Bacillus subtilis EzrA and FtsL synergistically regulate FtsZ ring dynamics during cell division

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Previous work has shown that the Bacillus subtilis EzrA protein directly inhibits FtsZ ring assembly, which is required for normal cell division, and that loss of EzrA results in hyperstabilization of the FtsZ polymer in vivo. Here, it was found that in ezrA-disrupted cells, artificial expression of YneA, which suppresses cell division during the SOS response, and disruption of noc (yyaA), which acts as an effector of nucleoid occlusion, resulted in accumulation of multiple non-constricting FtsZ rings, inhibition of cell division, and synthetic lethality. Overexpression of the essential cell division protein FtsL suppressed the effect of ezrA disruption. FtsL overexpression recovered the delayed FtsZ ring constriction seen in ezrA-disrupted wild-type cells. Conversely, the absence of EzrA caused lethality in cells producing a lower amount of FtsL than wild-type cells. It has previously been reported that FtsL is recruited to the division site during the later stages of cell division, although its exact role is currently unknown. The results of this study suggest that FtsL and EzrA synergistically regulate the FtsZ ring constriction in B. subtilis. Interestingly, FtsL overexpression also suppressed the cell division inhibition due to YneA expression or Noc inactivation in ezrA-disrupted cells.

INTRODUCTION

Cell division is tightly regulated to ensure accurate and efficient segregation of chromosomes and to maintain cell size and shape. In bacteria, the FtsZ protein plays a central role in regulating cell division. FtsZ forms a ring-like structure (the FtsZ ring) at the division site and triggers the assembly of the division machinery (divisome). Therefore, the nucleation of the FtsZ ring forms a primary point of control over the timing and positioning of the cell division septum (Harry, 2001; Errington et al., 2003). During the cell cycle, FtsZ exists in at least two states, monomeric and polymeric (FtsZ ring). Studies have shown that the transition between the two states is regulated by factors that influence FtsZ ring assembly and disassembly (Romberg & Levin, 2003), but the detailed mechanisms responsible for regulating FtsZ ring dynamics during the cell cycle have not yet been fully elucidated.

Earlier studies have identified a number of proteins that modulate FtsZ ring formation in Bacillus subtilis. The positive-acting factors, including FtsA and ZapA, promote FtsZ ring formation and stabilize the ring during assembly of the division apparatus (Romberg & Levin, 2003). FtsA is a widely conserved protein that binds directly to FtsZ and recruits other cell division proteins to the FtsZ ring (Romberg & Levin, 2003; Errington et al., 2003). Although the precise role of FtsA in cell division is not clear, the stability of the FtsZ ring is correlated with FtsA expression levels (Romberg & Levin, 2003). In contrast, ZapA promotes FtsZ ring formation by regulating the balance between the polymeric and monomeric states (Gueiros-Filho & Losick, 2002). The negative-acting factors, such as MinC, MinD, Noc (YyaA) and EzrA, are responsible for preventing FtsZ assembly at inappropriate locations and ensuring that the FtsZ ring is dynamic enough to respond to the signals governing cytokinesis (Romberg & Levin, 2003; Errington et al., 2003; Haeusser et al., 2004). MinC inhibits FtsZ polymerization (Hu et al., 1999), while MinD is an ATPase responsible for recruiting MinC to the membrane (Hu & Lutkenhaus, 1999; Marston & Errington, 1999; Raskin & de Boer, 1999). Together, they form a complex that prevents FtsZ ring formation and subsequent septation at the cell poles (Marston & Errington, 1999). Noc was recently identified as a specific effector of nucleoid occlusion, as it is capable of suppressing division in the vicinity of the nucleoid (Wu & Errington, 2004). Overexpression of Noc results in partial inhibition of cell division at the level of FtsZ ring assembly (Wu & Errington, 2004). EzrA directly interacts with FtsZ to prevent its assembly at aberrant locations along the cell membrane, possibly by blocking subunit addition or inhibiting lateral interactions of FtsZ filaments (Haeusser et al., 2004). Cells lacking EzrA form FtsZ rings at the cell poles as well as at medial sites, and lower the critical concentration of FtsZ required for ring formation (Levin et al., 1999). Moreover, an ezrA null mutation suppresses the inhibition of FtsZ polymer formation associated with minCD overexpression (Levin et al., 2001). Cells lacking
EzrA show partial inhibition of cell division (Levin et al., 1999; Chung et al., 2004), and a synergistic effect is observed when EzrA and ZapA are simultaneously underexpressed (Gueiros-Filho & Losick, 2002), suggesting that EzrA not only prevents polar FtsZ ring formation, but is also involved in the progression of mid-cell division. In addition, YneA is a cell division inhibitor induced during the SOS response in *B. subtilis*, and artificial expression of YneA in the absence of SOS induction results in partial cell elongation, although the precise mechanism of cell division suppression by YneA is not clear (Kawai et al., 2003).

In this study, we show that artificial expression of YneA and disruption of noc in the absence of EzrA lead to an accumulation of multiple non-constricting FtsZ rings and a lethal inhibition of cell division. Furthermore, we found that the defects were suppressed by the overexpression of FtsL, which is recruited to the division site during the later stages of cell division and is essential for the complete division of *B. subtilis*, and artificial expression of YneA in the absence of SOS induction results in partial cell elongation, although the precise mechanism of cell division suppression by YneA is not clear (Kawai et al., 2003).

To construct the ezrA inactivation mutants, insertion of pMutinT3 (Vagner et al., 1998) was also employed. Internal segments of ezrA were amplified using the primers specified in Table 2, digested at the BamHI or HindIII sites within the primers, and inserted between the BamHI and HindIII sites of pMutinT3. The resulting plasmids were used to transform CRK6000 by a single crossover, with selection of the erythromycin-resistant transformants.

The pX plasmid (Kim et al., 1996) was used to insert the yneA, ezrA and ftsZ coding regions, which contained the Shine–Dalgarno (SD) sequence fused to a xylose-inducible xylA promoter (Psyl), into the amyE locus of the *B. subtilis* chromosome, to generate YK016, YK017 and YK059, respectively.

The pAPlNC213-yfp-GW plasmid (T. Kaido & S. Ishikawa, unpublished data) was employed to insert the ftsZ gene translationally fused to the yfp gene for yellow fluorescent protein (YFP) under the control of an IPTG-inducible promoter Pspac into the aprE locus of the *B. subtilis* chromosome, to obtain YK019. When the merodiploid strain YK019 was grown in the presence of minimal inducer (200 μM IPTG), a limited amount of YFP–FtsZ fusion protein was produced, with no observable defects in cell growth or YFP–FtsZ localization (data not shown).

To generate *B. subtilis* cells with multiple mutations, chromosomal DNA from single mutants was employed for transformation, as indicated in Table 1.

**General methods.** *B. subtilis* strains were grown in antibiotic medium 3 (PAB, Difco) supplemented with adenine and guanosine (required for the growth of CRK6000; 20 μg ml⁻¹ each) at 37 °C. Where necessary, various concentrations of IPTG, xylose and/or antibiotics (chloramphenicol, tetracycline, spectinomycin, neomycin and erythromycin at 10, 5, 100, 2 and 0.5 μg ml⁻¹, respectively) were added to the *B. subtilis* cultures. Transformation of *B. subtilis* cells was performed according to the protocol previously reported (Moriya et al., 1998).

**Fluorescence microscopy.** Cell morphology and nucleoid distribution were examined under a fluorescence microscope after 4',6'-diamino-2-phenylindole (DAPI) staining, as described previously (Hassan et al., 1997). The membrane dye FM4-64 (Molecular Probes) was added to cultures at a final concentration of 0.5 μg ml⁻¹. To perform time-lapse microscopy, cells were grown to exponential phase in PAB containing 200 μM IPTG, and placed with 5 μl of fresh medium under a coverslip on an agarose slide (Price & Losick, 1999). Fluorescence images were captured every 20 min using a fluorescence microscope (DMRE-HC, Leica) with a digital cooled charge-coupled device (CCD) camera (1300Y, Roper Scientific) and a YFP filter set (Omega). Images were loaded onto a computer and the intracellular positions of the signals were analysed with the MetaMorph software (Universal Imaging).

**RESULTS**

**Expression of YneA in ezrA-disrupted cells results in synthetic lethality due to inhibition of cell division**

Positive and negative regulatory factors affecting FtsZ ring formation comprise a regulatory network that dictates the...
YneA expression in disruption mutants of ring formation, we investigated the effects of exogenous relationship between YneA and other factors affecting FtsZ cell division suppression by YneA is not clear. To clarify the et al.

*B. subtilis* spatial and temporal control of FtsZ assembly. We have previously reported that expression of exogenous YneA in wild-type *B. subtilis* cells suppresses cell division (Kawai et al., 2003; Fig. 1B, C), although the precise mechanism of cell division suppression by YneA is not clear. To clarify the

**Table 1. *B. subtilis* strains used in this study**

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significant changes in the rate of cell mass increase in cells expressing exogenous YneA (Kawai et al., 2003) or those with disruptions in zapA, minCD, noc and ezrA (Levin et al., 1999; Gueiros-Filho & Losick, 2002; Wu & Errington, 2004). Here, we examined whether a synergistic effect occurred when YneA was overexpressed in these disruption mutants. We replaced zapA, minCD, noc and ezrA with the spectinomycin or tetracycline resistance genes in YK005 cells containing an inducible copy of yneA under the control
of the IPTG-inducible Pspac promoter at the amyE locus. Parental YK005 cells (amyE::Pspac-ynmA) grew on solid medium containing IPTG (Fig. 1A, a), and inactivation of zapA, minCD or noc had no effect on growth (data not shown). In contrast, the ezrA-disrupted strain (YK045, ezrA::spec amyE::Pspac-ynmA) was unable to grow in the presence of IPTG (Fig. 1A, c). Consistent with previous findings for ezrA-disrupted cells (Levin et al., 1999; Chung et al., 2004), the mean length of YK045 cells grown in the absence of IPTG was slightly longer than that of control cells (Fig. 1B, c, C). In contrast, YK045 cells displayed severe filamentation at 90 min after addition of IPTG to the growth medium (Fig. 1B, g, C). These findings indicate that cell division was completely inhibited by the expression of YneA in ezrA-disrupted cells.

Formation of multiple FtsZ rings in YneA-expressing ezrA-disrupted cells

To determine the stage of cell division that was blocked in the double mutant, we examined FtsZ ring formation by fusing the yfp gene under the control of Pspac to the 5' terminus of the ftsZ gene, and integrating the construct into the aprE locus of the YK016 (amyE::Pxy1-ynmA) and YK018 (ezrA::tet amyE::Pxy1-ynmA) strains. Expression of YFP–FtsZ protein in wild-type cells did not impair cell growth and gave expected FtsZ localization patterns (Fig. 2A). In the YK026 (amyE::Pxy1-ynmA aprE::Pspac-yfp-ftsZ) cells cultivated in the presence of xylose, cell division in association with YneA expression was partially blocked (Fig. 2B). YK028 cells (ezrA::tet amyE::Pxy1-ynmA aprE::Pspac-yfp-ftsZ) cultivated without xylose exhibited a typical phenotype of the ezrA null mutation with regard to the position of FtsZ ring formation (Fig. 2C). Cells often had an FtsZ ring at one or both poles in addition to the medial ring (Levin et al., 1999). Interestingly, in the YK028 cells cultivated in the presence of xylose, which induces expression of YneA, assembly of FtsZ rings was observed at regular intervals in filamentous cells (Fig. 2D). The interval between FtsZ rings in the YneA-expressing ezrA-disrupted cells (~3·0 µm) was similar to that in wild-type cells (Fig. 2A, D), and was comparable to the size of newborn cells (~3·3 µm). Normal

### Table 2. Primers used in this study

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*Underlined sequences show introduced restriction enzyme sites: BamHI in ezrAR1, nocR1, minCDR1, divIBR1, tet2F, spec2F and ezrAT3R; HindIII in ezrAR2, nocF2, nocR2, minCDF2, divIBF2, tet2R, spec2R and ezrAT3R.
†Numbers are according to the Subtilist database at the Pasteur Institute (http://genolist.pasteur.fr/Subtilist/).
Fig. 1. Effects of exogenous YneA expression in ezrA-disrupted cells. (A) Growth of YK005 cells (amyE::Pspac-yneA) (a) and YK045 cells (ezrA::spec amyE::Pspac-yneA) (b, c) on PAB plates in the absence (b) or presence of 1 mM IPTG (a, c), which triggers YneA protein expression. (B) Membrane dye (FM4-64)-stained images (a–c, g) and DAPI-stained images (d–f, h) of YK005 (amyE::Pspac-yneA) (a, b, d, e) and YK045 (ezrA::spec amyE::Pspac-yneA) (c, f, g, h) cells, captured at 0 min (a, c, d, f) and 90 min (b, e, g, h) after addition of IPTG. Bar, 5 μm. (C) Cell elongation by the induction of YneA in the wild-type (YK005, white) and ezrA mutant (YK045, grey) cells. Phase-contrast images of cells were captured at 0, 45 and 90 min after addition of 1 mM IPTG, and the mean cell length was determined for more than 150 cells.

Fig. 2. Localization of FtsZ in YneA-expressing ezrA disruptants. YK026 (amyE::Pxyf-yneA aprE::Pspac-yfp-ftsZ) (A, B) and YK028 (ezrA::tet amyE::Pxyf-yneA aprE::Pspac-yfp-ftsZ) (C, D) cells were grown in PAB medium containing 200 μM IPTG. YFP–FtsZ fluorescence (lower) and phase-contrast (upper) images were captured at 0 min (A, C) or 90 min (B, D) after the addition of xylose, which induces expression of YneA. Bar, 5 μm.
FtsZ localization was additionally evident in YK045 cells (ezrA::spec amyE::Pspac-ynmA) in the presence of IPTG by immunofluorescence microscopy with antibodies against FtsZ, and the FtsZ level remained unchanged for up to 90 min following YneA induction in the presence or absence of EzrA (data not shown). These findings indicate that FtsZ ring assembly occurred at the potential division sites when overexpression of YneA was combined with an ezrA null mutation, but that the subsequent constricted and septation steps were inhibited.

**Overexpression of FtsL suppresses the division defect induced by YneA expression in ezrA-deleted cells**

Following assembly of the FtsZ ring, at least six proteins are required for septum formation (FtsA, FtsW, FtsL, DivIB, DivIC and PBP2B) are targeted to the division site to form the *B. subtilis* division machinery (Errington & Daniel, 2002; Errington et al., 2003). The exact roles of these proteins in septum formation remain unknown, with the exception of PBP2B, which participates in synthesis of the peptidoglycan cross-wall of the septum. In an effort to determine whether YneA overexpression in the ezrA mutant inhibits the function of one of these cell division proteins, we examined whether overexpression of FtsL, DivIB, DivIC, FtsW or PBP2B was capable of suppressing the synthetic lethality of YneA expression in ezrA-deleted cells. We cloned each division protein-encoding gene downstream of the Pspac promoter in a multicopy plasmid, pS12HC. We introduced the resultant plasmids (excluding that for FtsA, which would not correctly transform in *E. coli*) into strain YK018 (ezrA::tet amyE::Pxyl-ynmA) and examined whether the transformants were able to grow on a solid medium containing xylose and IPTG. Our results revealed that overexpression of FtsL rescued the growth of YneA-expressing ezrA mutants, whereas overexpression of DivIB, DivIC, PBP2B and FtsW had no such effect (Fig. 3A). To confirm the recovery of cell division, we examined the morphology of YK018 cells harbouring pS12HC-Pspac-ftsL. They formed long filaments when grown in the presence of xylose and absence of IPTG (i.e. with overexpression of YneA but not FtsL) (Fig. 3B, c), but the filamentous phenotype was suppressed when they were grown with both xylose and IPTG (i.e. with overexpression of both YneA and FtsL) (Fig. 3B, b).

**Overexpression of FtsL overcomes the division defect caused by simultaneous disruption of ezrA and noc**

A recent study showed severe inhibition of cell division due to the formation of multiple non-productive FtsZ rings in minD and noc double mutants, and suppression of the division defect by overproduction of FtsZ (Wu & Errington, 2004). In addition, Wu and Errington (2004) also reported that the simultaneous disruption of ezrA and noc was not successful. Here, we examined whether the synthetic lethality of the ezrA and noc inactivation could be suppressed by overproduction of FtsL. We first introduced an inducible copy of ezrA, Pxyl-eyrA, into the ezrA mutant, and then replaced the noc gene with a tetracycline resistance gene. The resulting strain (YK050, ezrA::spec noc::tet amyE::Pxyl-eyrA) divided normally in the presence of xylose (Fig. 4A, a), but when xylose was withdrawn (i.e. when EzrA was depleted), cell division was arrested and long filaments developed (Fig. 4A, b), confirming the synthetic lethality of ezrA noc double inactivation. Furthermore, excessive FtsZ ring formation, including ring formation that overlapped with the nucleoid, as specifically observed in the minD noc double disruptants (Wu & Errington, 2004), was observed in the long filaments of YK058 cells (ezrA::pMutinT3 noc::tet amyE::Pxyl-eyrA aprE::Pspac-yfp-ftsZ), when EzrA and Noc were simultaneously depleted (Fig. 4B).

To examine the effect of FtsL expression on this system, we introduced Pxyl-ftsL at the amyE locus of the ezrA mutant, and inactivated noc by selecting transformants in the presence of xylose, to obtain strain YK051 (ezrA::spec noc::tet amyE::Pxyl-eyrA). YK051 cells divided normally in the presence of xylose (Fig. 4C, a), but in the absence of xylose they formed long filaments (Fig. 4C, b). The dependence of cell division on the presence of xylose demonstrated that the severe division defect caused by the depletion of both ezrA and noc was suppressed by FtsL overproduction. In addition, we found that overproduction of FtsZ could also suppress the inhibition of cell growth and division in the ezrA noc double mutant (Fig. 5). In addition, overproduction of FtsL, as well as FtsZ, suppressed the defects of cell growth and division in the minCD noc double mutant (Fig. 5).

**Partial suppression of cell division in ezrA-disrupted cells due to delayed constriction following FtsZ ring formation is suppressed by exogenous FtsL**

Disruption of ezrA in wild-type cells results in slight cell elongation (Levin et al., 1999; Chung et al., 2004). We found that this cell elongation could be suppressed by the additional expression of FtsL. When YK032 (ezrA::tet amyE::Pxyl-ftsL) cells were cultivated to OD600 = 0.3 in the absence of xylose, the mean cell length and standard deviation were 7.2 ± 2.2 μm. In contrast, cells grown in the presence of 1% xylose showed a significant reduction in cell length (3.4 ± 1.2 μm). An earlier report has indicated that mini-cells constitute only 3-7% of the cells, although ~50% of the FtsZ rings are polar in ezrA null mutants grown in rich medium (Levin et al., 1999). When YK032 cells were cultivated without xylose to OD600 = 0.1 and then grown with 1% xylose for 90 min, the frequency of mini-cell formation was about tenfold that seen in control cells cultivated in the absence of xylose (Table 3), suggesting that expression of exogenous FtsL promoted cell division at the poles of ezrA mutant cells.

It has been suggested that the loss of EzrA stabilizes the FtsZ polymer (Levin et al., 2001), which could delay FtsZ ring disassembly, resulting in delayed and/or suppressed cell division. Accordingly, we analysed the time between FtsZ
ring assembly and disappearance in a single cell cycle, using time-lapse monitoring of YFP–FtsZ. YK019 (aprE::Pspac-yfp–ftsZ) and YK033 (ezrA::tet amyE::PxyI-yneA aprE::Pspac-yfp–ftsZ) cells were placed on agarose-containing PAB medium with IPTG in the presence or absence of xylose, and incubated at room temperature (24°C). Images were collected at 20 min intervals for up to 3 h. In control YK019 cells, new YFP–FtsZ signals appeared in the middle of newborn cells between 0 and 20 min (Fig. 6). This band subsequently dispersed to a dot pattern between 60 and 80 min and disappeared between 80 and 100 min. After septation, a new YFP–FtsZ signal appeared in the middle of each daughter cell. Statistical analyses indicated that in the control cells, FtsZ ring assembly and disappearance in a single cell cycle was completed within 100 min in more than 90% of the cells (Table 4). In contrast, in ezrA-deleted YK033 cells, the interval between FtsZ ring assembly and disappearance was more than 100 min in the majority of cells (Table 4). An earlier fluorescence photobleaching study has shown that the absence of EzrA has little effect on FtsZ assembly (Anderson et al., 2004). Consistent with this, our present results suggest that in ezrA mutant cells, delayed FtsZ ring disassembly extends the interval between FtsZ ring assembly and disappearance, leading to delayed cell division and elongation of cells. Consistent with this model, the delay of FtsZ ring disassembly in the ezrA mutant was suppressed by overexpression of FtsL (Table 4).

These results may suggest that EzrA and FtsL participate in the process of FtsZ ring disassembly or constriction. To examine this functional relationship in greater detail, we examined cells expressing a reduced amount of FtsL. We
Fig. 4. The ezrA noc double mutants show cell division defects that are rescued by exogenous expression of FtsL. (A) Membrane dye (FM4-64)-stained (a, b) and DAPI-stained images (c, d) of YK050 (ezrA::spec noc::tet amyE::Pxyl::ezrA) cells grown in the presence (a, c) and absence (b, d) of 1% xylose, which induces EzrA. Images of YK050 cells were captured at 0 min (a, c) and 120 min (b, d) after the removal of xylose from PAB liquid medium. Bar, 5 μm. (B) Localization of YFP–FtsZ in ezrA-disrupted cells lacking the noc gene (YK058, ezrA::pMutinT3 noc::tet amyE::Pxyl::ezrA aprE::Pspac-yfp-ftsZ). The panels show YFP fluorescence (a), DAPI fluorescence (b) and merged images (c), with YFP false-coloured green and DAPI in blue. Bar, 5 μm. (C) Membrane dye (FM4-64)-stained (a, b) and DAPI-stained images (c, d) of YK051 (ezrA::spec noc::tet amyE::Pxyl::ftsL) cells grown in the presence (a, c) and absence (b, d) of 1% xylose, which induces FtsL. Images of YK051 cells were captured at 0 min (a, c) and 90 min (b, d) after the removal of xylose. Bar, 5 μm.
constructed strain YK030 (ftsL::neo amyE::Pxyl-ftsL), in which FtsL expression was dependent on the Pxyl promoter. As shown in Fig. 7, YK030 cells grew in the presence of 0\%–1\% xylose, but further disruption of ezrA (strain YK034, ezrA::spec ftsL::neo amyE::Pxyl-ftsL) severely retarded cell growth under the same conditions, due to inhibition of cell division (data not shown). These findings demonstrated that higher amounts of FtsL are necessary for cell division in ezrA cells compared to ezrA+ cells. In contrast, inactivation of zapA and noc in strain YK030 (ftsL::neo amyE::Pxyl-ftsL) had little effect on the growth of cells expressing low concentrations of FtsL (data not shown). Inactivation of the DinR repressor of the SOS regulon, which induces constitutive YneA expression, also had no effect. Consistent with this result, cell elongation induced by the artificial expression of YneA in an ezrA+ background was not suppressed by additional expression of FtsL (data not shown).

**Table 3.** Mean cell length and frequency of mini-cells in the ezrA-disrupted cells with or without overexpression of FtsL

<table>
<thead>
<tr>
<th>Time* (min)</th>
<th>Mean cell length† (μm)</th>
<th>Frequency of mini-cells‡ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No xylose</td>
<td>1 % Xylose</td>
</tr>
<tr>
<td>0</td>
<td>7.49 ± 2.00</td>
<td>6.30 ± 1.63</td>
</tr>
<tr>
<td>45</td>
<td>7.21 ± 1.97</td>
<td>6.04 ± 1.68</td>
</tr>
<tr>
<td>90</td>
<td>6.75 ± 1.73</td>
<td></td>
</tr>
</tbody>
</table>

*DAPI-stained images of YK032 cells (ezrA::tet amyE::Pxyl-ftsL) were captured at 0, 45 and 90 min after the addition of 1 % xylose.
†The mean cell length was determined excluding mini-cells. The error value given for each cell length is the standard deviation.
‡Numbers in parentheses refer to the number of cells counted.

**YneA may interfere with the assembly process of late division proteins**

Induction of multiple abortive FtsZ rings at potential division sites by the YneA expression in ezrA-disrupted cells suggested that YneA acts to block cell division after the assembly of the FtsZ ring. In *B. subtilis*, late-assembling division proteins, FtsL, DivIC, DivIB, PBP2B and FtsW, are interdependent for their assembly at division sites (Errington & Daniel, 2002; Errington et al., 2003). The role of DivIB in cell division has been suggested to be the maintenance of FtsL stability at high temperature, and the growth of the divIVB null mutant is inhibited at 48 °C due to rapid degradation of FtsL (Daniel & Errington, 2000). To investigate the possibility that YneA expression affects the function of late-assembling division proteins, we introduced a divIB null mutation into YK005 cells (amyE::Pspac-yneA).

**Fig. 5.** Growth defect of the ezrA noc and minCD noc double mutants are suppressed by overproduction of FtsL or FtsZ. Growth of YK061 (minCD::spec noc::tet amyE::Pxyl-ftsZ), YK055 (minCD::spec noc::tet amyE::Pxyl-ftsL), YK062 (ezrA::spec noc::tet amyE::Pxyl-ftsZ) and YK051 (ezrA::spec noc::tet amyE::Pxyl-ftsL) cells on PAB plates in the presence or absence of xylose.
When YK064 cells (divIB::spec amyE::Pspac-yneA) were cultivated in PAB liquid medium with and without IPTG at 30°C, the rates of increase in cell mass (OD600) were similar to that of the parental YK005 cells (amyE::Pspac-yneA) (Fig. 8), and the mean cell lengths of YK005 and YK064 cells in the presence or absence of IPTG were similar (Table 5). However, at the higher temperature (42°C), growth of the YK064 cells (divIB::spec amyE::Pspac-yneA) was specifically inhibited in the presence of IPTG (Fig. 8). Consistent with this growth defect, after 45 min incubation at 42°C, the mean cell length of YK064 cells cultivated in the presence of IPTG (i.e. in the absence of DivIB and presence of YneA) was about two times longer than that in the absence of IPTG (i.e. in the absence of both DivIB and YneA) (Table 5). These results suggest that YneA interferes with the assembly process of late division proteins.

### Table 4. Time between FtsZ ring assembly and FtsZ ring disappearance

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>YK019 cells</th>
<th>YK033 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No xylose</td>
<td>1% Xylose</td>
</tr>
<tr>
<td>60</td>
<td>12.5</td>
<td>17.4</td>
</tr>
<tr>
<td>80</td>
<td>31.3</td>
<td>4.3</td>
</tr>
<tr>
<td>100</td>
<td>50.0</td>
<td>8.7</td>
</tr>
<tr>
<td>120</td>
<td>6.3</td>
<td>26.1</td>
</tr>
<tr>
<td>140</td>
<td>--</td>
<td>43.5</td>
</tr>
</tbody>
</table>

DISCUSSION

Earlier studies have shown that purified EzrA protein interacts directly with FtsZ to block its assembly in vitro, but that it is unable to disassemble preformed polymers (Haeusser et al., 2004). Inactivation of EzrA has been shown to induce additional formation of FtsZ rings at cell poles (Levin et al., 1999), lower the critical concentration of FtsZ required for ring formation (Levin et al., 1999), and stabilize the FtsZ polymer to an extent sufficient to overcome the inhibition of ring formation associated with minCD overexpression (Levin et al., 2001). Furthermore, overproduction of EzrA in vivo blocks FtsZ ring formation (Haeusser et al., 2004; Y. Kawai, unpublished data). These findings seem to indicate that EzrA acts to prevent FtsZ assembly at aberrant locations along the cell membrane, while also playing a role in mid-cell division. Here, we show for the first time that ezrA inactivation in wild-type cells stabilizes the FtsZ ring, lengthening the time between FtsZ ring formation and constriction, which results in cell elongation. Furthermore, we demonstrated that overexpression of the essential cell division protein FtsL overcomes the defects associated with ezrA inactivation. Conversely, the absence of EzrA caused lethality in cells underexpressing FtsL. These results indicate that the amount of FtsL is insufficient in the ezrA− background. Reduction of the FtsZ monomer pool due to aberrant FtsZ ring formation at cell poles is a possible reason for the delay in FtsZ ring formation and maturation in the ezrA mutant. However, complementation of the delay by overproduction of FtsL strongly suggests that the aberrant FtsZ ring formation results in insufficient FtsL to complete assembly of the division complex, thus prolonging the life of the FtsZ localization. EzrA protein has a single N-terminal membrane span and a large cytoplasmic domain (Errington et al., 2003). In contrast, the FtsL protein is a membrane protein with only a small part located in the cytoplasm. Thus, it is unlikely that EzrA and FtsL interact directly. EzrA is associated with the cell membrane and recruited to the
division site via the direct interaction with FtsZ (Levin et al., 1999; Haeusser et al., 2004). Our results indicate that EzrA not only inhibits aberrant FtsZ ring formation along the cell membrane but also participates in mid-cell division incorporated into the FtsZ ring, possibly by maintaining FtsZ ring dynamics and ensuring the proper timing of FtsZ ring constriction. In contrast, the FtsL protein is recruited to the division site at the late stage in cell division (Errington et al., 2003). No previous study has identified a precise function for FtsL; however, it is essential for the complete assembly of the late division complex. In the absence of FtsL, the FtsZ ring cannot progress from the initial ring and so will not disassemble (Daniel et al., 1998). In the absence of EzrA, multiple FtsZ rings are formed at the mid-cell and cell poles. It may lead to a limiting amount of FtsL for complete assembly of late division proteins following constriction of the divisome. A search for EzrA orthologues in the complete genome sequences of bacteria in the Microbial Genome Database (MBGD) (http://mbgd.genome.ad.jp/) revealed that EzrA is conserved only in bacilli. Even though the primary sequence similarity is weak (only 16% identity), the B. subtilis ftsL gene has been identified as a homologue of the E. coli ftsL, based on their similarities in size, predicted transmembrane topologies, mutant phenotypes, and genomic

![Fig. 7. Effect of the ezrA deletion in cells underexpressing FtsL. YK030 (ftsL::neo amyE::PxyL-ftsL), YK034 (ezrA::spec ftsL::neo amyE::PxyL-ftsL), YK035 (zapA::spec ftsL::neo amyE::PxyL-ftsL), YK036 (noc::tet ftsL::neo amyE::PxyL-ftsL) and YK037 (dinR::spec ftsL::neo amyE::PxyL-ftsL) cells were grown to stationary phase in the presence of 0.5% xylose, serially diluted (tenfold steps), and spotted onto PAB plates containing 0.5% (left) or 0.1% (right) xylose, to induce low-level expression of FtsL. The plates were incubated for ~16 h at 37 °C and photographed.](http://mic.sgmjournals.org)

![Fig. 8. Synthetic defect of cell growth of exogenous YneA expression in divIB null mutant. YK005 (amyE::Pspac-yneA, circles) and YK064 (divIB::spec amyE::Pspac-yneA, triangles) strains were grown in PAB in the presence (filled symbols) and absence (open symbols) of 1 mM IPTG at 30 or 42 °C.](http://mic.sgmjournals.org)
association with the \( \text{pbpB} \) gene (Daniel et al., 1998). Interestingly, \( \text{B. subtilis} \)-type FtsL is also conserved only in bacilli. The conservation of FtsL and EzrA in these common bacteria implies the functional correlation of these proteins.

We found that overexpression of FtsL suppresses the division defect due to multiple non-productive FtsZ ring formation that resulted from a combination of mild division inhibitions, \( \text{yneA} \) expression and \( \text{ezrA} \) disruption. Multiple non-productive FtsZ ring formation and inhibition of cell division has been reported to occur by the double disruption of negative regulators of FtsZ ring formation, \( \text{minD} \) and \( \text{noc} \), in \( \text{B. subtilis} \) (Wu & Errington, 2004). In an earlier report, the division defect in the \( \text{minD noc} \) double mutant was recovered by FtsL overproduction (Wu & Errington, 2004). We demonstrated that overproduction of FtsL could also suppress the division defect in the \( \text{minCD noc} \) double mutant. Additionally, we showed that multiple FtsZ rings and cell division inhibition are induced when Noc and EzrA are simultaneously depleted, probably due to hyperstabilization of excess FtsZ rings. This phenotype was also suppressed by the overproduction of FtsZ or FtsL. Wu & Errington (2004) have suggested that in the \( \text{minD noc} \) double mutant with multiple minor polymerization centres all competing for division proteins, none of these can recruit enough FtsZ protein to form a productive ring, and that this defect is recovered by increasing the concentration of FtsZ monomer by overproduction of FtsZ. Our findings may suggest that FtsL overproduction also increased the concentration of free FtsZ monomers by promoting the dissociation of abortive FtsZ rings to recover the normal number of FtsZ rings, and thus an apparently normal cell division frequency. On the other hand, the division defect caused by \( \text{yneA} \) expression in the \( \text{ezrA} \) mutant was not recovered by the overexpression of FtsZ, suggesting a difference in the mechanism of multiple Z-ring formation compared to those in the \( \text{minCD noc} \) and \( \text{ezrA noc} \) mutant cells. In the \( \text{yneA} \)-expressing \( \text{ezrA} \)-disrupted cells, the amount of FtsL may become limiting for complete assembly of the late division complex, as observed in the \( \text{ezrA} \)-disrupted cells. Although future work is necessary to elucidate the mechanisms by which multiple abortive FtsZ rings are formed in these cells, our results suggest that FtsL would have a role in promoting FtsZ ring disassembly and constriction, directly or indirectly.

Finally, we found that expression of \( \text{yneA} \) in \( \text{ezrA} \)-disrupted cells induced the formation of multiple abortive FtsZ rings at potential division sites, suggesting that \( \text{yneA} \) acts to block cell division after the assembly of the FtsZ ring in the absence of EzrA. Consistently, expression of \( \text{yneA} \) in \( \text{divIB} \)-disrupted cells cultivated at \( 42 \) °C resulted in a synthetic defect in cell division. DivIB protein is one of the late division proteins, and the main role of DivIB is to protect FtsL from degradation at higher temperatures (Daniel & Errington, 2000). This result further supports the idea that \( \text{yneA} \) interferes with the assembly process of the late division complex. FtsL is an intrinsically unstable protein. Upon repression of \( \text{ftsL} \) expression, FtsL rapidly disappears and division is quickly arrested (Daniel et al., 1998). We have not as yet succeeded in determining the cellular amount of FtsL, since effective antibodies against the protein are currently unavailable. Degradation of FtsL due to expression of \( \text{yneA} \) is thus a possibility. However, this is unlikely, because the amount of YFP–FtsL fusion protein, examined by using antibody against YFP, is not affected by the expression of \( \text{yneA} \) in wild-type or \( \text{ezrA} \)-disrupted cells (data not shown). On the other hand, localization of YEP–FtsL and YEP–DivIC, which interacts with FtsL directly (Errington et al., 2003), to the multiple FtsZ ring in filamentous cells of the \( \text{yneA} \)-expressing \( \text{ezrA} \) mutant significantly decreased at some places (data not shown). Such abnormal localization was not observed for other division proteins, FtsZ, FtsA, ZapA, DivIB and PBPB (Fig. 2 and data not shown). Thus, \( \text{yneA} \) may affect cell division in a later step by suppressing recruitment of FtsL and/or DivIC to the divisome, although the partial inhibition of cell division induced by \( \text{yneA} \) expression in wild-type cells was not suppressed by FtsL overproduction, and caused hypersensitivity to the reduction in the amount of FtsL.

In sum, the dynamics of FtsZ ring formation and disassembly are regulated by a sophisticated network of negative and positive regulatory factors. Here, we demonstrated for the first time that EzrA and FtsL participate in this network with a partly complementary function that promotes FtsZ ring disassociation and constriction.

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