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The LysR-Type Transcriptional Regulator YofA Controls Cell Division through the Regulation of Expression of *ftsW* in *Bacillus subtilis*▽

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We have carried out a functional analysis of LysR family transcriptional regulators in *Bacillus subtilis*. The cell density of cultures of a *yofA* insertion mutant declined sharply after the end of exponential growth, as measured by optical density at 600 nm. Complementation in *trans* and analysis of isopropyl-β-D-thiogalactopyranoside (IPTG)-dependent growth of an inducible *yofA* strain confirmed that YofA contributes to the cell density of a culture after the end of exponential growth. Microscopic observation suggested that cell division is inhibited or delayed in the *yofA* mutant during entry into stationary phase. Analysis of the transcription of cell division genes revealed that the expression of *ftsW* is inhibited in *yofA* mutants, and overexpression of *yofA*, driven by a multiple-copy plasmid, enhances the induction of *ftsW* expression. These results suggest that YofA is required for the final round of cell division before entry into stationary phase and that YofA positively regulates *ftsW* expression. The defects caused by mutation of *yofA* were suppressed in strains carrying P_{spac}-*ftsW* in the presence of IPTG. Furthermore, maximal expression of *yofA* was observed at the onset of stationary phase, which coincided with the maximal *ftsW* expression. Our data indicate that YofA is involved in cell division through positive regulation of the expression of *ftsW* in *B. subtilis*.

Cell division and growth phase (i.e., vegetative or stationary-phase growth) are coordinated processes in bacteria. In liquid culture, the gram-positive soil bacterium *Bacillus subtilis* enters a nondividing state after the end of exponential growth. Cells terminate cell division and enter into stationary phase to ensure survival under starvation conditions. Under certain conditions, the bacterium may receive a complex series of internal and external signals for sporulation and initiate a developmental program for spore formation (9, 19, 39) rather than complete cell division and enter into stationary phase (31). The mechanism of initiation of sporulation is well understood (9, 19, 39, 43). In contrast, the mechanism that regulates commitment to a final round of cell division and entry into stationary phase has not been well established.

The division of rod-shaped bacterial cells has been studied primarily in the model organisms *Escherichia coli* and *B. subtilis*. These bacteria grow by elongation of the long axis to form a rod, followed by cell division, which occurs at the midpoint of the rod. It has been demonstrated that septum assembly is mediated by a number of proteins that localize to the division site. Among the proteins known to localize to this site in *E. coli* are FtsZ, FtsA, FtsQ, FtsL, YqbQ, FtsW, FtsI (penicillin-binding protein B [PBPB]), FtsN, and ZipA (13, 44). In *B. subtilis*, eight cell division proteins have been shown to localize to the division site. They are FtsZ, FtsA, YtpT, FtsQ (DivIB), FtsL, DivIC, FtsW (YlaO), and PBPB (8, 10). The best-characterized protein component of the division site is FtsZ, which forms a cytoskeletal structure called the FtsZ ring. FtsZ is a highly conserved protein that appears to be widely present in prokaryotic cells (6, 16). The FtsZ ring and its associated proteins at the division site are called the divisome. Overall, the mechanisms of cell division are similar in *E. coli* and *B. subtilis*, although some aspects of the assembly of the protein complex at the division site differ. In *E. coli*, for example, FtsW is an integral membrane protein that is required for subsequent recruitment of its cognate transpeptidase, FtsI (32, 36). In contrast, *B. subtilis* FtsW is thought to be involved in the function of the PBP proteins, but the mechanism by which it does so is unknown.

Our lab is in the process of carrying out a functional analysis of LysR-type regulators in *B. subtilis* (30, 41). LysR family members typically contain an N-terminal helix-turn-helix motif and function as positive regulators of target promoters and negative autoregulators (41). In *B. subtilis* seven members of this family of regulators have been well investigated, whereas the functions of an additional 12 LysR-type regulators are unknown (Table 1) (30).

We report here the identification of a novel cell division-associated function for the LysR-type protein YofA, 1 of the 12 LysR-type regulators whose function was unknown. We show that YofA is essential for cell viability during stationary-phase growth of *B. subtilis*. We also show that maximal expression of *ftsW* at the transition from exponential growth to stationary phase is regulated by YofA and that the final round of cell division before entry into stationary phase is a prerequisite for cellular survival during stationary phase.

MATERIALS AND METHODS

Bacterial strains and plasmids. Most of the bacterial strains and plasmids used in this study are listed in Table 2. The exceptions are the *ycgK*, *yclA*, *yoaU*, *yraN*, *yrdQ*, *yusT*, *yvbU*, *ywbI*, *ywqM*, *yxjO*, and *yybE* mutant strains. These strains were constructed as part of European and Japanese projects for functional characterization of the *B. subtilis* genome and are listed on the following websites: http://locus.jouy.inra.fr/cgibin/genmic/madbase/progs/madbase.operl and http://bacillus.genome.ad.jp.

The oligonucleotide primers used for PCR amplification are listed in Table 3.

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TABLE 1. LysR-type regulator genes in *B. subtilis*

Gene	Function	Reference(s)
alsR	Activation of acetoin production genes (*alsSD*)	37
ccpC	Repression of the aconitase gene (*citB*)	28, 29
citR	Repression of the citrate synthase gene (*citA*)	26, 27
cysL	Activation of cysteine biosynthesis genes (*cysJI*)	15
gltC	Activation of glutamate biosynthesis genes (*gltAB*)	1, 3
gltR	Activation of glutamate biosynthesis genes (*gltAB*)	2
ytlI	Activation of the sulfur metabolism genes (*ytmI* operon)	5
ycgK	Unknown	
yclA	Unknown	
yoaU	Unknown	
yofA	This study	
yraN	Unknown	
yrdQ	Unknown	
yusT	Unknown	
yvbU	Unknown	
ywbI	Unknown	
ywqM	Unknown	
yxJO	Unknown	
yybE	Unknown	

E. coli JM105 was the host for all plasmid construction. *B. subtilis* strain 168 (wild type) served as the host for all strain construction. Transformation of *B. subtilis* was performed according to standard procedures (7).

Construction of plasmids and bacterial strains. pUCNyofA carries an internal fragment of *yofA*, which was amplified by PCR using primers yofAF and yofAR (Table 3). The amplified PCR product was digested with BamHI and HindIII and ligated into the corresponding sites of pUCN192 (20).

To construct the conditional *yofA* and *ftsW* mutants, DNA fragments that corresponded to nucleotides (nt) −22 to 315 of *yofA* and −21 to 435 of *ftsW* relative to the transcriptional start site at nt 1 were amplified by PCR with primers yofAF1 and yofAR1 and primers ftsWF1 and ftsWR1, respectively. After digestion with BamHI and HindIII, the PCR fragments were inserted into the corresponding sites of the P_{spac} integrational vector pMUTinT3 (35) to create pMUTinyofA and pMUTinftsW. Wild-type *B. subtilis* was transformed with pMUTinyofA and pMUTinftsW to generate the fusion strains ZL002 (P_{spac}-*yofA*) and ZL014 (P_{spac}-*ftsW*), in which expression of *yofA* and *ftsW*, respectively, was driven by the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible P_{spac} promoter.

To construct pTCCZ, the coding region of the *lacZ* gene from pMUTinT3 was subcloned into the *thrC* integration vector pTCC1 (24) using the PstI and XbaI restriction sites. To construct pTCCyofA, a DNA fragment containing the open reading frame and the promoter region of *yofA* was amplified with primers yofAfor and yofArev. The amplified PCR product was digested with PstI and XbaI and ligated into the corresponding sites of pTCC1. Before transformation of *B. subtilis*, the plasmid was linearized by digestion with ScaI.

To construct pTCCZ1, pTCCZ2, pTCCZ3, pTCCZ4, pTCCZ5, and pTCCZ6, DNA fragments of *ftsA* (nt −365 to −18), *divIB* (nt −2883 to −30), *divIC* (nt −643 to −20), *ftsL* (nt −1564 to −21), *rodA* (nt −711 to −31), and *ftsW* (nt −432 to −28) were amplified by PCR with primer pairs ftsAZF/ftsAZR, divIBF/divIBR, divICF/divICR, ftsLpbpBF/ftsLpbpBR, rodAF/rodAR, and ftsWF/ftsWR, respectively. Amplified PCR products were digested with PstI and XbaI and then ligated into the corresponding sites of pTCCZ. Before transformation into *B. subtilis*, the plasmids were linearized by digestion with ScaI.

To construct pHYXY1, the coding regions of P_{xyl} and *xylR* from pMF20 (34) were amplified with primers PXYP2 and PXYR. The amplified PCR product was completely digested with BamHI and EcoRI and ligated into the corresponding sites of pHY300PLK (25).

For overexpression of *yofA* in *B. subtilis*, the entire *yofA* open reading frame was amplified using primers yofABamHI and yofAHindIII. The amplified PCR product was digested with BamHI and HindIII and ligated into the corresponding sites of pHYXY1 to generate the multicopy plasmid pHYXYyofA, in which expression of *yofA* was driven by the xylose-inducible P_{xyl} promoter.

β-Galactosidase assay. Bacteria were grown in LB medium at 37°C and harvested at the indicated times by centrifugation. β-Galactosidase activity was assayed as described previously (33), using *o*-nitrophenyl-β-D-galactopyranoside as the substrate. Specific activity was calculated relative to the optical density at 600 nm (OD_{600}) of each sample and is expressed below in nanomoles of substrate (*o*-nitrophenyl-β-D-galactopyranoside) hydrolyzed per milligram of protein per minute.

Fluorescence microscopy. Cells were grown at 37°C in LB medium supplemented with FM4-64 (final concentration, 0.5 μg/ml; Molecular Probes) for labeling cell membranes and with SYTO16 (final concentration, 5 μg/ml; Molecular Probes) for labeling of cell nucleotides. A portion (2 μl) of each sample was mounted on a glass slide coated with 0.1% poly-L-lysine (Sigma), and slides were viewed by microscopy using an Olympus BX50 microscope with a 100× UplanApo objective lens. Images were captured using a SenSys charge-coupled camera device (Photometrics). FM4-64 and SYTO16 were visualized using a fluorescence isothiocyanate filter set (Olympus) and a wide interference green filter set (Olympus), respectively. Photos were viewed and analyzed using the Metamorph, version 6.1, software (Universal Image) and Adobe Photoshop, version 7.0.

RT-PCR experiments. Wild-type bacterial cells were grown in LB medium at 37°C, and samples were removed for analysis 2 and 1 h before the end of log phase and 1, 2, 3, 4, and 5 h after the end of log phase. Total RNA was extracted from the cells at the indicated time points, as described previously (22), and used as the template for reverse transcription (RT)-PCR analysis. Primer pairs yofARTF-yofARTR, ftsWRTF-ftsWRTR, and rpsRRTF-rpsRRTR were used to amplify *yofA*, *ftsW*, and *rpsR*, respectively (Table 3). Prior to RT-PCR, RNA was treated with DNase I (TAKARA) to remove any residual DNA. RT-PCR was performed using 0.5 μg of total RNA and an RNA PCR kit (TAKARA) according to the manufacturer's instructions. cDNA was amplified using an *Ex Taq* PCR kit (TAKARA). To obtain semiquantitative RT-PCR results, the number of PCR cycles was limited to 27, which is in the log-linear range of amplification. mRNA encoding ribosomal protein S18 (*rpsR*) was used as an internal standard to control for variations in the amount of total RNA used as the starting material. We assumed that the levels of *rpsR* mRNA were unaffected by the time of incubation of cells. The level of *rpsR* mRNA was also used to rule out the presence of contaminating chromosomal DNA (data not shown).

RESULTS

YofA is a regulator of cell growth in *B. subtilis*. As an initial step in the characterization of the 12 LysR-type regulators with unknown functions, we first examined the growth of 12 mutant strains of *B. subtilis* in LB medium at 37°C under nonsporulating conditions. These strains carried mutations in *ycgK*, *yclA*, *yoaU*, *yofA*, *yraN*, *yrdQ*, *yusT*, *yvbU*, *ywbI*, *ywqM*, *yxjO*, and *yybE* (30), and the growth rate was determined by monitoring the rate of increase of the OD_{600} of the cultures. Wild-type *B. subtilis* and all of the mutant strains except the *yofA* strain exhibited similar growth patterns (data not shown). The cell density of cultures of the *yofA* mutant strain declined sharply 14 to 24 h after inoculation (Fig. 1A). This decline continued up to 3 days after inoculation (OD_{600} at 3 days, ~0.8) (data not shown). The growth phenotype of the *yofA* mutant was confirmed by complementation experiments, in which *yofA* was supplied in *trans*, and in an inducible expression system, in which the gene was placed under the control of the P_{spac} promoter. An approximately 1.5-kb segment of DNA (nt −403 to 948) that contained the *yofA* locus was inserted into the *B. subtilis thrC* gene to generate the expression vector pTCCyofA. When pTCCyofA was introduced into *yofA* mutant cells (strain ZL004), normal growth was restored (Fig. 1B). We next examined the growth of a *yofA* conditional mutant. The *yofA* gene is located between *yogA* and *ggt* and is transcribed in a different direction than these two genes. Thus, it appears that *yofA* is monocistronic. We fused the ribosome binding site and the first 105 codons of the *yofA* gene to the inducible P_{spac} pro-

TABLE 2. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype, phenotype, and relevant characteristics	Source, reference, or construction[a]
E. coli JM105	*supE endA sbcB15 hsdR4 rpsL thi* Δ(*lac-proAB*) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]	45
B. subtilis strains		
168	*trpC2*	Laboratory stock
ZL001	*trpC2 yofA*::pUCN192	This study
ZL002	*trpC2 yofA*::pMUTinyofA (P_{spac}-*yofA*)	This study
ZL003	*trpC2 thrC*:: pTCCyofA (P_{yofA}-*yofA*)	This study
ZL004	*trpC2 yofA*:: pUCN192 *thrC*::pTCCyofA	ZL001 → ZL003
ZL005	*trpC2 thrC*::pTCCZ1 (*ftsAZ-lacZ*)	This study
ZL006	*trpC2 thrC*::pTCCZ2 (*divIB-lacZ*)	This study
ZL007	*trpC2 thrC*::pTCCZ3 (*divIC-lacZ*)	This study
ZL008	*trpC2 thrC*::pTCCZ4 (*ftsL-pbpB-lacZ*)	This study
ZL009	*trpC2 thrC*::pTCCZ5 (*rodA-lacZ*)	This study
ZL010	*trpC2 thrC*::pTCCZ6 (*ftsW-lacZ*)	This study
ZL011	*trpC2 yofA*::pUCN192 *thrC*::pTCCZ6	ZL001 → ZL010
ZL012	*trpC2* P_{xyl}-*yofA* (multicopy *yofA*)	This study
ZL013	*trpC2* P_{xyl}-*yofA thrC*::pTCCZ6	ZL012 → ZL010
ZL014	*trpC2 ftsW*::pMUTinftsW (P_{spac}-*ftsW*)	This study
ZL015	*trpC2 yofA*::pUCN192 *ftsW*::pMUTinftsW	ZL001 → ZL014
ZL016	*trpC2 yofA*::pUCN192 *thrC*::pTCCZ1	ZL001 → ZL005
ZL017	*trpC2 yofA*::pUCN192 *thrC*::pTCCZ2	ZL001 → ZL006
ZL018	*trpC2 yofA*::pUCN192 *thrC*::pTCCZ3	ZL001 → ZL007
ZL019	*trpC2 yofA*::pUCN192 *thrC*::pTCCZ4	ZL001 → ZL008
ZL020	*trpC2 yofA*::pUCN192 *thrC*::pTCCZ5	ZL001 → ZL009
Plasmids		
pMUTinT3	Vector carrying *bla erm*	35
pMUTinyofA	pMUTinT3 carrying P_{spac}-*yofA*	This study
pMUTinftsW	pMUTinT3 carrying P_{spac}-*ftsW*	This study
pUCN192	Vector carrying *bla neo*	20
pUCNyofA	pUCN192 carrying an internal region of *yofA* gene	This study
pTCC1	Integration vector at *thrC* carrying *bla cat*	24
pMF20	Vector carrying *bla cat*	34
pHY300PLK	Vector carrying *bla tet*	25
pTCCZ	pTCC1 carrying the coding region of *lacZ* gene	This study
pTCCZ1	pTCCZ carrying the upstream region of *ftsA* (*ftsAZ-lacZ*)	This study
pTCCZ2	pTCCZ carrying the upstream region of *divIB* (*divIB-lacZ*)	This study
pTCCZ3	pTCCZ carrying the upstream region of *divIC* (*divIC-lacZ*)	This study
pTCCZ4	pTCCZ carrying the upstream region of *ftsL* (*ftsL-pbpB-lacZ*)	This study
pTCCZ5	pTCCZ carrying the upstream region of *rodA* (*rodA-lacZ*)	This study
pTCCZ6	pTCCZ carrying the upstream region of *ftsW* (*ftsW-lacZ*)	This study
pTCCyofA	pTCC1 carrying native *yofA*	This study
pHYXY1	Vector carrying *bla tet*	This study
pHYXYyofA	pHYXY1 carrying the entire *yofA* open reading frame	This study

[a] Arrows indicate transformation from donor DNA to recipient strain.

moter, generating pMUTinyofA. Introduction of pMUTinyofA into wild-type strain 168 resulted in chromosomal integration of a full-length copy of *yofA* under the control of the P_{spac} promoter, which is repressed by the LacI repressor (strain ZL002). We then examined the growth of strain ZL002 (carrying P_{spac}-*yofA*) in LB medium in the absence and presence of various concentrations of inducer. An overnight culture grown in the presence of 0.05 mM IPTG was used to inoculate liquid cultures, and the growth rate was observed in the presence of 0.05 or 1 mM IPTG (Fig. 1C). The results showed that YofA is essential for cell viability during stationary phase and that a certain threshold level of YofA is required for cell survival during stationary phase.

To investigate whether YofA was required for sporulation, we examined the growth of the *yofA* mutant strain in DS medium (sporulating conditions) at 37°C. However, there was no difference in the growth curve during sporulation between wild-type and *yofA* mutant cells (data not shown). We also examined the effect of *yofA* mutation on the activation of the sporulation-specific sigma factors, σ^F and σ^E. We found no differences in the expression patterns of the σ^F-regulated gene *spoIIQ* and the σ^E-regulated gene *spoIID* in DS medium between wild-type and *yofA* mutant cells (data not shown). Thus, the effect of *yofA* appeared to be specific for the transition from log phase to stationary phase for cultures incubated in LB medium.

YofA controls cell viability and the formation of constrictions during cell division. To characterize the underlying defect in *yofA* mutant cells, we first performed a colony formation assay. During vegetative phase, wild-type and *yofA* mutant cells displayed similar numbers of viable cells (data not shown). However, as the *yofA* mutant strain entered stationary phase (12, 18, or 24 h after inoculation), there was a sharp decline in the number of CFU/ml, resulting in a 100-fold decrease 24 h

TABLE 3. Oligonucleotide primers used in this study

Primer	Sequence (5′ to 3′)[a]	Description	Location[b]	Restriction site
yofAF	**CCC**AAGCTTGGAAGCATAACGAAAGCA	*yofA* sense sequence	45	HindIII
yofAR	**CGC**GGATCCCACTGATAAATCCACTTC	*yofA* antisense sequence	369	BamHI
yofAF1	**CCC**AAGCTTCATAGCAAGCAGGTGAG	*yofA* sense sequence	−22	HindIII
yofAR1	**CGC**GGATCCCAGATGCGTTACCGCCAT	*yofA* antisense sequence	315	BamHI
yofAfor	**AAAA**CTGCAGAATCCCAGTTCAATGTCGG	*yofA* sense sequence	−403	PstI
yofArev	**TGC**TCTAGATACTCGCTTCAAATGAG	*yofA* antisense sequence	948	XbaI
yofABamHI	**CGC**GGATCCCATAGCAAGCAGGTGAG	*yofA* sense sequence	−22	BamHI
yofAHindIII	**CCC**AAGCTTTACTCGCTTCAAATGAG	*yofA* antisense sequence	948	HindIII
ftsAZF	**AAAA**CTGCAGGTTTCCGGTTTCTTTTTT	*ftsA* sense sequence	−365	PstI
ftsAZR	**TGC**TCTAGATTTCTATTCTATTATTTG	*ftsA* antisense sequence	−18	XbaI
divIBF	**AAAA**CTGCAGGACAGTTATGGTCGGAAC	*divIB* sense sequence	−2883	PstI
divIBR	**TGC**TCTAGACTGTTAAAAGTCTGTCTA	*divIB* antisense sequence	−30	XbaI
divICF	**AAAA**CTGCAGCGACACAATTCTATACAA	*divIC* sense sequence	−643	PstI
divICR	**TGC**TCTAGAATCTCTTCAAAACCGT	*divIC* antisense sequence	−20	XbaI
ftsLpbpBF	**AAAA**CTGCAGCTTTTTATGGGTAAACA	*ftsL* sense sequence	−1564	PstI
ftsLpbpBR	**TGC**TCTAGAATTTTGCGTTCGCGTTTC	*ftsL* antisense sequence	−21	XbaI
rodAF	**AAAA**CTGCAGTGCGGCGATATGAGCGAC	*rodA* sense sequence	−711	PstI
rodAR	**TGC**TCTAGATCACTAATGTTTATTATA	*rodA* antisense sequence	−31	XbaI
ftsWF	**AAAA**CTGCAGTGGACGCTGAGAAGATTT	*ftsW* sense sequence	−432	PstI
ftsWR	**TGC**TCTAGAATTATCTATGGTTTTTAT	*ftsW* antisense sequence	−28	XbaI
ftsWF1	**CCC**AAGCTTAGAAGCAGGGAAGAGGATG	*ftsW* sense sequence	−21	HindIII
ftsWR1	**CGC**GGATCCGACTACCGGAGGGCTACT	*ftsW* antisense sequence	453	BamHI
PXYP2	**CGC**GGATCCCCGGCATTCAAATACAG	*xylR* sense sequence	−322	BamHI
PXYR	**CCG**GAATTCTGCCATGTCACTATTGC	*xylR* antisense sequence	1133	EcoRI
yofARTF	GGAAGCATAACGAAAGCA	*yofA* sense sequence	46	
yofARTR	GCAGATGCGTTACCGCC	*yofA* antisense sequence	299	
ftsWRTF	CTCACTGATATTCGCAAT	*ftsW* sense sequence	32	
ftsWRTR	ACTACCGGAGGGGCTACT	*ftsW* antisense sequence	452	
rpsRRTF	GCAGAGGCGGTCGTGCGAAA	*rpsR* sense sequence	14	
rpsRRTR	ACGTGCGCGTTTGATCGCTGCA	*rpsR* antisense sequence	183	

[a] Additional sequences and restriction sites that do not correspond to the sequences of genes are indicated by boldface type and underlining, respectively.
[b] The locations are the 3′-end positions of the primers corresponding to the number of nucleotides from the initiation codons of the genes.

after inoculation compared to wild-type or ZL004 cells (Fig. 2A). Thus, the *yofA* mutant exhibited a decreased ability to grow in culture, most likely due to a loss of viability.

We next examined the cytological features of the *yofA* mutants. Samples were taken from early-stationary-phase cultures, and cells were double stained with SYTO16 and FM4-64 to visualize cytoplasmic nucleic acids and the cell membrane, respectively. Phase-contrast and fluorescence microscopy revealed that vegetative cells of the wild-type and *yofA* mutant strains were similar in shape and that the percentages of dividing cells were similar (Fig. 2B). Upon incubation for 4 h after entry into stationary phase, wild-type cells exhibited a dispersed distribution pattern, while *yofA* mutant cells displayed a highly filamentous morphology (Fig. 2B). The mutant cells at this time point were elongated with segments, and ghost cells were occasionally observed. In addition, the elongated mutant cells contained no visible constrictions of the cell wall and cell membrane at their septa, which suggested that *yofA* functions during cell division (38, 42). In wild-type *B. subtilis*, dividing cells can be identified by a visible constriction at the site of the septum, which is involved in the generation of two newborn daughter cells. We quantified the number of cells with constrictions in wild-type cells and *yofA* mutants harvested 0 to 12 h after the end of the exponential growth phase (Fig. 3). At the end of exponential phase, approximately 78% of wild-type and *yofA* mutant cells had no visible constrictions. However, 6 and 12 h after the end of log phase, the fraction of *yofA* mutant cells that had no visible membrane constrictions was larger than that of wild-type cells (45% compared to 20% at 6 h after the end of log phase and 33% compared to 5% at 12 h after the end of log phase) (Fig. 3). We also examined the population of dividing cells in the *yofA* conditional mutant strain ZL002. In the presence of 1 mM IPTG, the fraction of dividing cells without visible constrictions for strain ZL002 was similar to that for wild-type cells, whereas in the absence of IPTG, it was similar to that for the *yofA* mutant (Fig. 3). These results indicated that cell division is inhibited or delayed in a *yofA* mutant during entry into stationary phase. Thus, YofA appeared to be essential in the regulation of the final round of division before entry into stationary phase.

YofA controls the expression of *ftsW*, a cell division protein. The initiation of cell division involves the formation of a ring of FtsZ protein around the inner membrane of the cell at the midcell division site. Other division proteins, such as FtsA, FtsW (YlaO), FtsL, DivIB, DivIC, and PBPB, are believed to act after the formation of the FtsZ ring (10). RodA is a transmembrane protein that is involved in cell elongation and is required for the synthesis of peptidoglycan (18). To investigate whether depletion of YofA affected the expression of these division or elongation genes, we constructed several additional *yofA* mutant strains. The coding regions of *ftsA*, *ftsW*, *divIB*, *divIC*, *pbpB*, and *rodA-lacZ* were inserted into the *thrC* locus of the *yofA* mutant strain, which generated transcriptional *lacZ* fusions of each division or elongation gene. As a control, the gene fusions were also generated in a wild-type background. Strains were grown in LB medium at 37°C, and β-galactosidase

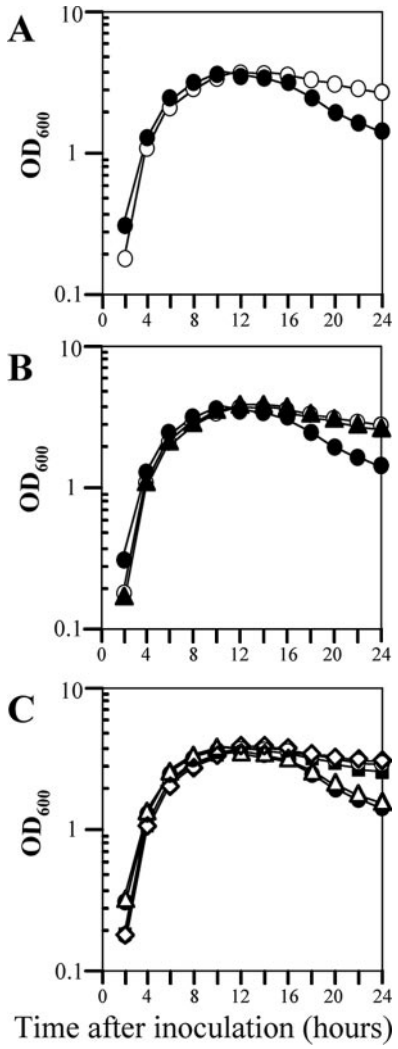

FIG. 1. Effect of *yofA* expression on cell growth. Cells were grown in LB broth at 37°C to stationary phase. (A) Growth assay of wild-type 168 (○) and ZL001 (*yofA*::*neo*) (●) cells. (B) Complementation of *yofA* deficiency by introduction of *thrC*::*yofA* in *trans*: growth assay of wild-type 168 (○), ZL001 (*yofA*::*neo*) (●), and ZL004 (*yofA*::*neo thrC*::*yofA*) (▲) cells. (C) Growth of wild-type 168 (○), ZL001 (*yofA*::*neo*) (●), and ZL002 (P_{spac}-*yofA*) cells in liquid LB medium in the absence of IPTG (△) or in the presence of the following concentrations of IPTG: 0.05 mM (■) and 1 mM (◇). Growth was determined by measuring the OD_{600}, and the data represent the means of three independent experiments.

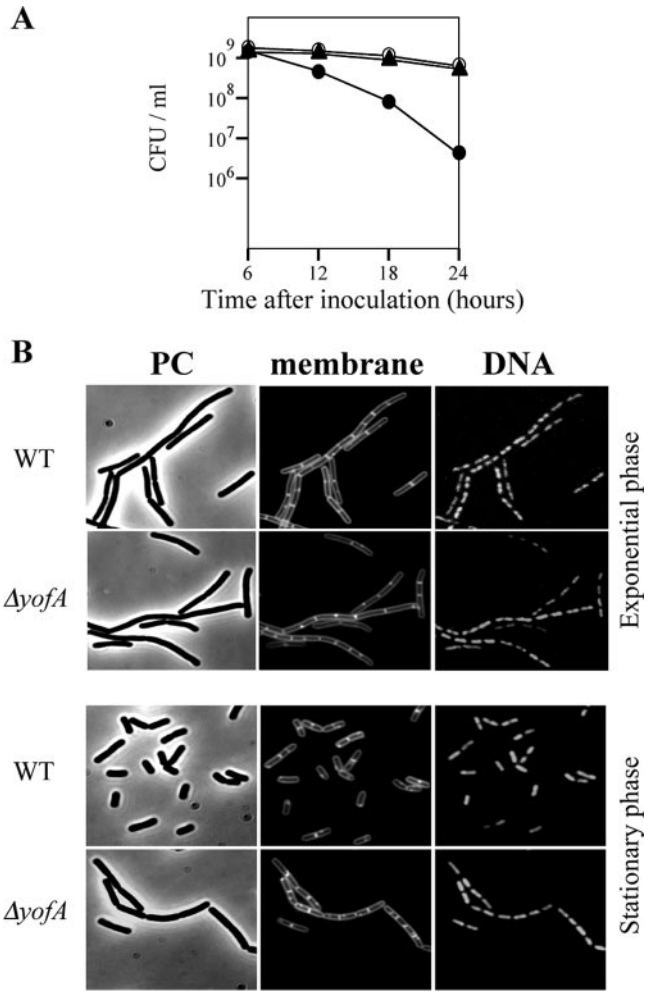

FIG. 2. Effect of deletion of *yofA* on the viability and morphology of *B. subtilis*. (A) Colony formation assay of wild-type 168 (○), ZL001 (*yofA*::*neo*) (●), and ZL004 (*yofA*::*neo thrC*::*yofA*) (▲) cells. The data represent the means of three independent experiments. (B) Fluorescence microscopy of ZL001 (Δ*yofA*) and wild-type 168 (WT) cells during exponential growth and at 4 h after the end of exponential phase. Cells were treated with FM4-64 and SYTO16 to visualize the membranes and DNA, respectively. PC, phase contrast.

activity was measured. As shown in Fig. 4, the expression of *ftsAZ*, *divIB*, *divIC*, *ftsL-pbpB*, and *rodA* in the *yofA* mutant background was similar to that in the wild-type cells. However, the transcription of *ftsW* was partially blocked in the *yofA* mutant. In wild-type cells, β-galactosidase activity of the *ftsW* gene fusion gradually increased during exponential phase and then declined during stationary phase. In the *yofA* mutant background, *ftsW* expression reached a peak level at the end of exponential phase and declined thereafter, but it was significantly lower at all time points than the expression in wild-type cells. These results suggested that the expression of *ftsW* is dependent on YofA. Of note, we observed a high level of expression of *rodA* in both wild-type and *yofA* mutant cells during vegetative phase, whereas transcription of the other five genes, including *ftsW*, reached a maximum level upon entry into stationary phase.

We next examined whether overexpression of YofA elevated the expression of *ftsW*. The wild type carrying the *ftsW-lacZ* fusion gene (strain ZL010, *thrC*::*ftsW-lacZ*) was transformed with a xylose-inducible *yofA* expression vector (pHYXYyofA). As shown in Fig. 5, induction of overexpression of *yofA* by incubation of the cells in 10 mM xylose resulted in enhanced expression of *ftsW* in both early stationary phase and late stationary phase. These results suggested that YofA regulates the transcription of *ftsW*.

Induction of FtsW overcomes the final cell division defect caused by *yofA* mutation during entry into stationary phase. We next examined whether the final cell division defect caused by *yofA* mutation at entry into stationary phase could be suppressed by induction of *ftsW*. The FtsW protein is an integral

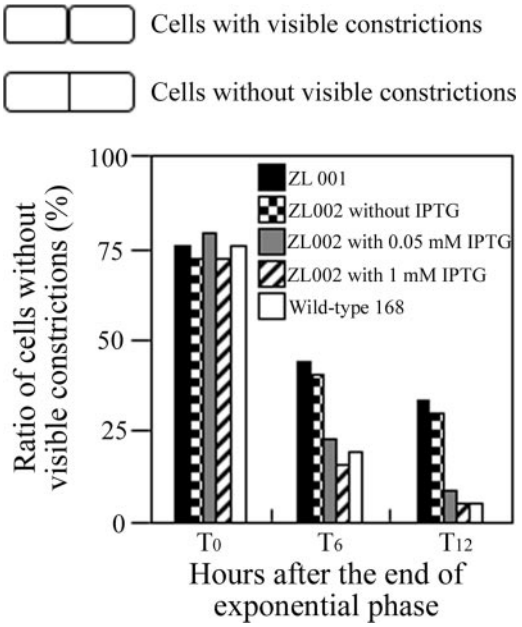

FIG. 3. Effect of *yofA* mutation on cell division: ratio of the number of cells lacking visible constrictions to the total number of cells in ZL001 (*yofA*::*neo*), wild-type 168, and ZL002 (P_{spac}-*yofA*) cultures in the absence of IPTG or in the presence of 0.05 and 1 mM IPTG. The data represent the means of three independent experiments.

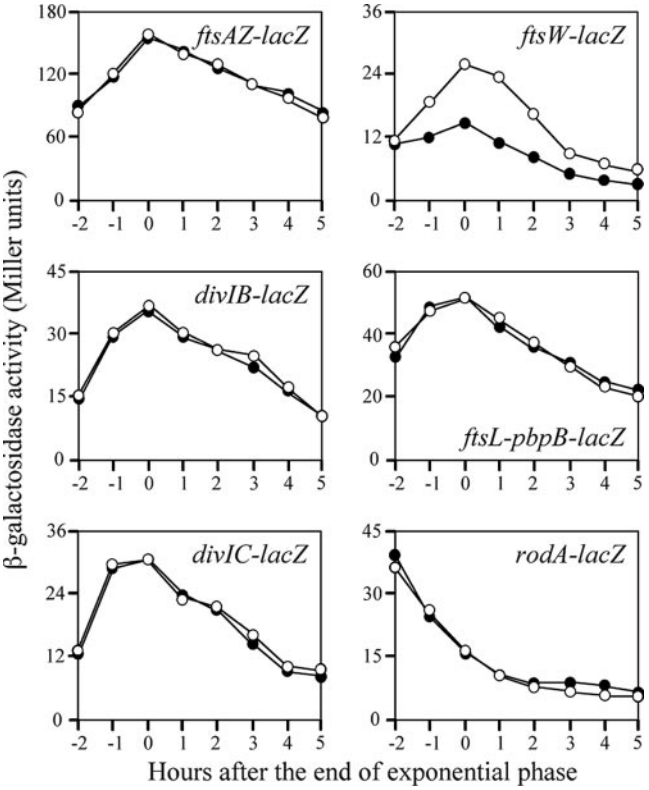

FIG. 4. Effect of *yofA* mutation on the expression of cell division genes. Wild-type (○) and *yofA*::*neo* (●) cells were grown in LB medium, and the β-galactosidase specific activities of the following reporter genes were examined: *ftsAZ-lacZ*, *divIB-lacZ*, *divIC-lacZ*, *ftsW-lacZ*, *ftsL-pbpB-lacZ*, and *rodA-lacZ*. The data represent the means of three independent experiments.

membrane protein, and its function in *B. subtilis* is poorly characterized. Therefore, we first examined the growth phenotype of an inducible *ftsW* strain of *B. subtilis*. We fused the ribosome binding site and the first 151 codons of *ftsW* to the P_{spac} promoter, generating the expression vector pMUTin ftsW. Integration of pMUTinftsW into the chromosome of wild-type 168 cells created a strain in which *ftsW* was under the control of the LacI-repressible, IPTG-inducible P_{spac} promoter (strain ZL014, P_{spac}-*ftsW*). The growth rate of strain ZL014 in the absence or presence of different concentrations of IPTG was determined by monitoring the rate of increase in the OD_{600}. As shown in Fig. 6A, there was a severe growth defect of the P_{spac}-*ftsW* cells in liquid medium in the absence of IPTG, suggesting that *ftsW* is essential for cell growth. We also examined ZL014 cells by microscopy after treatment with different concentrations of IPTG. Cells were double labeled with SYTO16 and FM4-64 to visualize cytoplasmic nucleic acids and the cell membrane, respectively. As shown in Fig. 7, ZL014 cells were extremely filamentous in the presence of low concentrations of IPTG (0.001 to ~0.01 mM), indicating that there was a block in cell division under these conditions. In contrast, in the presence of 0.1 mM IPTG, the shape of the cells was similar to the shape of wild-type cells. These results suggested that *ftsW* is required for cell division in *B. subtilis*.

To determine whether induction of FtsW overcomes the cell division defect caused by *yofA* mutation, we analyzed the cell growth (OD_{600}) and colony formation (CFU/ml) of strain ZL015 (P_{spac}-*ftsW yofA*::*neo*) in the presence or absence of IPTG. As shown in Fig. 6B, under inducing conditions (1 mM IPTG), the rate of increase of the OD_{600} of strain ZL015 was similar to that of wild-type cells. In the presence of 0.01 mM IPTG, there was a sharp decline in the OD_{600} of strain ZL014 compared to that of the wild type during stationary phase. The *yofA* mutant exhibited a similar pattern of growth. Under inducing conditions (1 mM IPTG), ZL015 cells also displayed a number of CFU/ml similar to that of wild-type cells (Fig. 6C), suggesting that the growth phenotype of the *yofA* mutant during stationary phase was completely suppressed by the induction of FtsW. We also examined the morphology of ZL015

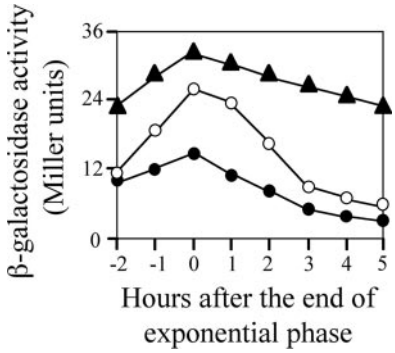

FIG. 5. Effect of *yofA* overexpression on the activity of an *ftsW-lacZ* reporter gene: β-galactosidase activity of ZL010 (wild-type) (○) and ZL011 (*yofA*::*neo*) (●) cells and of ZL013 (P_{xyl}-*yofA ftsW-lacZ*) cells supplemented with 10 mM xylose (▲). The data represent the means of three independent experiments.

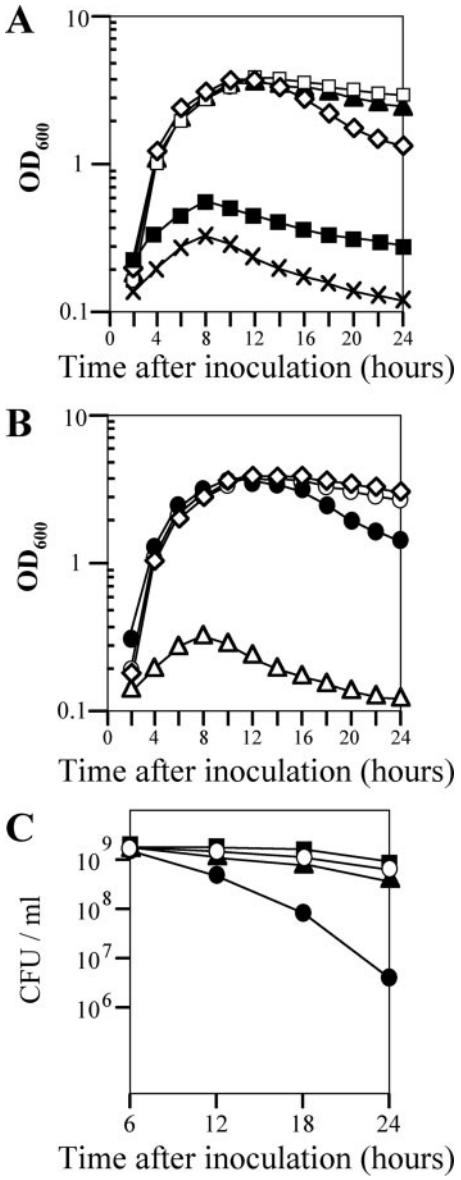

FIG. 6. Suppression of growth and viability defects of the *yofA* mutant upon induction of FtsW. (A) Effect of FtsW induction on growth. The growth of ZL014 (P_{spac}-*ftsW*) cells in liquid LB medium in the absence of IPTG (×) or in the presence of the following concentrations of IPTG was determined: 0.001 mM (■), 0.01 mM (◇), 0.1 mM (▲), and 1 mM (□). (B) Effect of FtsW induction on growth of *yofA* mutants: growth of wild-type 168 (○) and ZL001 (*yofA*::*neo*) (●) cells and of ZL015 (*yofA*::*neo* P_{spac}-*ftsW*) cells in liquid LB medium in the absence (△) or presence of 1 mM IPTG (◇). (C) Effect of FtsW induction on colony formation of *yofA* mutants: CFU/ml for wild-type 168 (○), ZL001 (*yofA*::*neo*) (●), ZL014 (P_{spac}-*ftsW*) in the presence of 1 mM IPTG (■), and ZL015 (*yofA*::*neo* P_{spac}-*ftsW*) supplemented with 1 mM IPTG (▲). The data represent the means of three independent experiments, and mean OD_{600} values are shown.

cells in the presence of IPTG by fluorescence microscopy. Induction of FtsW resulted in suppression of the final cell division defect caused by *yofA* mutation during entry into stationary phase (Fig. 7). These results indicated that cellular survival during stationary phase requires maximal expression of *ftsW*, which is controlled by YofA.

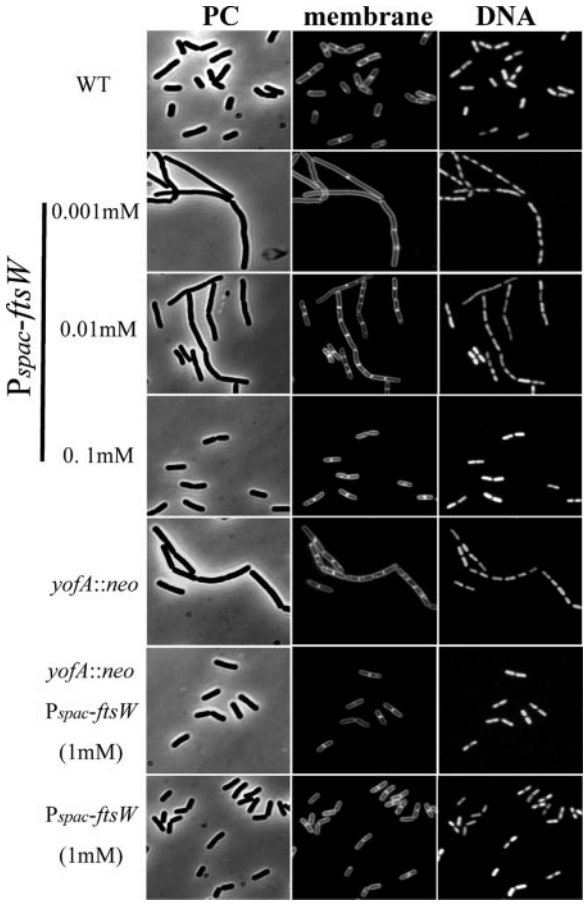

FIG. 7. Suppression of the cell division defect of *yofA* mutants by induction of FtsW: fluorescence micrography of wild-type 168 (WT), ZL001 (*yofA*::*neo*), ZL015 (*yofA*::*neo* P_{spac}-*ftsW*) in the presence of 1 mM IPTG, and ZL014 (P_{spac}-*ftsW*) cells in the presence of 0.001 mM, 0.01 mM, 0.1 mM, and 1 mM IPTG. Cells incubated until 4 h after the end of exponential phase were stained with FM4-64 and SYTO16 to visualize cell membranes and DNA, respectively. PC, phase contrast.

Transcription of *yofA* increased to the maximum level at entry into stationary phase. To examine the expression profile of *yofA*, we generated a strain carrying a *lacZ* gene fusion of *yofA*, in which the *lacZ* gene was integrated into the *yofA* locus. When we measured β-galactosidase activity in the cells, we observed very low or no activity (data not shown). Thus, we performed RT-PCR to detect *yofA* and *ftsW* transcription. As shown in Fig. 8, the transcription of *yofA* increased over time to a maximum level at entry into stationary phase, which coincided with the transcriptional profile of *ftsW*.

DISCUSSION

Simple unicellular organisms must undergo cell division in order to generate progeny. This is one of the most critical processes in biology. In the current study, we uncovered a novel role for *yofA* in septum formation during cell division in *B. subtilis*. We showed that *yofA* has a role in maintaining cell density after the end of exponential growth. Our analysis further indicated that YofA plays an important role in cell division through the regulation of expression of *ftsW*, which is an

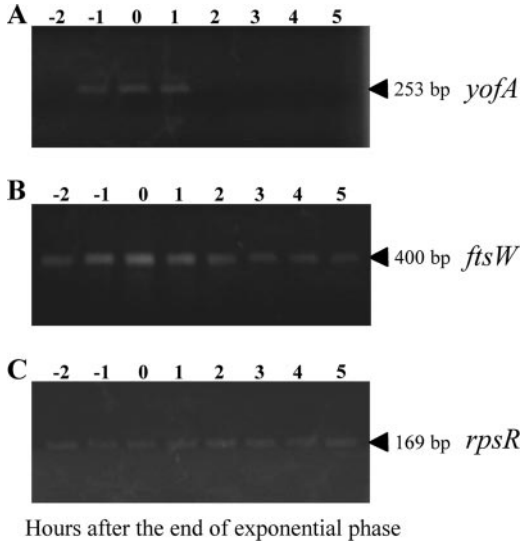

FIG. 8. RT-PCR analysis of expression of *yofA* and *ftsW*: time course of expression of *yofA* (A), *ftsW* (B), and *rpsR* (C). RNA (0.5 μg) was isolated at the indicated time points from LB medium cultures of wild-type strain 168 and then analyzed by RT-PCR as described in Material and Methods. The *rpsR* transcript encoding ribosomal protein S18 served as a control for fluctuations in total RNA amounts. The arrowheads indicate the positions of the expected RT-PCR products.

essential component of the cell division machinery in *B. subtilis*.

YofA is composed of 285 amino acids and shares sequence similarity with members of the LysR family of transcriptional regulators. Among the LysR family members in *B. subtilis* with known functions, AlsR (37), GltC (1, 3), and YtlI (5) act as positive regulators of target genes located close to them, whereas CysL (15) and CitR (26, 27) act as negative regulators that inhibit the transcription of neighboring genes. Other LysR-type regulators, such as GltR (2) and CcpC (28, 29), act in *trans* to regulate the expression of target genes, such as *gltA* and *citB*, respectively. *yofA* is a monocistronic operon, located 128 bp from the *yogA* gene, which encodes a putative alcohol dehydrogenase, and 107 bp from the *ggt* gene, which encodes gamma-glutamyltranspeptidase. The direction of transcription of *yofA* is opposite that of *yogA* and *ggt*. By analogy with the mechanisms of gene regulation of other LysR family members, YofA was thought to regulate the expression of neighboring genes, such as *yogA* and/or *ggt*. However, we found that YofA had no effect on the expression of these genes (data not shown). Thus, YofA is most likely to be a *trans*-acting regulatory protein, similar to GltR and CcpC. In the current study, we showed that the cell division gene *ftsW* is a putative target of YofA in *B. subtilis*.

While the role of cell division in *B. subtilis* has been clarified, the transcriptional regulatory proteins involved in cell division are not well understood. The only known transcriptional regulator involved in cell division is YycGF, a two-component regulatory protein that is essential for cell growth (12, 21). YycG and YycF function as a sensor kinase and response regulator, respectively (11). YycFG has been shown to play a role in cell division and in cell membrane and cell wall homeostasis. YycF binds to the P1 promoter of the *ftsAZ* operon, which is involved in cell division (12). Howell et al. (21) identified putative YycF binding sites in 14 genes, including *ykvT*, which encodes a putative cell wall hydrolase, and the *tagAB*/*tagDEF* divergon, which encodes essential components of the teichoic acid biosynthesis pathway. In the current study, we obtained evidence that mutation in a novel transcriptional regulator gene, *yofA*, inhibits the final round of cell division prior to entry into stationary phase.

Following exponential growth, most cells experience a significant reduction in the rate of cell division as they enter stationary phase. It has been proposed that this phenomenon is the result of the ability of the cells to detect certain signals (e.g., nutrient depletion and cell density) in the extracellular environment. The reduction in the division rate results in completion of the division process, a process that may be required for the accumulation of excess cell division proteins. For example, expression of *ftsAZ* reaches a maximum at the transition from exponential growth to stationary phase (14). One of the three *ftsAZ* promoters, P2, is recognized by σ^H-associated RNA polymerase, which is an alternative RNA polymerase sigma factor that directs the transcription of many genes that function at the transition state (4, 14). In a similar manner, we showed that expression of the division genes *ftsW*, *divIB*, *divIC*, and *pbpB*, which includes *ftsAZ*, was also maximal at the transition state (Fig. 4). These data imply that the expression of division genes increases until the end of log phase in order to increase the rate of division. The expression pattern of *rodA*, which is involved in cell elongation, was opposite that of the other division genes. Although we observed peak expression of *ftsW* at the transition state in the *yofA* mutant, the magnitude of expression appeared to be significantly enhanced by YofA, and the transcription profile of *yofA* correlated with that of *ftsW*. Thus, it appears that expression of *yofA* is modulated by nutritional status and that YofA in turn regulates the expression of *ftsW*.

FtsW is essential for septum formation; however, the function of *ftsW* has not been elucidated in *B. subtilis*. FtsW is a paralog of *B. subtilis* RodA and SpoVE (23, 30). RodA (18) and SpoVE (17, 40) are required for the maintenance of normal cell shape and the synthesis of spore cortex peptidoglycan, respectively. They are members of the SEDS family of proteins (shape, elongation, division, and sporulation) and contain 10 transmembrane-spanning segments (18). In *E. coli* FtsW is an essential gene for cell division and appears to be involved in the translocation of the lipid-linked peptidoglycan precursor through the cytoplasmic membrane. FtsW plays a role in the stabilization of the FtsZ ring and recruitment of the FtsW cognate transpeptidase FtsI (PBP3) to the division site during cell division (32). Based on these reports, the level of FtsW appears to be important for stabilization of the division machinery. We demonstrated that strain ZL014, which contained the inducible P_{spac}-*ftsW* expression construct, undergoes IPTG-dependent cell growth and division (Fig. 6 and 7). In addition, we showed that maximal expression of *ftsW* is dependent on YofA in *B. subtilis* (Fig. 5). Interestingly, the growth pattern of the *yofA* mutant was similar to that of strain ZL014 in the presence of 0.01 mM IPTG (Fig. 6). The reduced level of FtsW in the *yofA* mutant correlated with the level in ZL014 in the presence of 0.01 mM IPTG, which suggests that there is a threshold level of FtsW that is required for the final round of

cell division during entry into stationary phase. Of note, the OD_{600} and the number of CFU/ml declined in both the *yofA* mutant and strain ZL014 in the presence of 0.01 mM IPTG during stationary phase. This is may be due to a failure of chains of cells to separate, which may present a growth disadvantage under starvation conditions and eventually leads to cell lysis.

Taken together, our findings indicate that FtsW is specifically required for the formation of the division septum and that maximal expression of *ftsW* is required for cellular survival during stationary phase. In fact, we observed that decreased *ftsW* expression caused by *yofA* mutation led to a defect in septum formation and a growth defect after the end of exponential phase (Fig. 6 and Fig. 7).

To begin to characterize the phenotype of the *yofA* mutant during entry into stationary phase, we examined the effect of *yofA* mutation on the transition from logarithmic to stationary phase. We demonstrated that the effect of *yofA* mutation was specific for the stationary phase in cultures growing in LB medium (nonsporulating conditions). We speculate that the high rate of growth in LB medium requires cells to "change gears" at the transition, which requires YofA, whereas under sporulation conditions, the partial deficiency of FtsW caused by *yofA* mutation may be overcome during the early stage of sporulation.

The mechanism of activation of transcription of *ftsW* by YofA may involve binding of YofA to the *ftsW* promoter region. However, we have not yet determined if YofA binds to the promoter of *ftsW* or whether other ligands are involved. Another possibility is that YofA is involved in controlling the transcription of other genes, which affect the expression of *ftsW*. Thus, further studies, such as DNA microarray analysis, are needed to distinguish among these and other possibilities. Furthermore, investigation of growth state- and cell cycle-dependent fluctuations in the expression of *ftsW* will be essential for understanding the molecular mechanisms of cell division. The identification of *yofA* as a gene involved in cell division during entry into stationary phase provides a significant piece of information concerning the complex process of cell division. Further work on YofA should lead to a better understanding of the regulation of cell division in *B. subtilis*.

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