as the amounts of minicells are not as high as in a \textit{min} mutant strain (Marston et al., 1998). Alternatively, the mutant proteins might form more stable polymers, and thereby be more resistant to the action of MinC. It is interesting that all three mutated residues are located quite close together on the FtsZ protein (Fig. 5). Bi & Lutkenhaus (1990) isolated SulA-resistant \textit{ftsZ} mutants in \textit{E. coli} that also formed increased levels of minicells, but the mutations lie at different sites compared with the above-mentioned \textit{B. subtilis} \textit{ftsZ} mutations.

The \textit{ftsZ38} mutant (producing FtsZL302P-Q353R mutant...
protein) differed from all of the others in being drastically affected in sporulation and activation of \( \sigma^F \) (Fig. 4), even though preliminary light microscopy experiments suggested that medial and asymmetric division are near normal. Previously, an \( ftsA \) mutant with aberrant asymmetric septa, but no defect in vegetative growth, has been described (Kemp et al., 2002; Young, 1976), suggesting that \( FtsA \) protein has a distinct or additional role in asymmetric septation compared with vegetative division. The phenotype of the \( ftsZ38 \) mutation suggests that \( FtsZ \) may also have a modified role in asymmetric septation, either directly or indirectly via \( FtsA \). Alternatively, the \( FtsZL302P-Q353R \) mutant protein may be affected in binding, and thereby localizing the sporulation-specific division protein \( SpoIIE \) (Lucet et al., 2000).

In conclusion, \( B. \ subtilis \) appears to be a good organism in which to create and study mutations in the essential \( ftsZ \) gene. The easy integration of the \( ftsZ \) alleles into the chromosome, and thus disruption of the wild-type copy of \( ftsZ \), allows the viability and morphological phenotype of the mutants to be examined directly. Screening the mutant library for efficient sporulation (as an indication for changes in vegetative and/or asymmetric division) seemed to be quite sensitive, as we were able to isolate mutations with a range of mild phenotypes. Large-scale characterization of \( ftsZ \) mutants by this approach may help to shed light on the crucial questions of the role of \( FtsZ \) in assembly and constriction of the division machinery, and its coupling with cell growth, chromosome replication and segregation.

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REFERENCES


Ben-Yehuda, S. & Losick, R. (2002). Asymmetric cell division in \( B. \ subtilis \) involves a spiral-like intermediate of the cytokinetic protein \( FtsZ \). Cell 109, 257–266.


