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A sensor histidine kinase coordinates cell wall architecture with cell division in *Bacillus subtilis*

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Summary

The concerted interconnection between processes driving DNA synthesis, division septum formation and cell wall synthesis and remodeling in rapidly growing bacteria requires precise coordination by signaling mechanisms that are, for the most part, unknown. The YycG (sensor histidine kinase), YycF (response regulator/transcription factor) two-component system of *Bacillus subtilis* controls the synthesis of enzymes and their inhibitors that function in cell wall remodeling and cell separation. Here it is shown that the YycG sensor histidine kinase is a component of the division septum in growing cells. RT-PCR quantitation of YycF-PO₄ regulated gene transcription, in wild type and FtsZ-depleted, septum-less cells, indicated that YycG kinase activity on YycF is dependent on YycG localization to a division septum. The data support a model in which the YycG sensor kinase perceives information at the division septum and regulates the reciprocal synthesis of autolysins and autolysin inhibitors to coordinate growth and division with cell wall restructuring.

Keywords

Bacillus subtilis; YycFG; FtsZ; signal transduction; cell division

Introduction

Two-component and phosphorelay signal transduction systems play a major role in communicating environmental information to the cell and in coordinating cellular processes. Environmental signals are processed through these systems to influence transport and metabolism of catabolites, drugs and other compounds and to control motility systems to direct the chemotactic behavior of the cell, among other uses (reviewed in (Baker *et al.*, 2006; Mascher *et al.*, 2006)). These regulatory systems are key components in cell cycle signaling in *Caulobacter crescentus* and in sporulation in *Bacillus subtilis* indicating that they have been adopted to play more crucial roles in cell division and development (reviewed in (Holtzendorff *et al.*, 2006; Piggot and Hilbert, 2004)). However, deletion studies revealed only a few such signaling systems regulate essential cellular processes, thereby rendering the signal transduction system necessary for cellular survival.

One essential signal transduction system, the YycF (response regulator-transcription factor)-YycG (sensor histidine kinase) two-component system has been found to be conserved in most of the Firmicutes but is apparently absent from the genomes of Gram-negative bacteria (Fabret *et al.*, 1999; Ng and Winkler, 2004). Following the initial observation that this system was the

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only one of 34 two-component signal transduction systems required for cell survival by *Bacillus subtilis*, the essentiality of orthologs has been confirmed for the important Gram-positive pathogens *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Enterococcus faecalis* (Fabret *et al.*, 1999; Hancock and Perego, 2004; Martin *et al.*, 1999; Ng *et al.*, 2003).

The cellular role of the YycFG system has been assessed in strains where the cellular concentration of the proteins has been depleted by placing these genes under a controllable promoter. The phenotypes arising upon depletion include defects in cell division and cell wall architecture. *B. subtilis* strains depleted for YycFG form filamentous cells or chains of cells with empty sections (likely a result of cell lysis) whereas over-expression of *yycF* leads to the formation of mini-cells suggesting some component of cell division was regulated by this system (Fabret and Hoch, 1998). This notion was strengthened by the finding of genes, *ftsAZ*, important for cell division as part of the YycF regulon (Fukuchi *et al.*, 2000). Subsequent microarray and DNA-binding studies revealed genes coding for autolysins and autolysin activity modulating proteins to be under YycF expression control, thereby helping to explain cell wall defects in the depletion strain (Bisicchia *et al.*, 2007; Howell *et al.*, 2003). Similar types of gene products were identified to be under YycF control in *S. aureus* and *S. pneumoniae* as well as fatty acid biosynthesis genes in the latter organism (Dubrac *et al.*, 2007; Mohedano *et al.*, 2005; Ng *et al.*, 2003; Ng *et al.*, 2005). In summary, many of the products of the regulated genes play a role in cell wall metabolism even though the individual target genes differ from species to species.

While the number of genes defined by the regulon is near resolution, the signal input sensed by this system has yet to be defined, and therefore its actual role has been mysterious to this point. Whether YycG responds to a small molecule ligand, as do several other sensor kinases, remains unknown. However, the YycG kinase was shown to interact with two proteins YycH and YycI whose genes are commonly expressed from the same operon as *yycFG* (Szurmant *et al.*, 2007b). In both *yycH* and *yycI* deletion strains YycG activity appears constitutively up-regulated (Szurmant *et al.*, 2005; Szurmant *et al.*, 2007b). Crystal structures of the periplasmic domains of both YycH and YycI proteins revealed a common tertiary fold despite low sequence conservation (Santelli *et al.*, 2007; Szurmant *et al.*, 2006) but a function could not be deduced from the structures. Recent experiments indicate that YycG, YycH and YycI form a membrane bound ternary sensor complex that may serve to integrate signals designed to coordinate cell architecture and cell division (Szurmant *et al.*, 2008). What those signals are and from where they emanate are questions that address the integration of signal transduction in the overall homeostasis mechanisms that synchronize metabolism, biosynthesis and structure into cell division.

The studies reported here establish that the YycG sensor histidine kinase is localized to the division septum in growing cells. Inhibition of septum formation by FtsZ depletion affected the nucleoid location of the YycF response regulator suggesting that the YycG induced phosphorylation of YycF requires interaction with the division septum. RT PCR mRNA quantitation studies in FtsZ depleted cells showed that the formation of the division septum was necessary for YycG phosphorylation of YycF. The data support a model whereby the YycFG system receives its signal at the division septum and constitutes a positive feedback loop that serves to coordinate cell division with cell wall homeostasis.

Results

The YycG sensor histidine kinase co-localizes with FtsZ at the cell division site

The critical function of the YycFG two-component system in the homeostatic control of cell wall and surface constituents raised questions about its regulation; in particular, if the distribution of YycG in the cell membrane might play a role in this control. Initial studies to

determine the cellular location of YycG were carried out with green fluorescent protein (GFP) fusions to YycG but the expression of *yycG* was too low to visualize the GFP. To avoid probable artifacts from over expression of *yycG* to raise the cellular level of the GFP fusion, we chose to detect YycG with immunofluorescence in normal exponentially growing cells of *B. subtilis* strain JH642.

The cellular location of YycG was determined by a specific antibody followed by visualization with a fluorescent-labeled secondary antibody in confocal microscopy. From the images obtained (Fig. 1A-B) it was clear that YycG was located in regions corresponding to potential division sites between DAPI-stained nucleoids. Differential Interference Contrast (DIC) microscopy also revealed the YycG location at mid cell (Fig. 1E-F). In order to confirm the possible division site location of YycG, studies were begun to correlate the localization of YycG with FtsZ (Fig. 1C,G), which is well known to be localized with and crucial for the formation of the division septum (Bi and Lutkenhaus, 1991; Wang and Lutkenhaus, 1993). Overlaying the YycG and FtsZ images revealed that the two proteins co-localized (Fig. 1D,H). To quantify co-localization, 227 cells with visible FtsZ and YycG levels were analyzed for YycG and FtsZ localization to the septum. FtsZ appeared localized in all cells whereas YycG was localized in 224 cells and co-localization was observed in 98.7% of the cell population. Thus the YycG sensor kinase appears to be preferentially localized to the division septum and in the same general region occupied by FtsZ.

YycG localization is dependent upon FtsZ

In order to determine whether the observed localization of YycG was dependent on FtsZ, strain KP444, in which the cellular level of FtsZ could be controlled by the IPTG inducible *spac* promoter (Beall and Lutkenhaus, 1991), was used (Supplemental Fig. S1). This strain requires IPTG for division septum formation. Experiments designed to lower the cellular concentration of FtsZ were carried out by removal of IPTG from exponentially growing cells and observation of the positions of FtsZ and YycG one and three hours following IPTG removal (Fig. 2). At the earlier time the cells became elongated filaments with the residual FtsZ concentrated at a few possible division sites. However YycG was found spread out in the filament (perhaps in some aggregate or structure) and was not generally associated with a division site and was not concentrated at sites of residual FtsZ (Fig. 2A). At the later time point the remaining FtsZ appeared diffuse in the filaments along with YycG. The cellular level of YycG was unchanged (Fig. 2B). Thus, YycG localization was dependent on FtsZ to form a normal division septum and the two proteins did not co-localize.

YycG depletion does not interfere with FtsZ localization

A reciprocal experiment to the one described above was done using strain JH25033 in which the *yycG* gene was placed under control of the IPTG inducible *spac* promoter (Supplemental Fig. S1). Depletion of YycG had little effect on growth and after three hours filament formation was not observed consistent with what was previously observed by others utilizing a similar strain (Fukuchi *et al.*, 2000). The majority of the detectable FtsZ protein at this time was still localized to the division septum (Fig. 3A). Quantitative Western immunological analyses revealed a normal level of FtsZ after three hours and greatly diminished cellular concentration of YycG (Fig. 3B). It appears that FtsZ localization is independent of YycG protein levels. However due to the leakiness of the *spac* promoter sufficient YycG levels remain to sustain growth and although unlikely it cannot be ruled out that in the complete absence of YycG FtsZ localization might be effected.

Co-immunoprecipitation of YycG and FtsZ

While the co-localization of immunologically detected YycG and FtsZ proteins suggests they are in the same region of the cell at the same time, the results from this technique cannot prove

that these proteins interact or that they are part of a common protein complex. In order to further investigate their possible association, immunoprecipitation experiments were undertaken. Growing cells of wild type strain JH642 and the conditional *ftsZ* expression strain KP444 grown in the absence and presence of IPTG were exposed to formaldehyde to cross-link associated proteins. Extracts were prepared with detergent in a French Press to solubilize both membrane and cytosolic proteins. After mixing the extracts with antibody to FtsZ, the immune-complexes were purified, separated on SDS-PAGE and either YycG or FtsZ detected in a Western analysis. The results of these experiments are shown in Figure 4.

Immune-complexes initially precipitated with anti-FtsZ antibody were assayed for the presence of FtsZ and for co-precipitation of YycG. For KP444 grown in the presence of IPTG both, a clear FtsZ and a clear YycG band could be detected (Fig. 4) and similar results were obtained for the wild type strain JH642 (data not shown). When KP444 was grown in the absence of IPTG FtsZ was barely visible in the immune-complexes and YycG was not detectable. Additional controls with the antibody raised against the sporulation sensor kinase KinA and the DegS sensor kinase were not reactive on the cross-linked immunoprecipitation lanes (data not shown). The presence of YycG in an immune complex precipitated by anti-FtsZ antibody (Fig. 4) was consistent with the immunofluorescence results and suggested that these proteins either interact at the cell division septum or that both of them are part of a larger multiprotein complex present at that site.

YycG localization to the septum is independent of YycH and YycI

YycH and YycI are known to form a ternary complex with YycG (Szurmant *et al.*, 2007b; Szurmant *et al.*, 2008). To investigate a potential involvement of these auxiliary proteins in YycG localization, YycG positioning was probed in a *yycHI* double deletion strain JH25031 (Fig. S2). No difference in YycG localization was observed when compared to a wild type strain (data not shown) indicating YycG may localize to the division septum even in the absence of YycH and YycI.

Localization of the YycF response regulator to the nucleoid

The YycF response regulator is the transcription factor regulated by the YycG sensor kinase. It was of interest to determine the cellular location of YycF, if possible, under the conditions described above. To this end the immunofluorescent technique was used. In the wild type JH642 cells, YycF specific antibody detected YycF associated with the nucleoid. No YycF was found at the septum or other cytoplasmic locations (Fig. 5A). The effect of FtsZ depletion on YycF was determined with the conditional *ftsZ* expression strain KP444 used previously. In the presence of IPTG the localization of YycF was identical to that observed in JH642 (Fig. 5B). In FtsZ depletion conditions the cells formed filaments as before and YycF was found located throughout the filament and clearly in positions not occupied by the nucleoid (Fig. 5C). Thus the assembly of the Z-Ring by FtsZ and the subsequent septum are required for the observed localization of YycF to the nucleoid. Since binding of YycF to its chromosomal targets is likely to require phosphorylation and FtsZ depletion does not affect either YycF or YycG cellular levels, the results suggest that YycG is inactive as YycF kinase under these conditions.

A similar assay was performed utilizing the conditional *yycG* expression strain JH25033. Unexpectedly, depletion of YycG upon IPTG removal had no obvious effect on YycF localization (data not shown). As mentioned above as well as by others (Fukuchi *et al.*, 2000) low levels of YycG supplied by the leaky *spac* promoter are sufficient to sustain growth and it appears these levels are also sufficient to maintain YycF nucleoid localization. YycF localization was also tested in the *yycHI* deletion strain and did not change in respect to the wild type strain (Supplemental Figure S2).

YycG activation requires a division septum

The YycF localization data suggest that YycG is most active as a kinase of YycF when positioned at the division septum. The instability of the aspartyl-phosphate of YycF precludes direct measurement of phosphorylation levels of the response regulator but its transcriptional activity may be determined. To indirectly assess the phosphorylation of YycF, two genes were chosen that require YycF~PO₄ for regulation, *yocH* and *yjeA* (Bisicchia *et al.*, 2007). YycF~PO₄ is a positive activator of *yocH* transcription and a repressor of *yjeA* transcription. Thus, the messenger RNA levels for these genes should change in opposite directions as a function of the phosphorylation of YycF. As a control, to determine that this indeed happens, RT-PCR measurements of the transcript level of both genes was assayed in a strain, JH17040, in which both YycF and YycG levels were controlled by IPTG (Fig. 6, lanes 4,5 and 6). Shutting off the transcription of these genes by IPTG depletion should result in lowered *yocH* transcription and increased *yjeA* transcription. The anticipated result was obtained; both *yocH* and *yycG* transcripts were not detectable and the amount of *yjeA* transcript increased (Fig. 6). The same experiment was carried out in strain KP444 in which *ftsZ* transcription was regulated by IPTG. Depletion of IPTG and FtsZ in conditions known to prevent division septum formation resulted in identical results for *yocH* and *yjeA* transcripts as observed in the control strain (Fig. 6, lanes 1, 2 and 3). The *yycG* transcript was still produced in these conditions showing that FtsZ depletion did not affect transcription in general. Since the levels of YycG and YycF did not fluctuate under FtsZ depletion and the relevant transcripts mimicked the lowering of YycF~PO₄ levels, it appears that YycG is less active as a kinase of YycF in the absence of a division septum.

Discussion

A large number of cellular processes need to be precisely coordinated for a bacterium to repetitively grow and divide as rapidly as it does. A formidable challenge to this process is the need to coordinate internal events, DNA synthesis, septum formation, etc., with extracellular processes, the degradation and resynthesis of the cell wall at the division septum to insure cell separation while maintaining the structural barrier to lysis. The results reported here and elsewhere implicate the YycFG two-component system as a component in this multifactorial signaling process (Bisicchia *et al.*, 2007; Fukuchi *et al.*, 2000). Two-component systems generally control gene expression and it is enlightening to examine the genes that YycF regulates (Bisicchia *et al.*, 2007). YycF~PO₄ is a positive activator of the expression of genes for autolytic enzymes that cleave at several specific sites in the cell wall peptidoglycan matrix. Presumably these enzymes prepare the cell wall for restructuring and cell separation coincident with the synthesis of the division septum. YycF~PO₄ is also a repressor of genes for proteins that inhibit autolytic enzymes, *yoeB*, and that deacetylate peptidoglycan, *yjeA*, to inhibit the activity of autolysins. Rapidly growing cells depend on autolytic enzymes for cell division whereas these enzymes require inhibition in slow growth or non-growing conditions (Bisicchia *et al.*, 2007). The ratio of YycF~PO₄/YycF, determines the balance of synthesis of autolysins and their inhibitors. This ratio must be high in rapidly growing cells, to favor autolysin synthesis, and low in non-growing cells to favor synthesis of autolysin inhibitors. Thus, YycF may be thought of as a homeostatic regulator tuned to the division state of cells.

Since the phosphorylation state of YycF depends on the activity of the YycG sensor kinase, YycG kinase becomes an arbitrator of the state of the cell by responding to cellular signals. These signals are most likely recognized by the YycG periplasmic domain, its internal PAS and HAMP domains, and the YycH and YycI proteins with which it associates in a trans-membrane complex (Szurmant *et al.*, 2008). None of these signals are known and both agonists and antagonists probably exist. The experiments reported here show that YycG is located in the cell at the division septum in a position where it is positioned to interpret the state of septum

growth and cell division. Moreover, the RT-PCR experiments indicate that location at the division septum is a requirement for YycG phosphorylation of YycF. There are a number of mechanistic and signaling interpretations for this result that could be imagined. The simple, uncomplicated explanation is that YycG is most active as a kinase of YycF when associated with the division septum (Fig. 7).

The question of what drives the localization of YycG to the division septum remains unanswered. Data mentioned here indicate that YycG is still septum localized in mutants lacking both YycH and YycI indicating some domain of YycG must be involved. Equally elusive is the question of what YycG contacts when located at the septum.

Immunoprecipitation experiments showed that FtsZ antibody could coprecipitate YycG from extracts of cells in which the proteins were cross-linked, *in vivo*, with formaldehyde. Close association between proteins ($\sim 2\text{\AA}$) is needed for cross-linking with this agent, yet substantially more experimentation would be needed to distinguish between a direct interaction of YycG and FtsZ and indirect association with other proteins in common between the two. In any case, YycG joins an ever-growing number of proteins that are being found to localize to the septum in dividing cells since the initial discovery of the Z-ring and subsequent establishment of localization of essential cell divisional proteins to this cellular compartment (reviewed in (Dajkovic and Lutkenhaus, 2006; Goehring and Beckwith, 2005; Margolin, 2005)). In both *E. coli* and *B. subtilis*, the divisome assembles in a sequential manner and localization of later proteins to the complex depends on earlier ones (reviewed in (Errington *et al.*, 2003)). Association with the divisome could indicate the origin of signals for YycG activity if it were embedded within the divisome complex.

If the YycG kinase were an integral part of the divisome, required for its assembly, one would anticipate the protein's essentiality to be at least partially independent of its kinase activity. However Ogasawara and colleagues reported successful disruption of the YycG kinase in a *B. subtilis* strain over-expressing an assumed constitutively active YycF with a point mutation (D56H) (Fukuchi *et al.*, 2000). The strain was not further analyzed for phenotypes but the evidence suggested that the YycG protein was not essential for divisome assembly or its integrity.

The YycH and YycI auxiliary proteins are known to regulate kinase activity and assemble into a complex with the kinase, independent of FtsZ levels, suggesting that formation of this complex precedes participation in the divisome (unpublished data). Furthermore, neither YycH nor YycI are required for YycG localization to the septa as mentioned above. One can imagine two different mechanisms for YycG activation: one that involves localization of the entire YycGHI sensor complex to the septum and subsequent signal recognition; another in which YycG becomes activated by being physically separated from its regulatory proteins YycH and YycI during cell septation. The former model requires that YycH and YycI co-localize with YycG at the septum and the latter that they do not. This is currently under investigation.

A mechanism by which spatial localization plays a role in the activity of a signal transduction system has been well established for *Caulobacter crescentus*. Here a complex circuit of proteins is known to regulate the phosphorylation levels and proteolysis of the essential response regulator (RR) CtrA (Biondi *et al.*, 2006; McGrath *et al.*, 2006). Differential localization of the DivJ kinase and PleC phosphatase to opposite cell poles plays a role in asymmetric cell division (Wheeler and Shapiro, 1999). Both proteins act on the intermediary RR DivK, which in turn inhibits phosphorylation of CtrA indirectly via the kinase CckA (Biondi *et al.*, 2006). Indeed there are strong similarities between the *B. subtilis* YycFG system and the system controlling asymmetric development in *C. crescentus*. Aside from the fact that they are both essential systems they also regulate expression of operons encoding essential cell divisional

proteins, i.e. *ftsAZ* in *B. subtilis* and *ftsQA* in *C. crescentus* (Fukuchi *et al.*, 2000; Wortinger *et al.*, 2000). Not surprisingly phenotypes for depletion strains include elongated cells in both organisms. A model involving interaction of FtsQA with the DivK RR has recently been proposed (Biondi *et al.*, 2006). Therefore a number of protein interactions and auxiliary proteins render both pathways complicated control circuits of essential processes.

Recently, the MtrAB two-component system that is conserved in the high G+C Gram-positive actinomycetes was shown to be among the group of essential two-component systems (reviewed in (Hoskisson and Hutchings, 2006)). This system appears to serve a role in these organisms analogous to that of YycFG system in the low G+C Gram positive bacteria. Phenotypes observed upon depletion or deletion of this system include elongated cells for *Corynebacterium glutamicum* (where the system is not essential) and *Mycobacterium avium* (where only the RR is essential) and an increased sensitivity to antibiotics targeting cell wall synthesis (Cangelosi *et al.*, 2006; Moker *et al.*, 2004). In *Mycobacterium tuberculosis* (where MtrA and MtrB are both essential) the essential DNA replication gene *dnaA* appears to be under control of the MtrAB system, and links between *mtrAB* expression and FtsZ polymerization have also been reported (Fol *et al.*, 2006; Slayden *et al.*, 2006). Furthermore, the conserved lipoprotein LpqB, associated with MtrAB, was proposed to regulate the activity of the MtrB kinase. It is conceivable that this could serve a function similar to that of YycH and YycI in *B. subtilis* (Hoskisson and Hutchings, 2006).

It now appears that two-component signal transduction systems play a key role in the coordination of cell architecture and cell division in a wide variety of bacteria. Certainly this represents only a glimpse into the complexity of these controls and higher order regulatory processes will unquestionably be revealed.

Experimental Procedures

Culture media and growth conditions

All *Escherichia coli* strains were grown in Luria-Bertani (LB) broth and all *B. subtilis* strains were grown in Schaeffer's Sporulation Medium (SM), in the presence of appropriate antibiotics whenever necessary. Antibiotic concentrations were 100µg/ml ampicillin or 30µg/ml kanamycin for *E. coli* and 2µg/ml of kanamycin, 5µg/ml of chloramphenicol or 1µg/ml of phleomycin for *B. subtilis*.

Construction of strains and plasmids

E. coli strains used were DH5α and JM109 for cloning and propagation purposes. All *B. subtilis* strains were derivatives of JH642 except KP444. Plasmids and strains are listed in table 1.

To create an overexpression vector for a C-terminal 6xhis-tagged YycF protein, the *yycF* gene was PCR amplified with oligonucleotides introducing 5'NcoI and 3'XhoI sites. The resulting fragment was cloned in the respective sites of vector pET28a (Novagen) creating plasmid pJT04. Similarly, the gene fragment coding for the cytoplasmic domain of YycG (codons 213-stop codon) was PCR amplified introducing 5' NdeI and 3' BamHI sites and cloned in the same sites of vector pET28a. The resulting plasmid pJT05 expresses an N-terminal 6xhis-tagged cytoplasmic YycG fragment.

To create a maltose binding protein tagged FtsZ overexpression vector, the *ftsZ* gene was PCR amplified introducing 5' KpnI and 3' XbaI sites and the resulting product was cloned in the same sites of vector pMAL-c2E (New England Biolabs), resulting in plasmid pJT6.

Construction of a strain harboring an IPTG inducible *ycyG* gene

The *ycyG* coding sequence and ribosome binding site (*B. subtilis* 168 coordinates 4150876 to 4152743) was PCR amplified introducing 5' HindIII and 3' BamHI sites. The resulting fragment was cloned in the respective sites of the *amyE* integration vector pJM119 resulting in plasmid pJMS6. *B. subtilis* strain JH642 was transformed with ScaI linearized vector pJMS6 resulting in double-cross over integration into the *amyE* locus and producing strain JH25032. This strain harbors an additional copy of *ycyG* under control of the isopropyl- β -D-thiogalactopyranoside (IPTG) inducible *spac* promoter.

To delete the wild type copy of *ycyG* from strain JH25032, plasmid pJT03 was constructed in a multi-step process. First, the 3' portion of the *ycyF* gene (coordinates 4152711 to 4153154) was amplified by PCR and blunt-end ligated to ScaI-digested vector pJM117 resulting in pJMS1. Second, the 5' portion of the *ycyH* gene (coordinates 4150499 to 4150920) was PCR amplified introducing HindIII sites and ligated into HindIII digested pJMS1, producing plasmid pJMS3. Vector pJMS3 was partially digested with EcoRI and PvuII and the 3.2 kbp fragment was subcloned into EcoRI-NaeI-digested pBluescript KS(+) resulting in pJT01.

Next, the promoter region of *ycyF* (coordinates 4153014 to 4153936) was PCR amplified introducing 5' and 3' BamHI sites. This 0.9 kbp fragment was digested with BamHI and MboI and the resulting 0.5 kbp fragment was cloned into BglII digested vector pHY300PLK, producing vector pJT02. Lastly, the *XbaI-SmaI* fragment from pJT02 was subcloned into *XbaI-AfeI*-digested pJT01 thus replacing the P_{spac} promoter with the *ycyF* promoter, resulting in pJT03. The BglII linearized pJT03 was used for double-crossover integration in strain JH25032 at the *ycyG* locus, resulting in strain JH25033. This strain features a single IPTG inducible copy of *ycyG* (supplemental Figure S1B).

Production and purification of YcyF, YcyG and FtsZ

The YcyF-6xHis protein was over-expressed in LB medium by IPTG induction of the *E. coli* BL21(DE3) strain containing pJT04. Cells were broken with a French press, the cell lysate was centrifuged, and the supernatant was applied to a nickel-NTA agarose column (Qiagen). After YcyF-6xHis was eluted with a step gradient of imidazole, the protein was dialyzed against YcyF buffer (50mM Tris-HCl pH 7.6, 20mM KCl, 5mM β -mercaptoethanol). The solution was applied to Q-sepharose column (Amersham Bioscience), and the protein was eluted with YcyF buffer in a gradient of KCl (20 to 500 mM). Finally, the protein was dialyzed against borate buffer (20mM borate, 20mM NaCl, 5 mM β -mercaptoethanol, pH 9.0) to prepare the protein for affinity column purification.

The cytoplasmic region of YcyG fused to a histidine-tag (6xHis-YcyG_C) was induced by IPTG in LB medium from *E. coli* BL21(DE3) harboring pJT05. The protein was purified with a nickel-NTA agarose column as described for the purification of YcyF-6xHis. The eluted 6xHis-YcyG_C was dialyzed against borate buffer (20mM borate, 50mM NaCl, pH 9.0) to prepare the protein for affinity column purification.

The entire FtsZ protein fused to MalE (MBP-FtsZ) and the maltose binding protein (MBP alone) were over-expressed by IPTG induction from *E. coli* JM109 containing pJT06 and pMAL-c2E, respectively. Cells harvested by centrifugation were resuspended in TS buffer (20mM Tris-HCl pH 7.4, 0.2M NaCl) with phenylmethylsulfonyl fluoride (PMSF) and were broken with lysozyme and sonication. The cell lysate was centrifuged and the supernatant was applied to an amylose resin column (New England BioLabs). The MBP-FtsZ and MBP proteins were eluted with TS buffer containing 10mM maltose, and then both eluates were dialyzed against borate buffer (20mM borate, 50mM NaCl, pH 9.0).

Affinity purification of anti-YycF, anti-YycG and anti-FtsZ antibodies

For the preparation of the affinity columns for purification of anti-YycF, anti-YycG and anti-FtsZ antibodies, purified YycF-6xHis (4.7mg), 6xHis-YycG_C (3.8mg), MBP-FtsZ (0.8mg) and MBP (3.6mg) were cross-linked with 1 ml of Reacti-Gel (6x) Support (PIERCE) according to the manufacturer's instructions.

Antibodies raised against YycF or YycG were affinity purified from rabbit antiserum as previously described utilizing the appropriate affinity columns (Szurmant *et al.*, 2007a). Similarly, FtsZ antibody was affinity purified from sheep antiserum with the following modification. The eluate from the MBP-FtsZ column, concentrated and dialyzed against PBS was applied to the affinity column cross-linked with MBP in order to purify the IgG reacting with only FtsZ protein. The unbound IgG (anti-FtsZ antibodies) was collected as flow through.

Immunofluorescence microscopy

Wild-type (JH642) and conditional *yycG* and *ftsZ* mutant strains were incubated at 37°C in SM with or without 1 mM IPTG, and samples were taken at desired time points. YycG, YycF and FtsZ localization in fixed cells was detected immunologically as previously described (Szurmant *et al.*, 2007a). Primary purified antibodies were used at 1:20 (YycG), 1:50 (YycF) or 1:100 (FtsZ) dilutions. Secondary antibodies were Alexa Fluor goat anti-rabbit IgG or Alexa Fluor 546 donkey anti-sheep IgG (Molecular Probes) used at dilutions of 1:500 and 1:1000, respectively. The samples were observed and photographed with a Bio-Rad Radiance 2100 Rainbow laser scanning confocal microscope (LSCM) (running under Bio-Rad LaserSharp 2000 software) equipped with blue diode/argon/green HeNe/red diode laser and a Nikon TE2000-U microscope (X60 PlanApo oil-immersion objective; numerical aperture 1.4). A series of optical sections collected at 100 nm intervals in the z-axis was used to create the images. All pictures were processed with Adobe Photoshop software.

Immunoprecipitation experiments

B. subtilis cells for immunoprecipitation were prepared as described (Molle *et al.*, 2003). Briefly, cells were grown in 100 ml SM at 37°C until $A_{600\text{ nm}} = 0.3$. Cells were fixed with formaldehyde solution (1.33% final concentration) in 32 mM sodium phosphate buffer at pH 7.4 for 20-30 min. Following washing with phosphate buffered saline (PBS) cells were suspended in IP buffer (PBS containing 10mg/ml of lysozyme, 0.1M EDTA, 1% Triton X-100 and protease inhibitor cocktail [SIGMA]), the suspension was kept at 37°C for 1 h before a passage through a French press. Cell debris was removed by centrifugation and the cell extract was completely cleared by gentle shaking for 30 min followed by an additional centrifugation step.

1 ml of cross-linked cellular extract was subjected to immunoprecipitation by addition of purified antibody raised against FtsZ (1:150), or YycG (1:500) After 1 h incubation at room temperature (FtsZ) or overnight incubation at 4°C (YycG, YycH and YycI) the mixture was cleared by centrifugation, the supernatant was mixed with 40-60 μ l of recombinant Protein G agarose (Invitrogen) and gently shaken for 1 h (FtsZ) or 5 h (YycG, YycH and YycI) at room temperature. The resin was washed with PBS containing 1 % Triton X-100 and the immunocomplexes were eluted either with 0.2 M glycine pH 2.2 or by boiling with SDS sample buffer. Glycine eluted samples were neutralized by addition of 1M Tris-HCl (pH 8.0). Samples were used in immunoblot analysis in amounts corresponding to cell density $A_{600\text{ nm}} = 0.1$

Immunoblot analysis

YycG and FtsZ were visualized immunologically as described (Szurmant *et al.*, 2007a). Purified antibodies were used at 1:10,000 dilutions for rabbit anti-YycG antibody and at

1:30,000 dilutions for sheep anti-FtsZ antibody. Secondary horseradish peroxidase-labeled antibodies were used at 1:10,000 dilutions.

RT-PCR

Cells were grown to an OD_{525nm} of 0.3 in the presence of 1 mM IPTG, collected by centrifugation, washed and incubated in fresh media with or without 1 mM IPTG and incubated for an additional 3 h. 15-20 OD_{525nm} units of cells were collected, total RNA isolated, treated with DNAase I and quantified as described except that the cells were lysed with 6 mg/ml lysozyme (Farrell Jr., 2005). RNA (1 µg) was used as template to produce cDNA utilizing SuperScript III Reverse Transcriptase (Invitrogen), following manufactures recommendation in a total volume of 20 µl. 5 µl of the resulting cDNA was used in a standard PCR reaction, and resulting DNA was visualized by agarose gel electrophoresis and ethidium bromide staining. To assure absence of DNA contamination the same procedure was also performed for each pair of oligonucleotides and mRNA preparation in the absence of reverse transcriptase and in all experiments, no PCR product was detected (Supplemental Fig. S3).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Baker MD, Wolanin PM, Stock JB. Signal transduction in bacterial chemotaxis. *Bioessays* 2006;28:9–22. [PubMed: 16369945]
- Beall B, Lutkenhaus J. FtsZ in *Bacillus subtilis* is required for vegetative septation and for asymmetric septation during sporulation. *Genes Dev* 1991;5:447–455. [PubMed: 1848202]
- Bi EF, Lutkenhaus J. FtsZ ring structure associated with division in *Escherichia coli*. *Nature* 1991;354:161–164. [PubMed: 1944597]
- Biondi EG, Reisinger SJ, Skerker JM, Arif M, Perchuk BS, Ryan KR, Laub MT. Regulation of the bacterial cell cycle by an integrated genetic circuit. *Nature* 2006;444:899–904. [PubMed: 17136100]
- Bisicchia P, Noone D, Lioliou E, Howell A, Quigley S, Jensen T, Jarmer H, Devine KM. The essential YycFG two-component system controls cell wall metabolism in *Bacillus subtilis*. *Mol Microbiol* 2007;65:180–200. [PubMed: 17581128]
- Cangelosi GA, Do JS, Freeman R, Bennett JG, Semret M, Behr MA. The two-component regulatory system *mtrAB* is required for morphotypic multidrug resistance in *Mycobacterium avium*. *Antimicrob Agents Chemother* 2006;50:461–468. [PubMed: 16436697]
- Dajkovic A, Lutkenhaus J. Z ring as executor of bacterial cell division. *J Mol Microbiol Biotechnol* 2006;11:140–151. [PubMed: 16983191]
- Dubrac S, Boneca IG, Poupel O, Msadek T. New insights into the WalK/WalR (YycG/YycF) essential signal transduction pathway reveal a major role in controlling cell wall metabolism and biofilm formation in *Staphylococcus aureus*. *J Bacteriol* 2007;189:8257–8269. [PubMed: 17827301]

- Errington J, Daniel RA, Scheffers DJ. Cytokinesis in bacteria. *Microbiol Mol Biol Rev* 2003;67:52–65. [PubMed: 12626683] table of contents.
- Fabret C, Hoch JA. A two-component signal transduction system essential for growth of *Bacillus subtilis*: implications for anti-infective therapy. *J Bacteriol* 1998;180:6375–6383. [PubMed: 9829949]
- Fabret C, Feher VA, Hoch JA. Two-component signal transduction in *Bacillus subtilis*: how one organism sees its world. *J Bacteriol* 1999;181:1975–1983. [PubMed: 10094672]
- Farrell, RE, Jr.. *RNA Methodologies, A Laboratory Guide for Isolation and Characterization*. Elsevier Academic Press; Burlington: 2005.
- Fol M, Chauhan A, Nair NK, Maloney E, Moomey M, Jagannath C, Madiraju MV, Rajagopalan M. Modulation of *Mycobacterium tuberculosis* proliferation by MtrA, an essential two-component response regulator. *Mol Microbiol* 2006;60:643–657. [PubMed: 16629667]
- Fukuchi K, Kasahara Y, Asai K, Kobayashi K, Moriya S, Ogasawara N. The essential two-component regulatory system encoded by *yycF* and *yycG* modulates expression of the *ftsAZ* operon in *Bacillus subtilis*. *Microbiology* 2000;146(Pt 7):1573–1583. [PubMed: 10878122]
- Goehring NW, Beckwith J. Diverse paths to midcell: assembly of the bacterial cell division machinery. *Curr Biol* 2005;15:R514–526. [PubMed: 16005287]
- Hancock LE, Perego M. Systematic inactivation and phenotypic characterization of two-component signal transduction systems of *Enterococcus faecalis* V583. *J Bacteriol* 2004;186:7951–7958. [PubMed: 15547267]
- Holtzendorff J, Reinhardt J, Viollier PH. Cell cycle control by oscillating regulatory proteins in *Caulobacter crescentus*. *Bioessays* 2006;28:355–361. [PubMed: 16547950]
- Hoskisson PA, Hutchings MI. MtrAB-LpqB: a conserved three-component system in actinobacteria? *Trends Microbiol* 2006;14:444–449. [PubMed: 16934981]
- Howell A, Dubrac S, Andersen KK, Noone D, Fert J, Msadek T, Devine K. Genes controlled by the essential YycG/YycF two-component system of *Bacillus subtilis* revealed through a novel hybrid regulator approach. *Mol Microbiol* 2003;49:1639–1655. [PubMed: 12950927]
- Margolin W. FtsZ and the division of prokaryotic cells and organelles. *Nat Rev Mol Cell Biol* 2005;6:862–871. [PubMed: 16227976]
- Martin PK, Li T, Sun D, Biek DP, Schmid MB. Role in cell permeability of an essential two-component system in *Staphylococcus aureus*. *J Bacteriol* 1999;181:3666–3673. [PubMed: 10368139]
- Mascher T, Helmann JD, Uden G. Stimulus perception in bacterial signal-transducing histidine kinases. *Microbiol Mol Biol Rev* 2006;70:910–938. [PubMed: 17158704]
- McGrath PT, Iniesta AA, Ryan KR, Shapiro L, McAdams HH. A dynamically localized protease complex and a polar specificity factor control a cell cycle master regulator. *Cell* 2006;124:535–547. [PubMed: 16469700]
- Mohedano ML, Overweg K, de la Fuente A, Reuter M, Altabe S, Mulholland F, de Mendoza D, Lopez P, Wells JM. Evidence that the essential response regulator YycF in *Streptococcus pneumoniae* modulates expression of fatty acid biosynthesis genes and alters membrane composition. *J Bacteriol* 2005;187:2357–2367. [PubMed: 15774879]
- Moker N, Brocker M, Schaffer S, Kramer R, Morbach S, Bott M. Deletion of the genes encoding the MtrA-MtrB two-component system of *Corynebacterium glutamicum* has a strong influence on cell morphology, antibiotics susceptibility and expression of genes involved in osmoprotection. *Mol Microbiol* 2004;54:420–438. [PubMed: 15469514]
- Molle V, Nakaura Y, Shivers RP, Yamaguchi H, Losick R, Fujita Y, Sonenshein AL. Additional targets of the *Bacillus subtilis* global regulator CodY identified by chromatin immunoprecipitation and genome-wide transcript analysis. *J Bacteriol* 2003;185:1911–1922. [PubMed: 12618455]
- Ng WL, Robertson GT, Kazmierczak KM, Zhao J, Gilmour R, Winkler ME. Constitutive expression of PcsB suppresses the requirement for the essential VicR (YycF) response regulator in *Streptococcus pneumoniae* R6. *Mol Microbiol* 2003;50:1647–1663. [PubMed: 14651645]
- Ng WL, Winkler ME. Singular structures and operon organizations of essential two-component systems in species of *Streptococcus*. *Microbiology* 2004;150:3096–3098. [PubMed: 15470090]

- Ng WL, Tsui HC, Winkler ME. Regulation of the *pspA* virulence factor and essential *pcsB* murein biosynthetic genes by the phosphorylated VicR (YycF) response regulator in *Streptococcus pneumoniae*. *J Bacteriol* 2005;187:7444–7459. [PubMed: 16237028]
- Piggot PJ, Hilbert DW. Sporulation of *Bacillus subtilis*. *Curr Opin Microbiol* 2004;7:579–586. [PubMed: 15556029]
- Santelli E, Liddington RC, Mohan MA, Hoch JA, Szurmant H. The crystal structure of *Bacillus subtilis* YycI reveals a common fold for two members of an unusual class of sensor histidine kinase regulatory proteins. *J Bacteriol* 2007;189:3290–3295. [PubMed: 17307848]
- Slayden RA, Knudson DL, Belisle JT. Identification of cell cycle regulators in *Mycobacterium tuberculosis* by inhibition of septum formation and global transcriptional analysis. *Microbiology* 2006;152:1789–1797. [PubMed: 16735741]
- Szurmant H, Nelson K, Kim EJ, Perego M, Hoch JA. YycH regulates the activity of the essential YycFG two-component system in *Bacillus subtilis*. *J Bacteriol* 2005;187:5419–5426. [PubMed: 16030236]
- Szurmant H, Zhao H, Mohan MA, Hoch JA, Varughese KI. The crystal structure of YycH involved in the regulation of the essential YycFG two-component system in *Bacillus subtilis* reveals a novel tertiary structure. *Protein Sci* 2006;15:929–934. [PubMed: 16600972]
- Szurmant H, Fukushima T, Hoch JA. The Essential YycFG Two-Component System of *Bacillus subtilis*. *Methods Enzymol* 2007a;422:396–417. [PubMed: 17628151]
- Szurmant H, Mohan MA, Imus PM, Hoch JA. YycH and YycI interact to regulate the essential YycFG two-component system in *Bacillus subtilis*. *J Bacteriol* 2007b;189:3280–3289. [PubMed: 17307850]
- Szurmant H, Bu L, Brooks CL III, Hoch JA. An essential sensor histidine kinase controlled by transmembrane helix interactions with its auxiliary proteins. *Proc Natl Acad Sci U S A* 2008;105:5891–5896. [PubMed: 18408157]
- Wang X, Lutkenhaus J. The FtsZ protein of *Bacillus subtilis* is localized at the division site and has GTPase activity that is dependent upon FtsZ concentration. *Mol Microbiol* 1993;9:435–442. [PubMed: 8412693]
- Wheeler RT, Shapiro L. Differential localization of two histidine kinases controlling bacterial cell differentiation. *Mol Cell* 1999;4:683–694. [PubMed: 10619016]
- Wortinger M, Sackett MJ, Brun YV. CtrA mediates a DNA replication checkpoint that prevents cell division in *Caulobacter crescentus*. *Embo J* 2000;19:4503–4512. [PubMed: 10970844]

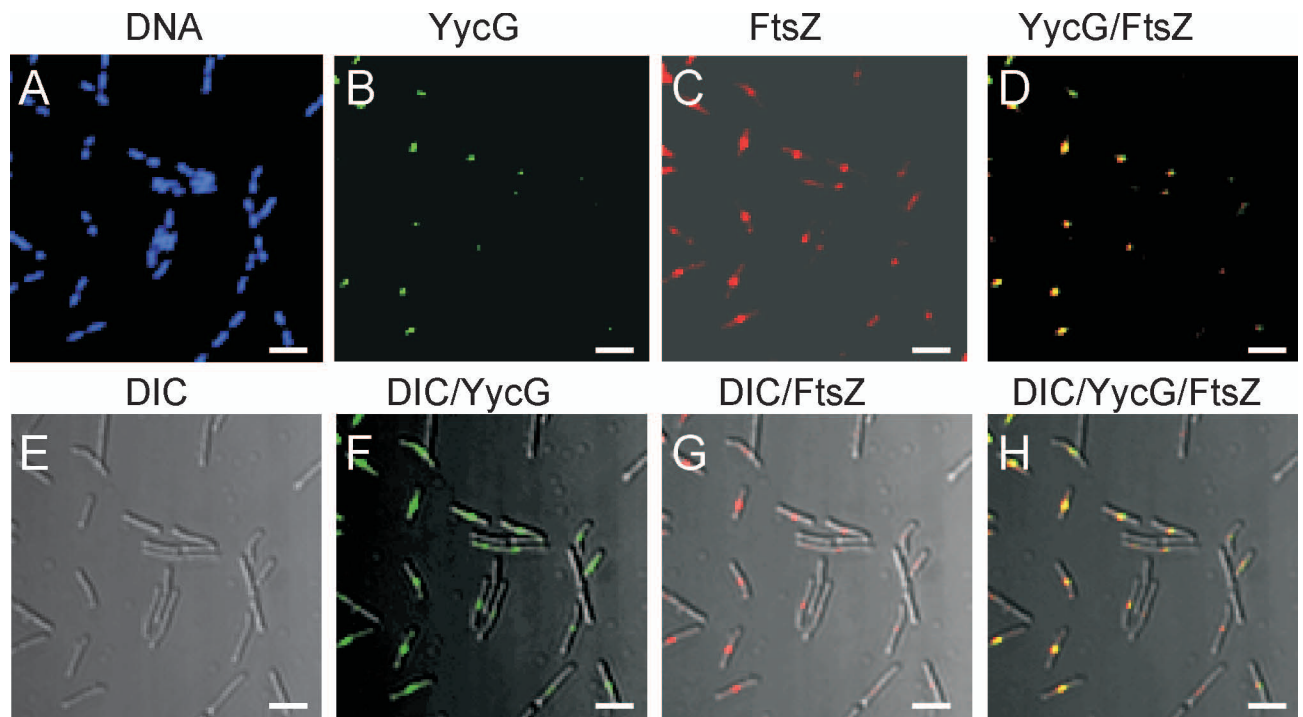


Figure 1.

YycG and FtsZ co-localize to the septum in the wildtype *B. subtilis* strain JH642. YycG (green) and FtsZ (red) proteins were (A-D) visualized immunologically by confocal microscopy and overlain with (E-H) differential interference contrast images, DIC, in exponentially growing cells of JH642 as outlined in Materials and Methods. DNA was visualized by DAPI staining (blue). Bars indicate 5 μm .

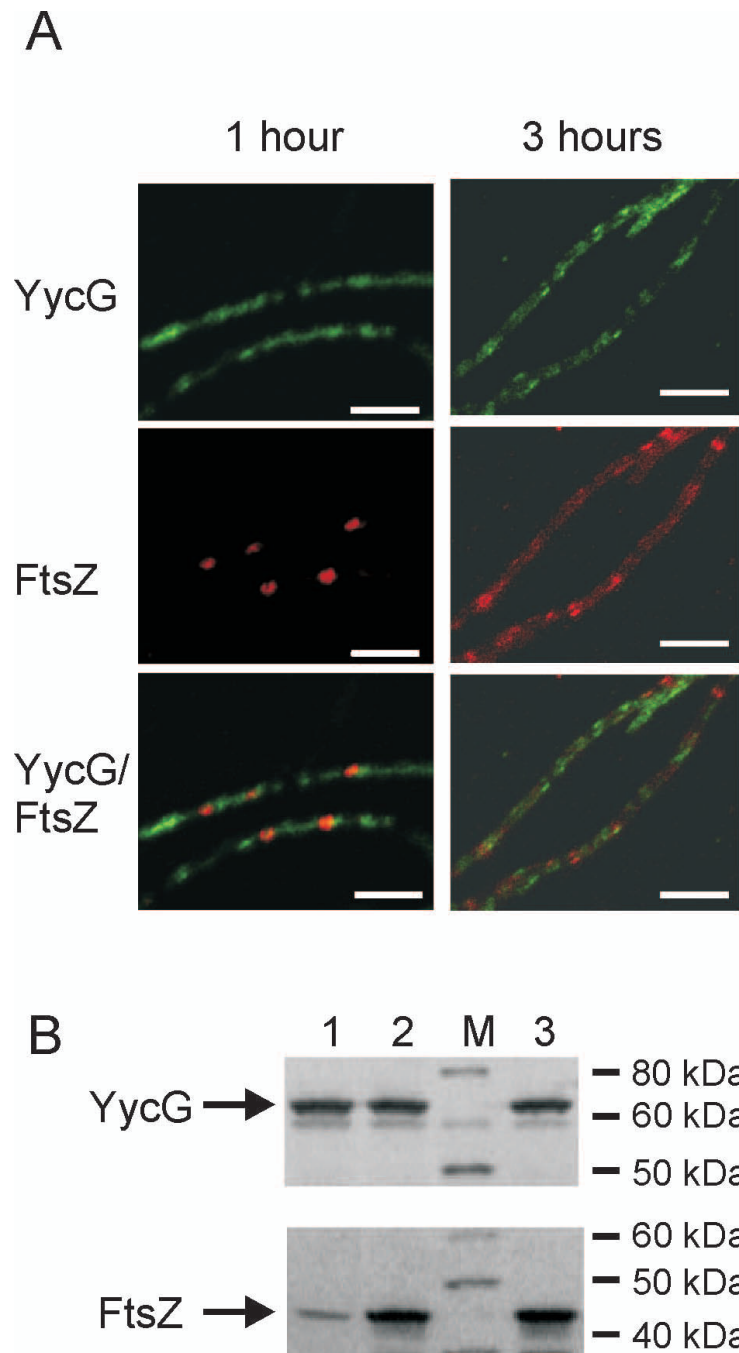


Figure 2.

YycG does not localize to the septum in the FtsZ-depleted strain. (A) YycG (green) and FtsZ (red) were visualized immunologically in KP444 cells following 1 and 3 hours growth at 37° C in the absence of IPTG to repress FtsZ expression. Bars indicate 5 μm. (B) Cellular protein levels of YycG and FtsZ were visualized by western blotting using whole cell protein extract derived from strain KP444 grown for one hour either in the absence (lane 1) or presence of 1 mM IPTG (lane 2) or from wild type strain JH642 (lane 3) as reference; Lane M, molecular weight standard.

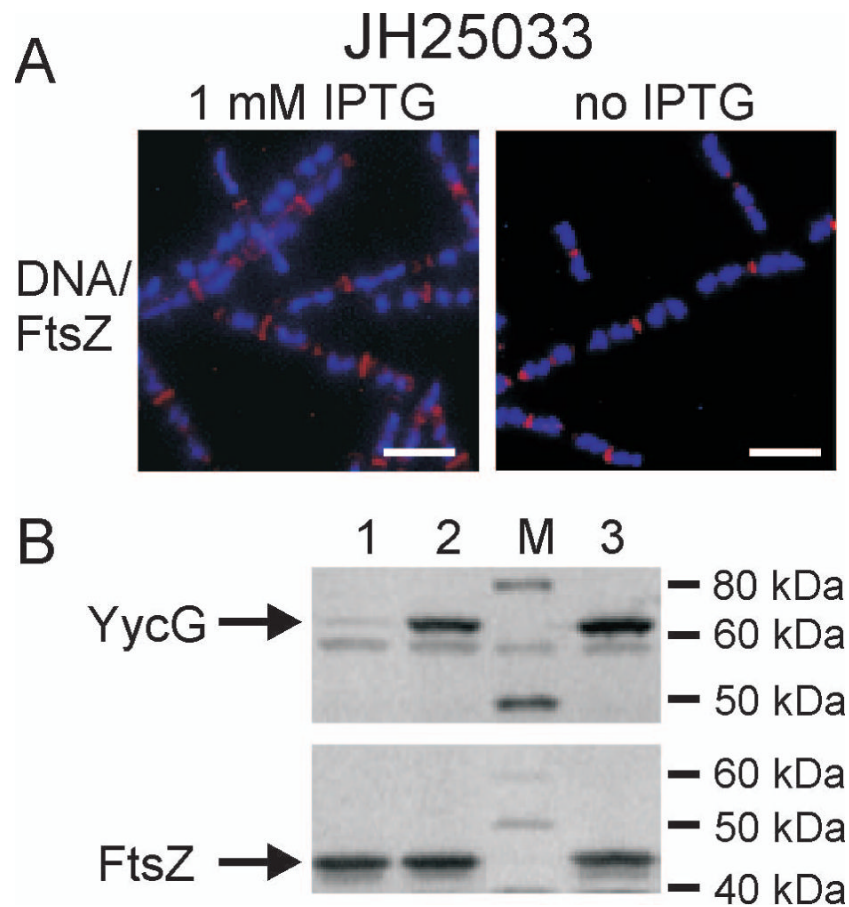


Figure 3.

FtsZ localizes to the septum independently of YycG. (A) FtsZ (red) localization was visualized immunologically in strain JH25033 grown three hours either in the presence of 1 mM IPTG (left panel) or in the absence of IPTG (right panel) to induce YycG depletion. DNA (blue) was visualized by DAPI staining. (B) Cellular protein levels of YycG and FtsZ were visualized by western blotting using whole cell protein extract derived from strain JH25033 grown three hours either in the absence (lane 1) or presence of 1 mM IPTG (lane 2) or from wild type strain JH642 (lane3) as reference; Lane M, molecular weight standard.

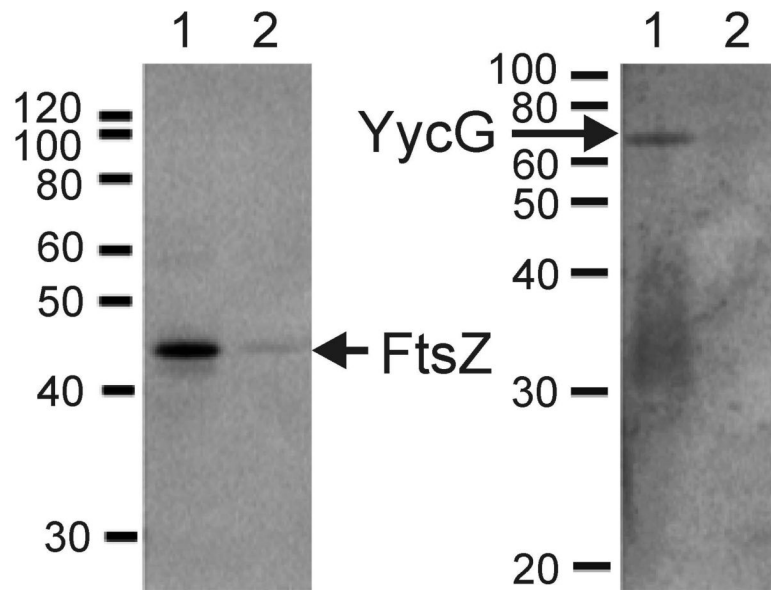


Figure 4.

YycG co-precipitates with FtsZ in an immunoprecipitation assay. Exponentially growing KP444 cells in the presence of IPTG ($OD_{525}=0.3$) were collected and suspended in media either supplemented with 1 mM IPTG or not. After 1 h, cultures were subjected to cross-linking and immunoprecipitation from cell extracts with purified anti-FtsZ antibodies as described in Material and Methods. FtsZ (left panel) and YycG (right panel) proteins were visualized immunologically by Western blot analysis using anti-YycG or anti-FtsZ antibody, respectively. Lanes 1 and 2 of each gel contain cross-linked sample immunoprecipitated with anti-FtsZ antibody from KP444 grown in the presence of 1 mM IPTG (lane 1) or in its absence (lane 2).

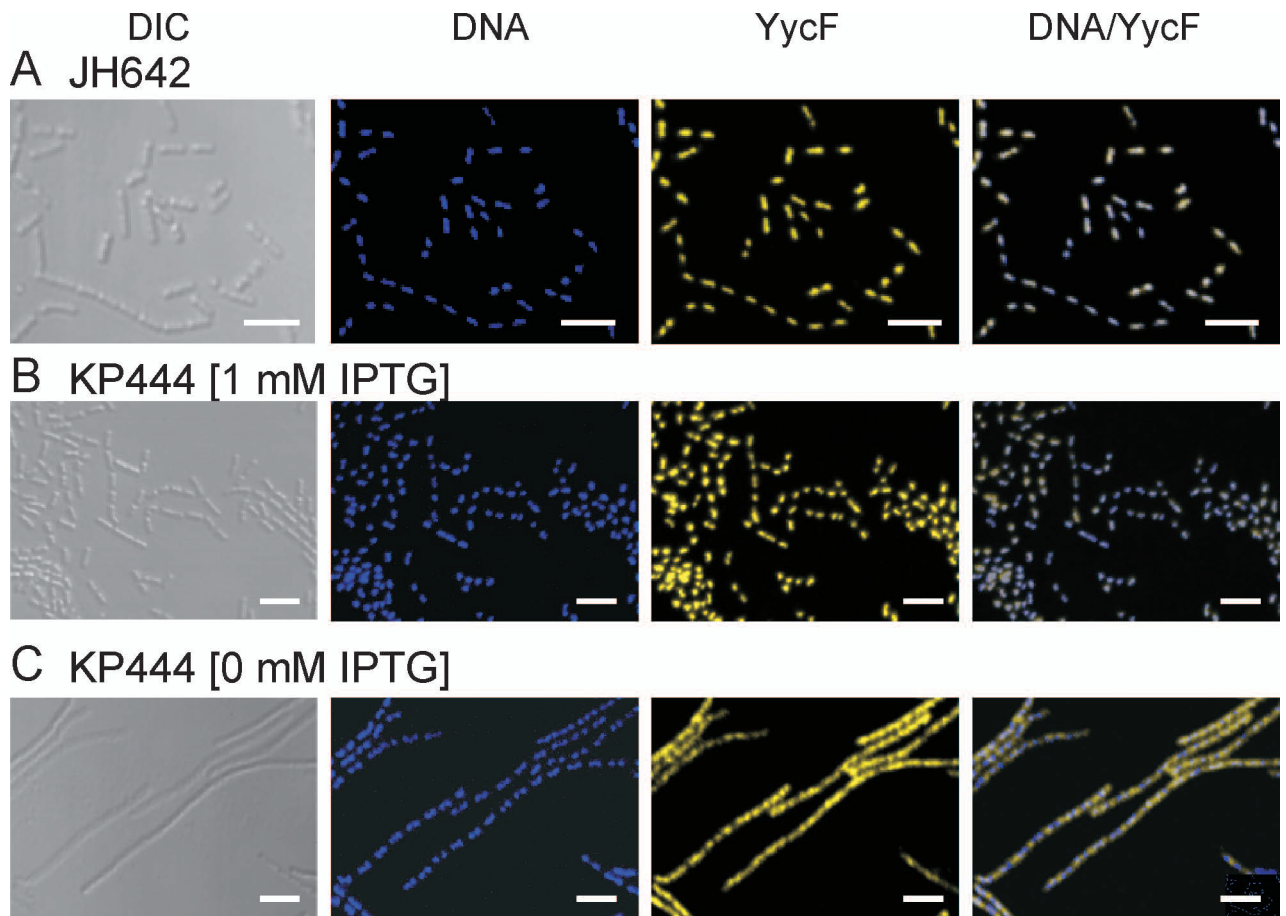
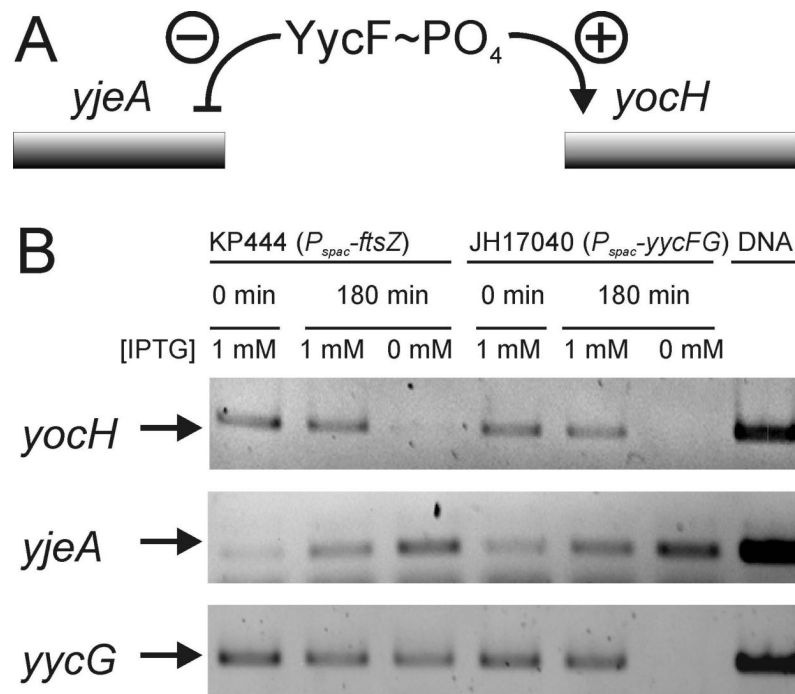


Figure 5. YycF localizes to the nucleoid in an FtsZ dependent manner. (A) YycF (yellow) was visualized immunologically in exponentially growing cells of wild type strain JH642. DNA (blue) was visualized by DAPI staining. The two figures were overlaid in DNA/YycF. Similarly DNA and YycF were visualized in the conditional FtsZ mutant strain KP444 grown for three hours at 37°C either in (B) the presence of 1 mM IPTG or (C) in the absence of IPTG to induce FtsZ depletion. DIC is differential interference microscopy.

**Figure 6.**

FtsZ depletion mimics YycF~PO₄ depletion in the regulation of the YycFG regulon. (A) A schematic showing regulation of two genes that are under negative (*yjeA*) or positive (*yocH*) transcription control by YycF~PO₄ (B) mRNA levels of *yocH*, *yjeA* and *yycG* were determined in an RT-PCR approach. RNA was extracted from strain KP444 grown in the presence of 1 mM IPTG or in its absence to induce FtsZ-depletion, as described in Materials and Methods. Similarly, RNA was extracted from strain JH17040 grown with or without IPTG to observe changes in mRNA levels of the respective genes in response to *yycFG* depletion. RNA was transcribed into cDNA and amplified by PCR. As a control the respective genes were also amplified directly from genomic DNA (right lane).

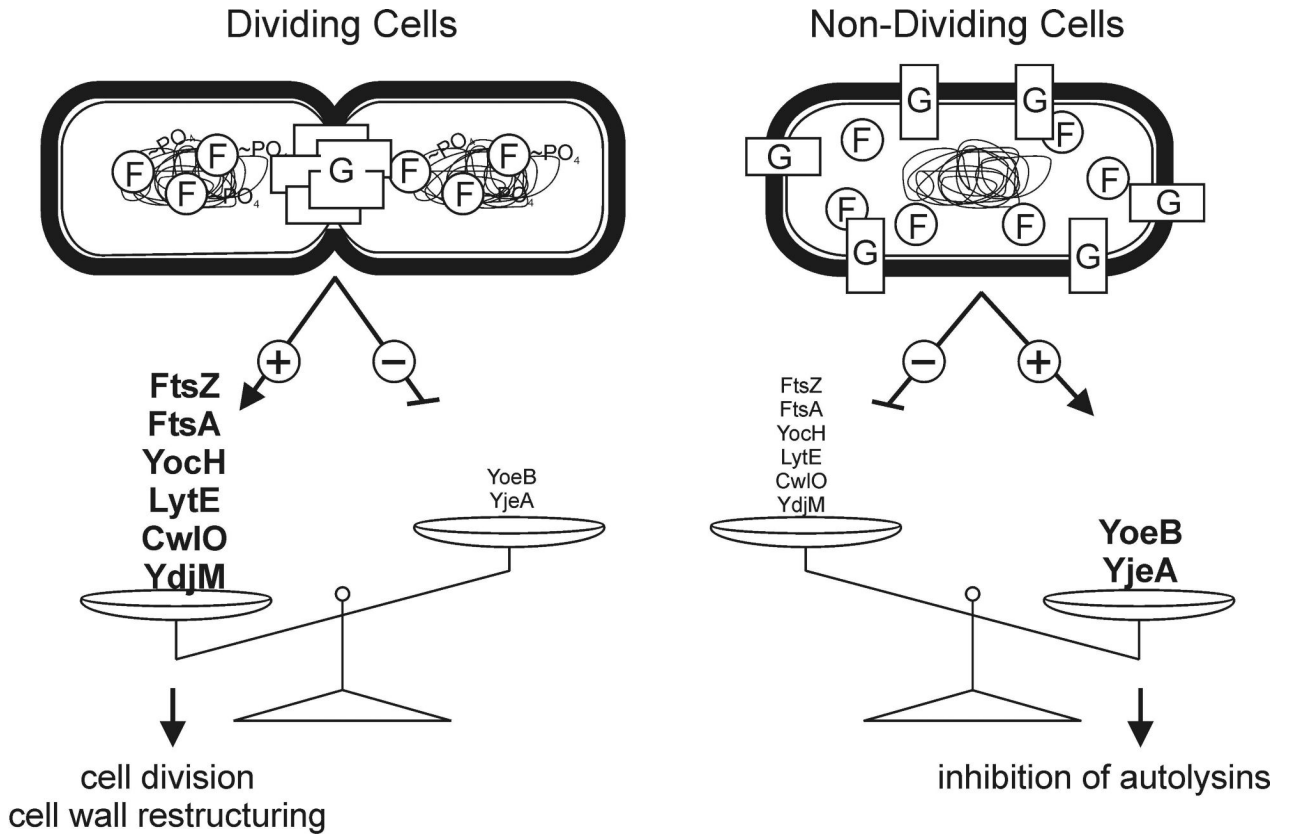


Figure 7. Model for a role of YycFG in coordinating cell division with cell wall homeostasis. The present data is consistent with a model in which YycG is activated upon localization to the septum in dividing cells. This activation increases the YycF~PO₄ levels and subsequently leads to enhanced expression of genes involved in cell wall remodeling (*yocH*, *lytE*, *cwlO* and *ydjM*) and cell division (*ftsA* and *ftsZ*) and repression of genes involved in inhibiting cell wall remodeling (*yoeB* and *yjeA*). In the absence of septa in non-dividing cells YycG fails to localize, adopts a state of low activity and hence the cellular YycF pool remains unphosphorylated. Under these conditions cell wall remodeling is not required and the expression balance is tipped towards the genes involved in autolysin inhibitory processes. The precise role of the involved genes of the YycF regulon, where known, has been addressed in (Bisicchia *et al.*, 2007).

Table 1
Plasmids and strains used in this study

Plasmid or strain	Relevant genotype or description	Source or reference
<u>Plasmids</u>		
pHY300PLK	<i>E. coli-B. subtilis</i> shuttle vector, Amp ^r , Tet ^r	Takara (Japan)
pBluescript-KS(+)	cloning vector, Amp ^r	Stratagene
pET28a	his-tag expression vector, Kan ^r	Navagen
pMAL-c2E	MBP-tag expression vector, Amp ^r	New England Biolabs
pJM115	<i>amyE</i> integration vector for <i>lacZ</i> fusions, Amp ^r , Kan ^r	M. Perego, unpublished
pJM117	pMUTIN4 derivative, Amp ^r , Cm ^r	M. Perego, unpublished
pJM119	<i>amyE</i> integration vector, P _{spac} , <i>lacI</i> , Amp ^r , Kan ^r	M. Perego, unpublished
pJMS1	pJM117-' <i>yycF-cat</i> -P _{spac}	This work
pJMS3	pJM117-' <i>yycF-cat</i> -P _{spac} - <i>yycH'</i>	This work
pJMS6	pJM119-P _{spac} - <i>yycG</i> , <i>lacI</i> Amp ^r , Kan ^r	This work
pJT01	pBluescript-KS(+)-' <i>yycF-cat</i> -P _{spac} - <i>yycH'</i>	This work
pJT02	pHY300PLK-P _{yycF}	This work
pJT03	pBluescript-KS(+)-' <i>yycF-cat</i> -P _{yycF} - <i>yycH'</i>	This work
pJT04	YycF-6xhis expression vector	This work
pJT05	6xhis-YycG expression vector	This work
pJT06	MBP-FtsZ expression vector	This work
<u>Strains</u>		
JH642	<i>pheA1 trpC2</i>	laboratory stock
JH17040	P _{spac} - <i>yycFG</i>	(Fabret and Hoch, 1998)
KP444	<i>ftsZ::phleo</i> P _{spac} - <i>ftsZ</i>	(Beall and Lutkenhaus, 1991)
JH25031	Δ <i>yycHI101 amyE::</i> (P _{yocH} - <i>lacZ aph3A</i>)	(Szurmant et al., 2007b)
JH25032	<i>amyE::</i> (P _{spac} - <i>yycG-lacZ aph3A lacI</i>)	This work
JH25033	<i>amyE::</i> (P _{spac} - <i>yycG-lacZ aph3A lacI</i>) <i>yycG::cat</i> P _{yycF} - <i>yycHIJK</i>	This work