

The ClpX chaperone modulates assembly of the tubulin-like protein FtsZ

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Summary

Assembly of the tubulin-like cytoskeletal protein FtsZ into a ring structure establishes the location of the nascent division site in prokaryotes. Factors that modulate FtsZ assembly are essential for ensuring the precise spatial and temporal regulation of cytokinesis. We have identified ClpX, the substrate recognition subunit of the ClpXP protease, as an inhibitor of FtsZ assembly in *Bacillus subtilis*. Genetic data indicate that ClpX but not ClpP inhibits FtsZ-ring formation *in vivo*. *In vitro*, ClpX inhibits FtsZ assembly in a ClpP-independent manner through a mechanism that does not require ATP hydrolysis. Together our data support a model in which ClpX helps maintain the cytoplasmic pool of unassembled FtsZ that is required for the dynamic nature of the cytokinetic ring. ClpX is conserved throughout bacteria and has been shown to interact directly with FtsZ in *Escherichia coli*. Thus, we speculate that ClpX functions as a general regulator of FtsZ assembly and cell division in a wide variety of bacteria.

Introduction

In bacteria the precise spatial and temporal regulation of cytokinesis is dependent on the assembly dynamics of the essential cell division protein FtsZ (Romberg and Levin, 2003). FtsZ, a GTPase with a tertiary structure similar to tubulin, assembles into a ring structure (the Z ring) at mid-cell. The Z ring establishes the location of the division site and is responsible for recruiting other cell division proteins to the nascent septum (Romberg and Levin, 2003). GTP

binding enhances FtsZ assembly (Mukherjee and Lutkenhaus, 1994) and dimerization leads to formation of a GTPase active site (Löwe and Amos, 1999; Scheffers *et al.*, 2002). As with tubulin, GTP hydrolysis *in vitro* causes a conformational change in FtsZ polymer structure and rapid disassembly (Mukherjee and Lutkenhaus, 1998; Romberg *et al.*, 2001). Photobleaching experiments indicate that the half-time for subunit replacement in the *Escherichia coli* Z ring is between 8 and 30 s (Stricker *et al.*, 2002). The observation that FtsZ levels are equivalent in cells cultured at different growth rates suggests that Z-ring formation is primarily governed at the level of FtsZ assembly (Weart and Levin, 2003).

FtsZ is thought to exist in two states: (i) a cytoplasmic pool of monomers, small multimers and potentially small polymers and (ii) the membrane-associated polymers that constitute the Z ring. A precisely balanced network of regulatory factors act in concert to modulate FtsZ assembly to ensure that the Z ring forms in the proper position and is tightly co-ordinated with DNA replication, chromosome segregation and cell growth (Romberg and Levin, 2003). The majority of cell division genes identified to date were isolated in screens for cells with gross defects in cell division either as conditional mutants that blocked division altogether (e.g. *ftsA*, *divIC*) or mutants that divided aberrantly (e.g. the *min* genes). However, recent work has led to the identification of several genes that appear to function in more subtle ways to maintain the balance between assembled and unassembled FtsZ, thereby contributing to the dynamic nature of the cytokinetic ring. One of these, the well-conserved protein ZapA, was isolated as a multicopy suppressor of the filamentation associated with overexpression of the cell division inhibitor MinCD (Gueiros-Filho and Losick, 2002). *In vitro*, ZapA interacts directly with FtsZ and induces protofilaments to associate into large bundles (Gueiros-Filho and Losick, 2002). Although not required for division in wild-type cells, ZapA becomes essential for efficient division under conditions in which the cytoplasmic pool of unassembled FtsZ is reduced, in particular in the absence of the division inhibitor EzrA or in cells in which the intracellular concentration of FtsZ has been artificially lowered (Gueiros-Filho and Losick, 2002). Similarly, a null mutation in *noc*, a division inhibitor associated with the bacterial nucleoid, does not significantly affect cell division under normal conditions. However, when combined with

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a mutation in the division inhibitor *minD*, the resulting double mutant cells fail to properly assemble FtsZ at putative division sites resulting in filamentation (Wu and Errington, 2004).

In an effort to identify additional modulators of FtsZ assembly in *Bacillus subtilis* we isolated transposon insertions that suppressed the heat sensitivity of a conditional allele of *ftsZ*, *ftsZts*. We previously employed a similar approach to identify the division inhibitor EzrA (Levin *et al.*, 1999). In this article we report the identification and characterization of ClpX as an inhibitor of FtsZ assembly. ClpX functions as the substrate recognition domain of the ClpXP protease. ClpX also functions in a ClpP-independent manner in which it can either prevent protein assembly/aggregation or remodel and disassemble pre-formed complexes/aggregates (Mhammedi-Alaoui *et al.*, 1994; Levchenko *et al.*, 1995; Wawrzynow *et al.*, 1995). Our data indicate that ClpX inhibits Z-ring formation through a ClpP-independent pathway. Characterization of *clpX* null and overexpression phenotypes in combination with a variety of cell division mutants suggest that ClpX is important for maintaining the pool of unassembled FtsZ. Intriguingly, our biochemical data indicates that ClpX inhibition of FtsZ assembly does not require nucleotide hydrolysis on the part of ClpX suggesting that ClpX inhibits FtsZ assembly by sterically interfering with FtsZ assembly.

Results

A genetic screen for negative regulators of FtsZ-ring assembly

Fusing *ftsZ* to the gene encoding the green fluorescent protein (*gfp*) renders the resulting fusion protein heat-sensitive for function (the *ftsZ-gfp* allele and gene product are hereafter referred to as *ftsZts* and FtsZts respectively) (Levin *et al.*, 1999). At the permissive temperature of 30°C most *ftsZts* cells have medial Z rings. In contrast, after 45 min at 45°C there are very few Z rings present. Not surprisingly, as Z-ring formation is an essential component of the division process, cells whose only source of FtsZ is the *ftsZts* allele are viable at 30°C but unable to form colonies at $\geq 45^\circ\text{C}$ (Levin *et al.*, 1999). The defect in FtsZ assembly most probably results from misfolding of the GFP moiety and potentially the entire fusion protein at higher temperatures and can be suppressed by increasing the intracellular concentration of FtsZts itself (Levin *et al.*, 1999).

Previously, we identified *ezrA*, an inhibitor of FtsZ assembly, as a spontaneous suppressor of *ftsZts* heat sensitivity (Levin *et al.*, 1999). In order to identify additional negative regulators of FtsZ assembly, we isolated extragenic Tn10 insertions that restored viability at 45°C

to a heat sensitive *ftsZts* strain (PL874) (see *Experimental procedures*). After screening $\sim 10^6$ colonies, we identified seven that had stable suppressor mutations linked to a Tn10 transposon insertion. Linkage analysis indicated that two of these insertions were in *ezrA* and two others appeared to correct the *ftsZts* defect by truncating the *gfp* moiety on the fusion protein. Sequencing the DNA flanking the transposon in the remaining strains indicated that one suppressor was linked to a gene of unknown function and two were insertions in *clpX*. *clpX* encodes the 420 residue (~ 46 kDa) substrate recognition component of the ClpXP protease.

A deletion in clpX restores viability and FtsZts assembly at 45°C to cells bearing the ftsZts allele

The isolation of *clpX::tn10* insertions in the above screen indicated that a null mutation in *clpX* restores function to the FtsZts protein at the restrictive temperature. To verify this, we proceeded to assess the ability of a previously characterized *clpX* deletion (*clpX::spc*; Liu *et al.*, 1999) to suppress the heat-sensitive defect of FtsZts.

Our initial attempts to characterize the *clpX::spc* phenotype were complicated by the fact that *B. subtilis clpX* and *clpP* null mutants possess defects in a host of cellular processes including growth and genetic competence (Gerth *et al.*, 1998; Msadek *et al.*, 1998; Liu *et al.*, 1999; Kruger *et al.*, 2000). Most confounding was the fact that *clpX* mutant strains quickly acquire extragenic suppressor mutations that restore normal growth. Strains bearing these suppressor mutations rapidly outgrow the parent strain in liquid culture, making phenotypic analysis difficult (Zuber, 2004).

A null mutation in *spx*, encoding a global transcriptional regulator, relieves many of the negative phenotypes associated with *clpX* and *clpP* deletions including the severe defects in growth and competence (Nakano *et al.*, 2001; 2003). We found that while an *spx* deletion (*spx::neo*) by itself was not sufficient to restore either viability or Z-ring formation at 45°C to *ftsZts* cells, an *spx::neo clpX::spc* double deletion restored ring formation and increased viability $\sim 10\,000$ -fold at the restrictive temperature (Table 1). Furthermore, these cells were proficient at assembling Z rings at the formerly restrictive temperature (Fig. 1). Thus, the *clpX* deletion restores FtsZts function at 45°C in a robust manner that is independent of the presence of SpX. Importantly, deletions in *clpX* and/or *spx* did not alter the intracellular concentration of either FtsZ or FtsZts as determined by quantitative immunoblot (data not shown). Based on these observations all subsequent analyses of the effect of *clpX* and *clpP* on FtsZ-ring assembly and cell division were conducted in the presence of the *spx* deletion.

Table 1. A *clpX* null mutation suppresses the lethal *ftsZts* defect at 45°C.

Strain	Plating efficiency (%)	Suppression (fold)
PL642 (<i>ftsZts cm</i>) ^a	0.01 ± 0.011	–
BW182 (Δ <i>spx ftsZts cm</i>)	0.01 ± 0.006	1
BW189 (Δ <i>clpX</i> Δ <i>spx ftsZts cm</i>)	108 ± 11.93	10 800
PL874 (<i>ftsZts spc</i>) ^a	0.10 ± 0.131	–
BW206 (Δ <i>clpP</i> Δ <i>spx ftsZts spc</i>)	0.30 ± 0.282	3

a. Reference strain.

ClpX inhibition of Z-ring formation is independent of *ClpP*

The best-characterized role for ClpX is that of the substrate recognition subunit of the ClpXP protease. The other principle component of this protease, ClpP, is the proteolytically active subunit (Gottesman *et al.*, 1993; Wojtkowiak *et al.*, 1993). ClpX also functions independently of ClpP to remodel and disassemble protein complexes (Mhammedi-Alaoui *et al.*, 1994; Levchenko *et al.*, 1995; Wawrzynow *et al.*, 1995). Given these two disparate functions, it was important to assess whether ClpX was regulating FtsZts assembly by a ClpP-dependent or ClpP-independent mechanism.

We hypothesized that if ClpP is required for the modulation of FtsZts activity by ClpX, then a *clpP* deletion should suppress the FtsZts defect at high temperature to the same degree as the *clpX* deletion (Table 1, Fig. 1). However, if ClpX regulates FtsZts assembly in a manner that is independent of ClpP, then a *clpP* null mutation should not restore either Z-ring assembly or viability to *ftsZts* cells. Consistent with this latter possibility, we observed that an *spx::neo clpP::cat* double mutation did

not restore either viability or Z-ring assembly to *ftsZts* cells at 45°C (Table 1, Fig. 1). These data argue that ClpX regulates Z-ring assembly in a manner that is independent of the presence of ClpP and that is therefore not related to ClpX's role in proteolysis.

Overexpression of *ClpX* inhibits cell division

To confirm that ClpX is a negative regulator of Z-ring formation *in vivo*, we assessed the effect of overexpressing ClpX within the cell, with the expectation that the overexpression of an FtsZ inhibitor would result in a block in FtsZ-ring formation. *clpX* was placed under the control of the IPTG-inducible P_{spachy} promoter (Quisel *et al.*, 2001), the strongest inducible promoter available for *B. subtilis*, at the native *clpX* locus. The presence of inducer resulted in the accumulation of intracellular ClpX to concentrations twice that of wild-type cells (data not shown). This modest overexpression of ClpX led to a 20% increase in average cell length [from 4.87 μ m ($n = 272$) to 5.87 μ m ($n = 300$)], consistent with a delay in Z-ring assembly and cell division.

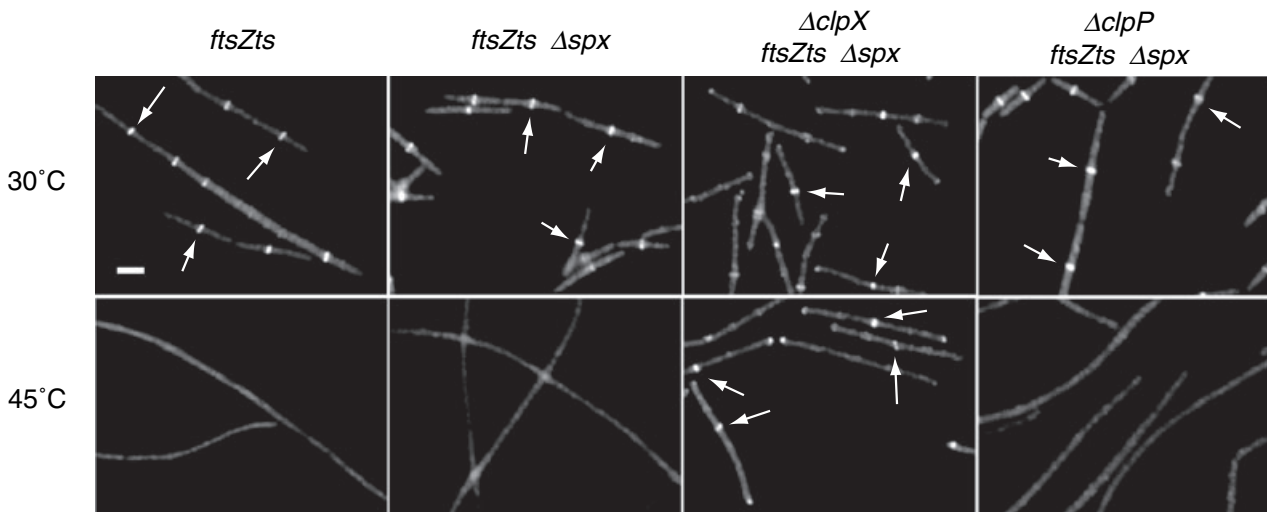


Fig. 1. ClpX modulates FtsZts assembly *in vivo*.

A. A *clpX* null mutation restores Z-ring formation to *ftsZts* cells at 45°C. PL642 (*ftsZts*), BW182 (*ftsZts spx::neo*), BW189 (*ftsZts spx::neo clpX::spc*) and BW206 (*ftsZts spx::neo clpP::cat*) cells were cultured in LB at 30°C to mid-exponential phase ($OD_{600} \sim 0.400$) and then back-diluted into LB at 30°C or 45°C and grown for ~ 2.5 h. FtsZts (FtsZ-GFP) was visualized in fixed cells using fluorescence microscopy. Arrows indicate examples of medial Z rings. Scale bar = 2.5 μ m.

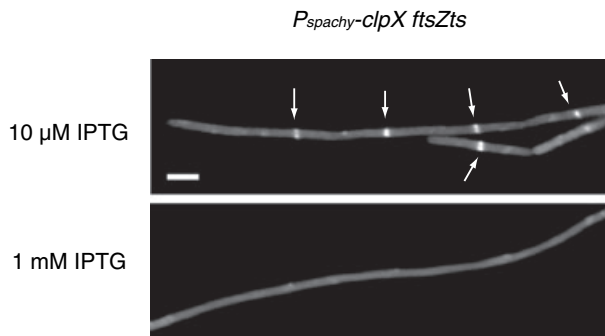


Fig. 2. ClpX overexpression blocks Z-ring assembly. A. ClpX overexpression increases cell length. Cells encoding both an IPTG-inducible *clpX* allele and an *ftsZts* allele (BW243) were grown in LB at 37°C with 10 μ M IPTG to maintain basal levels of ClpX expression. At mid-exponential phase ($OD_{600} \sim 0.400$) cultures were back-diluted into LB containing either 10 μ M IPTG (grey bars) or 1 mM IPTG (black bars) and grown for ~ 2.5 h prior. FtsZts (FtsZ–GFP) was visualized in live cells using fluorescence microscopy. Arrows indicate Z rings in cells grown in LB with 10 μ M IPTG. Scale bar = 2.5 μ m.

Although the limited overexpression we were able to achieve was insufficient to completely block assembly of wild-type FtsZ, we found that a twofold increase in ClpX levels had a dramatic effect on assembly of the defective FtsZ protein, FtsZts. Previous work indicates that the FtsZts protein is ‘sensitized’ to inhibitors of FtsZ assembly, presumably due to a mild defect in FtsZ polymerization even under permissive conditions (Haeusser *et al.*, 2004). When *P_{spachy}-clpX ftsZts* cells were grown in the presence of low levels of inducer (10 μ M IPTG was required to maintain basal ClpX expression to prevent the growth defects associated with a complete loss of ClpX), the cells were fully competent for FtsZts assembly and cell division (Fig. 2). However, full induction of ClpX by the addition of 1 mM IPTG, corresponding to a twofold increase in the intracellular concentration of ClpX, caused a severe block in FtsZts assembly and cell division that reduced viability ~ 1000 -fold (Fig. 2). Overexpression of *clpX* did not affect the intracellular concentration of FtsZts (data not shown). Furthermore, as a control we overexpressed *clpC* from *P_{spachy}* at its native locus. We found that in contrast to ClpX, overexpression of ClpC did not alter FtsZts-ring formation indicating that inhibition of FtsZts assembly is specific to ClpX and not a general feature of this class of proteins (data not shown). These findings support a model in which ClpX acts as an inhibitor of Z-ring formation *in vivo*.

ClpX helps maintain the balance between assembled and unassembled FtsZ

Recent work suggests that a host of regulatory factors act in concert to maintain the balance between assembled and unassembled FtsZ and, thus, ensure precise spatial and temporal regulation of Z-ring formation and

cytokinesis (Romberg and Levin, 2003). This regulatory network appears to be robust: null mutations in a number of cell division genes have no discernible cell division phenotype unless combined with mutations in other genes known to affect FtsZ assembly dynamics (Gueiros-Filho and Losick, 2002; Wu and Errington, 2004). As further evidence of the importance of maintaining the balance between assembled and unassembled FtsZ, overexpression of an inhibitor of Z-ring formation can be compensated for either by the loss of another FtsZ inhibitor or by the overexpression of a protein that stabilizes the Z ring (Levin *et al.*, 2001; Gueiros-Filho and Losick, 2002).

Our genetic data indicated that ClpX plays an important role in maintaining this balance between assembled and unassembled FtsZ. To confirm this possibility, we determined whether a null mutation in *clpX*, a putative inhibitor of FtsZ assembly, could compensate for the overexpression of another division inhibitor, MinCD. The MinCD complex is normally involved in preventing inappropriate Z-ring formation at the cell poles (Rothfield *et al.*, 1999). However, 15-fold overexpression of MinCD results in a lethal block in FtsZ-ring formation and cell division in *B. subtilis* (Levin *et al.*, 2001). This lethal overexpression of MinCD can be suppressed by a null mutation in a second inhibitor of Z-ring formation, EzrA (Levin *et al.*, 2001). We therefore hypothesized that if ClpX was a *bona fide* inhibitor of wild-type FtsZ assembly, like EzrA, then a *clpX* null mutation should suppress the lethal phenotype associated with MinCD overexpression by restoring the proper balance to FtsZ assembly dynamics.

Z-ring formation was blocked and viability reduced ~ 5000 -fold following the addition of IPTG to a strain encoding *P_{spachy}-minCD*. However, a *clpX::spx::neo* double mutation fully restored viability to *P_{spachy}-minCD* cells in the presence of inducer (Table 2). Moreover, immunofluorescence microscopy indicated that the presence of the *clpX::spx::neo* double mutation restored FtsZ assembly in cells overexpressing MinCD (Fig. 3). The *spx::neo* mutation alone had no effect on either viability or ring formation following MinCD overexpression (Fig. 3). In contrast to *clpX*, we found that the loss of *clpP* did not suppress the lethal block in Z-ring assembly caused by MinCD overexpression (Table 2, Fig. 3). The intracellular concentration of MinCD was not affected by the mutations in *spx*, *clpX* and/or *clpP* (data not shown).

As further support of ClpX as an inhibitor of Z-ring formation, we found that twofold overexpression of *clpX* from the inducible *P_{spachy}* promoter at its native locus (see above) reduced minicell formation from 21.2% to 7.7% and polar Z-ring assembly from 43.4% to 24.2% in a *minCD* null mutant. This result indicates that the additional ClpX is able to compensate partially for the loss of MinCD and prevent aberrant polar Z-ring formation and septation.

Table 2. A *clpX* null mutation suppresses the lethality of a ~15-fold MinCD overexpression.

Strain	Plating efficiency (%)	Suppression (fold)
PL1145 (<i>P_{spachy}-minCD</i>) ^a	0.02 ± 0.003	–
BW287 (Δ <i>spx P_{spachy}-minCD</i>)	0.01 ± 0.003	0.5
BW293 (Δ <i>clpX</i> Δ <i>spx P_{spachy}-minCD</i>)	101 ± 11.17	5050
BW220 (Δ <i>clpP</i> Δ <i>spx P_{spachy}-minCD</i>)	0.05 ± 0.085	2.5
BW299 (Δ <i>clpC P_{spachy}-minCD</i>)	0.07 ± 0.014	3.5

a. Reference strain.

These data support a model in which ClpX acts to modulate wild-type FtsZ assembly through a ClpP-independent mechanism. Moreover, these results indicate that ClpX, like EzrA, plays an important part in maintaining the balance between assembled and unassembled FtsZ within the cell.

ClpX inhibits FtsZ assembly in vitro

The genetic analyses described above establish that ClpX is an inhibitor of FtsZ assembly *in vivo*. To determine whether ClpX exerts its effects on FtsZ directly, we tested the ability of purified ClpX to inhibit assembly of purified wild-type FtsZ *in vitro* using a 90° angle light scattering assay (Mukherjee and Lutkenhaus, 1999).

All components in the reactions except GTP were mixed and incubated for 60 s to establish a baseline. FtsZ

assembly was initiated by the addition of 1 mM GTP and allowed to continue for another 340 s. Reactions typically reached steady state ~180 s after the addition of GTP (Fig. 4A). All reactions were performed in the presence of ATP unless otherwise noted. ATP and its derivatives had no effect on either the kinetics or maxima of FtsZ assembly.

To test the ability of purified ClpX to inhibit FtsZ assembly, we added increasing concentrations of ClpX to the light scattering reaction. FtsZ was kept at a constant concentration of 2.5 μ M. ClpX inhibited FtsZ assembly in a concentration-dependent manner with near maximal inhibition at 1.5 μ M ClpX, a ratio of 3 ClpX to 5 FtsZ (Fig. 4B). In light of these results and our difficulty obtaining pure and active ClpX at high concentration, all further experiments assessing the effect of ClpX on FtsZ assembly were performed at a 3:5 ratio of ClpX to FtsZ (concentra-

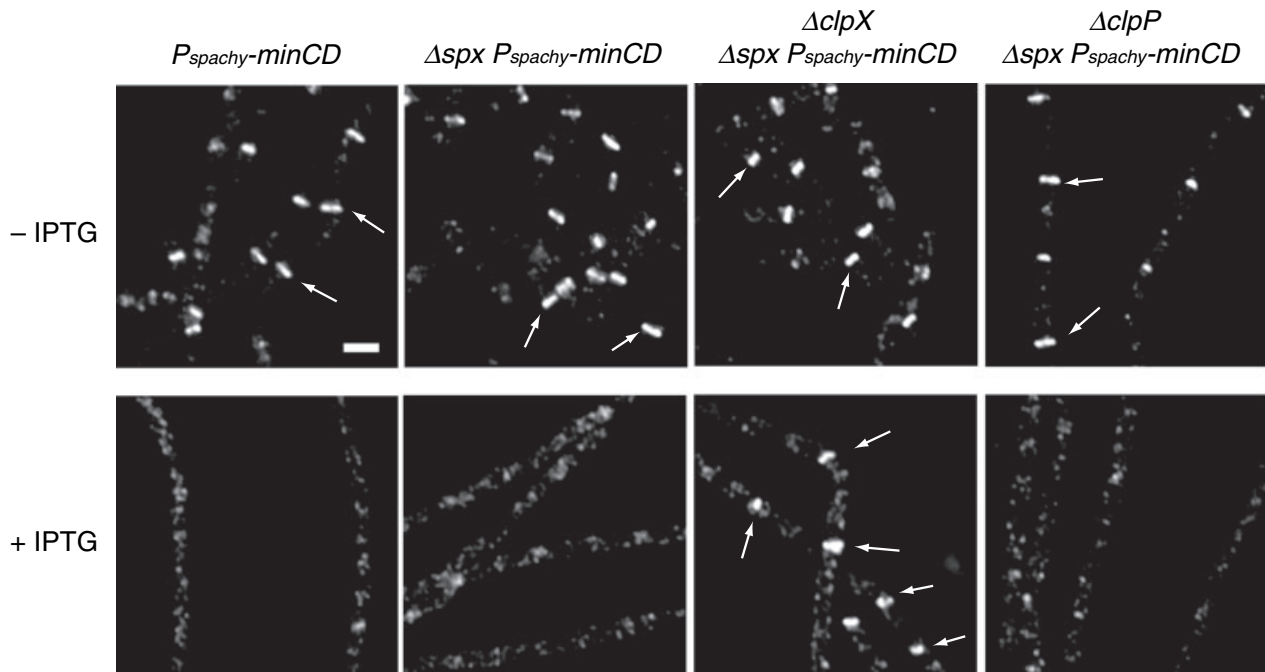


Fig. 3. A *clpX* null mutation restores Z-ring formation to cells overexpressing the FtsZ inhibitor MinCD. PL1145 (*thrC::P_{spachy}-minCD*), BW287 (*thrC::P_{spachy}-minCD spx::neo*), BW293 (*thrC::P_{spachy}-minCD spx::neo clpX::spx*) and BW220 (*thrC::P_{spachy}-minCD spx::neo clpP::cat*) cells were cultured in LB at 37°C to mid-exponential phase (OD_{600} ~0.400) and back-diluted into LB \pm 1 mM IPTG and grown for ~2.5 h. FtsZ was visualized by immunofluorescence microscopy. Arrows indicate examples of FtsZ rings. Scale bar = 2.5 μ m.

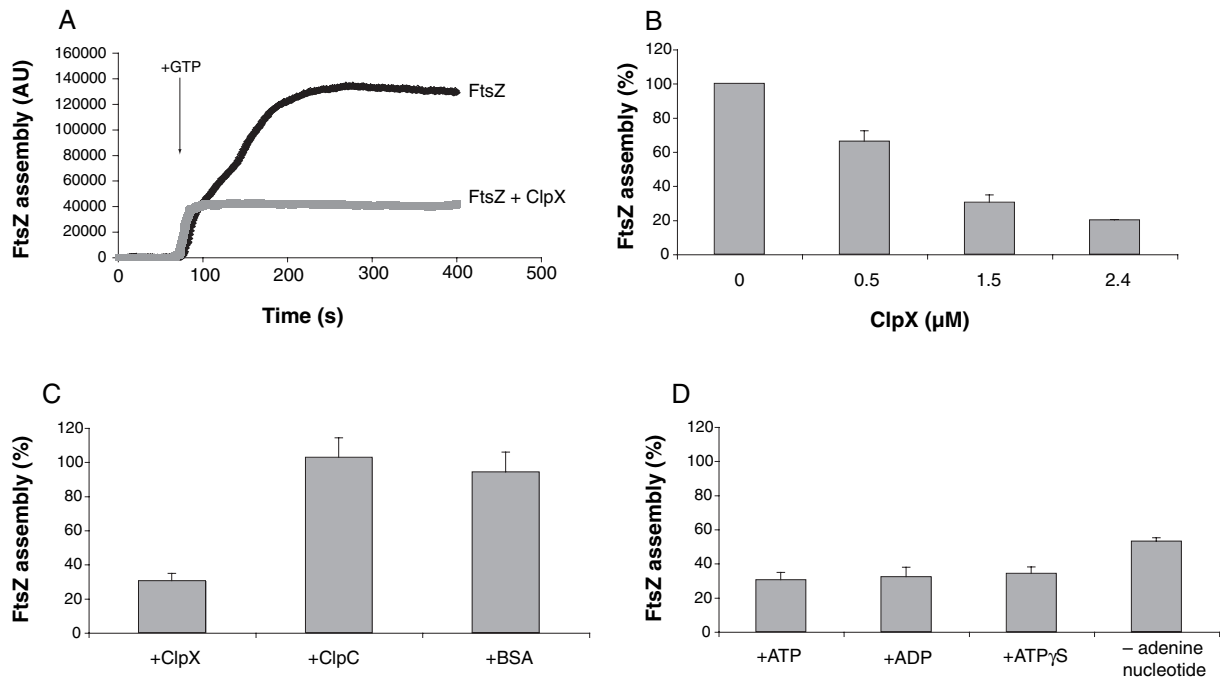


Fig. 4. ClpX inhibits FtsZ assembly *in vitro* in a 90° angle light scattering assay. Unless indicated otherwise, all reactions are performed in the presence of 1 mM ATP with an FtsZ concentration of 2.5 μM. Assembly values in (B), (C) and (D) represent maximal levels of FtsZ assembly in the presence of ClpX relative to FtsZ assembly in the absence of ClpX. Error bars represent standard deviation.

A. Representative light scattering plot of FtsZ (black trace) and FtsZ + ClpX (grey trace). FtsZ assembly was initiated by the addition of 1 mM GTP. B. Dose-dependent inhibition of FtsZ assembly by ClpX.

C. Inhibition of FtsZ assembly by ClpX is specific to ClpX. ClpX, ClpC and BSA were added to a final concentration of 1.5 μM.

D. ATP hydrolysis is not required for inhibition of FtsZ assembly by ClpX. ATP, ADP and ATP_γS were added to 1 mM where appropriate.

tions of 1.5 μM ClpX and 2.5 μM FtsZ respectively). The *in vivo* ratio of ClpX to FtsZ is predicted to be between 1:1 and 2:1 (Feucht *et al.*, 2001; Gerth *et al.*, 2004).

We observed that the addition of 1.5 μM ClpX to 2.5 μM FtsZ resulted in a ~70% inhibition of FtsZ assembly (Fig. 4A and B). As a negative control, we assayed the ability of the ClpX-related chaperone ClpC to inhibit FtsZ assembly. ClpC, like ClpX, functions as a substrate recognition domain for ClpP in the ClpCP protease in *B. subtilis* (Msadek *et al.*, 1994; Turgay *et al.*, 1998). However, genetic data indicate that ClpC is not a negative regulator of FtsZ-ring assembly; a *clpC* null mutation does not suppress the lethal effects of a MinCD overexpression (Table 2). Correspondingly, we found that 1.5 μM ClpC had no effect on FtsZ assembly *in vitro* (Fig. 4C). As an additional negative control, we also tested the ability of bovine serum albumin (BSA) to inhibit FtsZ assembly and found that 1.5 μM BSA did not affect FtsZ assembly (Fig. 4C). Thus, the ability of ClpX to inhibit FtsZ assembly appears to be specific to ClpX.

ClpX inhibition of FtsZ assembly does not require ATP hydrolysis

ClpX is an ATP-binding protein and hydrolysis of ATP is

required for many of ClpX's functions including both proteolysis and chaperone-like activities (Gottesman *et al.*, 1993; Wojtkowiak *et al.*, 1993; Kong and Dubnau, 1994; Mhammedi-Alaoui *et al.*, 1994; Levchenko *et al.*, 1995; Wawrzynow *et al.*, 1995; Singh *et al.*, 2000). We therefore tested the requirement for ATP hydrolysis in ClpX's ability to inhibit FtsZ assembly. Strikingly, we found that the level of inhibition by ClpX was virtually identical regardless of whether the reactions were performed in the presence of ATP, ADP or the non-hydrolysable ATP analogue ATP_γS (Fig. 4D). Neither ADP, ATP_γS nor GTP supports proteolysis of the known substrate Spx by ClpXP (data not shown), indicating that these nucleotides are not hydrolysed by ClpX to a significant degree. Therefore, these data suggest that nucleotide hydrolysis is dispensable for ClpX inhibition of FtsZ assembly. We also observed that ClpX was capable of inhibiting FtsZ assembly in the complete absence of adenine nucleotide, although at somewhat reduced levels when compared with reactions conducted in the presence of ATP, ADP or ATP_γS (Fig. 4D). The decrease in inhibition is potentially due to changes in ClpX's oligomerization state resulting from the absence of adenine nucleotide (Wawrzynow *et al.*, 1995; Grimaud *et al.*, 1998).

ClpX does not unfold FtsZ

ClpX's principle enzymatic activity – its ability to unfold proteins – is dependent on the energy derived from ClpX's hydrolysis of ATP. The finding that ClpX does not require ATP hydrolysis to inhibit FtsZ assembly suggests either that ClpX is incapable of unfolding FtsZ or that the unfolding of FtsZ is not involved in ClpX regulation of FtsZ assembly. To examine this issue, we assessed the ability of the ClpXP protease to degrade FtsZ. ClpX-dependent unfolding is required for substrate proteolysis by ClpXP. Thus, the ability of ClpXP to degrade a given substrate is diagnostic of the ability of ClpX to unfold that substrate.

Proteolysis reactions were assembled on ice. ClpX and ClpP were added to a final concentration of 2.4 μM and 3.6 μM respectively. Either Spx or FtsZ was added to 0.1 $\mu\text{g } \mu\text{l}^{-1}$. Reactions were initiated by the addition of 5 mM ATP and incubated at 37°C. Samples were removed at 0 and 60 min and analysed by SDS-PAGE. We found that ClpXP was unable to degrade FtsZ even under conditions that allowed the efficient degradation of a known ClpXP substrate, Spx (Fig. 5A). These data indicate that

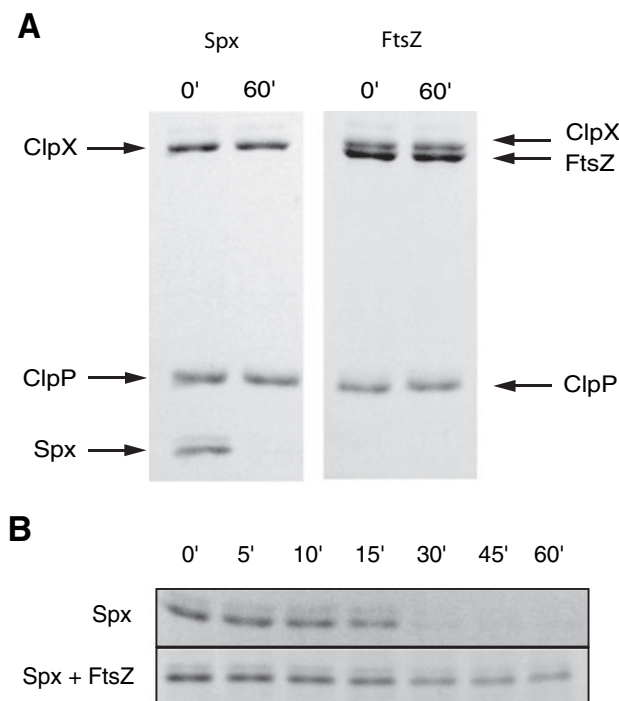


Fig. 5. FtsZ interacts with, but is not degraded by, ClpXP. **A.** ClpXP is able to degrade Spx but not FtsZ *in vitro*. Proteolysis reactions were performed at 37°C in the presence of 5 mM ATP and sampled at 0 and 60 min. **B.** The presence of 10-fold mass excess of FtsZ relative to Spx inhibits the degradation of Spx by ClpXP. Proteolysis reactions were performed at 30°C and sampled at 0, 5, 10, 15, 30, 45 and 60 min. Samples in (A) and (B) were analysed by SDS-PAGE and stained with coomassie Brilliant Blue and representative gels are shown.

ClpX cannot unfold FtsZ and is consistent with the finding that deletions in *clpX* and *clpP* do not affect intracellular levels of FtsZ.

While ClpXP does not degrade FtsZ, FtsZ does inhibit the ability of ClpXP to degrade other substrates. We found that a 10-fold mass excess of FtsZ (1.0 $\mu\text{g } \mu\text{l}^{-1}$) relative to the ClpXP substrate Spx (0.1 $\mu\text{g } \mu\text{l}^{-1}$) strongly inhibited the proteolysis of Spx by ClpXP (Fig. 5B). In contrast, a 10-fold mass excess of BSA (1.0 $\mu\text{g } \mu\text{l}^{-1}$) did not interfere with the degradation of Spx (data not shown). The simplest explanation for this finding is that the interaction between FtsZ and ClpX interferes with the docking between ClpX and either Spx or ClpP. FtsZ is unlikely to compete with ClpX for ATP as FtsZ does not possess an appreciable ATPase activity (data not shown).

ClpX does not affect FtsZ GTPase activity in vitro

Z-ring formation is thought to occur in two stages: (i) polymerization of FtsZ into single-stranded polymers or protofilaments and (ii) the assembly of FtsZ protofilaments into stable bundles via lateral interactions between polymers (Romberg and Levin, 2003). The light scattering assays discussed in the previous sections demonstrate that ClpX inhibits FtsZ assembly *in vitro*, but cannot distinguish which stage in FtsZ assembly is being affected. The GTPase active site is created by the interactions between the faces of two FtsZ subunits within FtsZ dimers or multimers (Erickson, 1998; Löwe and Amos, 1998; Sossong *et al.*, 1999). Therefore, factors that block multimerization by sequestering free FtsZ monomers inhibit GTP hydrolysis (Trusca *et al.*, 1998). To begin to elucidate which step in FtsZ assembly ClpX acts upon, we decided to examine whether ClpX alters FtsZ's intrinsic GTPase activity.

GTPase assays were performed under conditions analogous to those used for light scattering and all reactions contained 1 mM ATP. Components of the reactions were pre-assembled and assays were initiated by the addition of a mixture of unlabelled GTP and radioactively labelled [γ - ^{32}P]-GTP. Reactions were sampled every 5 min for 20 min, samples were separated by thin layer chromatography (TLC) and the generation of free radioactive phosphate was quantified by phosphorimaging coupled with densitometric analysis.

We found that the addition of 1.5 μM ClpX to a reaction containing 2.5 μM FtsZ, a condition that resulted in ~70% inhibition of FtsZ assembly (Fig. 4A and B), had no significant effect on FtsZ's GTPase activity (FtsZ: 0.15 ± 0.03 GTP FtsZ $^{-1}$ min $^{-1}$; FtsZ + ClpX: 0.18 ± 0.04 GTP FtsZ $^{-1}$ min $^{-1}$). ClpX on its own did not possess any detectable GTPase activity (data not shown). These data indicate that ClpX inhibits FtsZ assembly through a mechanism that does not affect FtsZ's intrinsic GTPase activity and

argue against it functioning as a monomer sequestering protein.

Discussion

Using a battery of genetic and biochemical assays, we have established that *B. subtilis* ClpX is a potent inhibitor of Z-ring formation *in vivo* and FtsZ assembly *in vitro*. Our data indicate that ClpX inhibits FtsZ assembly in the absence of ClpP. ClpP-independent roles for ClpX have been described for *E. coli* ClpX and its substrates MuA from Phage Mu and Protein O from Phage Lambda (Figs 1 and 3, Tables 1 and 2). In these cases ClpX modulates the assembly/aggregation state of the relevant substrate complexes (Levchenko *et al.*, 1995; Wawrzynow *et al.*, 1995). ClpX is thought to use ATP hydrolysis to power the partial unfolding of target substrates within the complex which in turn destabilizes the substrate complex as a whole (Burton *et al.*, 2001; Kenniston *et al.*, 2003). This allows either the remodelling or disassembly of the substrate complex.

We find that *B. subtilis* ClpX does not require ATP hydrolysis to block FtsZ assembly (Fig. 4D), suggesting that binding to FtsZ is sufficient to inhibit polymerization. A similar phenomenon has been observed with *E. coli* ClpX and its substrate Lambda Protein O. Protein O is susceptible to a heat-induced aggregation that is both preventable and reversible by ClpX (Wawrzynow *et al.*, 1995). Wawrzynow *et al.* found that ClpX requires ATP hydrolysis to reverse Protein O aggregation but not to prevent it. Thus, there is precedence for ClpX regulating protein complex assembly in the absence of ATP hydrolysis as our data suggest occurs between *B. subtilis* ClpX and FtsZ.

ClpX oligomerizes into a hexameric ring in the presence of ATP, ATP γ S and ADP and this oligomerization is important for ClpX's function as both a chaperone and subunit of the ClpXP protease. Interestingly, in the absence of exogenously added adenine nucleotide, we find that the inhibition of FtsZ assembly by ClpX is somewhat, but not completely, reduced. This result suggests that ClpX's effect on FtsZ is sensitive to the ClpX oligomerization state. Previous studies have suggested that ClpX remains partially assembled even in the absence of exogenously added nucleotide (Grimaud *et al.*, 1998), possibly due to contaminating ADP that co-purifies with ClpX (Kim and Kim, 2003). As such, it is not immediately clear whether unassembled ClpX remains partially active against FtsZ or whether a fraction of the ClpX remains assembled and, therefore fully active in the absence of added adenine nucleotide. Uncertainty regarding the functional ClpX unit combined with limitations in our assays' ability to distinguish between different oligomerization states of FtsZ (e.g. single-stranded protofilaments versus bundled poly-

mers; see below) confounds our ability to interpret the stoichiometric ratios at which ClpX inhibits FtsZ assembly without additional experimentation.

Current models suggest that FtsZ assembly commences with the polymerization of FtsZ monomers into single-stranded polymers or protofilaments followed by the assembly of these polymers via lateral interactions into protofilament bundles. Based on analogy with factors governing cytoskeletal assembly in eukaryotes, there are many possible mechanisms for ClpX inhibition of FtsZ assembly (Desai and Mitchison, 1997; Pollard *et al.*, 2000). One of the simplest models is that ClpX functions as a monomer-binding protein, sequestering free FtsZ monomers and preventing them from being added to growing polymers. This mode of action has precedence in the regulation of FtsZ assembly. The DNA damage-induced protein SulA inhibits Z-ring formation following DNA damage by sequestering FtsZ monomers (Trusca *et al.*, 1998; Cordell *et al.*, 2003). Because GTP hydrolysis is dependent on multimerization, SulA dramatically reduces GTPase activity when present at equimolar ratios by preventing interaction between FtsZ subunits (Trusca *et al.*, 1998). However, in contrast to SulA, we found that ClpX has no observable effect on FtsZ's GTPase activity, even when assayed under conditions in which ClpX blocked FtsZ assembly by > 70%. Thus, ClpX is unlikely to bind to and sequester FtsZ monomers with high affinity.

It also seems unlikely that ClpX regulates FtsZ assembly by unfolding FtsZ subunits within the polymer and thereby disassembling pre-formed polymers. First, ATP hydrolysis is not required to block FtsZ assembly (Fig. 4D), suggesting a non-enzymatic mechanism of inhibition. Moreover, ClpXP is unable to proteolyse FtsZ (Fig. 5A), suggesting that ClpX is incapable of unfolding FtsZ subunits (Fig. 5A).

Although ClpXP does not degrade FtsZ, the observed competition between FtsZ and the ClpXP substrate SpX for access to the protease (Fig. 5B) argues that FtsZ is capable of docking physically with ClpX. Indeed, the C-terminus of FtsZ is rich in basic amino acids and as such resembles signal sequence motifs involved in interacting with ClpX (Flynn *et al.*, 2003). Thus, the simplest model for the inhibition of FtsZ assembly by ClpX is that ClpX binds to FtsZ and sterically hinders FtsZ assembly. For example, ClpX might function to cap growing polymers of FtsZ rendering them too short to be incorporated efficiently into the FtsZ ring. Alternatively, ClpX may inhibit the lateral interactions between FtsZ protofilaments that lead to the formation of stable polymer bundles. Ultimately establishing the mechanism by which ClpX modulates FtsZ assembly will require extensive *in vitro* analysis of FtsZ assembly dynamics in the presence of the chaperone.

Bacterial cells maintain a reservoir of unassembled FtsZ throughout the cell cycle (Weart and Levin, 2003). Although FtsZ is present at concentrations 5- to 10-fold greater than the critical concentration required for assembly (Lu *et al.*, 1998; Feucht *et al.*, 2001), only 30% of available protein appears to be assembled into the Z ring at a given time (Stricker *et al.*, 2002). The presence of a large pool of cytoplasmic FtsZ is presumably important for the maintenance and control of the highly dynamic FtsZ ring *in vivo* (Stricker *et al.*, 2002). Our genetic data indicate that, like EzrA, ZapA and Noc, ClpX is important in maintaining the balance between assembled and unassembled FtsZ within the cell. Immunolocalization of ClpX indicates that it is distributed evenly throughout the cytoplasm (R.B. Weart, unpublished data), suggesting that ClpX impacts FtsZ assembly throughout the bulk of the cytosol.

ClpX is conserved among eubacteria and archaea and is therefore likely to be involved in the modulation of FtsZ assembly in organisms other than *B. subtilis*. Indeed, *E. coli* ClpX was recently shown to interact directly with FtsZ, suggesting that ClpX and/or ClpXP may play a role in regulating FtsZ assembly or stability in Gram-negative bacteria (Flynn *et al.*, 2003). These data, together with our observation that ClpX is a potent inhibitor of FtsZ assembly in *B. subtilis*, suggest that ClpX's activity as a modulator of FtsZ assembly may be a widely conserved mechanism for regulating cell division in prokaryotes.

Experimental procedures

General methods

Cloning and genetic manipulations were performed as described previously (Sambrook *et al.*, 1989; Harwood and Cutting, 1990). *B. subtilis* strains were grown in LB at either 37°C for strains bearing the wild-type *ftsZ*⁺ allele or 30°C for strains bearing the heat-sensitive *ftsZts* allele (Table 3). To assess viability, strains were grown to mid-exponential phase (OD₆₀₀ ~0.400), serially diluted from 10⁻³ to 10⁻⁶, and equal volumes plated at the permissive (30°C for *ftsZts* strains; no IPTG for MinCD overexpression strains; 10 µM IPTG for ClpX overexpression strains) and restrictive (45°C for *ftsZts* strains; 1 mM IPTG for MinCD and ClpX overexpression strains) conditions. 'Plating efficiency' was calculated as the ratio of colonies at the restrictive versus permissive conditions. 'Suppression' was calculated as the ratio of the plating efficiency of an experimental strain versus the plating efficiency of a reference strain. Results are averages for either two (MinCD overexpression strains) or three (*ftsZts* strains) experiments and error bars represent standard deviation.

Isolation of suppressor mutations

Heat-sensitive strain PL1296 encoding *ftsZts* (*ftsZ::ftsZ-gfp*) and the transposon bearing plasmid pHV1249 (Petit *et al.*, 1990) was taken from overnight culture and diluted 1:25 into fresh selective media (LB chloramphenicol 5 µg ml⁻¹, spectinomycin 100 µg ml⁻¹) at 30°C. When cell density reached OD₆₀₀ ~0.25 the culture was diluted twofold into pre-warmed media at 51°C and cultured for 2 h to select for loss of the plasmid and chromosomal integration of the transposon.

Table 3. Bacterial strains.

Strains	Genotype	Reference
<i>B. subtilis</i>		
PL642	<i>ftsZts cat</i>	Levin <i>et al.</i> (1999)
PL874	<i>ftsZts spc</i>	This work
PL990	<i>minCD::spc</i>	Levin <i>et al.</i> (1998)
PL1145	<i>thrC::P_{spachy}-minCD erm</i>	This work
PL1296	<i>ftsZts spc pHV1249</i>	This work
PL1610	<i>clpC::P_{spachy}-clpC</i>	This work
PL1612	<i>clpC::P_{spachy}-clpC ftsZts spc</i>	This work
BW182	<i>spx::neo ftsZts cat</i>	This work
BW189	<i>clpX::spc spx::neo ftsZts cat</i>	This work
BW206	<i>clpP::cat spx::neo ftsZts spc</i>	This work
BW220	<i>clpP::cat spx::neo thrC::P_{spachy}-minCD erm</i>	This work
BW239	<i>clpX::P_{spachy}-clpX</i>	This work
BW243	<i>clpX::P_{spachy}-clpX ftsZts spc</i>	This work
BW254	<i>minCD::spc clpX::P_{spachy}-clpX</i>	This work
BW287	<i>spx::neo thrC::P_{spachy}-minCD erm</i>	This work
BW293	<i>clpX::spc spx::neo thrC::P_{spachy}-minCD erm</i>	This work
BW299	<i>clpC::spc thrC::P_{spachy}-minCD erm</i>	This work
JRL672	<i>clpX::spc</i>	Liu <i>et al.</i> (1999)
BD2243	<i>clpC::spc amyE::comG-lacZ</i>	Kong and Dubnau (1994)
BD2590	<i>clpP::cat</i>	Turgay <i>et al.</i> (1998)
ORB3834	<i>spx::neo</i>	Nakano <i>et al.</i> (2001)
<i>E. coli</i>		
PL1184	ER2566 <i>pBS58 pCXZ</i>	Haeusser <i>et al.</i> (2004)
GZ1	ER2566 <i>pClpC</i>	Nakano <i>et al.</i> (2002)
MMN509	BL21(DE3) <i>pLysS pMMN509</i>	Nakano <i>et al.</i> (2003)
PP61	ER2566 <i>pTYB1-clpP</i>	D. Dubnau and P. Prepiak

Colony-forming units were determined by serial dilution at 30°C. The remaining culture was frozen in 1 ml aliquots with 10% glycerol at -80°C. Frozen aliquots were thawed and plated onto selective medium at 45°C to identify those colonies bearing second site suppressor mutations that restored growth to *ftsZts* cells at the restrictive temperature. We obtained resistant colonies at a frequency of $\sim 10^{-5}$. These colonies were passaged twice at 45°C to ensure that they retained their heat-resistant phenotype.

Linkage of suppressor mutations to the transposon was determined by transformation of chromosomal DNA into a fresh *ftsZts* background selecting for chloramphenicol resistance and screening for heat resistance. Further linkage analysis was used to eliminate transposon insertions in the previously identified cell division gene *ezaA* (Levin *et al.*, 1999) and *ftsZ*. Inverse polymerase chain reaction (PCR) was employed to amplify DNA flanking the transposon in the three suppressor bearing strains that did not show linkage to *ezaA* or *ftsZ*. Sequencing and comparison of the *B. subtilis* database (<http://genolist.pasteur.fr/SubtiList/>) revealed two insertions in *clpX* and one in a gene of unknown function.

Microscopy

Fluorescence microscopy was performed using an Olympus BX51 microscope and a Hamamatsu OrcaERG camera. Images were processed using Openlabs version 3.5 (Improvision) and Adobe Photoshop CS version 8.0 (Adobe Systems).

Immunofluorescence microscopy. Cells were prepared as described previously (Levin, 2002), except that lysozyme treatment was extended to 10 min. FtsZ was detected using affinity-purified polyclonal rabbit anti-FtsZ sera (Levin and Losick, 1996) in combination with either Donkey anti-rabbit sera conjugated to Cy-3 (Jackson Immunoresearch) or goat anti-rabbit sera conjugated to Alexa 488 (Molecular Probes). Cell walls were visualized with wheat germ agglutinin (WGA) conjugated to either fluorescein or tetramethylrhodamine (Molecular Probes). Cell length measurements were performed on cells stained with WGA-tetramethylrhodamine using Openlabs version 3.5 (Improvision). Statistical analysis of cell-length distributions was performed using a chi-squared test for homogeneity with six degrees of freedom (df) and a significance (α) of 0.001.

GFP. Cells were fixed in glutaraldehyde/paraformaldehyde (Levin, 2002) and adhered to the slide with 1% poly-L-lysine (Sigma). Alternatively, live cells were visualized after adherence to an agarose pad (Levin, 2002).

Protein purification

FtsZ was purified as described (Haeusser *et al.*, 2004) and stored at -80°C in 50 mM Mes pH 6.5, 2.5 mM Mg(CH₃COO)₂ and 1 mM EGTA. ClpX, ClpC and ClpP were purified using the Impact system (New England Biolabs). ClpX was subjected to further purification using Bio-Rad HighQ anion exchange column (Nakano *et al.*, 2003). ClpX, ClpC and ClpP were dialysed into 20 mM Tris pH 7.5,

100 mM KCl, 10% glycerol, 1 mM dithiothreitol (DTT) and stored at -80°C. Spx-his was purified using a Ni-NTA column (Qiagen) followed by a Bio-Rad HighQ anion exchange column (Nakano *et al.*, 2003). Spx-his was dialysed into 25 mM sodium phosphate pH 8.0, 100 mM KCl, 5 mM DTT, 1 mM TCEP, 5% glycerol and stored at -80°C.

90° angle light scattering

Light scattering assays were performed using a FluoroMax-2 fluorimeter. Assays were monitored at 310 nm with a slit width of 1 mm. Reaction components were at the following final concentrations: 25 mM Mes pH 6.5 and 5 mM Tris pH 7.5 to yield a final pH 6.5; 50 mM KCl; 0.25 mM DTT; 5% glycerol; 2.5 mM MgCl₂. FtsZ was added to 2.5 μM. GTP was added to 1 mM. ATP, ADP or ATPγS was added, where appropriate, to 1 mM.

Unless noted, all components except GTP were mixed in a quartz cuvette (Starna Cells) and a baseline was established for 60 s. Reactions were initiated by the addition of GTP and allowed to continue for an additional 340 s. Reactions containing FtsZ and either ClpX, ClpC or BSA were preceded by a reference reaction that contained FtsZ alone. Relative assembly was calculated by dividing the maxima for the ClpX-, ClpC- or BSA-containing reactions by the maxima for the FtsZ-only reference. Assembly values reflect an average of either two (ATPγS-containing reactions) or three (all other reactions) experiments and error bars reflect the standard deviations.

Proteolysis assay

Reactions were assembled on ice. Reaction components were added to the following concentrations: 4.6 mM Mes pH 6.5 and 14 mM Tris pH 7.5 and 2.7 mM sodium phosphate pH 8.0 to yield a final pH 7.5; 80.8 mM KCl; 0.38 mM DTT; 7.54% glycerol; 0.23 mM Mg(CH₃COO)₂, 5 mM MgCl₂, 0.09 mM EGTA. ClpX and ClpP were added to final concentrations of 2.4 μM and 3.6 μM respectively. Spx, FtsZ or BSA was added to final concentrations of 1 μg μl⁻¹. Reactions were initiated by the addition of 5 mM ATP and incubated at 37°C. Samples were taken at 0 and 60 min. Substrate competition assays were performed as above except that FtsZ was added to a final concentration of 10 μg μl⁻¹, reactions were incubated at 30°C and samples were taken at 0, 5, 10, 15, 30, 45 and 60 min. All samples were analysed by SDS-PAGE in conjunction with Kodak 1D version 3.5.3 after staining with coomassie Brilliant Blue.

GTPase assay

Reaction components were at the same final concentrations as described for the 90° angle light scattering assays except that Tris pH 7.5 was added to 10 mM yielding a final pH of ~6.5. All reaction components were assembled in the absence of GTP and reactions were initiated by the addition of a mixture of unlabelled GTP (1 mM final concentration) and radioactively labelled [γ -³²P]-GTP (0.5 μCi). Reactions were sampled at 5, 10, 15 and 20 min and samples were quenched using an equal volume of 8 M urea, 20 mM Tris

pH 7.5 and 20 mM EDTA. Samples were run on PEI-cellulose TLC plates (JT Baker) and the release of free phosphate was quantified using a Bio-Rad molecular Imager FX phosphorimager in conjunction with Quantity One version 4.2.1 (Bio-Rad). GTP hydrolysis rates represent an average of three experiments and error bars reflect the standard deviation.

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