University of Groningen

Balanced transcription of cell division genes in *Bacillus subtilis* as revealed by single cell analysis

Trip, Erik Nico; Veening, Jan-Willem; Stewart, Eric J.; Errington, Jeff; Scheffers, Dirk-Jan

Published in:
Environmental Microbiology

DOI:
[10.1111/1462-2920.12148](https://doi.org/10.1111/1462-2920.12148)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2013

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Trip, E. N., Veening, J-W., Stewart, E. J., Errington, J., & Scheffers, D-J. (2013). Balanced transcription of cell division genes in *Bacillus subtilis* as revealed by single cell analysis. *Environmental Microbiology*, 15(12), 3196-3209. <https://doi.org/10.1111/1462-2920.12148>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Balanced transcription of cell division genes in *Bacillus subtilis* as revealed by single cell analysis

Erik Nico Trip,^{1†} Jan-Willem Veening,^{2,*†}
Eric J. Stewart,³ Jeff Errington⁴ and
Dirk-Jan Scheffers^{1**}

¹Department of Molecular Microbiology and ²Molecular Genetics Group, Groningen Biomolecular Sciences and Biotechnology Institute, Centre for Synthetic Biology, University of Groningen, Nijenborgh 7, 9747 AG Groningen, the Netherlands.

³Department of Biology, Northeastern University, Boston, MA, USA.

⁴Centre for Bacterial Cell Biology, Newcastle University, Newcastle upon Tyne, UK.

Summary

Cell division in bacteria is carried out by a set of conserved proteins that all have to function at the correct place and time. A cell cycle-dependent transcriptional programme drives cell division in bacteria such as *Caulobacter crescentus*. Whether such a programme exists in the Gram-positive model organism *Bacillus subtilis* is unknown. Here, we investigate the role of gene transcription as a potential regulatory mechanism for control of division in *B. subtilis*. Transcriptional GFP fusions in combination with flow cytometry demonstrated a constitutive promoter activity, independent of growth rate, of nine tested cell division genes. These measurements were verified by quantitative real-time reverse-transcription PCR (qRT-PCR). Time-lapse fluorescence microscopy was performed on a set of selected reporter strains to test transcriptional regulation during the cell cycle. Interestingly, although the average fluorescence remained constant during cell-cycle progression, individual cells demonstrated a roughly twofold higher promoter activity at the end of the cell cycle. This cell cycle-dependent increased promoter activity can be partly explained by the doubled promoter copy number after DNA replication. Our results indicate that the transcriptional activity of promoters for cell division genes

remains constant regardless of growth rate and cell-cycle state, suggesting that regulation of cell division in *B. subtilis* predominantly takes place at the post-translational level.

Introduction

Cell division in bacteria is a dynamic event during which one cell divides into two. In Gram-positive bacteria, a physical barrier, the septum, is constructed at mid-cell across the diameter of the cell, to divide it in two compartments. Subsequently, the septum splits, thereby freeing the two cells. During division, different processes have to take place in a specific order; elongation of the cell, establishment of a division site at mid-cell, further elongation, constriction and septum formation, and finally separation. These different stages of division require the activity of many cell division proteins (CDPs). These CDPs form large protein complexes at mid-cell that drive cell division, collectively known as the divisome (Adams and Errington, 2009). A hierarchical two-step assembly of the divisome has been reported for the Gram-positive model organism *Bacillus subtilis*. During early division, FtsZ assembles into the Z-ring at mid-cell assisted by FtsA, ZapA and EzrA. At a later stage, a second group of CDPs including GpsB, FtsL, DivIB, FtsW, PBP2B (*pbpB*), PBP1 (*ponA*) and DivIVA localize at mid-cell to complete the divisome (Gamba *et al.*, 2009). Several of these second stage proteins, FtsL, DivIB, DivIC and PBP2B, are recruited cooperatively to the divisome; one missing CDP in this second stage of divisome assembly destabilizes interactions between all the other CDPs, stalling division (Errington *et al.*, 2003). So, in order for division to proceed correctly, the CDPs have to become active at the correct place and time.

The positioning of Z-ring formation at exactly mid-cell is ensured by the combined effort of the Min-system, nucleoid occlusion and a potential third, as yet unknown, mechanism (Wu and Errington, 2004; Dajkovic *et al.*, 2008; Rodrigues and Harry, 2012). The Min-system is a cell pole associated inhibitor of FtsZ polymerization that prevents initiation of a second round of division at a newly formed cell pole (Gregory *et al.*, 2008). In nucleoid occlusion, the non-specific DNA-binding protein Noc associates with the replicating nucleoid and inhibits FtsZ polymerization (Wu and Errington, 2004). During

Received 25 February, 2013; revised 18 April, 2013; accepted 22 April, 2013. For correspondence. *E-mail j.w.veening@rug.nl; Tel. (+31) (0)50 363 2408; Fax (+31) (0)50 363 2348 or **E-mail d.j.scheffers@rug.nl; Tel. (+31) (0)50 363 2319; Fax (+31) (0)50 363 2154. †These authors contributed equally.

segregation of the sister nucleoids to the two cell halves, the concentration of Noc at mid-cell is reduced, thus leaving space for Z-ring assembly (Hariharan *et al.*, 1982; Moriya *et al.*, 2010). The balance between polymerized and non-polymerized FtsZ seems to be maintained through ClpX, but in *B. subtilis* this seems to be through destabilization of FtsZ polymers without degradation of FtsZ (Weart *et al.*, 2005), whereas in *Escherichia coli* the mechanism is through proteolysis of polymeric FtsZ by ClpXP (Camberg *et al.*, 2009).

Another important factor that controls Z-ring formation is nutrient availability. *Bacillus subtilis* cells maintain a constant ratio of cell length per Z-ring (L/R) regardless of growth rate; the proportion of cells with Z-rings varies inversely with doubling time, ranging from 85% in LB to 50% in minimal sorbitol medium at mid-exponential growth phase (Weart *et al.*, 2007; Chien *et al.*, 2012). The L/R ratio remains constant because cells are significantly longer in rich medium compared with poor medium, while the diameter of the cells remains constant. The metabolic state of the cell provides a cue for Z-ring formation and subsequent division. *Bacillus subtilis* does this via the glycolipid biosynthetic pathway; PgcA and GtaB transmit carbon availability to the effector UgtP that inhibits Z-ring formation until the cell reaches a critical mass for division to commence (Weart *et al.*, 2007). The cell length increases with growth rate to facilitate multi-fork DNA replication, in order to speed up the cell-replication process (Sargent, 1975; Sharpe *et al.*, 1998).

Even before Z-ring formation and other division processes can take place, critical amounts of CDPs have to be present in the cell. FtsZ can only assemble into a ring and facilitate divisome formation when present above a certain concentration. In steady-state conditions, already a small reduction in FtsZ expression leads to a transient delay in division in *E. coli* (Rueda *et al.*, 2003; Weart and Levin, 2003). For *E. coli*, it was found that *ftsZ* expression oscillates during the cell cycle with a peak at the onset of chromosomal replication (Garrido *et al.*, 1993). The peak in expression of *ftsZ* before the start of division strongly suggests regulation of the start of division by transcription. However, the expression of genes encoding other (late stage) CDPs during the cell cycle was not studied. For *B. subtilis*, oscillating *ftsZ* expression during the cell cycle has never been described, but it was found that *ftsZ* expression is three to fourfold higher when this bacterium enters the developmental process of sporulation in which the cell divides asymmetrically to produce an endospore at one end of the cell pole. This burst in expression is not critical for sporulation, although it does increase sporulation efficiency (Gonzy-Treboul *et al.*, 1992). Despite the apparent transcriptional cell-cycle regulation in *E. coli*, other work describes constant protein levels of FtsZ at a range of growth rates in both *E. coli* and *B. subtilis* and

fluctuating protein levels of FtsZ during the cell cycle could not be found for these two model organisms (Weart and Levin, 2003). On a population level, analysis of transcriptomes by RNA tiling has shown a balanced expression of cell division genes in *B. subtilis* when cultured in a wide range of growth media and when exposed to stress such as temperature shifts, ethanol and various drugs. Cell division genes were shown to belong to the SigA promoter cluster that displays low variance in activity, ensuring stable transcription of genes that encode proteins with essential functions (Nicolas *et al.*, 2012). Still, the response of transcription of cell division genes to different growth rates (e.g. pace of division) was not studied.

Cell-cycle regulation of FtsZ protein levels as well as *ftsZ* transcription does occur in the differentiating bacterium *Caulobacter crescentus*. During division, a mature stalked cell releases a motile progeny swarmer cell that is temporarily inhibited for replication. Swarmer cells inherit high levels of the cell-cycle controller protein CtrA that inhibits *ftsZ* transcription, while the levels of inherited FtsZ protein are decreased due to proteolysis (Quardokus *et al.*, 1996; Kelly *et al.*, 1998). After the inherited levels of CtrA decrease, the transcription of *ftsZ* commences and the swarmer cell matures into a stalked cell that is able to divide.

Because of the observed differences in transcriptional regulation of CDPs in various bacteria, we were interested to study the transcriptional response of cell division genes to various growth rates in *B. subtilis*. We also wondered how transcription of cell division genes is organized during the cell cycle. Can transcription be the underlying process that speeds up or slows down division and can transcription regulate cell-cycle progression? To test this, we created transcriptional fusions to the promoter regions of nine cell division genes (comprising both early and late CDP) and monitored their transcriptional response to changes in growth rate by flow cytometry. In a complementary approach, the transcriptional response of cell division genes to growth rate was analysed at the mRNA level by rtPCR. The expression of cell division genes was also followed during the cell cycle using automated fluorescence time-lapse microscopy. Our findings demonstrate that, in general, cell division genes in *B. subtilis* are stably transcribed independent of growth rate and cell cycle.

Results and discussion

Experimental strategy

The aim of this work was to study the transcription of genes coding for CDPs, both at the population and single cell level at different growth rates, and at the single cell level during the cell cycle. To do so, we constructed a set of promoter-region fusions to *gfp* (Fig. 1, Table 1). These

A *PftsZ-gfp amyE* **B** *PftsA-gfp amyE* **C** *PftsA-gfp original*

Fig. 1. Schematic representations of a few transcriptional fusions to *gfp*.

A. The entire promoter region of the *ftsAZ* operon + the *ftsA* gene including a stop codon was taken as the *ftsZ* promoter and placed before *gfp* followed by a double terminator. This construct was integrated at the *amyE* region by a double-cross-over event.

B. The promoter region of the *ftsAZ* operon was taken as the *ftsA* promoter and placed before *gfp* followed by a double terminator. Also, this construct was integrated at the *amyE* region by a double-cross-over event.

C. The promoter region of the *ftsAZ* operon was taken as the *ftsA* promoter and placed before *gfp* followed by a single terminator. By a single-cross-over event this construct was integrated as a copy next to the original gene.

promoter-*gfp* fusions were integrated at the *amyE* locus, which provides a stable genetic environment that allows growth of the strains for analysis in the absence of antibiotics without the risk of loss of the fusion through recombination, which is especially important during single cell analysis by time-lapse microscopy. As a positive control a fusion to the *rrnB* promoter was included, as transcription from this promoter is strongly correlated to growth rate, while the constitutive *veg* promoter served as a control for unregulated expression (Krasny and Gourse, 2004). To make sure that the read-out from *amyE* reflected correct promoter activity, we also generated a subset of promoter fusions at the original locus with the pBaSysBioII transcriptional fusion integration vector (Botella *et al.*, 2010), again including the *rrnB* promoter as a control (Fig. 1). Populations growing at different rates were sampled at mid-exponential phase for each growth medium, when the greatest percentage of the culture undergoes division, and analysed by flow cytometry to determine promoter activity. *Bacillus subtilis* 168 1A700 was used as a host strain because this strain grows mainly as motile single cells, not chains, and therefore can be used for flow cytometry (Veening *et al.*, 2006). To verify the flow cytometry results by an independent technique, the transcription of genes coding for CDPs was also monitored

at different growth rates by quantitative real-time reverse-transcription PCR (qRT-PCR). For a valid comparison between populations cultured at different growth rates in different media the moment of sampling is critical; all cultures have to be sampled at the mid-exponential growth-phase. For this matching, we determined the specific mid-exponential growth-phases for cultures grown in all used media, and sampled at those moments. Finally, promoter activity in the *amyE* promoter-*gfp* fusion strains was monitored using time-lapse microscopy on growing microcolonies, which allowed the study of promoter activity in single cells during the cell cycle from birth of the cell to division.

Transcription of genes encoding cell division proteins is not strongly influenced by growth rate

The promoter-*gfp* fusions at *amyE* allow promoter activity to be monitored by the production of intracellular GFP. The reporter strains were cultured in three defined media with equal incubation temperature and aeration, and the differences in media composition resulted in three different doubling times (K) [$K = 0.5$, 1.0 and 2.0 mass doublings per hour (md h^{-1})]. Culturing in LB provided for a fast growth rate ($K = 2.0 \text{ md h}^{-1}$), culturing in adapted competence media provided for the medium and slow growth rates ($K = 1.0$ and 0.5 respectively) (see *Experimental procedures*: strains, media and growth conditions). Cells were sampled at mid-exponential phase and production of GFP was determined by flow cytometry for *gfp* fusions to the promoters of *ftsZ*, *ftsA*, *zapA*, *ftsW*, *noc*, *ezrA*, *minCD*, *dnaA* and *rrnB*. Promoter activities were normalized at a growth rate of 0.5 md h^{-1} . The activity of the *rrnB* promoter clearly increased with growth rate, as reported earlier (Krasny and Gourse, 2004) (Fig. 2A). The activity of promoters for genes that are directly involved in cell division (*ftsZ*, *ftsA*, *zapA*, *ftsW*) was found to be almost equal at all growth rates. The activity of the promoter for *minC* (which is encoded in an operon with *mreBCD* and *minD* (Lee and Price, 1993), slightly increased with growth rate, as did the activity of the *dnaA* promoter. However, this increase in promoter activity was never more than the twice the activity measured at the low growth rate of 0.5 md h^{-1} .

Stable integration of the promoter-*gfp* constructs at *amyE* allows the study of strains over long time intervals in the absence of antibiotics, but includes the risk that the influence of nearby genetic elements on the promoter activity is missed. To verify the results from the *amyE* integration strains, a subset of promoter-*gfp* fusions was created at the original locus. In addition, we extended our native locus promoter library with promoters of genes encoding the regulatory CDP DivIVA and the late CDP FtsL. These fusions were created using the BaSysBioII

Table 1. Relevant plasmids and bacterial strains used in this study.

Strain/plasmid	Relevant genotype ^a	Reference
<i>B. subtilis</i>		
168	Trp ⁻ , 1A700 variant	Veening <i>et al.</i> (2006)
JWV133	168, <i>amyE::P_{ezrA}-gfp</i> , <i>cm</i>	Gamba <i>et al.</i> (2009)
A::PftsZ-gfp	168, <i>amyE::P_{ftsZ}-gfp</i> , <i>cm</i>	This study
A::PftsA-gfp	168, <i>amyE::P_{ftsA}-gfp</i> , <i>cm</i>	This study
A::PzapA-gfp	168, <i>amyE::P_{zapA}-gfp</i> , <i>cm</i>	This study
A::PftsW-gfp	168, <i>amyE::P_{ftsW}-gfp</i> , <i>cm</i>	This study
A::PnoC-gfp	168, <i>amyE::P_{noc}-gfp</i> , <i>cm</i>	This study
A::PminC-gfp	168, <i>amyE::P_{minC}-gfp</i> , <i>cm</i>	This study
A::PdnaA-gfp	168, <i>amyE::P_{dnaA}-gfp</i> , <i>cm</i>	This study
A::PrnB16s-gfp	168, <i>amyE::P_{rmB16s}-gfp</i> , <i>cm</i>	This study
PftsA:PftsA-gfp	168, <i>PftsA::P_{ftsA}-gfp</i> , <i>spec</i>	This study
PdivIVA:PdivIVA-gfp	168, <i>PdivIVA::P_{divIVA}-gfp</i> , <i>spec</i>	This study
PftsL:PftsL-gfp	168, <i>PftsL::P_{ftsL}-gfp</i> , <i>spec</i>	This study
PftsW:PftsW-gfp	168, <i>PftsW::P_{ftsW}-gfp</i> , <i>spec</i>	This study
PrnB16s:PrnB-gfp	168, <i>PrnB16s::P_{rmB16s}-gfp</i> , <i>spec</i>	This study
<i>Escherichia coli</i>		
MC1000	F ⁻ , <i>araD139</i> (<i>ara ABC-leu</i>)7679, <i>galU</i> , <i>galK</i> , <i>lacX74</i> , <i>rspL</i> , <i>thi</i> ,	
BL21	F ⁻ , <i>dcm</i> , <i>ompT</i> , <i>hsdS</i> (<i>r_B⁻ m_B⁻</i>), <i>gal</i> , [<i>malB</i>] _{K-12} (λ^S)	Stratagene
TG1		Botella <i>et al.</i> (2010)
Plasmids		
pJWV017	<i>spec</i> , <i>amyE</i> , <i>gfp+</i> (F64L, S65T, F99S, M153T, V163A), <i>cat</i>	Veening <i>et al.</i> (2009)
pGFP-ftsZ	<i>spec</i> , <i>amyE</i> , <i>P_{ftsZ}-gfp</i> , <i>cat</i>	This study
pGFP-ftsA	<i>spec</i> , <i>amyE</i> , <i>P_{ftsA}-gfp</i> , <i>cat</i>	This study
pGFP-zapA	<i>spec</i> , <i>amyE</i> , <i>P_{zapA}-gfp</i> , <i>cat</i>	This study
pGFP-ftsW	<i>spec</i> , <i>amyE</i> , <i>P_{ftsW}-gfp</i> , <i>cat</i>	This study
pGFP-noc	<i>spec</i> , <i>amyE</i> , <i>P_{noc}-gfp</i> , <i>cat</i>	This study
pGFP-minC	<i>spec</i> , <i>amyE</i> , <i>P_{minC}-gfp</i> , <i>cat</i>	This study
pGFP-dnaA	<i>spec</i> , <i>amyE</i> , <i>P_{dnaA}-gfp</i> , <i>cat</i>	This study
pGFP-rnB	<i>spec</i> , <i>amyE</i> , <i>P_{rmB}-gfp</i>	Veening <i>et al.</i> (2009)
pBaSysBioII	<i>spec</i> , <i>LIC</i> transcriptional fusion vector	Botella <i>et al.</i> (2010)
GFP-ftsA	<i>spec</i> , <i>PftsA</i> , <i>P_{ftsA}-gfp</i>	This study
GFP-divIVA	<i>spec</i> , <i>PdivIVA</i> , <i>P_{divIVA}-gfp</i>	This study
GFP-ftsW	<i>spec</i> , <i>PftsW</i> , <i>P_{ftsW}-gfp</i>	This study
GFP-ftsL	<i>spec</i> , <i>PftsL</i> , <i>P_{ftsL}-gfp</i>	This study
GFP-rnB16s	<i>spec</i> , <i>PrnB16s</i> , <i>P_{rmB16s}-gfp</i>	This study

a. *cm*, chloramphenicol resistance marker; *tet*, tetracycline resistance marker; *amp*, ampicillin resistance marker; *km*, kanamycin resistance marker; *spec*, spectinomycin resistance marker; IPTG, isopropyl thio- β -D-galactoside.

vector that integrates as a copy next to the original locus in the *B. subtilis* chromosome by a single-cross-over event (Botella *et al.*, 2010). The resulting strains were grown identically to the *amyE* integration strains and the activity of the promoters for *ftsA*, *ftsW*, *ftsL*, *divIVA* and *rmB* were determined by flow cytometry. This selected group is representative for the cell division machinery since it is comprised of genes that encode early-, mid- and late-stage CDPs. Again, activity from the *rmB* promoter increased with growth rate, whereas the activity of the promoters for cell division genes hardly varied (Fig. 2B). This result confirms that the measurements from the *amyE* locus provide a valid indication of the activities of promoters for cell division genes.

The forward scatter value, as measured during flow cytometry, provides an indication of the size of the cells in the population. As expected, cell size increased with higher growth rates (not shown) (Weart *et al.*, 2007). It is known that the diameter of *B. subtilis* cells remains

constant at different growth rates (Sharpe *et al.*, 1998). Our data on the transcriptional activity of promoters for cell division genes suggests that *B. subtilis* needs a stable amount of CDPs at all growth rates. Indeed, equal amounts of FtsZ have been reported in both *B. subtilis* and *E. coli* cultured at different growth rates (Weart and Levin, 2003). The transcription of genes encoding proteins that are directly involved in division does not have to increase with growth rate, because the diameter of the cell, across which division takes place, remains constant while cell length increases. In contrast, regulatory proteins for cell division can be required in larger numbers when a cell is growing faster because cells are longer. To restrict the position of Z-ring formation at mid-cell, more MinC and MinD may be necessary, since the area in which Z-ring formation needs to be blocked is larger. In summary, the data from the promoter-*gfp* fusions indicate that the transcription of cell division genes in a bacterial population is constant over different growth rates. Transcription of the

Fig. 2. A. Normalized expression of *gfp* transcriptional fusions under the control of regulatory regions of various cell division genes and of *rrnB16s* (+) at different growth rates (slow ≈ 0.5 md h^{-1} , medium ≈ 1.0 md h^{-1} , fast ≈ 2.0 md h^{-1}). The reports come from transcriptional fusions to *gfp* integrated at the *amyE* locus. Total GFP fluorescence per cell was taken as a measure for transcription. By flow cytometry, 50,000 cells were measured; resulting in a normal distribution of which the peak-values were taken to calculate transcription as described in the *Experimental procedures*. The transcription values for the slow growth rate are set to 1.

B. Normalized expression of *gfp* transcriptional fusions under the control of regulatory regions of various cell division genes and of *rrnB16s* (+) at different growth rates (slow ≈ 0.5 md h^{-1} , medium ≈ 1.0 md h^{-1} , fast ≈ 2.0 md h^{-1}). The reports come from transcriptional fusions to *gfp* integrated as a copy next to the original gene. Data were collected as in (A).

mreB-mreC-mreD-minC-minD operon, which contains the genes coding for the MinC/D division site selection system, appears slightly upregulated at higher growth rates. This was also observed for *dnaA*.

Transcription of cell division genes monitored by qrtPCR

To verify the results from the promoter fusion experiments the transcription of several cell division genes was monitored by an independent method: qrtPCR. *Bacillus subtilis* 168 1A700 was grown at three different growth rates, exactly as described for the transcriptional fusion experiments, and at mid-exponential growth phase, total RNA was isolated. The RNA was used to generate complementary DNA (cDNA) to serve as a template for the qrtPCR. Equal amounts of cDNA were used as input material for

the qrtPCR. The ratios of cell division gene transcripts (*ftsZ*, *ftsA*, *zapA*, *ftsW*, *minC*, *dnaA*, *divIVA*, *ezrA* and *noc*) relative to their transcript level at the slowest growth rate were calculated, as well as the transcript level compared with a housekeeping gene (*veg*) at the different growth rates. The housekeeping gene *veg* was selected as this gene is constitutively transcribed during vegetative growth (Krasny and Gourse, 2004). As seen with the previous analysis, the amount of *rrnB* transcripts increases strongly with an increase in growth rate (Fig. 3A). Again the expression level of *minC* was found to slightly increase, but not as strongly as *rrnB16s* (Fig. 3A). The other tested cell division genes displayed hardly any variation with growth rate (Fig. 3A), similar to the results obtained with the transcriptional fusions. Comparing the amount of transcripts relative to those of the housekeeper gene *veg* that is strongly and constitutively transcribed in cells growing in a variety of media (Fukushima *et al.*, 2003; Nicolas *et al.*, 2012), *ftsA* and *ftsZ* were found to be almost equal and most abundant (Fig. 3B). Indeed, *FtsZ* and *FtsA*

Fig. 3. Amounts of mRNA transcripts as determined by qrtPCR of various cell division genes and *rrnB16s* (+). A. Transcript levels were normalized to the level of transcription of the same gene at the lowest growth rate. B. Transcript levels were normalized to the level of the housekeeping gene *veg* at different growth rates (slow ≈ 0.5 md h^{-1} , medium ≈ 1.0 md h^{-1} , fast ≈ 2.0 md h^{-1}).

Fig. 4. Time-lapse fluorescence microscopy to assess CDP gene expression as a function of the cell cycle.

A. Stills of a growing microcolony of *B. subtilis* cells harbouring the $P_{ftsZ-gfp}$ transcriptional fusion. GFP fluorescence remains constant during vegetative growth, but increases substantially upon entry into sporulation. Via computational analysis, data from individual cells in the growing microcolony such as fluorescence intensity and cell length and birth times (as a measure for cell-cycle progression) can be extracted.

B. A graphical representation of the time-lapse fluorescence microscopy images in the upper panel. The average GFP fluorescence of all the cells in the microcolony is displayed against time. Upon entry into sporulation, an increase in *ftsZ* transcription is clearly discernible.

protein copy numbers are relatively high in *B. subtilis*, estimated at 5000 and 1000 respectively (Feucht *et al.*, 2001). Also, relatively large amounts of transcripts were found for *minC*. As MinC binds to FtsZ in a 1:1 ratio (Okuno *et al.*, 2009) and MinC has to cover both poles of the cell, a similar number of MinC and FtsZ transcripts makes sense. Fewer transcripts were found for *dnaA*, *divIVA*, *ezrA* and *noc* and very little *zapA* and *ftsW* transcripts were present. It appears likely that either the proteins that are encoded by these genes are only required in small numbers or the mRNAs are very stable and/or efficiently translated.

Cell cycle-dependent increase in promoter activity ensures constant CDP concentrations

The results above show that transcription of the genes coding for CDPs is not significantly affected by the growth rate (Figs 1–3). However, these experiments do not exclude the possibility that there is cell cycle-dependent transcription. For example, using synchronized cultures, it

was suggested that transcription of *ftsZ* of *E. coli* oscillates during the cell cycle by two proximal promoters (Garrido *et al.*, 1993) and it is well documented that *C. crescentus ftsZ* is transcribed in a cell cycle-dependent manner (Sackett *et al.*, 1998; Quardokus *et al.*, 2001). To examine whether transcription of *ftsZ* shows cell cycle-dependent oscillations, we turned to automated live cell time-lapse fluorescence microscopy. With this technique, cells are grown directly under a microscope and phase contrast and GFP fluorescence images are taken at intervals and movies can be generated and analysed (de Jong *et al.*, 2011). As shown in Fig. 4A and Movie S1, a few cells grow out and divide, and within 12 h form small monolayered microcolonies consisting of a couple of hundred cells. In the case of the $P_{ftsZ-gfp}$ reporter strain, a clear increase in average population fluorescence can be observed after approximately 600 min of growth, coinciding with the onset of sporulation (Fig. 4B). As P_{ftsZ} contains a second, sporulation-dependent, promoter (Gonzy-Treboul *et al.*, 1992), this further highlights the strength of this technique.

To assess whether transcription of *ftsZ* shows any cell cycle-dependent expression pattern, we first had to examine whether the *B. subtilis* cell-cycle progresses linearly or exponentially in time relative to the cell length. To do so, we collected more than 900 data points of exponentially growing *B. subtilis* cells and plotted cell length as a function of the cell-cycle progression in time (see *Experimental procedures*). Interestingly, the data points are much a better fit to an exponential curve than to a line (Fig. S1; $R^2 = 0.997$ versus 0.982 respectively), indicating that, as cells progress in the cell cycle, they more rapidly increase in cell length. Interestingly, this also holds true for *E. coli* (Wang *et al.*, 2010) suggesting that rod-shaped cells that can also elongate along the periphery of the cell have an exponential mode of cell length growth as was shown previously using more invasive techniques that require cell fixation such as transmission electron microscopy (Hariharan *et al.*, 1982; Burdett *et al.*, 1986; Kirkwood and Burdett, 1988; Koch, 1993). These results indicate that cell-cycle progression in *B. subtilis* can best be plotted as a function of cell length (Fig. S1).

Having established a robust way to categorize cells into their correct cell-cycle progression state (Fig. S1 and *Experimental procedures*), we collected data from three independent movies of the *PftsZ-gfp* strain. Only exponentially growing cells were taken into account (generally cells between approximately 250 and 500 min after the start of the experiment). Plotting all data points as cell length versus cell-cycle progression (as a function of cell length) showed a straight line, both for the *B. subtilis* *PftsZ-gfp* data set and for a large *E. coli* data set for an inducible, non-cell-cycle-regulated promoter (Stewart *et al.*, 2005), again verifying that our mathematical routine to analyse single cells from movies is robust (Fig. S2A and B). Plotting the average GFP fluorescence (mean of the fluorescence level per pixel) from the *ftsZ* promoter as a function of cell-cycle progression showed a straight line (Fig. 5A). This indicates that the concentration of GFP protein, and by inference FtsZ protein, does not fluctuate during the cell cycle. Doing the same analysis on a movie of a late cell division reporter, *PftsW-gfp*, showed the same result indicating the absence of a cell cycle-dependent manner of transcriptional regulation (Fig. 5C). We have shown previously that the *sda* gene is cell cycle regulated via transcriptional activation of DnaA (Veening *et al.*, 2009). Analysing a movie of a strain carrying a *Psda-GFP* fusion showed that the concentration of GFP fluctuates as a function of the cell cycle, as expected (Fig. 5E).

While average GFP levels are a good indication for the concentration of the protein, it does not provide direct information for the promoter activity (Rosenfeld *et al.*, 2005; 2006; Zaslaver *et al.*, 2009). Therefore, we plotted the normalized promoter activity of *PftsZ-gfp* as a func-

tion of cell-cycle progression. As shown in Fig. 5C, *ftsZ* promoter activity gradually increases with increasing cell-cycle progression. At the end of the cell cycle, *ftsZ* promoter activity is roughly twofold higher than at the beginning of the cell cycle, while this is not the case for the cell cycle-dependent *sda* promoter which is already high at the beginning of the cell cycle (Fig. 5F). The trend of higher promoter activity at the end of the cell cycle also holds true for the *ftsW* promoter but to a lesser extent than for *PftsZ* (Fig. 5D). This might be because less data points were available for this strain compared with the *PftsZ* strain.

To examine whether increased promoter activity is a general phenomenon for constitutive promoters, we analysed a large *E. coli* data set of cells harbouring a *yfp* fusion driven by the IPTG inducible P_{lacO1} promoter fully induced with 1 mM IPTG (Stewart *et al.*, 2005) using our image analysis algorithms. As shown in Fig. S2C, this *E. coli* promoter is solely controlled by inducer level, and the concentration of YFP is constant regardless of the cell-cycle state. Importantly, P_{lacO1} promoter activity is also approximately twofold higher at the end of the cell cycle than at cell birth (Fig. S2D).

Besides the presence of a greater number of RNA polymerases and ribosomes within a cell at the end of its cell cycle, a likely explanation for the enhanced promoter activity is the increase in chromosome copy number, as suggested previously (Elowitz *et al.*, 2002). After DNA-replication but before cell division, cells at the end of the cell cycle contain twice as many promoters as at the beginning of the cell cycle. Thus, in the absence of any cell-cycle control (i.e. in the case of constitutive promoters), promoter activity is twice as high at the end of the cell cycle compared with the beginning.

Concluding remarks

In this report, we show that the transcription of cell division genes in *B. subtilis* is not regulated by growth rate both on a population and on a single cell level, and not cell cycle regulated on the single cell level. Stable transcription of cell division genes independent of growth rate ensures constant translation and a fixed amount of CDPs per cell. The cell length of a rapidly growing *B. subtilis* cell can be twice that of a slower counterpart, but its diameter is fixed (Sharpe *et al.*, 1998; Weart and Levin, 2003), so the amount of CDPs required to form a single septum does not have to change when the cell increases its size.

By balancing its numbers of CDPs, *B. subtilis* demonstrates efficient division; it produces the critical amounts of proteins needed for this process, not more. Studies in which mild overexpression of an inhibitor or absence of a stabilizer of the divisome can be counteracted by mild overexpression or a gain of function mutation in another

Fig. 5. A, C and E. Average GFP fluorescence plotted against cell cycle progression (based on cell length) for the *PftsZ-gfp*, *PftsW-gfp* and *Psda-gfp* transcriptional fusions. B, D and E. Promoter activity (arbitrary units per minute) plotted against cell cycle progression (based on cell length) for the *PftsZ-gfp*, *PftsW-gfp* and *Psda-gfp* transcriptional fusions. For details, see Fig. S2 and *Experimental procedures*.

divisome component also point to a minimal amount of each CDP being produced (Dai and Lutkenhaus, 1992; Gueiros-Filho and Losick, 2002; Geissler *et al.*, 2003; Kawai and Ogasawara, 2006). Constant transcription,

which we confirmed with quantitative rtPCR could be followed by constant translation. The transcriptional fusion strains provide evidence for this as this technique measures both transcription and translation of GFP. The levels

of GFP remain fairly constant over various growth rates in the transcriptional fusion strains for genes encoding CDPs. Other studies also demonstrated equal levels of the main CDP, FtsZ, in *E. coli* and *B. subtilis* across a wide range of growth rates (Weart and Levin, 2003). Furthermore, on a population level, within the transcriptomes of *B. subtilis* cultured at a wide range of growth-conditions, the amounts of transcripts for cell division genes remain constant. Medium composition, temperature and stress hardly influence their quantities, highlighting the importance of a balanced transcription for these genes that encode for proteins with an essential function (Nicolas *et al.*, 2012).

Since the levels of mRNA and CDPs remain unchanged at various growth rates, other factors must dictate the pace of division. A richer medium allows for faster growth and rapid metabolism. Substrate and energy for building the septum may be more abundant and more readily available. In *B. subtilis* the metabolic sensor UgtP couples carbon availability to cell division by inhibiting the formation of the Z-ring until the cell reaches a critical mass (Weart *et al.*, 2007). Such a mechanism, combined with concentration gradients of division inhibitors such as Min and Noc, would indeed be sufficient to activate a pool of CDPs already present, to perform a round of division.

Interestingly, the transcription of genes encoding the cell division regulatory proteins MinCD is increasing slightly with growth rate, as measured by levels of GFP (Fig. 2A). Because of the increase in cell length, *B. subtilis* could need a higher amount of negative regulators to prevent the formation of a division site at the wrong place. Negative regulators are also required to prevent the dilution of the pool of FtsZ so that functional rings can be formed at every potential division site – Z-ring frequency is greatly decreased in a *min/noc* double knockout (Wu and Errington, 2004; Rodrigues and Harry, 2012). Also, MinC prevents formation of a second Z-ring right after division at the new cell pole (Gregory *et al.*, 2008). With new-born cells being significantly larger at a fast growth rate there may be a need for a higher concentration of MinC to counteract the formation of Z-rings immediately after division. In addition, the expression of *minCD* is regulated by the same promoter as that for *mreBCD*, genes that play a role in cell wall synthesis during elongation (Typas *et al.*, 2011). Upregulation of *mreBCD* could be essential for the increase in cell length at high growth rates. The molecular mechanism driving the potential growth rate-dependent transcription of the *mreBCD-minCD* operon remains elusive.

Our single cell experiments confirmed that *ftsZ* expression is upregulated during sporulation and shows that regulatory events can be identified with the methods we used. The finding that the transcription of cell division genes in *B. subtilis* is balanced both at different growth

rates at a population level and throughout the cell cycle on the single cell level, resulting in constant quantities of cell division protein, strongly suggests cell division is regulated by other factors that do fluctuate and that can better pace division like the metabolic state and substrate availability. Because of their stable quantities, CDPs can respond more rapidly and in a tightly organized fashion to these fluctuating factors, resulting in intricate interplay and specific activity that determines the cell-cycle progression.

Experimental procedures

Strains, media and growth conditions

Bacterial strains are listed in Table 1. *Bacillus subtilis* 168 1A700 cells were grown with various doubling times in adapted *Bacillus* competence medium (CM) for slow ($K = 0.5 \text{ md h}^{-1}$) and medium growth rates ($K = 1.0 \text{ md h}^{-1}$) and in Luria–Bertani broth (LB) for a fast growth rate ($K = 2.0 \text{ md h}^{-1}$). Adapted CM for slow growth is Spizizen's salt medium (SSM) containing glucose (0.1% w/v), tryptophan (0.2 mg ml⁻¹), MgSO₄ (3 mM) and FeNH₄ citrate ($5.5 \times 10^{-5}\%$ w/v) (Spizizen, 1958). Adapted CM for medium growth is SSM containing glucose (1.0% w/v), tryptophan (0.4 mg ml⁻¹), casamino acids (0.2% w/v), MgSO₄ (3 mM) and FeNH₄ citrate ($11.0 \times 10^{-5}\%$ w/v). When required, spectinomycin (50 µg ml⁻¹) and chloramphenicol (5 µg ml⁻¹) was added. During culture for analysis, no antibiotics were added to ensure normal ribosomal activity. Overnight cultures were diluted to an optical density of 0.02 at 600 nm (OD₆₀₀) in the adapted competence medium or to 0.05 in LB. The cells were incubated at 37°C with shaking and grown until mid-exponential growth phase (OD₆₀₀ ≈ 0.15–0.4) for sampling.

Construction of *amyE* integration plasmids

To construct plasmid pGFP_{ssrA}-ftsA, a PCR using primers ftsA-F + EcoRI and ftsA-R + NheI was performed, using chromosomal DNA of *B. subtilis* 168 as a template. The amplified fragment was subsequently cleaved with EcoRI and NheI and ligated into the corresponding sites of pGFP_{ssrA} (Veening *et al.*, 2009), resulting in plasmid pGFP_{ssrA}-ftsA. To construct plasmid pGFP_{ssrA}-ftsZ, carrying the *B. subtilis* ftsA promoter region and a strong RBS fused with *gfp+* (F64L, S65T, F99S, M153T, V163A (Scholz *et al.*, 2000; Zellmeier *et al.*, 2006), flanked by *amyE* regions, a PCR with the primers gfp-R + FseI and ter-F + FseI was performed, using plasmid pGFP_{ssrA}-ftsA as a template. Next the template was digested with DpnI and the amplified fragment was subsequently cleaved with FseI and self-ligated resulting in plasmid pGFP_{ssrA}-ftsZ.

To construct plasmid pGFP_{ssrA}-ftsZ, a PCR using primers ftsA_{stop}-F + NheI and ftsA_{stop}-R + NheI was performed, using chromosomal DNA of *B. subtilis* 168 as a template. The amplified fragment was subsequently cleaved with NheI and ligated into the corresponding site of pGFP_{ssrA}-ftsA, resulting in plasmid pGFP_{ssrA}-ftsZ. Note that the forward primer introduces a stop codon directly after the

ATG-start codon of *ftsA* and introduces a frameshift ensuring that there is only a single functional copy of *ftsA* present in the genome, when integrated at *amyE*. To construct plasmid pGFP-ftsZ, carrying the *B. subtilis* *ftsA* promoter region, a non-functional *ftsA* gene, and the intergenic region between *ftsA* and *ftsZ* (together here called P_{ftsZ}), fused to *gfp+*, flanked by *amyE* regions, a PCR with the primers *gfp*-R + FseI and *ter*-F + FseI was performed, using plasmid pGFP-ssrA-ftsZ as a template. Next the template was digested with DpnI and the amplified fragment was subsequently cleaved with FseI and self-ligated resulting in plasmid pGFP-ftsZ.

To construct plasmid pGFP-ssrA-zapA, a PCR using primers zapA-F + EcoRI and zapA-R + NheI was performed, using chromosomal DNA of *B. subtilis* 168 as a template. The amplified fragment was subsequently cleaved with EcoRI and NheI and ligated into the corresponding sites of pGFP-ssrA, resulting in plasmid pGFP-ssrA-zapA. To construct plasmid pGFP-zapA, carrying the *B. subtilis* *zapA* promoter region and a strong RBS fused with *gfp+*, flanked by *amyE* regions, a PCR with the primers *gfp*-R + FseI and *ter*-F + FseI was performed, using plasmid pGFP-ssrA-zapA as a template. Next the template was digested with DpnI and the amplified fragment was subsequently cleaved with FseI and self-ligated resulting in plasmid pGFP-zapA.

To construct plasmid pGFP-ftsW, carrying the *B. subtilis* *ftsW* promoter region and a strong RBS fused with *gfp+*, a PCR with the primers *ftsW*-F + EcoRI and *ftsW*-R + NheI was performed, using chromosomal DNA of *B. subtilis* 168 as a template. The amplified fragment was subsequently cleaved with EcoRI and NheI and ligated into the corresponding sites of pJWV017 (Veening *et al.*, 2009), resulting in plasmid pGFP-ftsW.

To construct plasmid pGFP-ssrA-noc, a PCR using primers noc-F + EcoRI and noc-R + NheI was performed, using chromosomal DNA of *B. subtilis* 168 as a template. The amplified fragment was subsequently cleaved with EcoRI and NheI and ligated into the corresponding sites of pGFP-ssrA, resulting in plasmid pGFP-ssrA-noc. To construct plasmid pGFP-noc, carrying the *B. subtilis* *noc* promoter region and a strong RBS fused with *gfp+*, flanked by *amyE* regions, a PCR with the primers *gfp*-R + FseI and *ter*-F + FseI was performed, using plasmid pGFP-ssrA-noc as a template. Next the template was digested with DpnI and the amplified fragment was subsequently cleaved with FseI and self-ligated resulting in plasmid pGFP-noc.

To construct plasmid pGFP-ssrA-minC, a PCR using primers minC-F + NotI and minC-R + NheI was performed, using chromosomal DNA of *B. subtilis* 168 as a template. The amplified fragment was subsequently cleaved with NotI and NheI and ligated into the corresponding sites of pGFP-ssrA, resulting in plasmid pGFP-ssrA-minC. To construct plasmid pGFP-minC, carrying the *B. subtilis* *minCD* promoter region and a strong RBS fused with *gfp+*, flanked by *amyE* regions, a PCR with the primers *gfp*-R + FseI and *ter*-F + FseI was performed, using plasmid pGFP-ssrA-minC as a template. Next the template was digested with DpnI and the amplified fragment was subsequently cleaved with FseI and self-ligated resulting in plasmid pGFP-minC.

To construct plasmid pGFP-ssrA-dnaA, a PCR using primers dnaA-F + EcoRI and dnaA-R + NheI was performed

was performed, using chromosomal DNA of *B. subtilis* 168 as a template. The amplified fragment was subsequently cleaved with EcoRI and NheI and ligated into the corresponding sites of pGFP-ssrA, resulting in plasmid pGFP-ssrA-dnaA. To construct plasmid pGFP-dnaA, carrying the *B. subtilis* *dnaA* promoter region and a strong RBS fused with *gfp+*, flanked by *amyE* regions, a PCR with the primers *gfp*-R + FseI and *ter*-F + FseI was performed, using plasmid pGFP-ssrA-dnaA as a template. Next the template was digested with DpnI and the amplified fragment was subsequently cleaved with FseI and self-ligated resulting in plasmid pGFP-dnaA.

All constructs were sequence verified.

Construction of *amyE* integrated *B. subtilis* strains

Bacillus subtilis strains A::P-gfp, A::PftsZ-gfp, A::PftsA-gfp, A::PzapA-gfp, A::PftsW-gfp, A::Pnoc-gfp, A::PminC-gfp, A::PdnaA-gfp and A::PrnB-gfp were obtained by a double-cross-over recombination event between the *amyE* regions located on the pJWV017, pGFP-ftsZ, pGFP-ftsA, pGFP-zapA, pGFP-ftsW, pGFP-noc, pGFP-minC, pGFP-dnaA and pGFP-rnB plasmids and the chromosomal *amyE* gene of strain 168 respectively (Veening *et al.*, 2009). Transformants were selected on LB agar plates containing chloramphenicol (Cm) after overnight incubation at 37°C. Correct integration into the *amyE* gene was tested and confirmed by lack of amylase activity upon growth on plates containing 1% starch.

Construction of strains with a transcriptional fusion reporter at the original locus

The pBaSysBioII transcriptional fusion vector was used to construct the plasmids for transcriptional fusions that integrate as copies at the original locus (Botella *et al.*, 2010). This plasmid has a SmaI restriction site, flanked by ligase independent cloning (LIC) sites, upstream of *gfp*. After restriction by SmaI, promoter regions of interest can be cloned into this site by LIC to control the transcription of *gfp*. The promoter region of interest targets the integration of the pBaSysBioII transcriptional fusion vector into the *B. subtilis* chromosome as a copy next to the original promoter region by a single-cross-over event. The promoter regions of *ftsA*, *divIVA*, *ftsW*, *ylbB* (*ftsL* operon) and *rrnB16s* were amplified by PCR, using chromosomal DNA from *B. subtilis* 168 as a template, with the appropriate primers listed in Table 2. By primer-design, LIC-sites were added at the ends of the fragments. Also, strong identical ribosome binding sites (RBS) were placed between the 3' ends of the promoter-regions and the 3' LIC site. For LIC, 0.2 pmol of each fragment was incubated with 2.5 mM dTTP and T4 DNA polymerase. The pBaSysBioII plasmid was linearized using SmaI, gel-purified, and treated with T4 DNA polymerase and 2.5 mM dATP. A mix of 5 ng prepared plasmid with 15 ng of prepared fragment was used to transform *E. coli* TG1, using standard techniques. Transformants were selected on LB plates containing spectinomycin (50 µg ml⁻¹). Subsequently, these plasmids were isolated from *E. coli* TG1 and correctness of the inserts was verified by sequencing. The correct plasmids were introduced to a non-chaining *B. subtilis* 168 strain (1A700) (Veening *et al.*, 2006), using standard procedures

Table 2. Oligonucleotides.^a

Primer	Sequence (5' to 3')	Restriction or LIC site
ftsA-F + EcoRI	GCGCGAATTCAGACCACTGGACGTACAACAGC	EcoRI
ftsA-R + NheI	GCGCGCTAGCCATAGTAGTTCTCTTACACATTTTCGATCATTTCTATTCTATTATTG	NheI
gfp-R + FseI	GCTCAATGGCCGGCCTTATTATTTGTAGAGCTCATCCATGCCATG	FseI
ter-F + FseI	CGATAGGCCCGGCCAATAAAGATCCTAGGACGCCGCCAAG	FseI
ftsA_stop-F + NheI	GCGCGCTAGCATGTAACAACAATGAACTTTACG	NheI
ftsA_stop-R + NheI	GCGCGCTAGCCATAGTAGTTCTCTTATCTGCGAATGACTATCTATCTATTCC	NheI
zapA-F + EcoRI	GCGCGAATTCAGCAGGCTGTACGGAATGG	EcoRI
zapA-R + NheI	GCGCGCTAGCCATAGTAGTTCTCTTACCGCGAGAATCCTAGTTCTTATC	NheI
ftsW-F + EcoRI	CGCAATTAAGGTCGGAATTCCTGC	EcoRI
ftsW-R + NheI	GCGCGCTAGCCATAGTAGTTCTCTCTTAGCTTCTAACTTCCATTATCTATGG	NheI
noc-F + EcoRI	GCGCGAATTCGGTGAACACGATTTGTG	EcoRI
noc-R + NheI	GCGCGCTAGCCATAGTAGTTCTCTCTTATTAATAAAGTCCATTTTATAATTG	NheI
minC-F + NotI	GCATGCGGCCGCTGTCAAGCATCCCGGGAATC	NotI
minC-R + NheI	GCGCGCTAGCCATAGTAGTTCTCTCTTATTTCTAAAGCAAAAATACC	NheI
dnaA-F + EcoRI	GCGCGAATTCGATCCACAGAGGTTATCGACAAC	EcoRI
dnaA-R + NheI	GCGCGCTAGCCATAGTAGTTCTCTCTTACCTTTTCTTAGAAAATGGCGTCTTTCTC	NheI
ftsA-F + LIC-F	CCGCGGGCTTTCCAGCCAGACCACTGGACGTACAAC	LIC-F
ftsA-R + LIC-R	GTTCTCTCTTTCCACCCACATTTTCGATCATTTCTATTCTATTATTG	LIC-R
divIVA-F + LIC-F	CCGCGGGCTTTCCAGCCGCTCGGTTATGCTGACAAGT	LIC-F
divIVA-R + LIC-R	GTTCTCTCTTTCCACCATTTTACATTTTCAGTACGGTTATCTAG	LIC-R
ftsW-F + LIC-F	CCGCGGGCTTTCCAGCCGCAATTAAGGTCGGAATTC	LIC-F
ftsW-R + LIC-R	GTTCTCTCTTTCCACCACTTCTAACTTCCATTATCTATGG	LIC-R
yliB-F + LIC-F	CCGCGGGCTTTCCAGCCATTTCTTTTCAAATCAACATCAAG	LIC-F
yliB-R + LIC-R	GTTCTCTCTTTCCACCACTTCACTATTCACCACTTC	LIC-R
rrnB16s-F + LIC-F	CCGCGGGCTTTCCAGCACATCCCTACGCCA	LIC-F
rrnB16s-R + LIC-R	GTTCTCTCTTTCCACCGCTTTACTAATATAACATTCAGC	LIC-R
Upstream P _{ftsA} -F	TAAAGTGCTTGCCAAAGAT	
Upstream P _{divIVA} -F	CTGCTGTTACGGGACA	
Upstream P _{ftsW} -F	CGGATCTGTATTCGCATT	
Upstream P _{yliB} -F	GGTGATGGGCTTCAAAA	
Upstream P _{rrnB16s} -F	GTTCCACGCACTGC	
<i>gfpmut3</i> -R	TTCTCCTTTACGCATAGTAG	
<i>ftsA</i> 250 bp fragment-F	AACAACAATGAACTTTACGTC	
ftsA-R	GATATATTTCCATTAACGCCG	
ftsZ-F	TTGGAGTTGAAACAAACATA	
ftsZ-R	TTCTTCAGCGGCTTTTTT	
zapA-F	TCTGACGGCAAAAAAACA	
zapA-R	TCCTTTTCTTTAAGCTGACG	
minC-F	AAGACCAAAAAGCAGCAATA	
minC-R	ATGAACAAATCTTTCTTTGA	
ftsW-F	TTAAAAAATGCTAAATCTTATGATTA	
ftsW-R	AAGCAGTATCCCCTTCTG	
divIVA-F	CCATTAACGCCAAATGATATTC	
divIVA-R	ATTGCGTTTACGTCTTCA	
rrnB16s-F	AAGTCGAGCGGACAGGTG	
rrnB16s-R	AGTGTGGCCGATCAC	
<i>noc</i> -F	CCGCGCAATTATTAAGGATTTT	
<i>noc</i> -R	GGTGATTTTCTTCTCCATAATC	
<i>veg</i> -F	GCGAAGACGTTGTCCGATATT	
<i>veg</i> -R	CTCTGAGCTTGCGGCGTCATC	

a. Relevant restriction or LIC sites are underlined.

(Anagnostopoulos and Spizizen, 1961) with selection for spectinomycin (50 µg ml⁻¹). The integration of the vectors at the desired locus (as a copy next to the original) was verified by PCR using primers designed for a location upstream of the promoter region and for *gfp* on the vector (Table 2).

Flow cytometry

The various promoter-reporter strains were cultured at the conditions as described above; for each strain, cells were

obtained from cultures with slow ($K = 0.5$ md h⁻¹), medium ($K = 1.0$ md h⁻¹) and fast growth rate ($K = 2.0$ md h⁻¹) at mid-exponential growth phase (OD₆₀₀ ≈ 0.15–0.4). The cells were washed and diluted in cold, sterile PBS and measured immediately. Flow cytometry was performed with a FACSCANTO flow cytometer (BD Biosciences). Parameters that were measured are forward scatter (FSC), side scatter (SSC), GFP fluorescence (FL-1) and the ratio FL-1/FSC. 50,000 cells were measured. The flow cytometry data were analysed with the WinMDI 2.9 software. From the resulting normal

distribution (of 50,000 measurements) the peak median values for total GFP per cell (FI-1) were taken as a measure for transcription. These values were normalized to the average value for the slow growing culture ($K = 0.5 \text{ md h}^{-1}$) set to one.

Quantitative real-time reverse-transcription PCR

RNA was isolated from *B. subtilis* 168 1A700wt. This strain was cultured at the conditions as described above; cells were obtained from cultures with slow ($K = 0.5 \text{ md h}^{-1}$), medium ($K = 1.0 \text{ md h}^{-1}$) and fast growth rate ($K = 2.0 \text{ md h}^{-1}$) at mid-exponential growth phase ($\text{OD}_{600} \approx 0.15\text{--}0.4$). The cells were disrupted with glass beads ($\varnothing 0.25 \text{ mm}$) using the FP120 FastPrep shaker (ThermoSavant). Total RNA was isolated using TRIzol reagent (Chomczynski and Sacchi, 1987), and purified using the TURBO DNA-free KIT (Ambion). cDNA was prepared from 500 ng of this DNA-free RNA with the iSCRIPT cDNA synthesis kit (Bio-Rad). The cDNA was used as template for qrtPCR. For all qrtPCR reactions, equal amounts of cDNA were used. Primers were designed to have identical T_m s of 57°C and to multiply 250 bp fragments from *ftsA*, *ftsZ*, *minC*, *ftsW*, *divIVA*, *ezrA*, *noc*, *dnaA*, *veg* and *rrnB16s*. qrtPCRs were performed in triplo for each gene and every growth condition with the SensiMix SYBR Hi-ROX kit (Bioline) in a Mini Opticon real-time PCR system (Bio-Rad). The quantities of mRNA from specific cell division genes were compared with the housekeeper *veg* per growth condition. These ratios were then compared between the different growth rates.

Time-lapse fluorescence microscopy

Cells were grown at 30°C in Spizizen minimal medium and inoculated onto a thin semi-solid agarose matrix based on a chemically defined medium and attached to a microscope slide as described (Veening *et al.*, 2008). Microscope slides were incubated on a temperature controlled automated Deltavision RT microscope (Applied Precision). Images were obtained up to 24 h with a CoolSNAP HQ2 (Princeton Instruments) at $63\times$ or $100\times$ magnification. Fluorescent and phase-contrast images were recorded at specified intervals, basically as described (de Jong *et al.*, 2011). To prevent phototoxicity, the excitation light (480–500 nm for GFP) was limited to 32% of the output of a 100 W Hg-vapour lamp by neutral density filters with an exposure time of 0.8 s. Emission wavelengths were 509–547 nm (GFP) (filters from Chroma). Microscopy images were prepared for publication using ImageJ (<http://rsb.info.nih.gov/ij/>) and CorelDRAW X3 (Corel Corporation).

Microscopy data analysis

Cells were automatically segmented and measured using custom BHV software (Stewart *et al.*, 2005; de Jong *et al.*, 2011) and information regarding cell length, width, fluorescence intensity, orientation, geographical location within the colony, and the complete lineage history of each cell was recorded. The software was supplemented for this paper with

new Python routines to calculate promoter activity and to define cell-cycle progression. The Python code is available upon request.

Promoter activity

To calculate the specific promoter activity for each reporter at each cell-cycle progression category, we used an approach similar as pioneered by the Alon and Elowitz laboratories (Rosenfeld *et al.*, 2005; 2006). In brief, to measure the average GFP production rate between movie frames (promoter activity), we took the difference in total GFP level per cell between consecutive time points and divided that by their separating time interval (8 min). This approach assumes that the half-life or photobleaching of GFP is insignificant within the timescale of a cell's life and that GFP is only diluted by cell growth. Western blot analysis has shown a half-life of 1 h for GFP in *B. subtilis* while the *in vivo* half-life or photobleaching properties of GFP under our imaging conditions were neglectable (Veening *et al.*, 2009). Thus, the abovementioned approach is a valid one.

Cell-cycle progression

To determine the cell-cycle state (or cell-cycle progression) of each data point within a movie, only data from cells growing exponentially were taken into account for the analysis (typically data of cells between 250 and 550 min after start of the experiment, after cells have adapted and before cells enter sporulation). Since the birth and division of each cell in the data set is known, a cell's cell-cycle progression can be defined and binned in categories relative to the total time it took this cell to complete one full cell cycle (from birth to division, Fig. S1). For instance, the data of all newly born cells within the first 10% of their total cell-cycle progression time (the relative time it takes cells to divide again) are plotted in the 10% category in Fig. S1, and all cells between 10% and 20% of their cell-cycle progression are plotted as the 20% category. Since *B. subtilis* shows an exponential increase in cell length progression during its cell cycle, we plotted cell-cycle progression as a function of cell length (Fig. S1). For every GFP-reporter movie, the binned cell-cycle progression data set can be plotted against mean fluorescence (which reflects CDP concentration), the total fluorescence (which reflects total CDP levels) or the promoter activity (reflecting transcription of CDPs).

Acknowledgements

We would like to thank Emma Denham for providing us with the pBaSysBioII vector, the accessory *E. coli* TG-1 cloning strain and protocols.

Work in the Errington lab was funded by Grant BB/G015902/1 from the UK Biotechnology and Biological Sciences Research Council. Work in the Veening lab is funded by a Veni-fellowship from the Netherlands Organisation for Scientific Research (NWO-ALW). Work in the Scheffers lab is funded by a Vidi career award from NWO-ALW.

References

- Adams, D.W., and Errington, J. (2009) Bacterial cell division: assembly, maintenance and disassembly of the Z ring. *Nat Rev Microbiol* **7**: 642–653.
- Anagnostopoulos, C., and Spizizen, J. (1961) Requirements for transformation in *Bacillus subtilis*. *J Bacteriol* **81**: 741–746.
- Botella, E., Fogg, M., Jules, M., Piersma, S., Doherty, G., Hansen, A., et al. (2010) pBaSysBioll: an integrative plasmid generating gfp transcriptional fusions for high-throughput analysis of gene expression in *Bacillus subtilis*. *Microbiology* **156**: 1600–1608.
- Burdett, I.D., Kirkwood, T.B., and Whalley, J.B. (1986) Growth kinetics of individual *Bacillus subtilis* cells and correlation with nucleoid extension. *J Bacteriol* **167**: 219–230.
- Camberg, J.L., Hoskins, J.R., and Wickner, S. (2009) ClpXP protease degrades the cytoskeletal protein, FtsZ, and modulates FtsZ polymer dynamics. *Proc Natl Acad Sci USA* **106**: 10614–10619.
- Chien, A.C., Zareh, S.K., Wang, Y.M., and Levin, P.A. (2012) Changes in the oligomerization potential of the division inhibitor UgtP co-ordinate *Bacillus subtilis* cell size with nutrient availability. *Mol Microbiol* **86**: 594–610.
- Chomczynski, P., and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**: 156–159.
- Dai, K., and Lutkenhaus, J. (1992) The proper ratio of FtsZ to FtsA is required for cell division to occur in *Escherichia coli*. *J Bacteriol* **174**: 6145–6151.
- Dajkovic, A., Lan, G., Sun, S.X., Wirtz, D., and Lutkenhaus, J. (2008) MinC spatially controls bacterial cytokinesis by antagonizing the scaffolding function of FtsZ. *Curr Biol* **18**: 235–244.
- Elowitz, M.B., Levine, A.J., Siggia, E.D., and Swain, P.S. (2002) Stochastic gene expression in a single cell. *Science* **297**: 1183–1186.
- Errington, J., Daniel, R.A., and Scheffers, D.J. (2003) Cytokinesis in bacteria. *Microbiol Mol Biol Rev* **67**: 52–65.
- Feucht, A., Lucet, I., Yudkin, M.D., and Errington, J. (2001) Cytological and biochemical characterization of the FtsA cell division protein of *Bacillus subtilis*. *Mol Microbiol* **40**: 115–125.
- Fukushima, T., Ishikawa, S., Yamamoto, H., Ogasawara, N., and Sekiguchi, J. (2003) Transcriptional, functional and cytochemical analyses of the *veg* gene in *Bacillus subtilis*. *J Biochem (Tokyo)* **133**: 475–483.
- Gamba, P., Veening, J.W., Saunders, N.J., Hamoen, L.W., and Daniel, R.A. (2009) Two-step assembly dynamics of the *Bacillus subtilis* divisome. *J Bacteriol* **191**: 4186–4194.
- Garrido, T., Sanchez, M., Palacios, P., Aldea, M., and Vicente, M. (1993) Transcription of *ftsZ* oscillates during the cell cycle of *Escherichia coli*. *EMBO J* **12**: 3957–3965.
- Geissler, B., Elraheb, D., and Margolin, W. (2003) A gain-of-function mutation in *ftsA* bypasses the requirement for the essential cell division gene *zipA* in *Escherichia coli*. *Proc Natl Acad Sci USA* **100**: 4197–4202.
- Gonzy-Treboul, G., Karmazyn-Campelli, C., and Stragier, P. (1992) Developmental regulation of transcription of the *Bacillus subtilis* *ftsAZ* operon. *J Mol Biol* **224**: 967–979.
- Gregory, J.A., Becker, E.C., and Pogliano, K. (2008) *Bacillus subtilis* MinC destabilizes FtsZ-rings at new cell poles and contributes to the timing of cell division. *Genes Dev* **22**: 3475–3488.
- Gueiros-Filho, F.J., and Losick, R. (2002) A widely conserved bacterial cell division protein that promotes assembly of the tubulin-like protein FtsZ. *Genes Dev* **16**: 2544–2556.
- Hariharan, I.K., Czolij, R., and Wake, R.G. (1982) Conformation and segregation of nucleoids accompanying cell length extension after completion of a single round of DNA replication in germinated and outgrowing *Bacillus subtilis* spores. *J Bacteriol* **150**: 861–869.
- de Jong, I.G., Beilharz, K., Kuipers, O., and Veening, J.W. (2011) Live cell imaging of *Bacillus subtilis* and *Streptococcus pneumoniae* using automated time-lapse microscopy. *J Vis Exp* **53**: pii: 3145. doi:10.3791/3145.
- Kawai, Y., and Ogasawara, N. (2006) *Bacillus subtilis* EzrA and FtsL synergistically regulate FtsZ ring dynamics during cell division. *Microbiology* **152**: 1129–1141.
- Kelly, A.J., Sackett, M.J., Din, N., Quardokus, E., and Brun, Y.V. (1998) Cell cycle-dependent transcriptional and proteolytic regulation of FtsZ in *Caulobacter*. *Genes Dev* **12**: 880–893.
- Kirkwood, T.B., and Burdett, I.D. (1988) Estimating the growth pattern of micro-organisms in distinct stages of the cell cycle. *J Theor Biol* **130**: 255–273.
- Koch, A.L. (1993) The growth kinetics of *Bacillus subtilis*. *Antonie Van Leeuwenhoek* **63**: 45–53.
- Krasny, L., and Gourse, R.L. (2004) An alternative strategy for bacterial ribosome synthesis: *Bacillus subtilis* rRNA transcription regulation. *EMBO J* **23**: 4473–4483.
- Lee, S., and Price, C.W. (1993) The minCD locus of *Bacillus subtilis* lacks the minE determinant that provides topological specificity to cell division. *Mol Microbiol* **7**: 601–610.
- Moriya, S., Rashid, R.A., Rodrigues, C.D., and Harry, E.J. (2010) Influence of the nucleoid and the early stages of DNA replication on positioning the division site in *Bacillus subtilis*. *Mol Microbiol* **76**: 634–647.
- Nicolas, P., Mader, U., Dervyn, E., Rochat, T., Leduc, A., Pigeonneau, N., et al. (2012) Condition-dependent transcriptome reveals high-level regulatory architecture in *Bacillus subtilis*. *Science* **335**: 1103–1106.
- Okuno, T., Ogoh, M., Tanina, H., Funasaki, N., and Kogure, K. (2009) Direct monitoring of interaction between *Escherichia coli* proteins, MinC and monomeric FtsZ, in solution. *Biol Pharm Bull* **32**: 1473–1475.
- Quardokus, E., Din, N., and Brun, Y.V. (1996) Cell cycle regulation and cell type-specific localization of the FtsZ division initiation protein in *Caulobacter*. *Proc Natl Acad Sci USA* **93**: 6314–6319.
- Quardokus, E.M., Din, N., and Brun, Y.V. (2001) Cell cycle and positional constraints on FtsZ localization and the initiation of cell division in *Caulobacter crescentus*. *Mol Microbiol* **39**: 949–959.
- Rodrigues, C.D., and Harry, E.J. (2012) The Min system and nucleoid occlusion are not required for identifying the division site in *Bacillus subtilis* but ensure its efficient utilization. *PLoS Genet* **8**: e1002561.
- Rosenfeld, N., Young, J.W., Alon, U., Swain, P.S., and Elowitz, M.B. (2005) Gene regulation at the single-cell level. *Science* **307**: 1962–1965.

- Rosenfeld, N., Perkins, T.J., Alon, U., Elowitz, M.B., and Swain, P.S. (2006) A fluctuation method to quantify *in vivo* fluorescence data. *Biophys J* **91**: 759–766.
- Rueda, S., Vicente, M., and Mingorance, J. (2003) Concentration and assembly of the division ring proteins FtsZ, FtsA, and ZipA during the *Escherichia coli* cell cycle. *J Bacteriol* **185**: 3344–3351.
- Sackett, M.J., Kelly, A.J., and Brun, Y.V. (1998) Ordered expression of ftsQA and ftsZ during the *Caulobacter crescentus* cell cycle. *Mol Microbiol* **28**: 421–434.
- Sargent, M.G. (1975) Control of cell length in *Bacillus subtilis*. *J Bacteriol* **123**: 7–19.
- Scholz, O., Thiel, A., Hillen, W., and Niederweis, M. (2000) Quantitative analysis of gene expression with an improved green fluorescent protein. p6. *Eur J Biochem* **267**: 1565–1570.
- Sharpe, M.E., Hauser, P.M., Sharpe, R.G., and Errington, J. (1998) *Bacillus subtilis* cell cycle as studied by fluorescence microscopy: constancy of cell length at initiation of DNA replication and evidence for active nucleoid partitioning. *J Bacteriol* **180**: 547–555.
- Spizizen, J. (1958) Transformation of Biochemically Deficient Strains of *Bacillus subtilis* by Deoxyribonucleate. *Proc Natl Acad Sci USA* **44**: 1072–1078.
- Stewart, E.J., Madden, R., Paul, G., and Taddei, F. (2005) Aging and death in an organism that reproduces by morphologically symmetric division. *PLoS Biol* **3**: e45.
- Typas, A., Banzhaf, M., Gross, C.A., and Vollmer, W. (2011) From the regulation of peptidoglycan synthesis to bacterial growth and morphology. *Nat Rev Microbiol* **10**: 123–136.
- Veening, J.W., Kuipers, O.P., Brul, S., Hellingwerf, K.J., and Kort, R. (2006) Effects of phosphorelay perturbations on architecture, sporulation, and spore resistance in biofilms of *Bacillus subtilis*. *J Bacteriol* **188**: 3099–3109.
- Veening, J.W., Stewart, E.J., Berngruber, T.W., Taddei, F., Kuipers, O.P., and Hamoen, L.W. (2008) Bet-hedging and epigenetic inheritance in bacterial cell development. *Proc Natl Acad Sci USA* **105**: 4393–4398.
- Veening, J.W., Murray, H., and Errington, J. (2009) A mechanism for cell cycle regulation of sporulation initiation in *Bacillus subtilis*. *Genes Dev* **23**: 1959–1970.
- Wang, P., Robert, L., Pelletier, J., Dang, W.L., Taddei, F., Wright, A., and Jun, S. (2010) Robust growth of *Escherichia coli*. *Curr Biol* **20**: 1099–1103.
- Weart, R.B., and Levin, P.A. (2003) Growth rate-dependent regulation of medial FtsZ ring formation. *J Bacteriol* **185**: 2826–2834.
- Weart, R.B., Nakano, S., Lane, B.E., Zuber, P., and Levin, P.A. (2005) The ClpX chaperone modulates assembly of the tubulin-like protein FtsZ. *Mol Microbiol* **57**: 238–249.
- Weart, R.B., Lee, A.H., Chien, A.C., Haeussler, D.P., Hill, N.S., and Levin, P.A. (2007) A metabolic sensor governing cell size in bacteria. *Cell* **130**: 335–347.
- Wu, L.J., and Errington, J. (2004) Coordination of cell division and chromosome segregation by a nucleoid occlusion protein in *Bacillus subtilis*. *Cell* **117**: 915–925.
- Zaslaver, A., Kaplan, S., Bren, A., Jinich, A., Mayo, A., Dekel, E., *et al.* (2009) Invariant distribution of promoter activities in *Escherichia coli*. *PLoS Comput Biol* **5**: e1000545.
- Zellmeier, S., Schumann, W., and Wiegert, T. (2006) Involvement of Clp protease activity in modulating the *Bacillus subtilis* sigma^w stress response. *Mol Microbiol* **61**: 1569–1582.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Defining cell-cycle progression and exponential length growth.

A. Cell-cycle progression defined as a function of time since birth and new cell division (left). Cell-cycle progression defined as a function of cell length (% of maximal cell length at time prior to new cell division) (right).

B. More than 900 data points of exponentially growing *B. subtilis* cells are plotted as a function of the cell-cycle progression in time (see *Experimental procedures*). The data points are much a better fit to an exponential curve than to a line ($R^2 = 0.997$ versus 0.982 respectively).

Fig. S2. Validation of our cell-cycle progression software routines.

A. All data points of *B. subtilis* *PftsZ-gfp* (~ 900) were plotted as a function of cell-cycle progression based on cell length versus cell length. A clear straight line is visible demonstrating that the binning procedure works as expected. Error bars represent standard error of the mean.

B. Same as for (A) but now on a large (15593 data points) *E. coli* data set (Stewart *et al.*, 2005).

C. Average fluorescence from cells carrying an IPTG inducible YFP construct grown in the presence of 1 mM IPTG as a function of cell-cycle progression. Same data set as used for (B). Error bars indicate standard error of the mean.

D. Promoter activity (normalized GFP production per minute) as a function of cell-cycle progression for the *E. coli* data set. Error bars indicate standard error of the mean. Note the approximate twofold increase in PA at the end of the cell cycle.

Movie S1. This film shows approximately 20 h of the growth of a *B. subtilis* microcolony harbouring the *PftsZ-gfp* construct. Images were taken every 8 min. Scale bar: 10 μ m.