Two-Step Assembly Dynamics of the *Bacillus subtilis* Divisome^{∇†}

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Cell division in bacteria is carried out by about a dozen proteins which assemble at midcell and form a complex known as the divisome. To study the dynamics and temporal hierarchy of divisome assembly in *Bacillus subtilis*, we have examined the in vivo localization pattern of a set of division proteins fused to green fluorescent protein in germinating spores and vegetative cells. Using time series and time-lapse microscopy, we show that the FtsZ ring assembles early and concomitantly with FtsA, ZapA, and EzrA. After a time delay of at least 20% of the cell cycle, a second set of division proteins, including GpsB, FtsL, DivIB, FtsW, Pbp2B, and DivIVA, are recruited to midcell. Together, our data provide in vivo evidence for two-step assembly of the divisome. Interestingly, overproduction of FtsZ advances the temporal assembly of EzrA but not of DivIVA, suggesting that a signal different from that of FtsZ polymerization drives the assembly of late divisome proteins. Microarray analysis shows that FtsZ depletion or overexpression does not significantly alter the transcription of division genes, supporting the hypothesis that cell division in *B. subtilis* is mainly regulated at the posttranscriptional level.

Cell division in bacteria is initiated by the assembly of a multiprotein complex known as the divisome (Fig. 1). The most critical component of the divisome is FtsZ, a structural homologue of eukaryotic tubulin (33). FtsZ can polymerize in a GTP-dependent manner (5, 37), and the resulting FtsZ ring (Z ring) functions as a scaffold for all other known proteins responsible for cell division. Being the first and most crucial event in cytokinesis, Z-ring formation is strictly regulated in space and time. Two mechanisms are thought to determine the precise medial localization of the Z ring: the Min system and nucleoid occlusion (10, 53). The regulation of initiation of Z-ring assembly is less clear and probably involves multiple points of coordination with the DNA replication cycle and the growth rate (21). Recently, close links have been shown between cell size and growth rate, which involve the direct interaction of a metabolic enzyme, UgtP, with FtsZ, resulting in nutrient-dependent inhibition of FtsZ assembly (51).

Once the Z ring is assembled, it remains at midcell (52) and supports the assembly of at least 10 other proteins (for reviews, see references 15 and 48). FtsA tethers FtsZ to the membrane (28), and ZapA promotes Z-ring assembly (20), whereas EzrA and GpsB are involved in the cell cycle-dependent localization of PBP1, the major transglycosylase/transpeptidase, thereby controlling the site of cell wall synthesis (8). SepF might have a role that overlaps that of FtsA (23, 27). FtsL, DivIB, DivIC, and PBP-2B all have a single transmembrane span and a substantial extracellular domain (15). PBP-2B is the transpepti-

dase proposed to introduce cross-links into septal peptidoglycan (12, 35). FtsL has been pointed out as a possible key regulator of cell division in *Bacillus subtilis* (7, 11). FtsW may be involved in the translocation of peptidoglycan precursors (15). Finally, DivIVA is involved in the positioning of the Min system (14).

The way in which the divisome assembles has been studied extensively in *Escherichia coli*, leading to an assembly pathway which requires the sequential assembly of three subcomplexes. First, FtsZ-ZapA-ZipA assembles, allowing the assembly of the FtsL-FtsQ-FtsB complex (homologues of *B. subtilis* FtsL, DivIB, and DivIC, respectively), and then the FtsW-FtsI complex (homologues of *B. subtilis* FtsW and PBP-2B) associates with it (17). It has recently been shown that a time delay separates the assembly of FtsZ and the recruitment of FtsQ, FtsW, FtsI, and FtsN (1).

In *B. subtilis*, the hierarchical dependency of divisome assembly is less clear, but differences from the *E. coli* system have been observed. FtsL, DivIB, DivIC, and PBP-2B were shown to be interdependent for assembly, suggesting a cooperative rather than a sequential pathway of recruitment (15). Moreover, regulation of protein stability seems to have a significant role in the control of *B. subtilis* cell division (13), and a possible linear pathway of assembly could be masked by degradation. Interestingly, Gonzalez and Beckwith recently showed that in *E. coli* there may be degradation of FtsB when cell division is perturbed (18). Little is known about the dynamics of cell division in *B. subtilis*, although a time dissociation between the assembly of FtsZ and that of DivIVA has been inferred from colocalization studies with germinating spores (26).

To study the dynamics and hierarchy of divisome assembly in *B. subtilis*, we set out to analyze the timing of recruitment to midcell of several key divisome components. To compare the differences in time of localization between these proteins, a spore germination system was used. This provides the advan-

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FIG. 1. Schematic representation of the *B. subtilis* divisome. The classical view of the *B. subtilis* divisome is represented; protein names have been abbreviated by excluding Fts and Div (Z, FtsZ; A, FtsA; L, FtsL; W, FtsW; IB, DivIB; IC, DivIC; IVA, DivIVA).

tage of cell cycle synchrony, enabling us to directly compare the temporal order of divisome assembly. To examine divisome dynamics in vegetatively growing cells, time-lapse microscopy was performed. Since this is a single-cell technique whereby we can track individual cell lineages, synchronization of the population is not required. With these techniques, we show that there is a considerable delay between the assembly of early and late cell division proteins. Interestingly, FtsZ overexpression changes the timing of localization of early but not late division proteins. This overexpression had no effect on the expression of known division genes. Our data suggest the existence of distinct signals for the assembly of early and late division proteins and are in favor of a posttranscriptional control mechanism for cell division.

MATERIALS AND METHODS

Media, strains, and general methods. The bacterial strains and plasmids used in this study are listed in Table 1. *B. subtilis* strains were grown at 30°C or 37°C on solid nutrient agar (Oxoid) plates, in Difco antibiotic medium 3, or in casein hydrolysate medium supplemented where required with 0.4% xylose or 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside). *E. coli* DH5 α was grown at 37°C in LB medium and used as a cloning intermediate. Molecular cloning, PCRs, and *E. coli* transformations were carried out by standard techniques. *B. subtilis* chromosomal DNA was purified as described by Ward and Zahler (49) when used for transformation or as described by Venema et al. (47) when required for PCRs. Transformation of competent *B. subtilis* cells was performed by the method of Anagnostopoulos and Spizizen (2) as modified by Hamoen et al. (24). Selection for *E. coli* or *B. subtilis* transformants was carried out on nutrient agar (Oxoid) supplemented with 100 μ g/ml ampicillin, 5 μ g/ml chloramphenicol, 50 μ g/ml spectinomycin, or 1 μ g/ml phleomycin.

Plasmid and strain constructions. To construct plasmid pRD410, a 468-bp C-terminal fragment of the coding sequence of *ezrA* was amplified with oligonucleotides *ezrA*-2 (AAAGGTACCCTTCAGGCGAGGGAGACGCTCAG) and *ezrA*-3 (GATCTCGAGAGCGGATATGTCAGCTTTGATTTTCAAC TGCACC), carrying KpnI and XhoI restriction sites (underlined), respectively, and the PCR fragment was double digested (31) and ligated to similarly digested pSG1151.

Plasmid pPG2 was constructed to allow C-terminal green fluorescent protein (GFP) fusions to be placed at the *amyE* locus under the control of the native promoter. This plasmid was made in two stages. First, pJS2 was cut with SalI to

remove a small region containing a few restriction sites and religated. The resulting plasmid, pJS2 Δ S, was cut with BamHI, blunted, and then digested with SpeI. The DNA fragment encoding GFP, liberated from pSG1164 (31) by KpnI, treatment to blunt the restriction site, and SpeI digestion, was then ligated to digested pJS2 Δ S to generate pPG2. Plasmid pPG3 was constructed by insertion of a PCR fragment containing *ftsW* and 150 bp of the upstream region into XhoI- and HindIII-digested pPG2. The PCR fragment was amplified from 168 chromosomal DNA with oligonucleotides PG17 (CGGCTCGAGTCTCATCTCAT AAAAGACGG) and PG18 (GCCAAGCTTCA GATAAACAGTTTTTTTGA GC), carrying XhoI and HindIII restriction sites (underlined), respectively. Plasmid pPG4 was constructed by insertion of a PCR fragment encoding *divIB* into XhoI- and HindIII-digested pSG1729. The PCR fragment was amplified from 168 chromosomal DNA with oligonucleotides PG53 (GACCTCGAGATG AACCCGGGTCAAGACCG) and PG54 (GCCAAGCTTATTTTCATCTTCC TTTTAGCAGC), carrying XhoI and HindIII restriction sites (underlined), respectively.

To construct plasmid pGFP-*divIVA*, carrying the *B. subtilis* *divIVA* promoter region, with a strong ribosome binding site linked with *gfp*, PCR was used to amplify the promoter region (with primers *divIVA*-F+EcoRI [GCG CGAATTCGTCGTTATGCTGACAAGTTTGTC] and *divIVA*-R+NheI [GCGCGCTAGCCATAGTAGTTCCTCCTTAATTTTACATTTTCAGTA CGGTTATCTAGTTTAC 3']) from chromosomal DNA of *B. subtilis* 168. The amplified fragment was subsequently cleaved with EcoRI and NheI and ligated into the corresponding sites of the *amyE* integration vector pJWV017 (J.-W. Veening et al., submitted for publication), resulting in plasmid pGFP-*divIVA*.

Strain 3312 was constructed by transforming competent cells of 168 with plasmid pRD410 and selecting for chloramphenicol resistance. Strain PG8 was constructed by transforming strain 168 with chromosomal DNA of strain YK059 and selecting for chloramphenicol resistance. Strain PG10 was constructed by transforming competent cells of strain PG8 with the replacing cassette plasmid pCm::Spc (44), selecting for spectinomycin resistance, and screening the transformants for the loss of chloramphenicol resistance. Strain PG17 was obtained by a double-crossover recombination event between the *amyE* regions located on plasmid pPG3 and the chromosomal *amyE* gene of strain 168, respectively. Transformants were selected on nutrient agar plates containing spectinomycin. Correct integration into the *amyE* gene was tested and confirmed by the absence of amylase activity upon growth on plates containing 1% starch. Plasmid integration or transfer of genetic markers by transformation was used to construct all other strains, as described in Table 1. Strain constructions were confirmed by PCR or by phenotypic and genetic tests.

As detailed in Table 1, strains 1803, 4224, and PG17 contain GFP fusions of *divIVA*, *gpsB*, and *ftsW*, respectively, as second copies of the genes under the control of the native promoters, at the wild-type locus (1803 and 4224) or at the *amyE* locus for PG17. Strains 2012, 2020, PG62, PG67, and PG117 contain *gfp* fusions of *ftsL*, *ftsZ*, *ftsA*, *zapA*, and *divIB*, as second copies of the genes at the *amyE* locus under the control of the inducible *P_{xyI}* promoter (*ftsL*, *ftsZ*, *pbpB*, and *divIB*) or at the nonessential *aprE* locus under the control of the inducible *P_{spac}* promoter (*ftsA* and *zapA*). Strains 3122 and 3312 contain *P_{xyI}*-*gfp*-*pbpB* and *ezrA*-*gfp* fusions, respectively, present as the only copies of the genes at the endogenous locus.

For time-lapse microscopy, agarose was supplemented with 0.3 to 1% xylose or 0.3 mM IPTG where required. Control experiments showed that the presence of these inducers had no effect on the cell cycle of strains not requiring these inducers.

Sporulation and germination conditions. *B. subtilis* spores were prepared as previously described (36), with minor modifications. Briefly, strains were grown at 37°C in liquid sporulation medium until they reached an optical density at 600 nm (OD_{600}) of approximately 0.6 and then plated on sporulation agar plates after four serial fivefold dilutions. Sporulation medium was adapted from that of Schaeffer et al. (41) and contained dehydrated Nutrient Broth (0.8%; Difco), $MgSO_4$ (1 mM), KCl (13 mM), $CaCl_2$ (1 mM), and $MnSO_4$ (0.13 mM). Plates were incubated at 30°C for 7 days. Spores were then scraped off the plates and suspended in sterile water. After a wash step in sterile water, spores were suspended in TE (Tris-Cl at 10 mM [pH 8], EDTA at 1 mM) supplemented with 1.5 mg/ml lysozyme and incubated at 37°C for 1 h on a shaker. Sodium dodecyl sulfate (SDS) was added to a final concentration of 5%, and the suspensions were incubated at 37°C for 30 min with continuous shaking. Spores were then collected by centrifugation and washed four times with sterile water prior to being stored in water at 4°C. Spore preparations were subsequently washed twice a day for a week to avoid spontaneous germination. The purity of the purified spores was evaluated by phase-contrast microscopy, and all preparations were free (99%) of vegetative cells and cell debris.

Spore germination was performed by the method described by Hamoen and

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant features or genotype	Construction, source, or reference
<i>B. subtilis</i>		
168	<i>trpC2</i>	Laboratory stock
1801	<i>P_{spac}-ftsZ ble</i>	34
1803	<i>divIVA::</i> (<i>P_{divIVA}-divIVA-gfp divIVA⁺ cat</i>)	45
2012	<i>amyE::spc</i> (<i>P_{xyI}-gfpmut1-ftsL</i>)	43
2020	<i>amyE::</i> (<i>P_{xyI}-gfpmut1-ftsZ spc</i>)	J. Sievers (unpublished)
3122	<i>pbpB::pSG5061</i> (<i>cat P_{xyI}-gfp-pbpB</i>)	42
3312	<i>ezrA-gfp cat</i>	pRD410 integration into strain 168
4224	<i>gpsB::</i> (<i>P_{gpsB}-gpsB-gfpmut1 gpsB⁺ cat</i>)	8
YK020	CRK6000 <i>aprE::P_{spac}-yfp-ftsA</i>	Y. Kawai and N. Ogasawara (unpublished)
YK021	CRK6000 <i>aprE::P_{spac}-yfp-ftsA</i>	Y. Kawai and N. Ogasawara (unpublished)
YK059	CRK6000 <i>amyE::</i> (<i>P_{xyI}-ftsZ cat</i>)	29
PG8	<i>amyE::</i> (<i>P_{xyI}-ftsZ cat</i>)	YK059 DNA transformed into strain 168
PG10	<i>amyE::</i> (<i>P_{xyI}-ftsZ cat::spc</i>)	pCm::Spc integration into PG8
PG13	<i>divIVA::</i> (<i>P_{divIVA}-divIVA-gfp divIVA⁺ cat</i>) <i>ΩamyE::</i> (<i>P_{xyI}-ftsZ cat::spc</i>)	PG10 DNA transformed into strain 1803
PG14	<i>ezrA-gfp cat ΩamyE::</i> (<i>P_{xyI}-ftsZ cat::spc</i>)	PG10 DNA transformed into strain 3312
PG17	<i>amyE::</i> (<i>P_{ftsW}-ftsW-gfpmut1 spc</i>)	pPG3 integration into strain 168
PG51	<i>ezrA-gfp cat Ωchr::</i> (<i>P_{spac}-ftsZ ble</i>)	1801 DNA transformed into strain 3312
PG62	<i>aprE::P_{spac}-yfp-ftsA</i>	YK020 DNA transformed into strain 168
PG67	<i>aprE::P_{spac}-yfp-zapA</i>	YK021 DNA transformed into strain 168
PG117	<i>amyE::</i> (<i>P_{xyI}-gfpmut1-divIB spc</i>)	pPG4 integration into strain 168
JWV132	<i>amyE::</i> (<i>P_{divIVA}-gfp cat</i>)	pGFP-divIVA integration into 168
JWV133	<i>amyE::</i> (<i>P_{ezrA}-gfp cat</i>)	J.-W. Veening (unpublished)
<i>E. coli</i> DH5α	F [−] <i>φ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17</i> (<i>r_K[−] m_K⁺</i>) <i>phoA supE44 λ[−] thi-1 gyrA96 relA1</i>	Invitrogen
Plasmids		
pCm::Spc	<i>cat::spc</i>	44
pSG1151	<i>bla cat gfpmut1</i>	31
pSG1164	<i>bla cat P_{xyI}-gfpmut1</i>	31
pSG1729	<i>bla amyE3' spc P_{xyI}-gfpmut1' amyE5'</i>	31
pJS2	<i>bla amyE3' spc amyE5'</i>	31
pJS2ΔS	<i>bla amyE3' spc amyE5'</i>	pJS2 cut with SalI
pRD410	<i>bla cat'ezrA-gfpmut1</i>	468-bp C-terminal fragment of <i>ezrA</i> into KpnI-XhoI-cut pSG1151
pPG2	<i>bla amyE3' spc gfpmut1 amyE5'</i>	KpnI-SpeI fragment of pSG1164 into BamHI-SpeI-cut pJS2ΔS
pPG3	<i>bla amyE3' spc P_{ftsW}-ftsW-gfpmut1 amyE5'</i>	<i>P_{ftsW}-ftsW</i> into XhoI-HindIII-cut pPG2
pPG4	<i>bla amyE3' spc P_{xyI}-divIB-gfpmut1' amyE5'</i>	<i>divIB</i> into XhoI-HindIII-cut pPG4
pJWV017	<i>bla amyE3' cat gfp amyE5'</i>	Veening et al. (unpublished)
pGFP-divIVA	<i>bla amyE3' cat P_{divIVA}-gfp amyE5'</i>	<i>P_{divIVA}</i> into EcoRI-NheI-cut pJWV017

Errington (22), modified as follows. Spores were diluted in germination medium (22), heat shocked for 30 min at 70°C, and quickly cooled on ice. The spore mixtures were then diluted in germination medium to an OD₆₀₀ of 0.3 and incubated at 30°C with continuous shaking. At intervals of 30 min, starting from the beginning of the heat shock treatment (*t* = 0), samples were collected for microscopy. Unless stated otherwise, experiments were repeated at least three times with at least two independent spore preparations. The data shown are averages of these replicates with the error bars indicating the standard errors.

Microscopic imaging. Samples of germinating spores were mounted on microscope slides coated with a thin layer of 1% agarose. Images were acquired with a Zeiss Axiovert 200M microscope coupled to a Sony Cool-Snap HQ cooled charge-coupled device camera (Roper Scientific) and with Metamorph imaging software (Universal Imaging). For the germination analysis, at least three different fields of germinating spores were imaged and about 200 cells were analyzed for each time point. For membrane staining, cells were mounted on slides coated with 1% agarose supplemented with the membrane dye Nile Red (0.1 μg/ml; Invitrogen). For time-lapse microscopy, spores of strains EzrA-GFP and DivIVA-GFP were mixed at a 1:1 ratio, heat shocked as described above, and inoculated onto a thin semisolid matrix made of germination medium supplemented with 1.5% low-melting-point agarose attached to a microscope slide. Microscope slides were prepared as described by Veening et al. (46) and contained the FM5-95 fluorescent membrane stain (0.6 μg/ml; Invitrogen). Slides were incubated in a temperature-controlled chamber (30°C) on a Deltavision RT

automated microscope (Applied Precision). Phase-contrast, FM5-95, and GFP pictures were taken every 10, 13, or 15 min. Images were analyzed with ImageJ (<http://rsb.info.nih.gov/ij/>) and prepared for publication with Adobe Photoshop 8.0 CS and Adobe Illustrator 10.

RNA extraction. For RNA isolation, spores were heat shocked as described and diluted in 30 ml of germination medium to an initial OD₆₀₀ of 0.3 in the presence or absence of the appropriate inducer (see Results). After 220 or 300 min, cultures were blocked by adding an equal volume of an RNA stabilization reagent (RNAlater; Ambion). The bacterial suspensions were spun at 4,000 rpm for 20 min, washed once in 0.5 ml of RNAlater, and then processed with the FastRNA Pro Blue kit (Qbiogene-MP Biomedicals). Samples were shaken for 50 s in the FastPrep machine at a setting of 6.0, and RNA was isolated according to the manufacturer's recommendations. The RNA was then immediately further purified by using the Qiagen RNeasy kit and eluted in RNase-free water with 20 U of SUPERase-In RNase inhibitor (Ambion). The quantity and quality of all preparations were determined first with a NanoDrop ND-1000 spectrophotometer and subsequently with an Agilent 2100 Bioanalyzer (Agilent Technologies). For each strain, three independent preparations were used to extract RNA to allow triplication of the experimental data.

cDNA synthesis and slide hybridization. Enzymes and reagents used for microarray hybridization were provided by the 3DNA Array 900MPX kit for bacteria (Genisphere), unless otherwise stated.

cDNA was synthesized from 2 to 3 μg of total RNA by using Superscript III

FIG. 2. Synchronized germination of *B. subtilis* spores. Spores of EzrA-GFP-, GFP-FtsZ-, FtsW-GFP-, GpsB-GFP-, and DivIVA-GFP-expressing strains were heat shocked and germinated as described in Materials and Methods. (A) Morphological stages of spore germination and outgrowth. Phase-contrast (T_0 , T_{60}) and fluorescence (membrane staining, Nile Red, $T_{240-300}$) images of spores are shown (time in minutes). (B to D) Synchrony of the spore preparations was assessed by examining the fraction of phase-bright spores at the early time points (B, average of six independent replicates), the increase in optical density (C, average of six independent replicates), and the appearance of a septal membrane (D, average of two independent replicates). Symbols: $\circ$, GFP-FtsZ; $\blacktriangle$, EzrA-GFP; $\bullet$, DivIVA-GFP; $\blacklozenge$, GpsB-GFP; $\blacksquare$, FtsW-GFP. Error bars indicate standard errors.

reverse transcriptase (Invitrogen) at 42°C for 2 h and subsequently purified with Clean & Concentrate-5 columns (Zymo Research). cDNA was then poly(T) tailed with terminal deoxynucleotidyl transferase and ligated to dye-specific capture sequences. Tagged cDNA was purified again as described above prior to being hybridized to microarray slides. For this experiment, a 70-mer oligonucleotide-based array designed for *B. subtilis* strain 168 and printed with nonadjacent triplicate replicates was used (ArrayExpress accession no. A-MEXP-839). The microarray is available on a collaborative basis on request from N.J.S. Slides were first prehybridized in 3.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS–10 mg/ml bovine serum albumin at 65°C for 20 min, washed with MilliQ water and isopropanol, and dried with an airbrush. Slides were then scanned at 50- μ m resolution with a GenePix 4000B Scanner (Axon Instruments) and checked for defects such as smears or streaks. The tagged cDNA was hybridized to the microarray slides for 16 h at 55°C with a SlideBooster (Advantix) with a mixing pulse of 7:3 and a power setting of 27. After the first hybridization, slides were washed first in prewarmed (55°C) 2× SSC–0.2% SDS for 10 min, at room temperature in 2× SSC for 10 min, and finally in 0.2× SSC for another 10 min. Slides were immediately dried with an airbrush and hybridized to fluorescently labeled DNA dendrimers, which could bind the dye-specific target sequences on the cDNA molecules. The second hybridization was performed at 50°C for 4 h at the same mixing settings. Finally, slides were washed as before but by performing the first wash step at 50°C and then dried and immediately scanned.

Slide scanning and data analysis. The microarrays were scanned with either a ScanArrayExpress HT scanner (Perkin-Elmer) with autocalibration (to a maximum saturation of 96%) or a GenePix 4000B scanner (Axon Instruments) with manual calibration. All images were acquired at a resolution of 5 μ m.

Scanned pictures were analyzed with BlueFuse for Microarrays (BlueGnome). Spot data were extracted from images, and then all scanned arrays were manually flagged to remove artifacts and unreliable or damaged spots. Finally, data for the

three replicates on each slide were combined by using the BlueFuse fusion algorithm.

Data analysis was performed with BASE (39). Normalization, cross-channel correction, and the Cyber-T statistical test (32) were carried out with custom-made tools within BASE which are available from N.J.S. on request, and the gene expression mean n -fold changes were calculated with the MGH fold-change algorithm. A P value of 0.01 was used to assess the significance of the results.

Microarray data accession number. Microarray data have been deposited in ArrayExpress (www.ebi.ac.uk/microarray-as/ae/) under accession no. E-MEXP-1929.

RESULTS

Synchronizing populations by spore germination. To accurately compare the assembly patterns of the different divisome components, we synchronized cells by adopting a spore germination assay. The use of germinating spores has the advantages that all cells are in the same cell cycle state and the localization of division proteins is not influenced by previous cell division events (Fig. 2A) (25). First, we tested the variability in germination rate between spores of different reporter strains prepared under our experimental conditions (Fig. 2B to D). As a preliminary index of the germination rate, the percentage of phase-bright (nongerminated) versus phase-dark (germinated) spores, as judged by phase-contrast microscopy, was plotted (Fig. 2B). This shows relatively little differences in germination

efficiency between different spore preparations of the same or different strains, as the germination curves are largely superimposable and are similar to that of a wild-type strain without a GFP fusion (data not shown). Thus, the GFP reporters used in this study are considered to represent the wild-type situation. The synchronous progression of the outgrowth was further assessed by measuring the OD₆₀₀ of the cultures (Fig. 2C). Again, this showed uniform germination of our spore preparations. By staining cells with a membrane dye (Nile Red), followed by fluorescence microscopy, we investigated the frequency of outgrowing cells showing the formation of the first septal membrane after germination (Fig. 2D). A significant degree of synchrony was observed at the first time points ($T_{240-300}$), since the percentage of cells undergoing the first septation increased at comparable rates in different cell populations. Synchrony appeared to decrease over time, probably because of subsequent cell division events. This was particularly evident after T_{300} , when differences between cultures became too large for a reliable comparison (Fig. 2D).

Divisome assembly occurs in two distinct steps. To follow the dynamics of the *B. subtilis* divisome, we examined the localization pattern of five division proteins, FtsZ, EzrA, GpsB, FtsW, and DivIVA, representing early to late division proteins. To do so, we used different GFP fusions. The EzrA-, GpsB-, FtsW-, and DivIVA-GFP fusions were expressed under the control of their respective native promoters, while FtsZ-GFP, present as a second copy at the *amyE* locus, was under the control of the inducible P_{xyt} promoter (see Materials and Methods).

To determine the temporal sequence of recruitment of the different fusion proteins, spores of the GFP reporter strains were germinated in liquid medium and fluorescence images were analyzed over time. We scored a dividing cell when a fluorescent signal from the GFP fusion was clearly visible at the midcell position. As shown in Fig. 3, FtsZ and EzrA assemble early after spore germination to the midcell position, while FtsW, GpsB, and DivIVA are recruited approximately 40 min later to the midcell position. Differences between the curves of the three late proteins were not considered significant because of the variation observed in different experiments (Fig. 3 and data not shown). For early division proteins, the localization frequency determination was not extended after 270 min because of the increasing percentage of second division rings, which render the analysis less accurate.

Two-step assembly is not caused by transcriptional regulation. Recently, it was shown that a large set of genes are expressed earlier than others during spore germination and outgrowth (30). For instance, germinating spores first activate genes involved in DNA repair and sequentially express DNA replication and cell division genes (30). To test whether the delay in the localization of late proteins was due to the delayed transcription of their genes during spore germination, we analyzed the expression levels of *ezrA* and *divIVA* by fusing their promoter regions to the *gfp* gene. Germination of spores of strains JWV132 (P_{divIVA} -*gfp*) and JWV133 (P_{ezrA} -*gfp*) was followed by time-lapse microscopy. Images were acquired every 15 min, and the mean GFP fluorescence was recorded. To define the cell cycle, the outgrowth of the spore was set to 0% and the first appearance of a septum was set to 100% of the cell cycle progression. The average cell cycle time under these conditions was 151 min (standard deviation = 24 min), and cell

FIG. 3. A time delay separates the assembly of FtsZ and EzrA from the recruitment of FtsW, GpsB, and DivIVA. Spores of the five GFP fusion strains were heat shocked and germinated as described in Materials and Methods. GFP pictures were taken every 30 min, starting at 210 min after the beginning of the heat shock. Percentages of cells showing a septal GFP signal were determined for the first round of cell division. At least 150 to 200 cells were analyzed at each time point. The values shown are averages of three replicates from two independent spore preparations. Symbols: ○, GFP-FtsZ; ▲, EzrA-GFP; ●, DivIVA-GFP; ◆, GpsB-GFP; ■, FtsW-GFP. Error bars indicate standard errors.

cycle progressions were binned into categories of 10%. Trajectories of at least six outgrowing spores of each strain were analyzed. Plots of expression against time were made. These clearly show that both promoters are transcribed immediately after germination (Fig. 4), and since GFP is a stable molecule, the linear increase in GFP concentration indicates that the *ezrA* and *divIVA* promoters are constitutively expressed during spore germination and outgrowth.

Time-lapse imaging of dividing cells. To rule out the possibility that the observed two-step assembly pattern of the divisome was specific to the spore germination process and to the five selected division proteins, we analyzed the cell cycles of vegetatively growing cells by using time-lapse microscopy on a large set of division proteins. We used *gfp* or *yfp* fusions of eight key divisome components: FtsZ, FtsA, ZapA, EzrA, Pbp2B, FtsL, DivIB, and DivIVA. Spores were germinated as described in Materials and Methods and applied to a microscope slide coated with semisolid medium containing membrane stain (FM5-95) and the appropriate inducer. As an additional control, spores carrying EzrA-GFP and DivIVA-GFP fusions were mixed at an equal ratio and applied to the same microscope slide, since after outgrowth the two strains were easily distinguishable due to the midcell localization pattern of

FIG. 4. *divIVA* and *ezrA* are transcribed immediately after spore germination. Germination of spores of strains with *P_{divIVA}-gfp* (●) and *P_{ezrA}-gfp* (▲) was followed by time-lapse microscopy. Images were acquired every 15 min, and the mean GFP fluorescence was recorded. The outgrowth of the spore was set to 0%, while 100% indicates the first appearance of a septum as determined by membrane staining. Trajectories of at least six outgrowing spores of each strain were analyzed, and the highest recorded mean fluorescence value within a single cell trajectory was set to 1 to calculate the relative expression level. Error bars show standard errors. The broken vertical line indicates the time of first appearance of a septum.

EzrA and the unique polar localization of DivIVA. The growth of several microcolonies was followed by taking phase-contrast and fluorescence images every 10, 12, or 13 min (for two examples, see Movies S1 and S2 in the supplemental material). From the images obtained, 25 cell cycles (of cells in the second, third, or fourth round of cell division after spore germination) were followed for each strain. To determine when in the cell cycle a given division protein localizes at the division site, FM5-95 and GFP images were analyzed in parallel. The fluorescent membrane stain allowed the evaluation of the cell cycle length, which was defined as the time period between subsequent division events. The first occurrence of the GFP signal in a given cell cycle was then obtained from the GFP images. Values were expressed as a percentage of the cell cycle progression, where 0% indicates a newborn cell and 100% is the time at which a septal membrane separating two sister cells can be detected. A representative cell cycle for the bright EzrA-GFP and DivIVA-GFP fusions is shown in Fig. 5A and B. The single-cell analysis is summarized in Fig. 5C (individual scatter plots) and D (averaged values). Two clusters of division proteins were clearly distinguishable: FtsZ, FtsA, ZapA, and EzrA were localized between 23% and 25% of the cell cycle, whereas Pbp2B, FtsL, DivIB, and DivIVA arrived at the new cell division site between 45% and 60% of the cell cycle. Thus, a significant period of at least 20% of the cell cycle separates the assembly of early and late division proteins ($P < 0.01$, t test). It is of interest that DivIVA localized slightly but significantly later to the midcell position than the other late cell division proteins ($P < 0.05$, t test). To test whether the delay between early and late division proteins was dependent on the cell cycle length, 100 cell cycles each of the EzrA-GFP and

DivIVA-GFP strains were analyzed (see Fig. S2 in the supplemental material). Under these microcolony conditions, the cell cycle length, determined for individual cell cycles by analyzing FM5-95 images as described, varied quite significantly from 40 to 110 min. However, the timing of localization as a function of cell cycle duration appeared constant (see Table S1 in the supplemental material).

FtsZ overexpression changes the timing of divisome assembly. To determine how this two-stage assembly may be regulated, we looked at the effects of FtsZ overexpression on the assembly timing of EzrA and DivIVA assembly. For both *E. coli* and *B. subtilis*, it was shown that increased cellular FtsZ expression causes the formation of polar septa and minicells (50) and a reduction in the average cell length by 10% (52). An additional copy of *ftsZ* placed under the control of an inducible *P_{xyI}* promoter was inserted at the *amyE* locus of strains carrying either an *ezrA-gfp* or a *divIVA-gfp* fusion. Spores of strains PG13 (*divIVA-gfp P_{xyI}-ftsZ*) and PG14 (*ezrA-gfp P_{xyI}-ftsZ*) and of parental strains 1803 (*divIVA-gfp*) and 3312 (*ezrA-gfp*) were grown in germination medium supplemented with 0.4% xylose. Western blotting analysis of samples taken at 220 and 300 min confirmed that FtsZ was clearly overexpressed (data not shown), and subsequent microarray experiments quantified the overexpression as 1.61 times (see next section). Fluorescence microscopy of the germinating spores showed that the localization of EzrA-GFP occurred significantly earlier when FtsZ was overexpressed (Fig. 6A and B). Even in germinating spores that were still relatively small, rings of EzrA-GFP were already visible (Fig. 6B, PG14). Surprisingly, FtsZ overexpression had no effect on the timing of DivIVA-GFP (Fig. 6A), and the delay between EzrA localization and DivIVA localization was widened by approximately 20 min.

Transcriptional effects of altered FtsZ levels. As there was a significant alteration in the timing of EzrA assembly due to the overexpression of FtsZ, it was possible that high levels of FtsZ lead to altered transcription of early cell division genes. To examine this, we performed a microarray analysis of germinating spores that either overexpressed FtsZ or were depleted of FtsZ. To examine the genome-wide transcriptional response upon FtsZ overproduction, spores of strain PG14 (*ezrA-gfp P_{xyI}-ftsZ*) were germinated and grown in the presence or absence of 0.4% xylose. After 220 min, samples were taken for microarray analyses as described in Materials and Methods. Three biological replicates were used for each experiment, with three independent spore preparations. The complete data sets of the transcriptome analyses may be accessed at Array-Express (www.ebi.ac.uk/microarray-as/ae/) under accession no. E-MEXP-1929.

As expected, *ftsZ* was found to be significantly overexpressed with a mean fold ratio (MFR) of 1.61 (overexpression/wild type) and a P value of <0.00001 . Another 39 genes were found to be significantly altered (P value, ≤ 0.01 ; MFR, <0.8 and >1.2). All genes enhanced in expression by more than twofold encode proteins involved in xylose transport and metabolism (*xylA*, *xylB*, *xylR*, *xynB*, and *xynP*). The other significantly affected genes were enhanced in expression to a lesser extent and are either uncharacterized or have no known relationship with cell division (*abh*, *abrB*, *aroK*, *cspD*, *dps*, *fer*, *gamP*, *rpmB*, *rpsR*, *smpB*, *veg*, *ycbC*, *ybyB*, *ycbP*, *ydaS*, *ydbN*, *ydjM*, *ydjN*, *yfiE*,

FIG. 5. Two clusters of division proteins can be distinguished by time-lapse microscopy. (A and B) Representative cell cycles of EzrA-GFP- and DivIVA-GFP-expressing strains. Spores of EzrA-GFP (A)- and DivIVA-GFP (B)-expressing strains were heat shocked and then germinated on a microscope slide. GFP and FM5-95 pictures were taken every 10 min. The images shown are overlays of both channels. Percentages indicate cell cycle progression, as determined by membrane staining. Asterisks indicate the cells considered in this montage. Arrows indicate the first appearance of EzrA-GFP or DivIVA-GFP at the midcell location. (C and D) Assembly initiation times of eight division proteins (EzrA, FtsZ, FtsA, ZapA, Pbp2B, FtsL, DivIB, and DivIVA) expressed as percentages of cell cycle progression. Growth was followed by time-lapse microscopy as described, and 25 cell cycles were analyzed for each strain. Single-cell values (C) and average values (D) are shown. Error bars indicate standard errors.

yheA, *yheI*, *yjgD*, *yjqA*, *ykpC*, *yktA*, *ykve*, *ylaF*, *yoaF*, *yorD*, *yqgX*, *yrzA*, *yutI*, *ywdA*, and *ywjC*).

For *ftsZ* depletion, the wild-type gene was placed under the control of an IPTG-inducible promoter. Spores of the resulting PG51 strain were grown in the presence or absence of 1 mM IPTG, and samples were taken after 220 and 300 min. At the early time point (220 min), *ftsZ* was found significantly depleted, with an MFR of 54.48 (wild type/depletion) and a *P* value of <0.00000001 . Surprisingly, only three other genes were found to be affected: *yvfC* and *yngH*, with unknown function, and *cgeC*, which is involved in the maturation of the outermost layer of the spore (38). At the late time point, *ftsZ* was reduced to a lesser extent, with an MFR of 10.76 and a *P* value of <0.00000001 . Here, nine other genes also differed (*uvrX*, *ydjM*, *yeeF*, *yfkD*, *ykrP*, *ykzE*, *yoeB*, *yqjX*, and *yurM*). The downregulation of *yoeB* (MFR = 2.64), a gene involved in autolysin activity modulation (40), and the upregulation of

ydjM (MFR = 0.58) are potentially interesting observations, as both genes are part of the YycFG regulon, an essential two-component system presumed to coordinate cell wall architecture with cell division in *B. subtilis* (6, 16). However, taking all of the microarray results together, we did not observe a clear effect in the expression profiles of known cell division genes. Therefore, the effect on EzrA localization upon FtsZ overexpression is not due to altered transcriptional regulation.

DISCUSSION

By examining the first round of cell division after spore germination, we found a significant time delay between the assembly of FtsZ and EzrA and that of FtsW, GpsB, and DivIVA. This delay does not seem to depend on a delayed transcription of the late division genes since both the *ezrA* and

FIG. 6. FtsZ overexpression affects the assembly of EzrA but not of DivIVA. (A) Spores of wild-type strains 3312 (*ezrA-gfp*) and 1803 (*divIVA-gfp*) and of FtsZ-overexpressing strains PG13 (*divIVA-gfp P_{xyI}-ftsZ*) and PG14 (*ezrA-gfp P_{xyI}-ftsZ*) were germinated as described in Materials and Methods. The percentage of cells showing a septal GFP signal is shown. Symbols: ▲, EzrA-GFP; △, EzrA-GFP (*P_{xyI}-ftsZ*); ●, DivIVA-GFP; ○, DivIVA-GFP (*P_{xyI}-ftsZ*). (B) Representative micrographs of germinating spores of EzrA-GFP and EzrA-GFP (*P_{xyI}-ftsZ*) at 210 min after germination. Phase-contrast and GFP channels are shown. Arrows indicate Z rings assembled in short cells immediately after germination.

divIVA promoters are active from the beginning of spore outgrowth.

Using time-lapse microscopy of vegetative cells, we identified two clusters of division proteins, FtsZ-FtsA-ZapA-EzrA and Pbp2B-FtsL-DivIB-DivIVA, whose localization at midcell is delayed by at least 20% of the cell cycle. Previous colocalization studies inferred a delay in DivIVA localization compared to FtsZ localization (26). However, it was unclear if DivIVA bound to the newly formed cell poles without having contact with the divisome or after the actual division (4, 22). Here we show that Pbp2B, FtsL, and DivIB have a localization time comparable to but perhaps slightly earlier than that of DivIVA and prior to the appearance of a membrane septum. However, single-cell analysis of DivIVA-GFP showed less variation in the localization time of DivIVA than in that of the others and we cannot exclude the possibility that DivIVA arrives at the membrane as septation is initiated, due to the detection limit of the membrane dye.

A two-step assembly model appears to be conserved in other bacterial divisomes as well. In *E. coli*, for instance, the delay between Z-ring formation and late protein assembly was quantified as 15% of the cell cycle in slow-growing cells, whereas at fast growth rates it extended up to 34% (1). Our experimental conditions are compatible with a moderate growth rate, and despite the high heterogeneity of cell cycle length, the delay appeared surprisingly constant and comparable to the one found in *E. coli*. Furthermore, the localization time of the two clusters appears to be well defined, as in *E. coli*, and in contrast to the more dynamic divisome assembly recently observed in *Caulobacter crescentus* (9).

Interestingly, the timing of EzrA localization could be ad-

vanced by FtsZ overexpression, whereas DivIVA localization was not altered in time (Fig. 6). These results suggest that the assembly of the Z ring stimulates the assembly of the early proteins. However, the time of assembly of late divisome proteins is unaltered, indicating that assembly of this subcomplex is stimulated by a signal other than the assembly of the inner-ring complex.

Since the timing of Z-ring assembly can be changed by alteration of its expression levels, we wanted to know whether this might affect the transcription of other division genes. Therefore, we performed a microarray analysis on germinating spores after FtsZ depletion and overexpression. However, we could only see moderate effects on the transcriptome in both conditions, although we cannot exclude the possibility that even moderate changes (lower than the distinguishable 1.2 MFR) might affect cell division. We noticed a significant alteration of *yoeB* and *ydjM* transcripts after extended depletion of FtsZ. *yoeB* is involved in the modulation of autolysin activity (40), whereas the role of *ydjM* is still unknown. Interestingly, both genes are part of the YycFG regulon, an essential two-component system whose sensor kinase YycG localizes to the division site (6, 16). The effect on their transcription can be explained by the facts that YycG cannot localize at the midcell position in the absence of Z rings and, as a consequence, it is less efficient in the activation of the response regulator YycF, as recently proposed (16). However, it should be said that the effects we noticed are the opposite of those observed by Bisicchia et al. upon YycFG depletion (6).

From the transcriptome analysis, it is clear that the FtsZ level is not a signal for transcriptional regulation of any known cell division or cell wall synthesis genes, in accordance with what was observed in *E. coli* (3). This is perhaps not surprising, as FtsZ appears to be abundantly present throughout the cell cycle (37).

The timing of cytokinesis has to respond to multiple dynamic processes such as growth rate changes, cell wall elongation, and DNA replication/segregation. We could not detect any cell cycle-dependent regulation in our promoter-GFP study, and our microarray data show that FtsZ levels are not a signal for transcriptional regulation of cell division genes. Transcriptional control of cytokinesis might only be effective when the proteins involved are intrinsically unstable, as is the case of for FtsL (11). In this respect, it is interesting that a link between DNA replication perturbations and the expression of *ftsL* and *pbbB* has been reported (19).

In conclusion, we have shown that the assembly of the divisome in *B. subtilis* proceeds in two steps and that earlier localization of FtsZ does not alter the localization of late proteins or the timing of cytokinesis. Such temporal dissociation between early and late divisome components appears to be conserved in bacteria, but the signal which drives late protein assembly remains to be discovered.

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