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Coordination of Cell Division and Chromosome Segregation by a Nucleoid Occlusion Protein in *Bacillus subtilis*

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Summary

A range of genetical and physiological experiments have established that diverse bacterial cells possess a function called nucleoid occlusion, which acts to prevent cell division in the vicinity of the nucleoid. We have identified a specific effector of nucleoid occlusion in *Bacillus subtilis*, Noc (YyaA), as an inhibitor of division that is also a nonspecific DNA binding protein. Under various conditions in which the cell cycle is perturbed, Noc prevents the division machinery from assembling in the vicinity of the nucleoid. Unexpectedly, cells lacking both Noc and the Min system (which prevents division close to the cell poles) are blocked for division, apparently because they establish multiple nonproductive accumulations of division proteins. The results help to explain how *B. subtilis* specifies the division site under a range of conditions and how it avoids catastrophic breakage of the chromosome by division through the nucleoid.

Introduction

Proper timing and positioning of the division plane is a crucial aspect of the cell cycle throughout biology. In higher organisms, it is generally assumed that the position of the interphase nucleus is used as a topological signal that determines the site and orientation of the division plane. In most cases, however, little is known about the molecular basis for division site specification. In fission yeast, several genes required for division site positioning have been identified, including *mid1*, *plo1*, and *pom1*, but details of how their gene products work in this context remain hazy (Rajagopalan et al., 2003). In the rod-shaped bacteria *E. coli* and *Bacillus subtilis*, division occurs with exquisite precision at mid-cell, and the timing of division is regulated so that it follows the replication and segregation of sister chromosomes. However, despite decades of study, the mechanisms underlying division timing and positioning in these organisms remain poorly understood. The best-characterized components of this process are the widely conserved MinC and MinD proteins, which form a complex that acts as an inhibitor of division (de Boer et al., 1990, 1992; Hu et al., 1999). The activity of MinCD is restricted to the vicinity of the cell poles by a topological specificity factor (de Boer et al., 1989). In many bacteria, this factor is a protein called MinE, which works by driving a remarkable pole-to-pole oscillation of the MinCD inhibitor (Fu et al., 2001; Hale et al., 2001; Hu et al., 2002; Hu and Lutkenhaus, 2001; Shih et al., 2003). The net result of this oscillation appears to be that the inhibitor spends most of its time, and is therefore most active, near the cell poles, leaving the mid-cell region available for division. In *B. subtilis* and related Gram-positive bacteria, a different topological specificity factor, DivIVA, achieves the same result by the apparently simpler approach of directly recruiting the inhibitor to the cell poles and retaining them there in a static fashion (Cha and Stewart, 1997; Edwards and Errington, 1997; Marston et al., 1998). In both *E. coli* and *B. subtilis*, mutants that lack MinCD function have a phenotype in which division occurs either at the normal mid-cell position or at sites located very close to the cell pole; this division produces small spherical anucleate "minicells." The positioning of division in Min^- mutants raises the important question as to whether there is an additional underlying mechanism that can prevent division at sites between the pole and mid-cell. A range of physiological and genetical experiments suggest that this other hypothetical effector of division-site selection could be the nucleoid (reviewed by Harry, 2001; Margolin, 2001). The nucleoid is the bacterial equivalent of the nucleus and comprises a highly compacted and organized chromosome but with no surrounding nuclear membrane. During DNA replication and segregation, the nucleoids occupy positions corresponding to the forbidden intermediate sites between the pole and mid-cell that are predicted on the basis of the Min mutant phenotype. Thus, combination of nucleoid occlusion and the Min system could provide an effective dual system for directing division to the correct mid-cell, internucleoid site in both *B. subtilis* and *E. coli* (Marston et al., 1998; Yu and Margolin, 1999). The "nucleoid occlusion" effect, first postulated by Woldringh and coworkers more than a decade ago (Mulder and Woldringh, 1989; Woldringh et al., 1990), is attractive because it could not only contribute to positioning division at mid-cell but might also provide a checkpoint-like mechanism for preventing division before the replication and segregation of the sister chromosomes is complete. In Woldringh's view, the nucleoid occlusion effect is mediated by molecular crowding effects arising from the activity of the nucleoid in terms of the linked transcription, translation, and translocation of secreted proteins in the vicinity of the nucleoid (Woldringh, 2002). An alternative view would be that one or more specific proteins associated with the nucleoid acts directly to inhibit assembly of the division machinery around the nucleoid. Unfortunately, genes specifically affected in nucleoid occlusion have not been found. Although mutations in the *mukB* gene of *E. coli* and its equivalent, *smc*, in *B. subtilis*, allow guillotining of the nucleoid in some cells (e.g., Niki et al., 1991; Moriya et al. 1998), these mutations almost certainly affect nucleoid occlusion indirectly by their profound effects on the condensation or organization of nucleoid structure. An alternative model for division site selection supposes that the middle of the cell is somehow marked as a site for nucleation of the division machinery (e.g., Foley et al., 1989; Re-

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gamey et al., 2000). Again, the molecular mechanisms that might underlie this kind of model remain ill defined.

About eight widely conserved proteins are known to be required for cell division in bacteria (reviewed recently: Errington et al., 2003). The key player in the machinery is a tubulin homolog, FtsZ, that polymerizes to form a ring-like structure, the Z ring, at the site of cell division. The other division proteins, most of which are integral membrane proteins, are recruited to the Z ring in a hierarchy that is quite well characterized in both *E. coli* and *B. subtilis*. Formation of the Z ring is regulated by a number of proteins. The MinCD protein mentioned above is an inhibitor of Z ring formation (Hu et al., 1999). In *E. coli*, FtsA and ZipA have partially redundant functions, at least one of which needs to be present to allow formation of the Z ring (Pichoff and Lutkenhaus, 2002). In *B. subtilis*, EzrA (Levin et al., 1999) and ZapA (Gueiros-Filho and Losick, 2002) have been identified as likely negative and positive regulators of FtsZ polymerization or Z ring formation. However, how the functions of all of these proteins are integrated to control the timing and spatial localization of Z ring formation is not yet clear. In particular, none of these proteins appears to be capable of coupling Z ring formation to the position of the nucleoid.

We have now identified a *B. subtilis* protein, YyaA, that associates nonspecifically with the chromosome and is required to block cell division in the vicinity of the DNA, finally providing a specific protein with a role in nucleoid occlusion. Under various conditions, YyaA contributes to correct placement of the division site, reducing the risk of chromosome damage during division.

Results

Cell Division Is Inhibited in *yyaA minD* Double Mutants

In genetic crosses designed to construct strains with which to investigate the role of the Soj-Spo0J and DivIVA systems in chromosome segregation in *B. subtilis* (Wu and Errington, 2003), we serendipitously discovered that certain combinations of mutations seriously compromised cell viability. A series of genetic crosses established that the effect was actually due to simultaneous loss of the Min system (*divIVA*, *minC*, *minD*, or *ezrA*) and the product of a poorly characterized gene called *yyaA* (see Supplemental Table S1 at http://www.cell.com/cgi/content/full/117/7/915/DC1). *yyaA* lies just upstream of the *soj-spo0J* locus and encodes a product that is about 40% identical to Spo0J over much of its length. We previously reported that *yyaA* mutants do not have an obvious growth, segregation, or division phenotype, though overexpression impairs sporulation (Sievers et al., 2002).

Prolonged low temperature incubation (30°C or less) of transformation plates bearing potential double mutants of the poorly viable class, e.g., *minD yyaA*, eventually resulted in colonies that contained both incoming and resident markers (Figure 1A; compare with the abundant uniform colonies in the control transformation in Figure 1B). To examine the mutant phenotype under more controlled conditions, a repressible allele of *minD* (dependent on an inducer, IPTG) was introduced into a *yyaA* null mutant background. This strain grew normally in the presence of inducer (data not shown). Removal of the inducer to deplete MinD in the presence of YyaA resulted, as expected, in the production of minicells (Figure 1C). However, when MinD was depleted in a *yyaA* mutant, division was arrested and the cells grew into long filaments (Figure 1D). Similar results were obtained when YyaA was depleted in a *minD* background (data not shown).These results showed that absence of both the Min system and the YyaA protein results in a severe defect in cell division.

yyaA minD Double Mutants Fail to Concentrate FtsZ at Potential Division Sites

To test at what level the block in cell division was occurring, we examined the effect of loss of YyaA and MinD on localization of cell division proteins. FtsZ is a tubulin homolog that lies at the top of the hierarchy of division proteins. In cells containing a disruption of *yyaA*, cell length was normal and FtsZ-GFP was targeted correctly to bands located at nascent and future division sites, as in wild-type cells (not shown). In *yyaA*$^+$ cells depleted for MinD, again discrete bright fluorescent bands were detected, some of which were adjacent to each other due to minicell formation but all were correctly interpolated between segregating nucleoids (Figure 2A). However, when MinD was depleted for 3–4 generations at 30°C in the Δ*yyaA* background, bright transverse bands of FtsZ were formed only rarely in the elongating filaments; instead, the protein was distributed much more widely in multiple weak bands (Figure 2B). Similar results were obtained when a nonessential but early assembling (*ftsZ*-dependent) component of the division machinery, EzrA (Levin et al., 1999), was examined. Thus, single bright transverse bands of EzrA-GFP were seen when MinD was depleted in the presence of YyaA (Figure 2C), but these were replaced by scattered spots, weak tilted bands, and helices in the double mutant (Figure 2D).

YyaA Colocalizes with the Nucleoid and Inhibits Division when Overproduced

YyaA was shown previously to be a relatively non-sequence-specific DNA binding protein, and immunofluorescence microscopy suggested that it colocalized with the nucleoid (Sievers et al., 2002). Unfortunately, the preparation of specimens for immunofluorescence microscopy does not preserve the normal morphology of the nucleoid well. To assess the localization of YyaA in living cells, a GFP fusion was constructed. The *gfp-yyaA* fusion construct was functional because, when present as the only expressed copy of *yyaA* in a *minD* mutant background, the cell length was typical of that of a *min* mutant, and the colonial growth rate was normal. As shown in Figures 3A–3C, the GFP-YyaA fusion colocalized with the nucleoid, in confirmation of the previous immunofluorescence data (Sievers et al., 2002). Interestingly, the GFP-YyaA signal did not always cover the whole nucleoid and a gap was often evident near the middle of longer nucleoids (arrows in Figure 3C).

In the light of the effects of *yyaA* mutation on division (in combination with *min*), it was important to look

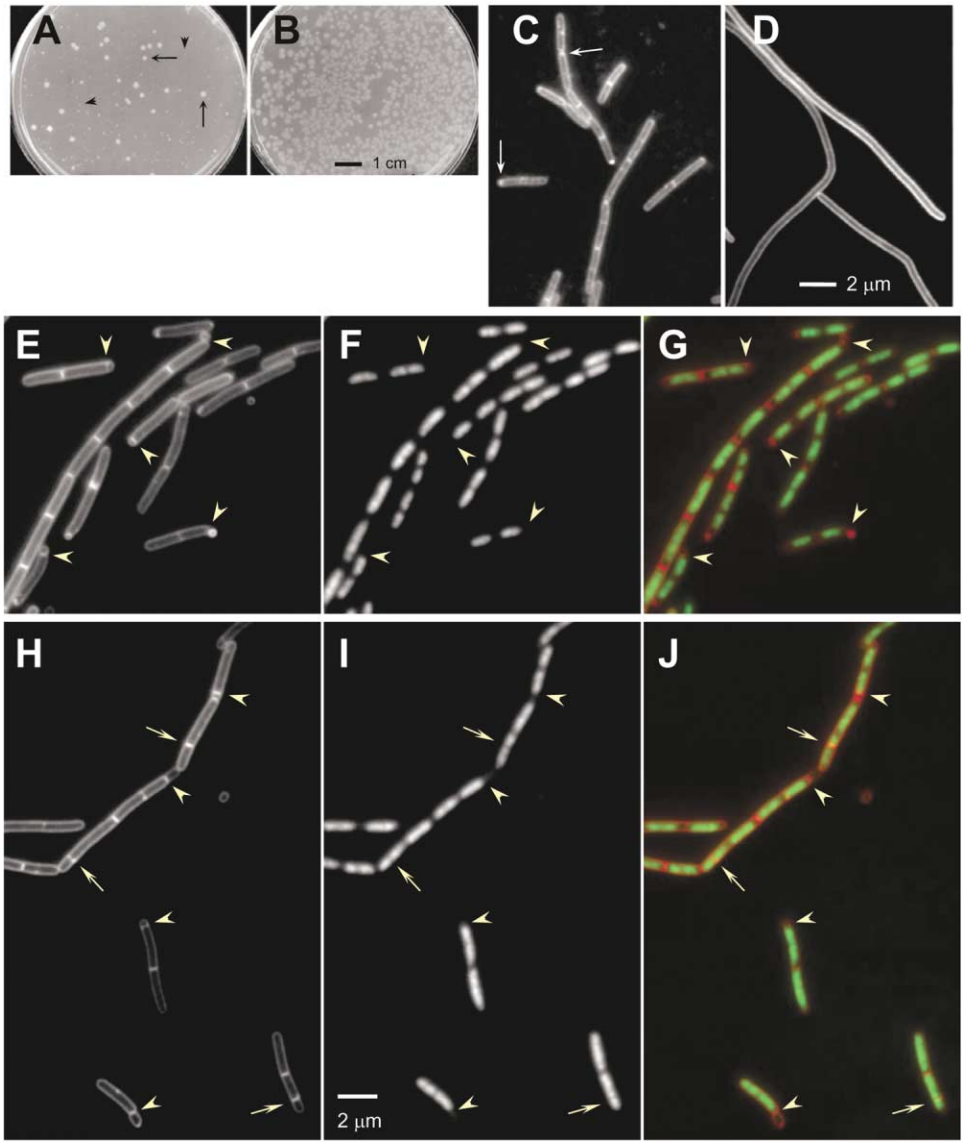

Figure 1. Severe Cell Division Defect of *yyaA minD* Double Mutants and Rescue by Overproduction of FtsZ

(A and B) Small colony phenotype and poor recovery of *yyaA minD* double mutants in a transformation cross. A *minD* mutant (strain 1901) was transformed with equal concentrations of DNA from strains carrying a tetracycline resistance cassette inserted into the *yyaA* gene (A) or into an intergenic site (*yxeDC*; B). The treated recipient cells were plated in the presence of tetracycline and incubated for 1 day at 37°C then 1 day at 30°C. The control (B) showed large numbers of uniformly sized colonies. The *yyaA*-transformed cells gave only a few large colonies (arrows) that usually had lost the resident *minD* mutation. Small colonies (arrowheads) retained both resident (*minD*) and incoming (*yyaA*) mutations but appeared only after incubation at 30°C and were extremely underrepresented.

(C and D) Filamentous phenotype arising from depletion of *minD* in a Δ*yyaA* background (D; strain 1284) but not in a *yyaA*$^+$ background (C; strain 1998). Strains were grown at 30°C and 2 hr after removal of the inducer (IPTG), cells stained with the membrane dye FM5-95 were examined directly by fluorescence microscopy. Arrows in (C) point to examples of minicells.

(E–J) Partial rescue of a *yyaA minD* double mutant by overproduction of FtsZ.

FtsZ was overproduced by adding both IPTG (1 mM) and xylose (0.5%) to cultures containing inducible copies of wild-type and *gfp* fusion derivatives of *ftsZ*. The strains used carried mutations in *minD* (strain 1296; E–G), or *minD* and *yyaA* (strain 1297; H–J). The panels show typical fields of cells stained to visualize the cell membrane (FM5-95; E and H), the DNA (DAPI; F and I), and a false color merge with membrane in red and DNA in green (G and J). Arrows point to septa that appear to have bisected a nucleoid, and arrowheads to septa that formed anucleate cells (mainly minicells in the *minD* single mutant and a range of cell sizes in the double mutant).

closely at the phenotype of *yyaA* single mutants. In our previous characterization of *yyaA*, we reported that overexpression of *yyaA* inhibited sporulation (Sievers et al., 2002). One contributing factor appeared to be a slight increase in cell length. The previous experiments were done with multicopy plasmids, which provide a variable gene dosage, and for which controls are difficult because the vector plasmids with no inserts tend to have effects on the growth and cell cycle progression of the host cells. To overexpress *yyaA* under more controlled

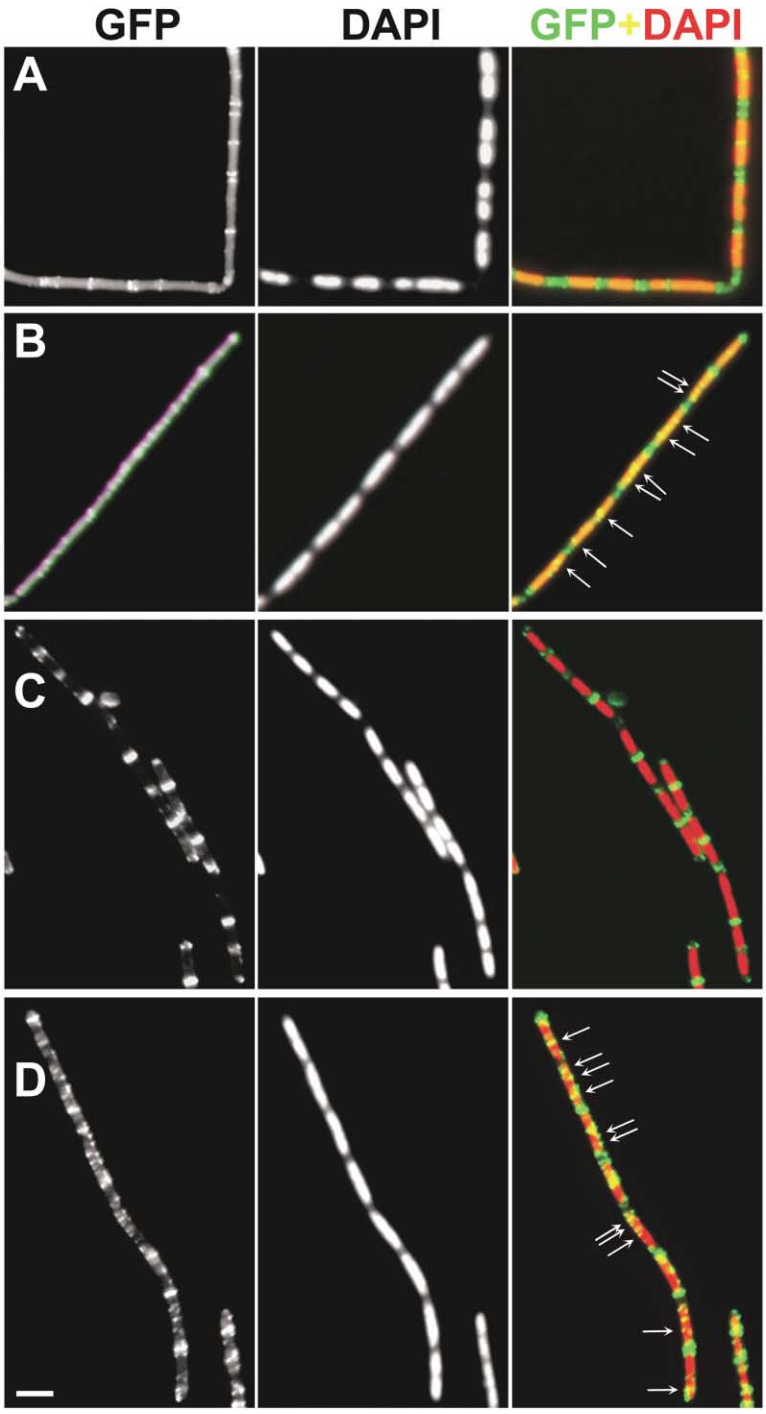

Figure 2. Double Defect in *yyaA* and *minD* Blocks Cell Division at the Level of Z Ring Formation

Localization of GFP-FtsZ (A and B) or EzrA-GFP (C and D) in MinD-depleted cells with (A, strain 1291; C, strain 1286) or without (B, strain 1292; D, strain 1287) a functional *yyaA* gene. The panels show GFP fluorescence (left), DAPI (DNA; middle), and a false color merge of the two channels (right) with GFP in green and DAPI in red. Arrows point to GFP bands that overlap the nucleoids. Scale bar, 2 μm.

conditions, we introduced into otherwise wild-type cells a second copy of *yyaA* under the control of the strong IPTG-inducible $P_{spac(hy)}$ promoter (Quisel et al., 2001), which gave about an 8-fold increase in transcription, as judged from microarray data (data not show). Figures 3D and 3E show that cell length was normal in the control strain with no overexpressed *yyaA*, but that in the overexpressing strain, the cells were about 2- to 4-fold longer (Figures 3F and 3G). Thus, overexpression of YyaA resulted in a partial inhibition or delay in cell division. Western blot analysis showed that FtsZ levels were unaffected by the overexpression (not shown). However, immunofluorescence microscopy revealed a reduction in FtsZ bands in the elongated cells (1 band per 13.2 μm) compared with the control cells (1 band per 6.3 μm), showing that the inhibition of division occurred at the level of Z ring assembly.

yyaA Disruption Allows Division through the Nucleoid

The above and previous experiments established that YyaA is a relatively nonspecific DNA binding protein and

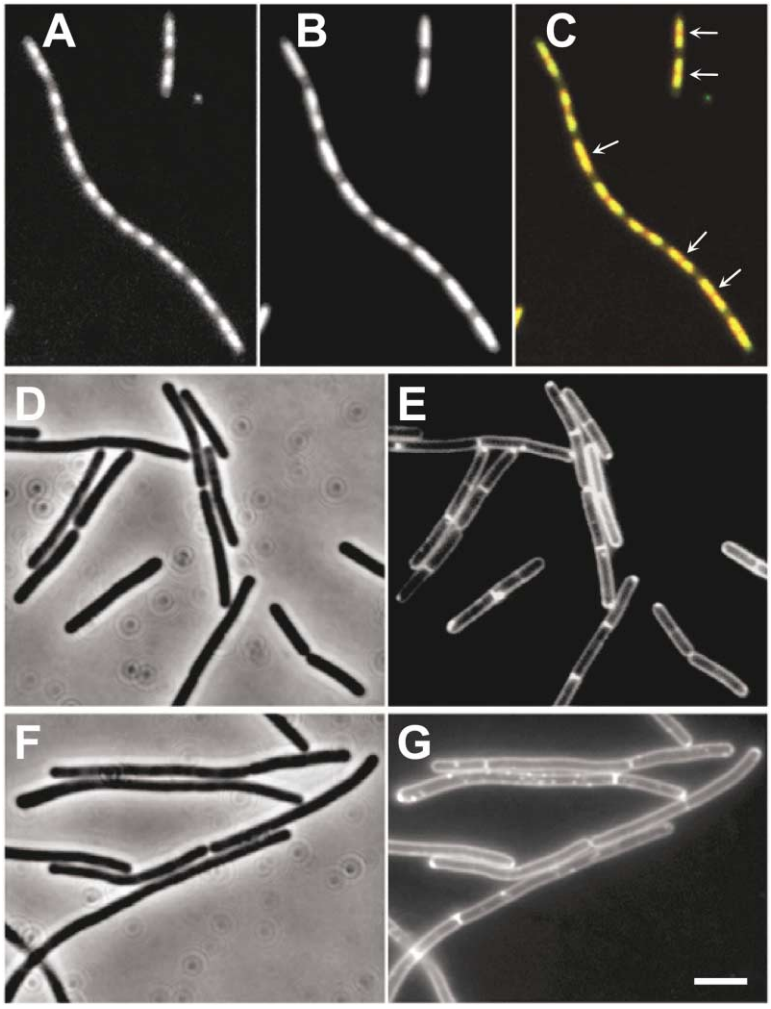

Figure 3. Localization of GFP-YyaA over the Nucleoid and Weak Filamentous Phenotype upon YyaA Overproduction

(A–C) Strain 1288 with a *gfp-yyaA* fusion growing exponentially in CH medium containing 0.3% xylose was examined by fluoresence microscopy. Panels show the GFP signal (A), DAPI (B), and a merge of the two images (C) with GFP false colored green and DAPI in red. The arrows in (C) point to central regions of the longer nucleoids where the GFP signal appears to be reduced relative to that of the DNA.

(D–G) Isogenic strains with the strong $P_{spac(hy)}$ promoter inserted into the *amyE* locus with no additional gene (D and E; strain 1289) or upstream of a copy of the *yyaA* gene (F and G; strain 1290). Images shown are phase contrast (D and F) and membrane fluorescence (FM5-95; E and G). Scale bar, 2 μm.

that it can delay cell division when overexpressed. These properties were suggestive of a role in nucleoid occlusion. In support of this idea, we noticed that in the earlier experiments with *yyaA minD* double mutants, the aberrant accumulations of FtsZ or EzrA protein frequently overlapped the nucleoid (arrows in Figures 2B and 2D), in contrast to the discrete bands located precisely between nucleoids in *yyaA*$^+$ cells (Figures 2A and 2C). To test directly for a role in nucleoid occlusion, we used a well-characterized system based on the manipulation of germinating spores. Spores contain a single replicated chromosome, and the first cell cycle following germination and outgrowth of the spore can be followed under well-characterized and relatively synchronous conditions. The timing of various events is well known and the effects of addition of various inhibitors of DNA replication have been extensively documented. In particular, Wake and coworkers have shown that if the round of DNA replication is blocked at the initiation stage, e.g., by early addition of the replication elongation inhibitor HPUra, the cells elongate at the normal rate but division is delayed. Importantly, when division does eventually occur, it is asymmetric and positioned to one side of the centrally located nucleoid (e.g., Harry et al., 1999; Rowland et al., 1997), presumably as a result of nucleoid occlusion. We therefore tested whether *yyaA* mutants are affected in division septum positioning under these conditions. Wild-type and Δ*yyaA* spores were germinated and cells were allowed to outgrow. After a time interval sufficient to complete one or two rounds of cell division, samples of the cultures were examined microscopically to determine the numbers of septa, and their position relative to the nucleoid and the cell poles. In the absence of HPUra, there was no detectable difference between the wild-type and the Δ*yyaA* cells; all of the outgrowing cells divided at the expected size and at mid-cell (Figures 4A–4D). As expected, when HPUra was added to prevent DNA replication at a very early stage in outgrowth, wild-type outgrowing spores showed mainly acentral division septa (Figures 4E–4H). Thus, only 19% of septa were within 10% of cell length from the midpoint of the cell (Figure 4H). In contrast, most of the *yyaA* septa (85%) were close to mid-cell (Figures 4I–4L). The frequency of division was also greater in *yyaA* cells (51% of cells) than in wild-type cells (32%). Importantly, the septa of the *yyaA* mutant cells frequently had DNA on both sides of them (28% of all cells), indicating that the nucleoid had been bisected, whereas in the wild-type only 5.4% of cells had DNA visible on both sides of a septum. In the *yyaA* mutant, cells with central septa

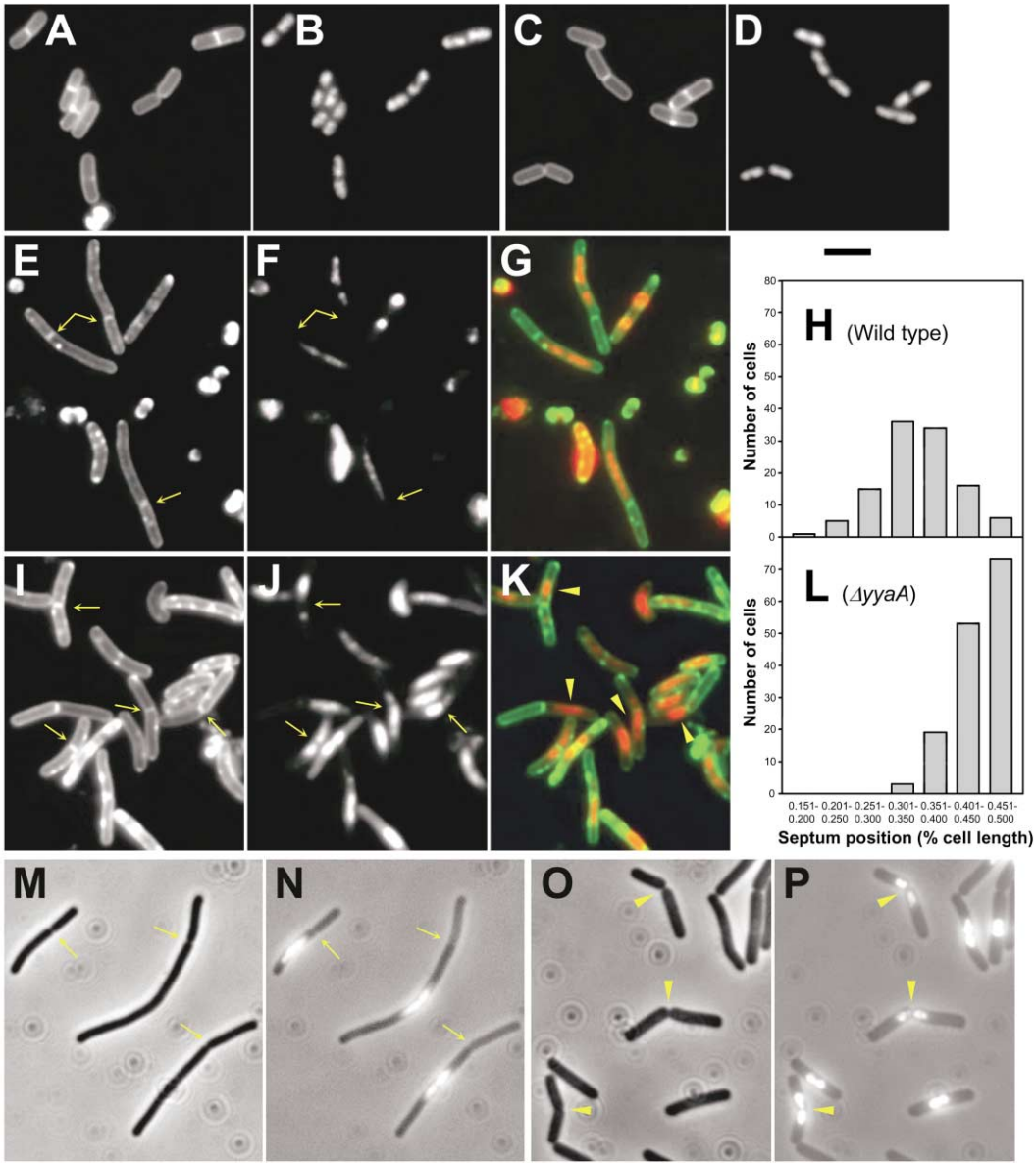

Figure 4. Impaired Nucleoid Occlusion in the Absence of YyaA

(A–L) Spores of wild-type *B. subtilis* (A, B, and E–H; strain 168) or an isogenic strain with a disruption of *yyaA* (C, D, and I–L; strain 1282) were germinated and allowed to outgrow with (E–L; for 342 min) or without (A–D; for 210 min) HPUra inhibition of DNA replication. (A), (C), (E), and (I) show FM5-95 (membrane) stain; (B), (D), (F), and (J) show DAPI (DNA); (G) and (K) are false color merges of the membrane (green) and DNA (red) images to their left. Arrows in (E) and (F) point to asymmetric septa formed outside the nucleoids, and in (I) and (J), arrows point to mid-cell septa that have bisected a nucleoid. Arrowheads in (K) point to highly asymmetrical nucleoids that lie to one side of a mid-cell septum. The positions of the septum in about 150 dividing cells of the wild-type (H) and mutant (L) spores were measured relative to the nearest cell pole and scored as a percentage of total cell length. Note that the bright green ovoid objects are spore coats or nongerminated spores.

(M–P) Isogenic strains containing an IPTG-inducible *dnaA* gene and either wild-type *yyaA* (M and N; strain PL10) or a deletion of *yyaA* (O and P; strain 1295) were grown in the presence of IPTG, then the inducer was withdrawn to allow ongoing rounds of DNA replication to be completed but no new rounds to be started. Cells were imaged after a time sufficient to allow approximately 2 doublings in cell length. Shown are phase contrast (M and O) and combined phase contrast and DAPI images (N and P). Nucleoids are visible as white areas against the dark cell background. Arrows point to asymmetrical septa that lie distant to the nucleoid and arrowheads to medial septa that have bisected a nucleoid. Scale bar, 2 μm.

were often seen to have a nucleoid positioned asymmetrically, lying mostly or completely on one side of the septum (arrowheads in Figure 4K). We assume that this is a secondary effect due to SpoIIIE-mediated translocation of the nucleoid after septation (Sharpe and Errington, 1995). This experiment showed that *yyaA* plays an important role in preventing division at mid-cell when wild-type spores outgrow under conditions of DNA replication inhibition.

As an alternative way of testing for a defect in nucleoid occlusion, we examined the effects of depleting cells for the key initiator protein for DNA replication, DnaA,

in exponentially growing cells. The cultures induced to express *dnaA* had a wild-type phenotype, and *yyaA*$^+$ and Δ*yyaA* strains were indistinguishable in terms of cell length and cell cycle progression (data not shown). After about two generations of growth in the absence of inducer, DnaA-depleted cells of the *yyaA*$^+$ strain were elongated and tended to contain a single nucleoid, corresponding to a single chromosome (Figures 4M and 4N). Where division septa were visible, they were always located asymmetrically, away from the nucleoid (arrows). In contrast, the DnaA-depleted cells of the *yyaA* mutant were shorter (Figures 4O and 4P), as if division was not inhibited, and division frequently occurred right through the nucleoid at mid-cell (arrowheads in Figure 4P). Thus, under two quite different experimental conditions, *yyaA* mutant cells apparently lacked the nucleoid occlusion function that normally prevents cell division from occurring through the nucleoid.

YyaA-Dependent Positioning of Z Rings between Nucleoids in Elongated Cells

Several authors have reported that cells blocked late in division form Z rings at regular intervals, "potential division sites," which generally correspond to internucleoid spaces. In *E. coli*, the oscillating Min system can notionally identify intermediate division sites in such filaments (Raskin and de Boer, 1997), but the fixed polar Min system of *B. subtilis* should not have this capability (Harry, 2001; Margolin, 2001). It seemed possible that YyaA might be involved in directing Z ring assembly to internucleoid spaces in abnormally long cells. To test this, we used a strain in which expression of two "late" cell division genes, *ftsL* and *pbpB*, could be simultaneously repressed (Daniel et al., 1996), leading to elongated cells in which Z rings would be expected to form but no division septa. In the initial experiments, EzrA-GFP was used as a surrogate marker for FtsZ ring formation. As shown in Figures 5A–5D, when the *yyaA*$^+$ cells were depleted, the cell filaments contained EzrA-GFP bands positioned precisely in internucleoid spaces. Some of the bands appeared to correspond to double ring structures or short helices, but these structures were always localized in the internucleoid spaces. In sharp contrast, in the equivalent filaments of the Δ*yyaA* derivative (Figures 5E–5H), clusters of EzrA-GFP bands, some of which were slightly tilted, suggestive of an overall helical configuration, were located in broad regions, which substantially overlapped the nucleoids. The nucleoids had their normal regular spacing in the cell but no longer apparently excluded FtsZ polymerization. Experiments looking directly at FtsZ localization by immunofluorescence microscopy in fixed cells gave results in accordance with those of EzrA-GFP (not shown). These results provided further evidence that *yyaA* is required for specification of Z ring position independently of the Min system.

Interestingly, in the above experiments, it was evident that the accumulations of FtsZ tended not to occur close to the poles of the filaments, consistent with these regions being protected by MinCD. In a further repetition of the above experiment, we examined cells containing an FtsZ-GFP fusion, soon after depletion, at a time when the cells were just beginning to become elongated. As shown in Figure 5I, the *yyaA*$^+$ cells tended to have a single bright Z band at mid-cell, and in longer cells, further weak Z bands were evident at approximately 1/4 and 3/4 positions, as expected. In contrast, the *yyaA* mutant cells tended to have helical or multibanded systems occupying the medial portion of the cell but not in the polar regions (compare with the poles of cells of a *min* mutant in Figure 2B). These results were consistent with the fact that the Min system is capable of protecting an extended polar zone of the cell from FtsZ polymerization but YyaA becomes increasingly important as the cell elongates.

Division through the Nucleoid in *yyaA minD* Double Mutants Overproducing FtsZ

On the basis of the above results, it seemed likely that the filamentous phenotype of *yyaA minD* double mutants was due to delocalized or uncoordinated polymerization of FtsZ. If so, it might be possible to partially rescue division by overproduction of FtsZ. We therefore built a strain with two inducible copies of *ftsZ* (one also fused to *gfp*) in which this could be tested. The experiment shown in Figure 1 (E–J) demonstrates that overproduction of FtsZ did indeed lead to a partial restoration of division in cells impaired in *yyaA* and *minD* function. Figures 1E to 1G show a control experiment with a *minD* single mutant overproducing FtsZ. The overproduction had little effect on the cells, which were of relatively normal length and produced anucleate minicells at a substantial frequency (arrowheads), as expected. When the overproduction was done in a *yyaA minD* background (Figures 1H–1J), the long aseptate filaments normally seen in the double mutant (e.g., Figure 1D) were resolved into much shorter units (Figure 1H), due to a substantially increased rate of division. However, unlike the equivalent *minD* single mutant (Figures 1E–1G), in which division occurred neatly in the nucleoid free spaces, many cell divisions were clearly asymmetrical and occurred over or through nucleoids (arrows). Many of the divisions again produced anucleate "minicells" (arrowheads), but these showed an unusually variable length compared with the tiny spherical minicells produced by the *minD* single mutant.

Although these experiments again highlighted the need for YyaA protein to prevent division through the nucleoid, it was notable that a substantial proportion of the cell divisions that occurred under these conditions were located in internucleoid positions, and this also applied to the positioning of Z rings as visualized by FtsZ-GFP (not shown). This raises the possibility that under these conditions, a *yyaA*-indepependent system can bias the division machinery away from the bulk of the nucleoid.

Discussion

A Gene for Nucleoid Occlusion

The nucleoid occlusion model was first promulgated by Woldringh and coworkers (Mulder and Woldringh, 1989; Woldringh et al., 1990) to explain their observation that in *E. coli* cell division did not occur in the vicinity of the nucleoid under various conditions in which DNA replication was inhibited. Rather, it occurred in the flank-

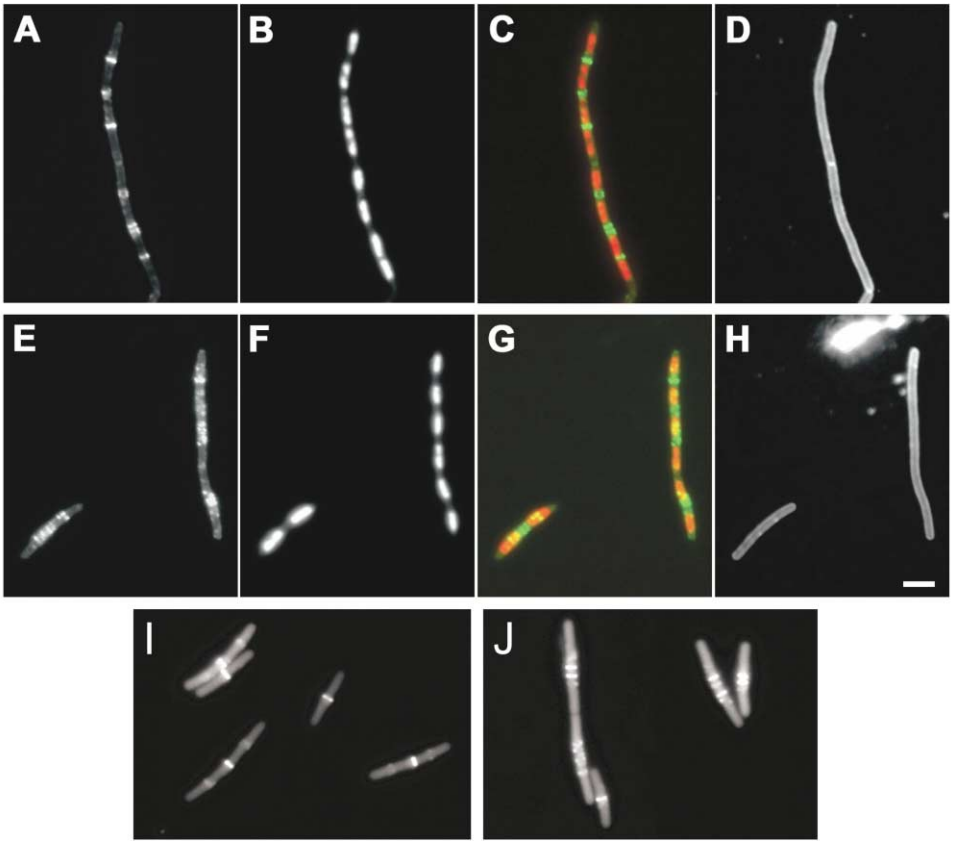

Figure 5. Decreased Ability of *yyaA* Mutants to Direct the Division Machinery to Internucleoid Spaces in Filamentous Cells

Cells bearing an IPTG-inducible promoter driving expression of the essential division genes *ftsL* and *pbpB* were grown in the presence of IPTG, then the inducer was removed to block division. After sufficient time to allow about 2 doublings in cell length, cell filaments were imaged. In (A)–(H), the strains also contained an *ezrA-gfp* fusion to monitor FtsZ assembly and either wild-type *yyaA* (A–D; strain 1293) or a deletion mutation (E–H; strain 1294). The panels show GFP fluorescence (A and E), DNA (B and F), a false color merge with GFP in green, DNA in red (C and G), and membrane (Nile Red) (D and H). (I) and (J) show the localization of GFP-FtsZ in *yyaA*$^+$ (I; strain 1298) or Δ*yyaA* mutant background (J; strain 1299) when FtsL and PbpB were depleted, as for panels (A)–(H). Scale bar, 2 μm.

ing regions of the filamentous cells, away from the nucleoid. Similar conclusions had been drawn in earlier extensive work on *B. subtilis*, by Wake and coworkers (see Regamey et al. [2000] and references therein). Outgrowing spores initiate a relatively synchronous round of DNA replication that can be readily manipulated. In this system, it was shown that an early block in chromosome replication prevents division through the nucleoid, although this inhibitory effect begins to disappear when the round of DNA replication has progressed beyond about 60% toward completion. In terms of nucleoid occlusion, the latter observation could be explained by the switch in configuration of the nucleoid to a bilobed shape, which occurs at about that time (Sharpe et al., 1998). The bilobed nucleoid could allow Z ring formation in the space between the two major lobes of DNA ahead of the completion of DNA replication. In support of this notion, in various other conditions in which division has been observed to occur through the nucleoid, the effect seems to be associated with a localized reduction of DNA concentration (see Margolin [2001]). Recent work on both *E. coli* and *B. subtils* has confirmed and extended the work on division by demonstrating that nucleoid occlusion is mediated at some step in FtsZ ring assembly (reviewed by Harry [2001]; Margolin [2001]). Despite these numerous observations, the molecular basis for nucleoid occlusion has remained unclear, mainly because no genes or proteins directly responsible for this function had been identified (see Introduction). We have now identified the YyaA protein as an important effector of nucleoid occlusion in *B. subtilis*. We propose to rename this protein Noc, to reflect its role in *n*ucleoid *oc*clusion.

Several lines of evidence demonstrated impaired nucleoid occlusion in the *noc* mutant. When initiation of DNA replication was blocked, either by HPUra treatment of outgrowing spores or by depleting DnaA in batch-grown cells, division occurred much more frequently in the *noc* mutant than in the equivalent wild-type strain. Crucially, many divisions occurred through the nucleoid in the *noc* mutant, whereas the nucleoid is usually missed in the wild-type (Figure 4). When *noc* and *min* mutations were combined, the filamentous cells frequently showed concentrations of FtsZ (or EzrA) as spots or bands over the nucleoid, whereas in isogenic *noc*$^+$ cells, the FtsZ assemblies were almost always in the internucleoid spaces (Figure 2). The clearest indication of the function of Noc protein in directing FtsZ

assembly away from the nucleoid was seen in the experiments illustrated in Figure 5. In elongated filaments generated by a late block in cell division, the Z rings that normally form accurately in internucleoid spaces no longer did so in the absence of Noc. Instead, elongated structures, probably helical in gross architecture, were formed at sites that extensively overlapped the nucleoid. Noc is therefore required to protect the nucleoid from division, and it does so apparently by inhibiting FtsZ accumulation or polymerization in the vicinity of the nucleoid; it functions, directly or indirectly, as a nucleoid occlusion protein. The importance of this protein to cells is best illustrated by the experiments in Figure 4 showing that Noc has a critical role as the first line of defense in protecting the nucleoid from damage by the division septum. It might also be thought of as a checkpoint protein that helps to prevent division from occurring until the chromosomal DNA has properly cleared from the mid-cell site for division.

Noc may act directly because it has the two key properties potentially needed for a nucleoid occlusion protein. First, it is a relatively nonspecific DNA binding protein that localizes almost uniformly over the nucleoid (Sievers et al., 2002 and Figure 2A). This contrasts with the small discrete foci of its close relative, Spo0J protein, which colocalizes with *oriC* regions (Glaser et al., 1997; Lin et al., 1997). Second, when overproduced, it partially inhibits division. The fact that inhibition is partial is probably due to Noc localization still being restricted to the vicinity of the nucleoid. Overproduction may simply increase the concentration of Noc on the nucleoid and delay the time at which division at mid-cell, between segregating nucleoids, occurs. It will be interesting to carry out a finer detail analysis of the distribution of the protein on the nucleoid (e.g., by chromatin immunoprecipitation). Inspection of the images shown in Figures 3A–3C suggests that the protein may be less abundant in the medial part of the longer nucleoids, corresponding to the unreplicated terminus region. This might allow assembly of the Z ring ahead of the complete termination of replication, as observed in the experiments by the Wake lab described above.

Several lines of argument support the notion that Noc acts directly, rather than at the level of transcriptional regulation of a division inhibitor. First, although Noc appears to localize all over the nucleoid, preliminary microarray experiments have shown that very few genes change in expression in the absence of Noc. In particular, we found no significant change in the expression of any known gene involved in cell division or its regulation (L.J.W., unpublished data). This includes the recently described *yneA* gene, which seems to be the *B. subtilis* functional equivalent of the SOS-responsive division inhibitor, *sulA* of *E. coli* (Kawai et al., 2003). Second, the protein is apparently expressed constitutively and is present on the nucleoid independent of cell cycle progression or DNA damage (Sievers et al., 2002; Figure 2A; L.J.W., unpublished data), providing no hint for a role in a putative signal transduction pathway. Third, the results shown in Figures 2 and 5 strongly suggest that the loss of Noc function affects the spatial organization of Z rings rather than conferring a general inhibition of polymerization, and the location of the inhibitory effect in wild-type cells (i.e., over the nucleoid) is where the Noc protein localizes. Finally, when overproduced, Noc caused a reduction in cell division (Figure 3) that occurred at the level of FtsZ assembly.

Implications for the Mechanism of Z Ring Assembly and Positioning

This investigation of *noc* function was prompted by the near lethal filamentous phenotype of the *noc minD* mutant (Figure 1). This phenotype was initially confusing because we had anticipated that combination of a nucleoid occlusion mutation with a *min* mutation would give rise to uncontrolled division by elimination of two negative regulators. Why should the loss of two division inhibition systems result in a block in division? The images shown in Figure 2 suggest that the failure to divide in the double mutant arises because FtsZ polymerization occurs at multiple sites, most of which do not lead to the formation of a productive Z ring structure. We suggest that with multiple minor polymerization centers all competing for protein, none of these can recruit enough FtsZ protein to form a productive ring. In support of this interpretation, we showed that the division frequency could be substantially recovered by overproduction of FtsZ (Figures 1E–1J). This provides strong support for the notion that topological restriction of Z localization or accumulation is normally crucial for driving the formation of a complete, functional Z ring. Similar conclusions have been drawn for *E. coli* based on analysis of the effects of combining deletion of the *min* system with an *ftsZ* mutation that reduces FtsZ polymerization (Yu and Margolin, 2000). Conversely, these kinds of observations suggest that condensation of FtsZ into a defined region could be sufficient to drive the formation of an active division machine, eliminating the need for a predetermined site for Z ring assembly, as has been postulated previously (e.g., Cook and Rothfield, 1999; Regamey et al., 2000).

If nucleoid occlusion was important in selecting the mid-cell site for division, *noc* mutants should exhibit some loss of specificity of division site placement, but this was not apparent in unperturbed cells. We think that there are at least two explanations for this. First, there is some evidence for a weak *noc*-independent system that biases division to internucleoid spaces, as evident in the successful divisions that occur in Figures 1D–1F. Second, the Min system may be capable of preventing division at a significant distance from the cell poles in *B. subtilis*, rather than acting only to prevent minicell divisions close to existing cell poles. This was particularly evident in the experiment shown in Figures 5I and 5J. We previously noted that MinD localization, though concentrated at the cell poles, appears to emanate out from the poles toward mid-cell (Marston et al., 1998). In retrospect, it appears to make sense for the Min system to prevent division from occurring within about one nucleoid-length equivalent from each cell pole (Figures 6A and 6B). Under normal conditions, this could be sufficient to allow division only at mid-cell and in cells that had reached the appropriate length. In preliminary experiments, we found that even slight overproduction of MinCD increases the cell length distribution (L.J.W., unpublished results), consistent with an increase in the length of the pole-proximal part of the cell

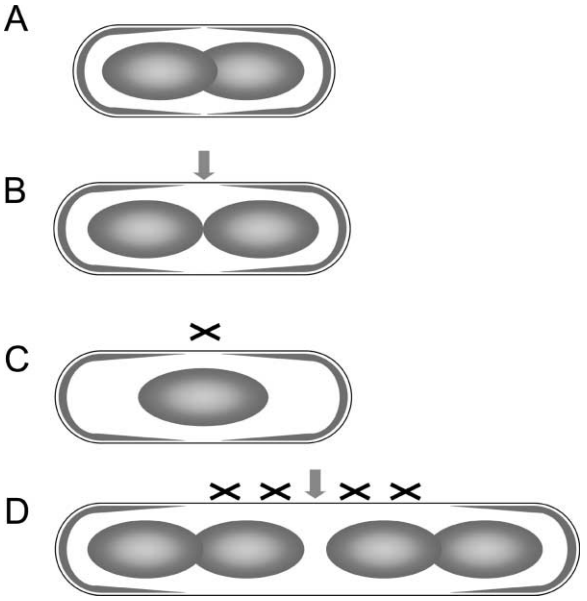

Figure 6. An Extended Role for the Min System in *B. subtilis*

The MinCD division inhibitor activity (gray shading) accumulates at the cell poles and extends out in a diminishing gradient toward mid-cell.

(A and B) Under normal conditions, the Min system is sufficient to specify the correct timing and placement of division. In young (short) cells that have not yet replicated and segregated their chromosome (gray ovals) (A), the Min system prevents division throughout the length of the cell. As the cells grow, and replication and segregation are completed (B), a Min-free space appears at mid-cell, allowing division to occur in the correct place (arrow).

(C and D) Under certain abnormal conditions, nucleoid occlusion, via Noc protein, is needed to prevent inappropriate division (indicated by crosses). (C) Impaired chromosome replication or segregation. (D) Elongated cells with a large Min-free zone.

protected from division. However, in otherwise wild-type cells, the Min system cannot direct proper placement of the division site in cells that are abnormally long or inhibit division in cells that have failed to correctly segregate their replicated chromosomes (Figures 6C and 6D). Here a nucleoid occlusion system becomes crucial. Many bacteria do not contain either a Min system or a recognizable homolog of Noc. It follows that bacteria may have evolved a range of different systems to regulate the correct timing and positioning of their division septum.

Experimental Procedures

Bacterial Strains and General Methods

These are detailed in the Supplemental Data on the *Cell* website.

Depletion of MinD, FtsL/PbpB, or DnaA by Removal of IPTG

To deplete MinD, FtsL/PbpB, or DnaA, cells grown in either PAB or CH with 0.5 mM IPTG were diluted (1 in 100) into the same medium and grown to midexponential phase. Cells were then pelleted, washed once, and diluted in the medium without IPTG to an OD_{600nm} of 0.05 or 0.1. The culture was then divided into two and IPTG was added into one of the cultures and omitted from the other.

Overexpression of YyaA

Strains 1289 and 1290 were grown at 37°C in PAB to exponential phase, diluted 1 in 30 into PAB containing 1 mM IPTG, and continued to grow for 95 min. Cells were then diluted once more into PAB containing 1 mM IPTG to an OD_{600nm} of 0.05 and grown for a further 65 min before examination by fluorescence microscopy.

Spore Germination, Outgrowth, and Inhibition of DNA Replication using HPUra

Spore (2×10^8) germination and outgrowth were performed at 30°C in S medium (Sharpe et al., 1998) supplemented with 0.2 mg/ml of alanine and 0.002% Tween as described by Hamoen and Errington (2003). After 65 min, the DNA polymerase III inhibitor 6-(p-hydroxyphenylazo)-uracil (HPUra: Brown, 1971) was added to a final concentration of 200 μM to block DNA replication.

Overexpression of *ftsZ*/*ftsZ-gfp*

Strains 1296 and 1297 were grown in CH medium containing 0.4 mM IPTG at 30°C for 90 to 120 min then diluted into the same medium to an OD_{600nm} of 0.1. The diluted culture was divided into two, one was supplemented with 0.5% xylose and an extra amount of IPTG (to a final concentration of 1 mM) while the concentration of IPTG of the other remained at 0.4 mM. The cultures were then grown at 37°C and at intervals portions were removed for staining with DAPI and FM5-95.

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References

Brown, N.C. (1971). Inhibition of bacterial DNA replication by 6-(*p*-hydroxyphenylazo)-uracil: differential effect on repair and semiconservative synthesis in *Bacillus subtilis*. J. Mol. Biol. *59*, 1–16.

Cha, J.-H., and Stewart, G.C. (1997). The *divIVA* minicell locus of *Bacillus subtilis*. J. Bacteriol. *179*, 1671–1683.

Cook, W.R., and Rothfield, L.I. (1999). Nucleoid-independent identification of cell division sites in *Escherichia coli*. J. Bacteriol. *181*, 1900–1905.

Daniel, R.A., Williams, A.M., and Errington, J. (1996). A complex four-gene operon containing essential cell division gene *pbpB* in *Bacillus subtilis*. J. Bacteriol. *178*, 2343–2350.

de Boer, P.A.J., Crossley, R.E., and Rothfield, L.I. (1989). A division inhibitor and a topological specificity factor coded for by the minicell locus determine proper placement of the division septum in E. coli. Cell *56*, 641–649.

de Boer, P.A.J., Crossley, R.E., and Rothfield, L.I. (1990). Central role for the *Escherichia coli minC* gene product in two different cell division-inhibition systems. Proc. Natl. Acad. Sci. USA *87*, 1129–1133.

de Boer, P.A.J., Crossley, R.E., and Rothfield, L.I. (1992). Roles of MinC and MinD in the site-specific septation block mediated by the MinCDE system of *Escherichia coli*. J. Bacteriol. *174*, 63–70.

Edwards, D.H., and Errington, J. (1997). The *Bacillus subtilis* DivIVA protein targets to the division septum and controls the site specificity of cell division. Mol. Microbiol. *24*, 905–915.

Errington, J., Daniel, R.A., and Scheffers, D.J. (2003). Cytokinesis in bacteria. Microbiol. Mol. Biol. Rev. *67*, 52–65.

Foley, M., Brass, J.M., Birmingham, J., Cook, W.R., Garland, P.B., Higgins, C.F., and Rothfield, L.I. (1989). Compartmentalization of the periplasm at cell division sites in *Escherichia coli* as shown by fluorescence photobleaching experiments. Mol. Microbiol. *3*, 1329–1336.

Fu, X., Shih, Y.-L., and Rothfield, L.I. (2001). The MinE ring required for proper placement of the division site is a mobile structure that

changes its cellular location during the *Escherichia coli* division cycle. Proc. Natl. Acad. Sci. USA *98*, 980–985.

Glaser, P., Sharpe, M.E., Raether, B., Perego, M., Ohlsen, K., and Errington, J. (1997). Dynamic, mitotic-like behaviour of a bacterial protein required for accurate chromosome partitioning. Genes Dev. *11*, 1160–1168.

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.

Hale, C.A., Meinhardt, H., and de Boer, P.A. (2001). Dynamic localization cycle of the cell division regulator MinE in *Escherichia coli*. EMBO J. *20*, 1563–1572.

Hamoen, L.W., and Errington, J. (2003). Polar targeting of DivIVA in *Bacillus subtilis* is not directly dependent on FtsZ or PBP 2B. J. Bacteriol. *185*, 693–697.

Harry, E.J. (2001). Bacterial cell division: regulating Z-ring formation. Mol. Microbiol. *40*, 795–803.

Harry, E.J., Rodwell, J., and Wake, R.G. (1999). Co-ordinating DNA replication with cell division in bacteria: a link between the early stages of a round of replication and mid-cell Z ring assembly. Mol. Microbiol. *33*, 33–40.

Hu, Z., and Lutkenhaus, J. (2001). Topological regulation of cell division in *E. coli*. spatiotemporal oscillation of MinD requires stimulation of its ATPase by MinE and phospholipid. Mol. Cell *7*, 1337–1343.

Hu, Z., Mukherjee, A., Pichoff, S., and Lutkenhaus, J. (1999). The MinC component of the division site selection system in *Escherichia coli* interacts with FtsZ to prevent polymerization. Proc. Natl. Acad. Sci. USA *96*, 14819–14824.

Hu, Z., Gogol, E.P., and Lutkenhaus, J. (2002). Dynamic assembly of MinD on phospholipid vesicles regulated by ATP and MinE. Proc. Natl. Acad. Sci. USA *99*, 6761–6766.

Kawai, Y., Moriya, S., and Ogasawara, N. (2003). Identification of a protein, YneA, responsible for cell division suppression during the SOS response in *Bacillus subtilis*. Mol. Microbiol. *47*, 1113–1122.

Levin, P.A., Kurtser, I.G., and Grossman, A.D. (1999). Identification and characterization of a negative regulator of FtsZ ring formation in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA *96*, 9642–9647.

Lin, D.C.-H., Levin, P.A., and Grossman, A.D. (1997). Bipolar localization of a chromosome partition protein in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA *94*, 4721–4726.

Margolin, W. (2001). Spatial regulation of cytokinesis in bacteria. Curr. Opin. Microbiol. *4*, 647–652.

Marston, A.L., Thomaides, H.B., Edwards, D.H., Sharpe, M.E., and Errington, J. (1998). Polar localization of the MinD protein of *Bacillus subtilis* and its role in selection of the mid-cell division site. Genes Dev. *12*, 3419–3430.

Moriya, S., Tsujikawa, E., Hassan, A.K.M., Asai, K., Kodama, T., and Ogasawara, N. (1998). A *Bacillus subtilis* gene-encoding protein homologous to eukaryotic SMC motor protein is necessary for chromosome partition. Mol. Microbiol. *29*, 179–187.

Mulder, E., and Woldringh, C.L. (1989). Actively replicating nucleoids influence positioning of division sites in *Escherichia coli* filaments forming cells lacking DNA. J. Bacteriol. *171*, 4303–4314.

Niki, H., Jaffe, A., Imamura, R., Ogura, T., and Hiraga, S. (1991). The new gene *mukB* codes for a 177 kd protein with coiled-coil domains involved in chromosome partitioning of *E. coli*. EMBO J. *10*, 183–193.

Pichoff, S., and Lutkenhaus, J. (2002). Unique and overlapping roles for ZipA and FtsA in septal ring assembly in *Escherichia coli*. EMBO J. *21*, 685–693.

Quisel, J.D., Burkholder, W.F., and Grossman, A.D. (2001). In vivo effects of sporulation kinases on mutant Spo0A proteins in *Bacillus subtilis*. J. Bacteriol. *183*, 6573–6578.

Rajagopalan, S., Wachtler, V., and Balasubramanian, M. (2003). Cytokinesis in fission yeast: a story of rings, rafts and walls. Trends Genet. *19*, 403–408.

Raskin, D.M., and De Boer, P.A.J. (1997). The MinE ring: an FtsZ-independent cell structure required for selection of the correct division site in *E. coli*. Cell *91*, 685–694.

Regamey, A., Harry, E.J., and Wake, R.G. (2000). Mid-cell Z ring assembly in the absence of entry into the elongation phase of the round of replication in bacteria: co-ordinating chromosome replication with cell division. Mol. Microbiol. *38*, 423–434.

Rowland, S.L., Katis, V.L., Partridge, S.R., and Wake, R.G. (1997). DivIB, FtsZ and cell division in *Bacillus subtilis*. Mol. Microbiol. *23*, 295–302.

Sharpe, M.E., and Errington, J. (1995). Postseptational chromosome partitioning in bacteria. Proc. Natl. Acad. Sci. USA *92*, 8630–8634.

Sharpe, M.E., Hauser, P.M., Sharpe, R.G., and Errington, J. (1998). *Bacillus subtilis* cell cycle as studied by fluorescence microscopy: constancy of the cell length at initiation of DNA replication and evidence for active nucleoid partitioning. J. Bacteriol. *180*, 547–555.

Shih, Y.L., Le, T., and Rothfield, L. (2003). Division site selection in *Escherichia coli* involves dynamic redistribution of Min proteins within coiled structures that extend between the two cell poles. Proc. Natl. Acad. Sci. USA *100*, 7865–7870.

Sievers, J., Raether, B., Perego, M., and Errington, J. (2002). Characterization of the *parB*-like *yyaA* gene of *Bacillus subtilis*. J. Bacteriol. *184*, 1102–1111.

Woldringh, C.L. (2002). The role of co-transcriptional translation and protein translocation (transertion) in bacterial chromosome segregation. Mol. Microbiol. *45*, 17–29.

Woldringh, C.L., Mulder, E., Valkenburg, J.A., Wientjes, F.B., Zaritsky, A., and Nanninga, N. (1990). Role of the nucleoid in the toporegulation of division. Res. Microbiol. *141*, 39–49.

Wu, L.J., and Errington, J. (2003). RacA and the Soj-Spo0J system combine to effect polar chromosome segregation in sporulating *Bacillus subtilis*. Mol. Microbiol. *49*, 1463–1475.

Yu, X.C., and Margolin, W. (1999). FtsZ ring clusters in *min* and partition mutants: role of both the Min system and the nucleoid in regulating FtsZ ring localization. Mol. Microbiol. *32*, 315–326.

Yu, X.C., and Margolin, W. (2000). Deletion of the *min* operon results in increased thermosensitivity of an *ftsZ84* mutant and abnormal FtsZ ring assembly, placement, and disassembly. J. Bacteriol. *182*, 6203–6213.