The *Bacillus subtilis* essential gene *dgkB* is dispensable in mutants with defective lipoteichoic acid synthesis

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The *dgkB* gene is essential for the growth of *Bacillus subtilis*. It encodes a diacylglycerol (DG) kinase that converts DG to phosphatidic acid to reintroduce it into the phospholipid synthesis pathway. Repression of the *dgkB* gene placed under a regulatable promoter causes accumulation of DG and leads to lethality. DG is formed as a byproduct of the synthesis of lipoteichoic acid (LTA), a polyanionic component of the cell envelope. *B. subtilis* synthesizes LTA by polymerizing the glycerophosphate moiety of phosphatidylglycerol (PG) onto a glucolipid membrane anchor, and releasing the DG moiety of PG. *B. subtilis* has four genes homologous to *Staphylococcus aureus* *ltaS*, which encodes LTA synthase. Disruption of either or both of two genes, *yflE* and *yfnI*, whose products show higher homology with *S. aureus* LtaS among the four homologues, suppressed the lethality caused by *dgkB* repression. In cells with *dgkB* repression, DG was accumulated to $43 \pm 3\%$ of total lipids, about three times the content of wild type cells ($13 \pm 1\%$). Disruption of *yfnI* in the *dgkB*-repressed cells reduced the DG content to $15 \pm 2\%$, but *yflE*-disruption did not ($42 \pm 1\%$); this was probably due to efficient LTA synthesis by YfnI in the *yflE*-disrupted cells. Further introduction of a disrupted allele of *ugtP*, encoding glucolipid synthase that consumes DG as a substrate, partially lowered the colony forming capacity in strains with *yflE*-disruption. A disrupted *dgkB* allele was successfully introduced into strains disrupted for either or both of *yflE* and *yfnI*, indicating that the essential gene *dgkB* is dispensable in mutants defective in LTA synthesis.

Key words: *Bacillus subtilis*, *dgkB*, diacylglycerol kinase, lipoteichoic acid

INTRODUCTION

Lipoteichoic acid (LTA) is a polyanionic component of the cell envelope of Gram-positive bacteria (Neuhaus and Baddiley, 2003). It is synthesized by polymerizing the *sn*-glycerol-1-phosphate (G1P) moiety of phosphatidylglycerol (PG) onto a diglucosyldiacylglycerol (DGDG) membrane anchor (Fig. 1). We first became interested in the LTA synthetic pathway when we found that *Bacillus subtilis* *pgsA* is an essential gene. *pgsA* encodes a phosphatidylglycerophosphate synthase that is a key enzyme for the synthesis of PG. It could not be disrupted, and its placement under an isopropyl- β -D-thiogalactoside (IPTG)-inducible P_{spac} promoter resulted in bacteria whose growth was dependent on the inducer (Kobayashi et al., 2003; Hashimoto et al., 2009). This stands in contrast to

the dispensability of the *pgsA* gene in *Escherichia coli* (Matsumoto, 2001). Since in *B. subtilis* the *pgsA* gene is required for the synthesis of PG, a substrate for LTA, it would seem natural to ascribe its essentiality to its role in LTA synthesis. Gründling and Schneewind (2007) have identified the *ltaS* gene encoding LTA synthase in *Staphylococcus aureus*. Growth of an *S. aureus* P_{spac} -*ltaS* strain was dependent on IPTG for LTA synthesis and growth, indicating that LTA is essential at 37°C. Oku et al. (2009) have shown that *S. aureus* *ltaS*-null mutations result in severe thermosensitivity: the mutants grow very poorly even at 30°C. Because *B. subtilis* has four homologues of *ltaS* (Gründling and Schneewind, 2007), it was not surprising that none of the homologues were among the essential genes identified by Kobayashi et al. (2003), yet it seemed possible that defective mutations of the homologues might be synthetically lethal. However, Schirner et al. (2009) have reported that a quadruple mutant defective in all the homologues could be constructed, although it grew slowly and had a cell division

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Fig. 1. Synthetic pathways for phospholipids, glucolipids and LTA in *B. subtilis*. The diacylglycerol cycle (Jerga et al., 2007) for LTA synthesis is depicted with thick arrows. The genes encoding the enzymes relevant to the present study are indicated. The genes for PA and PGP phosphatase have not yet been identified. PA, phosphatidic acid; PS, phosphatidylserine; PE, phosphatidylethanolamine; PGP, phosphatidylglycerophosphate; PG, phosphatidylglycerol; lysylPG, lysylphosphatidylglycerol; CL, cardiolipin; DG, diacylglycerol; MGDG, monoglucosyldiacylglycerol; DGDG, diglucosyldiacylglycerol; TGDG, triglucosyldiacylglycerol; LTA, lipoteichoic acid.

and separation defect. Recently, Wörmann et al. (2011) have shown that LTA is absent in the quadruple mutant. Therefore, the essentiality of the *B. subtilis* *pgsA* gene cannot be ascribed to its indispensability for LTA synthesis.

The synthesis of LTA releases diacylglycerol (DG) moieties of PG. Since LTA is a long polymer, synthesis of a single LTA molecule results in the production of dozens of DG molecules. DG is converted to phosphatidic acid (PA) by DG kinase and reused in the synthesis of phospholipids, among them including PG (Fig. 1). Thus, LTA is synthesized by a cyclic reaction in which DG kinase plays a key role. The *dgkB* gene encoding DG kinase is essential in *B. subtilis* (Kobayashi et al., 2003), and repression of P_{spac} -*dgkB* leads to lethality. Jerga et al. (2007) have reported cessation of LTA synthesis and accumulation of DG in the *dgkB*-repressed cells. Since LTA is not essential in *B. subtilis*, it is unlikely that the defect in LTA synthesis is responsible for the death of the repressed cells, and the accumulation of DG is rather more likely the reason. In this study, we show that disruption of either or both of two LTA synthesis genes, *yflE* and *yfnI*, suppresses the lethality of the *dgkB*-repression. Accumulation of DG after repression of *dgkB* is suppressed by the disruption of *yfnI* but not *yflE*, suggesting that the DG accumulation is involved in, but not the

immediate and sole cause of, the non-viability of *B. subtilis* lacking *DgkB*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media *B. subtilis* 168 derivative strains and plasmids used in this study are listed in Table 1. Deletion mutants of *yflE* and *yfnI* were constructed by replacement with spectinomycin and tetracycline resistant cassettes, respectively. To construct these mutants, *yflE* and *yfnI*, including flanking sequences extending about 800 and 1100 bp upstream, and 900 and 1100 bp downstream of the genes, were amplified by PCR using appropriate primer sets. The amplified PCR fragments were digested with appropriate restriction enzymes (for *yflE*, *ApaI*, *BamHI*, and *PstI*; for *yfnI*, *ApaI*, *BamHI*, and *ClaI*), then cloned into pBlue-script II SK(+). The resulting plasmids (for *yflE* and *yfnI*) were digested with *PstI* and *ClaI*, then *spc* and *tet* genes cut out from pDG1726 and pDG1513, respectively, were ligated to produce pMH05, in which the entire coding region of *yflE* was replaced with *spc*, and pMT11, in which the region 270 bp downstream from the start codon and 170 bp upstream from the stop codon of *yfnI* was deleted and replaced with *tet*. These plasmids were used for the construction of strains MBS10 (*yflE::spc*) and MBS11 (*yfnI::tet*). Disruption of *dgkB* was by integration of a pMUTIN-derived plasmid (pMUTINCC) containing an internal fragment of the gene (Vagner et al., 1998) and the disrupted allele was denoted as *dgkB::pMUTINCC* (*erm*). Placement of *dgkB* under P_{spac} was by integration of a pMUTIN3 derivative containing the ribosome-binding site (RBS) and the 5' end of the gene and this was denoted as P_{dgkB} (YERQp). Xylose-inducible P_{xyt} promoter-controlled genes with or without the 5'-terminally fused green fluorescent protein (GFP)-coding sequence were constructed on pSG1729 (Lewis and Marston, 1999) or pX (Kim et al., 1996) and integrated into the chromosomal *amyE* locus by selecting for resistance to spectinomycin or chloramphenicol, respectively. The genes without GFP-fusion were designed to be expressed using the RBS on the vector. The allele *ugtP::kan* in which *ugtP* was replaced with a *kan* cassette was from strain KP261 (Price et al., 1997).

Luria-Bertani (LB) medium and buffered LB medium (Shiba et al., 2004) were used. For plates LB medium was solidified with 1.5% agar. When appropriate, antibiotics were included at the following concentrations (in µg/ml): erythromycin, 0.5; tetracycline, 10 or 15; spectinomycin, 50 or 100; chloramphenicol, 5; kanamycin, 5. Inducer IPTG was used at 1 mM, and xylose at 0.05% or 1%. Cell growth was monitored with a Klett-Summerson colorimeter (with no. 54 filter) or Shimadzu Bio Spec-mini (at OD 600 nm).

Table 1. *B. subtilis* strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Construction, source or reference ^b
<i>B. subtilis</i>		
168	<i>trpC2</i>	Laboratory stock
YERQp ^a	168 P _{spac} - <i>dgkB</i> (<i>erm</i>)	K. Asai
KP261	PY79 <i>ugtP::kan</i>	Price et al. (1997)
YTB019	168 <i>ugtP::kan</i>	KP261 → 168
TMB001	168 <i>amyE::(spc P_{xyl}-gfpmut1'-dgkB)</i>	pHR788 → 168
TMB003	168 <i>amyE::(spc P_{xyl}-gfpmut1'-dgkB)</i> <i>dgkB::pMUTINCC</i> (<i>erm</i>)	pTM000 → TMB001
MBS10	168 <i>yflE::spc</i>	pMH05 → 168
MBS11	168 <i>yfnI::tet</i>	pMT11 → 168
MBS12	168 <i>yflE::spc yfnI::tet</i>	MBS11 → MBS10
MBS13	168 <i>yflE::spc P_{spac}-dgkB</i>	YERQp → MBS10
MBS14	168 <i>yfnI::tet P_{spac}-dgkB</i>	YERQp → MBS11
MBS15	168 <i>yflE::spc yfnI::tet P_{spac}-dgkB</i>	YERQp → MBS12
MBS16	168 <i>yflE::spc P_{spac}-dgkB ugtP::kan</i>	YTB019 → MBS13
MBS17	168 <i>yfnI::tet P_{spac}-dgkB ugtP::kan</i>	YTB019 → MBS14
MBS18	168 <i>yflE::spc yfnI::tet P_{spac}-dgkB ugtP::kan</i>	YTB019 → MBS15
GFP-YflE	168 <i>amyE::(spc P_{xyl}-gfpmut1'-yflE)</i>	pGFP-YflE → 168
GFP-YfnI	168 <i>amyE::(spc P_{xyl}-gfpmut1'-yfnI)</i>	pGFP-YfnI → 168
UB62	168 Δ <i>amyE::(spc P_{xyl}-yflE)</i>	pHR790 → 168
UB64	168 Δ <i>amyE::(spc P_{xyl}-yfnI)</i>	pHR792 → 168
MBS21	168 P _{spac} - <i>dgkB yflE::spc amyE::(cat xylR P_{xyl}-yflE)</i>	pMT12 → MBS13
MBS22	168 P _{spac} - <i>dgkB yflE::spc amyE::(cat xylR P_{xyl}-yfnI)</i>	pMT13 → MBS13
MBS23	168 P _{spac} - <i>dgkB yfnI::tet amyE::(spc xylR P_{xyl}-yflE)</i>	UB62 → MBS14
MBS24	168 P _{spac} - <i>dgkB yfnI::tet amyE::(spc xylR P_{xyl}-yfnI)</i>	UB64 → MBS14
Plasmid		
pSG1729	pBR322 Δ <i>amyE::(spc P_{xyl}-gfpmut1-MCS^c)</i>	Lewis and Marston (1999)
pMUTINCC	pMUTIN3 having two Oid operator sequences	Hashimoto et al. (2009)
pHR788	pSG1729 P _{xyl} - <i>gfpmut1'-dgkB</i>	This work
pHR790	pSG1729 P _{xyl} - <i>yflE</i>	This work
pHR792	pSG1729 P _{xyl} - <i>yfnI</i>	This work
pTM000	pMUTINCC ' <i>dgkB</i> '	This work
pGFP-YflE	pSG1729 P _{xyl} - <i>gfpmut1'-yflE</i>	This work
pGFP-YfnI	pSG1729 P _{xyl} - <i>gfpmut1'-yfnI</i>	This work
pBluescript II SK(+)	cloning vector	Stratagene
pMH05	pBluescript II SK(+)- <i>yflE:: spc</i>	This work
pMT11	pBluescript II SK(+)- <i>yfnI:: tet</i>	This work
pDG1513	<i>tet</i> cassette	Guérout-Fleury et al. (1995)
pDG1726	<i>spc</i> cassette	Guérout-Fleury et al. (1995)
pX	pDG364 <i>amyE::(cat xylR P_{xyl}-MCS^c)</i>	Kim et al. (1996)
pMT12	pX P _{xyl} - <i>yflE</i>	This work
pMT13	pX P _{xyl} - <i>yfnI</i>	This work

^a. see <http://bacillus.genome.jp/> and <http://www.shigen.nig.ac.jp/bsub/>.^b. →, transformation with chromosomal or plasmid DNA.^c. MCS, multiple cloning site.

Genetic and recombinant DNA procedures These were based on standard methods. Competent cells for transformation were prepared by one-step (Wilson and

Bott, 1968) or two-step (Anagnostopoulos and Spizizen, 1961) methods. PCR primers used in this study are listed in Table 2.

Table 2. PCR primers used in this study

Primer	Sequence ^a	Description
pHV-F	5'-GTGTGGAATTGTGAGCGG-3'	For confirmation of gene disruption with pMUTIN
pHV-R	5'-AGTGTATCAACAAGCTGG-3'	
yflE5A	5'-agattcctaggATGAAAACATTTATAAAAGAAA-GAGG-3'	For cloning of <i>yflE</i> on pSG1729
yflE3B	5'-cgggatCCGAATGTGGAATTTGCAGC-3'	
yfnI5A	5'-agattcctaggATGAAGAACTTTTTTCTTACAA-AC-3'	For cloning of <i>yfnI</i> on pSG1729
yfnI3B	5'-cgggatCCTTGAGCGAAGGCAATGCG-3'	
yflEXhoIs	5'-GAGTcTcgagATGAAAACATTTATAAAAG-3'	For cloning of <i>gfp-yflE</i> on pSG1729
yflEEcoRIas	5'-CCGCgaaTTCTTATTTATCTTCGTTA-3'	
yfnIXhoIs	5'-AGAGcTcGagATGAAGAACTTTTTTCTTA-3'	For cloning of <i>gfp-yfnI</i> on pSG1729
yfnIEcoRIas	5'-CTTTgaatTCTTATTTGATTTCTTTC-3'	
yerQ-F3	5'-ctctcGAGATAATGAAACGTGCACGC-3'	For cloning of <i>gfp-dgkB</i> into pSG1729
yerQ-R3	5'-aagaaGCTTACCAAGCCAAGTTTGTAG-3'	
amyE-front-fwd	5'-CCAAACTGGACACATGGAAACACAC-3'	For confirmation of <i>amyE</i> disruption
amyE-back-rvs	5'-CCTGAACGAGAAGCTATCACCGCCC-3'	
yerQ-F0	5'-aagaagcttGCATCTTGCCAGGTCCT-3'	For disruption of <i>dgkB</i>
yerQ-R0	5'-ggaggatcCTGGCCGTTGACCTGTCC-3'	
dgkB check F	5'-CCGCTCCAATGCTCCCCCTT-3'	For confirmation of <i>dgkB</i> disruption, used together with pHV-R and Em-F
dgkB check R	5'-CACGTCGTACGTCAGCTCCG-3'	
yflEupsApaI	5'-GCAGGATGATTAGCGGCGGgCCCAC-3'	For disruption of <i>yflE</i> by Sp ^R cassette insertion
yflEupsPstI	5'-AATATAACACGAATcGCAGATTAG-3'	
yflEdownsPstI	5'-TCTCCGCTTTTTTATTTGACTGCAG-3'	
yflEdownsBamHI	5'-GTTTGGAGAGTGGATcCTCGGGCT-3'	
yfnIupsApaI	5'-AATTATTATGGTGGGCGCcGGCACCG-3'	For disruption of <i>yfnI</i> by Tc ^R cassette insertion
yfnIup-i-asClaI	5'-AAATGTCATCAAGAAATCGATC-3'	
yfnIdown-i-sClaI	5'-AAAAACTGAAAATCGATTcTGAAGAA-3'	
yfnIdownsBamHI	5'-GTAATGGCGGTACACAGCAGGaTCC-3'	
yflEF(Bam)	5'-ggaggatccATGAAAACATTTATAAAAGAA-3'	For cloning of <i>yflE</i> into pX
yflER(Bam)	5'-ggaggatccTTATTTATCTTCGTTATCCTT-3'	
yfnIF(Bam)	5'-ggaggatccATGAAGAACTTTTTTCTTAC-3'	For cloning of <i>yfnI</i> into pX
yfnIR(Bam)	5'-ggaggatccATGAAGAACTTTTTTCTTAC-3'	
pX checkF	5'-GAGATAAAGTTAGTTTATTGGATA-3'	For confirmation of gene direction cloned into pX
pX checkR	5'-AACTATTGCCGATGATAAGCTGTC-3'	

^a. Additional or unmatched nucleotides are in lowercase; restriction sites are italicized.

Radioactive labeling and analysis of lipids Cells were labeled with 3.75×10^4 Bq/ml of [1^{14} C] acetic acid (Amersham; 2.12×10^9 Bq/mmol) in LB medium at 37°C, continuously from initial inoculation for precultures to cell harvesting. Mid-exponential phase cells grown in the presence of 1 mM IPTG were washed with LB medium and diluted into LB medium with and without 1 mM IPTG to 5 Klett units. After 3 h cultivation cells were harvested, and lipids were extracted by a modified Bligh and Dyer's method (Ames, 1968). In order to assay DG accumulation, the extracts were subjected to TLC using TLC Silica Gel 60 plates (Merck) developed with hexane/diethylether/acetic acid (50:50:1, v/v/v). Mobili-

ties of DG and fatty acids (FA) were confirmed in preliminary experiments in which nonradioactive samples were analyzed with authentic DG (1, 2-dioleoyl-*sn*-glycerol; Avanti Polar Lipids) and FA (oleic acid; Wako) as standards and stained with primuline. Phospholipids and glucolipids were analyzed by two-dimensional TLC as described previously (Hashimoto et al., 2009). Lipid bands/spots were detected and quantified using an FLA-7000 image analyzer (Fujifilm).

Transformation efficiency assay for introduction of the disrupted *dgkB* allele into *yflE*- and *yfnI*-disrupted strains The chromosomal DNA of the strain

TMB003 [*amyE::(spc P_{xyt}-gfp-dgkB) dgkB::pMUTINCC(erm)*] was used to transform strains disrupted for either or both of *yflE* and *yfnI*, designated as MBS10 (*yflE::spc*), MBS11 (*yfnI::tet*), and MBS12 (*yflE::spc yfnI::tet*), selecting for erythromycin resistance of the *dgkB::pMUTINCC(erm)* allele. The transformation efficiencies were compared with the efficiencies at which the erythromycin resistance of *P_{spac}-dgkB(erm)* allele from DNA of strain YERQp was introduced into the same competent cell preparations in the presence of IPTG. The transformation efficiencies of the *P_{spac}-dgkB(erm)* allele were $1.8 \times 10^4 - 9.7 \times 10^5/\mu\text{g}$ DNA.

Subcellular localization of proteins Subcellular localization of GFP-fused proteins was observed under a fluorescence microscope as described previously (Nishibori et al., 2005). GFP fusions were confirmed by Western immunoblotting using anti-GFP mouse monoclonal antibody (Santa Cruz Biotechnology), horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (Novagen) and ECL Advance Kit (Amersham).

RESULTS AND DISCUSSION

Lethality due to *dgkB* repression was suppressed by *yflE* and *yfnI* disruption In this study we focused on the *yflE* and *yfnI* genes, whose products show higher homologies to *S. aureus* LtaS among the four homologues (Schirner et al., 2009). Gründling and Schneewind (2007) have reported that, when expressed from a chromosomally integrated copy in *ltaS*-repressed *S. aureus* cells, *yflE* restored growth and LTA synthesis. By contrast, *yfnI* restored the staphylococcal growth only partially and LTA synthesis was observed but the product showed a retarded electrophoretic mobility. The other two more distant homologues, *yqgS* and *yvgJ*, did not restore growth or LTA synthesis.

When the *dgkB* gene was placed under the *P_{spac}* promoter, cell growth became dependent on IPTG, indicating that the *dgkB* repression leads to lethality (Fig. 2), as had been shown by Jerga et al. (2007). We introduced disruptions of either or both of *yflE* and *yfnI* into the *P_{spac}-dgkB* strain and found that the lethal effect was suppressed: the cells of all three transformed strains were able to grow well in the absence of IPTG (Fig. 2a). Although *yfnI* cannot fully substitute for *ltaS* in *S. aureus* (Gründling and Schneewind, 2007), the suppression by the *yfnI* single disruption (Fig. 2a) suggests that YfnI produces DG and thus synthesizes LTA in *B. subtilis* cells as recently shown by Wörmann et al. (2011).

The growth of cells with *yflE*-disruption was slightly poorer (or colonies were smaller) than that of the parental *P_{spac}-dgkB* cells in the presence of the inducer. This was probably because the defect in *yflE* is slightly deleterious to cell growth. Poor growth of a *yflE* mutant has previ-

ously been reported by Schirner et al. (2009). When the *P_{xyt}* promoter-controlled *yflE* integrated in the *amyE* locus was expressed by an addition of 1% xylose, the growth of the *yflE*-disrupted strain became IPTG-dependent again (Fig. 2b). The *yflE* disruption was complemented by ectopic expression of *yflE*. The expression of *yfnI* from *P_{xyt}* promoter, however, did not cause the growth of the *yflE*-disrupted strain to become IPTG-dependent, i.e. cross-complementation was not observed (Fig. 2b). In the *yfnI*-disrupted strain, the ectopic expression of *yflE* from *P_{xyt}* promoter did not, while ectopic *yfnI* expression did return the growth to IPTG-dependent, indicating that there was no cross-complementation in the opposite direction either. The results suggest that the two gene products, YflE and YfnI, are functionally different, although the two enzymes have the same predicted membrane topology and domain structure (Wörmann et al., 2011).

Accumulation of DG in *dgkB*-repressed cells and its suppression by *yfnI* disruption Repression of *dgkB* in the *P_{spac}-dgkB* strain led to a markedly increased cellular level of DG, as revealed by continuous labeling with radioactive acetic acid followed by TLC analysis of the extracted lipids: three hours after IPTG removal the DG level had increased to $43 \pm 3\%$ of total lipids from a level of $26 \pm 5\%$ in the cells incubated with IPTG and very much higher than the $13 \pm 1\%$ observed in wild type cells (Fig. 3a). The higher DG level of *P_{spac}-dgkB* cells in the presence of IPTG indicates that *P_{spac}* is a weaker promoter, even when induced with 1 mM IPTG, than the native promoter for the *dgkB* gene. DG accumulation in *dgkB*-repressed cells was also shown in a pulse-chase experiment by Jerga et al. (2007). Disruption of *yfnI* in the *dgkB*-repressed cells reduced the DG content to $15 \pm 2\%$, which is close to the wild type level, but unexpectedly *yflE*-disruption did not ($42 \pm 1\%$) in cells whose growth defect was noticeably suppressed to the level of the wild type (Fig. 3b). The fact that no suppression of DG accumulation was observed in *yflE*-disrupted cells indicates that YflE may have little effect, as opposed to YfnI, on the accumulation and further that the growth defect after *dgkB* repression is not directly linked to the observed DG accumulation. Wörmann et al. (2011) have reported that strains expressing YfnI in the absence of YflE in both *B. subtilis* and *S. aureus* cells produced LTA of retarded mobility (this was first observed in Fig. 4G of Gründling and Schneewind, 2007), presumably caused by an increase ($\times 1.6$) in glycerolphosphate chain length compared with LTA produced by YflE. They suggested that YfnI becomes more efficient (or activated) in LTA synthesis when YflE is absent. The DG accumulated in *yflE*-disrupted cells (Fig. 3a), may thus possibly result from the efficient synthesis of LTA, yielding a large amount of DG, by the more efficient YfnI that has been freed from an as yet unknown control by YflE.

Fig. 2. Suppression of the lethality due to *dgkB* repression by *yfIE* and *yfNI* disruptions (a) and a complementation test of the suppressive effect by ectopically expressed homologues (b). Overnight cultures in buffered LB medium containing 1 mM IPTG and appropriate antibiotics were washed, diluted to $OD_{600}=0.05$ and then tenfold serially down to 10^{-4} with saline. One microliter each was spotted on LB agar plates containing no antibiotic with or without 1 mM IPTG (a); LB agar contained 1% xylose in (b). The plates were incubated at 37°C for ca. 9 h and then at room temperature overnight. Strains used in (a) were 168, YERQp, MBS13, MBS14, and MBS15. Strains used in (b) upper panel were 168, YERQp, MBS13, MBS21, and MBS22 and those in (b) lower panel were 168, YERQp, MBS14, MBS23, and MBS24.

The results shown in Fig. 3 also indicate that each of YqgS and YvgJ makes only a small contribution to DG accumulation, although they may be partly responsible, in addition to PA phosphatase, for the DG accumulation in cells doubly disrupted for *yfIE* and *yfNI* ($18 \pm 3\%$). The expression levels of the *yqgS* and *yvgJ* genes are low during vegetative growth, and these genes seem to play important roles during sporulation: a *yfIE yqgS*-doubly disrupted strain did not sporulate, and a *yfIE yvgJ*-doubly disrupted

strain showed a reduced sporulation efficiency, while a *yfIE*-disrupted strain sporulated normally (Schirner et al., 2009). Although YqgS has LTA-synthesizing activity (Wörmann et al., 2011), its contribution to DG production is probably very small during vegetative growth. YvgJ is likely to function as an LTA primase transferring a single G1P to the membrane anchor (Wörmann et al., 2011), which would produce a much smaller amount of DG than the LTA polymerization performed by the other enzymes.

Fig. 3. Effect on DG accumulation of disruption *yfIE* and *yfjI* in the *dgkB*-repressed cells. Cells of the disrupted strains and their parental strain YERQp (*P_{spac}-dgkB*) were continuously labeled with [^{14}C] acetic acid and harvested 3 h after removal of 1 mM IPTG (–) or without its removal (+). Cells of wild-type strain 168 were labeled in the absence of the inducer but subjected to the same washing and diluting procedures. Lipids were extracted and analyzed for DG content by TLC as described in MATERIALS AND METHODS. (a). The position of 1, 2-diacylglycerol is indicated by 1, 2-DG. The position of the 1, 3-isomer of DG, which is likely formed by spontaneous isomerization, is indicated by 1, 3-DG. The 1, 3-isomer was also detected when authentic DG (1, 2-dioleoyl-*sn*-glycerol; Avanti Polar Lipids) was subjected to TLC and stained with primuline. The position of fatty acids is indicated by FA. Other polar lipids (phospholipids and glucolipids) remain at the chromatographic origin indicated by O. The percentage of DG (1, 2-DG plus 1, 3-isomer) to total lipids is shown above each lane. Mean values of experiments with three different cultures are given with standard deviations. Growth of the cells of the disrupted strains and their parental strain used for the lipid extraction are shown (b). Strains 168 (*), YERQp (□, ■), MBS13 (◇, ◆), MBS14 (△, ▲), and MBS15 (○, ●) in the absence (open symbols) or presence (closed symbols) of 1 mM IPTG.

Since DG kinase reintroduces DG into the phospholipid synthetic pathway (Fig. 1), a lower supply of PA due to *dgkB* repression may result in reduced synthesis of

phospholipids. Reduced PG content might then lead to a growth defect as it does in *pgsA*-repressed cells (Hashimoto et al., 2009), although the direct cause for the defect is

Fig. 4. Effect of *dgkB* repression on phospholipids. The lipid extracts of the P_{spac} -*dgkB* cells prepared as described in the legend to Fig. 3 were analyzed by two-dimensional TLC, first (x dimension) with chloroform/methanol/water (65:25:4, v/v/v) and then (y dimension) with chloroform/methanol/acetic acid (65:25:10, v/v/v), as described in MATERIALS AND METHODS. O, chromatographic origin. See legends to Fig. 1 for abbreviations.

still unclear. Two-dimensional TLC analyses of the labeled lipid extracts of the P_{spac} -*dgkB* cells (YERQp strain) at 3 h after IPTG removal, however, showed that PG content was not reduced (31.5% to 30.9% of total lipids), while the contents of phosphatidylethanolamine (PE), cardiolipin (CL) and lysylphosphatidylglycerol (lysylPG) were greatly decreased (5.9% to 0.4%, 5.8% to 0.7%, and 6.0% to 0.6%, respectively) (Fig. 4, Table 3). Note that both CDP-DG and PA, the essential intermediates for the synthesis of phospholipids and others, were not decreased (1.8% to 2.2% and 0.4% to 0.4%, respectively). Disruption of *yflE* largely mitigated the decrease of PE, CL, and lysylPG (13.7% to 6.7%, 2.8% to 2.1%, and 4.9% to 3.4%, respectively). Disruption of *yfnI*, both in the cases with and without *yflE* disruption, fully mitigated the reduction of these phospholipids.

Mutants singly or multiply defective in the synthesis of phospholipids other than PG grow almost normally (Matsumoto et al., 1998; Kawai et al., 2004, 2006; Salzberg and Helmann, 2008). Thus, it is unlikely that the growth defect of the *dgkB*-repressed cells is due to interrupted reintroduction of DG into the phospholipid synthetic pathway. The result of Table 3 suggests that the imbalance of phospholipid composition induced after the interrupted reintroduction into the phospholipid synthetic pathway and simultaneous consumption of PG through LTA synthesis, which causes the large increase in DG content, but not the increase in DG content alone, might be the cause for the growth stasis of *dgkB* repressed cells.

The large reduction of PE content in contrast to the small PG reduction may suggest a possible lower affinity with the substrate CDP-DG of phosphatidylserine synthase, which catalyzes the committed step for PE synthesis, compared to that of phosphatidylglycerophosphate

synthase, which catalyzes the committed step for PG synthesis. However, comparably large reductions of CL and lysylPG content, both synthesized downstream from PG, suggest that it is not simply ascribable to a possible difference in affinity. It seems that *B. subtilis* cells have a mechanism that minimizes the reduction of PG content at the expense of other phospholipids, PE, CL and lysylPG, when PA is in short supply.

Disruption of *ugtP* partially weakened the suppression by *yflE* disruption of the lethality of *dgkB* repression Glucolipid synthase encoded by the *ugtP* gene processively transfers glucose from UDP-glucose to DG (Jorasch et al., 1998a), thus draining DG into the glucolipid synthetic pathway (Fig. 1). Disruption of *ugtP* is nonlethal in a wild-type background (Price et al., 1997; Jorasch et al., 1998b), but how deleterious is it in a *dgkB*-repressed background? We introduced a *ugtP*::*kan* disrupted allele into P_{spac} -*dgkB* strains carrying disruptions of either or both of *yflE* and *yfnI*. The P_{spac} -*dgkB* *yflE* strain became partially IPTG-dependent for its capacity to form colonies (that is, the suppression by *yflE* disruption was weakened) when the disrupted allele of *ugtP* was introduced, whereas the growth of the P_{spac} -*dgkB* *yfnI* *ugtP* strain was almost the same in the presence and absence of the inducer (Fig. 5). Inactivation by *ugtP* disruption of the DG-draining pathway presumably increases DG accumulation to a deleterious level upon prolonged incubation on plate in P_{spac} -*dgkB* *yflE* cells, which retain efficient LTA synthetic activity via YfnI (Fig. 3a).

Introduction of disrupted *dgkB* into strains defective in LTA synthesis Although the *dgkB*-repressed cells were viable if either or both of *yflE* and *yfnI* were dis-

Table 3. Effect on lipid composition of disruption of *yflE* and *yfnI* in *Pspac-dgkB* cells

	% of total lipid ^a										
	PG	PE	CL	PA	CDPDG	LysylPG	MGDG	DGDG	TGDG	DG	others
168	37.5	30.5	4.5	0.6	1.8	7.8	0.3	4.9	0.3	9.6	2.2
	±6.2	±3.8	±5.5	±0.5	±0.2	±3.7	±0.2	±0.9	±0.1	±4.7	±1.8
<i>Pspac-dgkB</i>	31.5	5.9	5.8	0.4	1.8	6.0	1.5	11.1	0.3	34.3	1.4
+ IPTG	±3.3	±2.1	±3.8	±0.1	±0.4	±0.7	±0.3	±1.0	±0.1	±3.7	±1.3
<i>Pspac-dgkB</i>	30.9	0.4	0.7	0.4	2.2	0.6	1.8	13.1	0.2	49.5	0.2
– IPTG	±6.4	±0.2	±0.3	±0.3	±0.7	±0.1	±0.3	±2.0	±0.1	±8.8	±0.2
<i>Pspac-dgkB yflE</i>	23.6	13.7	2.8	0.4	7.9	4.9	0.9	3.9	0.3	41.4	0.2
+ IPTG	±2.3	±2.1	±0.2	±0.1	±1.1	±1.0	±0.1	±0.4	±0.1	±5.1	±0.2
<i>Pspac-dgkB yflE</i>	23.9	6.7	2.1	0.1	8.3	3.4	1.3	7.2	0.3	46.2	0.5
– IPTG	±4.7	±1.8	±0.2	±0.0	±0.9	±1.1	±0.2	±0.6	±0.1	±8.2	±0.2
<i>Pspac-dgkB yfnI</i>	35.0	25.2	3.6	0.9	2.1	8.9	0.5	10.7	0.3	12.1	0.7
+ IPTG	±2.8	±10.5	±3.4	±0.9	±1.3	±1.3	±0.2	±4.7	±0.1	±6.6	±0.6
<i>Pspac-dgkB yfnI</i>	37.2	23.3	3.1	0.8	2.0	8.8	0.5	10.5	0.3	13.1	0.4
– IPTG	±4.0	±7.8	±1.7	±0.8	±1.4	±0.9	±0.2	±5.0	±0.1	±6.4	±0.4
<i>Pspac-dgkB yflE yfnI</i>	27.5	15.4	8.1	1.7	7.2	7.4	0.8	3.7	0.3	27.0	0.9
+ IPTG	±1.9	±0.3	±1.3	±1.6	±1.8	±0.7	±0.1	±0.4	±0.1	±3.0	±0.2
<i>Pspac-dgkB yflE yfnI</i>	26.0	14.0	7.6	1.6	6.8	6.6	0.9	4.5	0.3	31.1	0.6
– IPTG	±1.3	±1.6	±1.0	±1.6	±2.2	±1.3	±0.1	±0.9	±0.0	±5.3	±0.2

^a. Membrane lipids of the strains YERQp (*P_{spac}-dgkB*), MBS13 (*yflE::spc P_{spac}-dgkB*), MBS14 (*yfnI::tet P_{spac}-dgkB*), MBS15 (*yflE::spc yfnI::tet P_{spac}-dgkB*) and wild type 168 were labeled with [¹⁴C] acetic acid. The labeled acetic acid was continuously included in LB medium containing 1 mM IPTG. At 3 h after removal of the inducer (–) or in its presence (+) the cells were harvested to extract membrane lipids. For details of lipid extraction and two-dimensional TLC, see MATERIALS AND METHODS. Percentage of [¹⁴C]-labeled spot to total lipid developed on TLC plate was calculated from PSL units obtained with a FLA-7000 bioimaging analyzer. A representative TLC plate of YERQp (*P_{spac}-dgkB*) cells is shown in Fig. 4. Mean values of the experiments with three different cultures are shown with standard deviations below. Abbreviations: PG, phosphatidylglycerol; PE, phosphatidylethanolamine; CL, cardiolipin; PA, phosphatidic acid; CDPDG, CDP-diacylglycerol; lysylPG, lysylphosphatidylglycerol; MGDG, monoglucosyldiacylglycerol; DGDG, diglucosyldiacylglycerol; TGDG, triglucosyldiacylglycerol; DG, diacylglycerol.

Fig. 5. Effect of deletion of *ugtP* on the suppression of lethality due to *dgkB* repression by *yflE* and *yfnI* disruption. For the experimental procedure see the legend to Fig. 2. Strains used were 168, YERQp, MBS16, MBS17, MBS18, and YTB019.

Fig. 6. Subcellular localization of GFP-YflE (a), GFP-YfnI (b), and GFP-DgkB (c) fusion proteins. The fusion genes under P_{xyl} control were integrated into the *amyE* locus of strain 168 and induced with 0.05% xylose in LB medium. Cells were harvested at ca. 100 Klett units, and fluorescence and phase-contrast micrographs were taken with exposure times of 4 s and 0.04 s, respectively. Scale bar, 1 μ m.

rupted, basal level expression of P_{spac} -*dgkB* in the absence of IPTG might possibly contribute to the viability. To test for this possibility, we constructed a disrupted *dgkB* allele and introduced it into *yflE*- and *yfnI*-disrupted strains.

First, we integrated a pMUTINCC plasmid having an internal fragment of the *dgkB* gene into the chromosome of a strain that harbors a gene encoding N-terminally GFP-fused DgkB under the control of P_{xyl} at the *amyE* locus in the presence of xylose, and screened the integrants by PCR for those where integration occurred at the native *dgkB* locus, not in the ectopic *gfp*-fused locus at *amyE*. The resultant *dgkB*::pMUTINCC (*erm*) mutant harboring *amyE*::(*spc* P_{xyl} -*gfp*'-*dgkB*), designated as strain TMB003, was dependent on xylose for growth.

The disrupted allele *dgkB*::pMUTINCC (*erm*) of the chromosomal DNA of TMB003 was introduced into the recipient strains disrupted for either or both of *yflE* and *yfnI* at transformation efficiencies only slightly lower than those of the introduction of the P_{spac} -*dgkB* allele in the presence of IPTG; the relative transformation efficiencies of the strains MBS10 (*yflE*::*spc*), MBS11 (*yfnI*::*tet*), and MBS12 (*yflE*::*spc* *yfnI*::*tet*) were 0.44 ± 0.2 , 0.45 ± 0.11 , and 0.35 ± 0.12 , respectively. In the control experiment in which wild-type strain 168 was transformed with the TMB003 DNA, a few minute colonies appeared on the erythromycin-containing selection plates, but they did not continue to grow when plated on LB agar containing erythromycin. To confirm that the introduced *dgkB*::pMUTINCC (*erm*) was not coupled with the P_{xyl} -*gfp*'-*dgkB* allele, we picked up eight erythromycin-resistant transformants for each recipient strain and checked whether the *amyE*::(*spc* P_{xyl} -*gfp*'-*dgkB*) allele was simultaneously introduced. No such transformant was obtained, indicating that the P_{xyl} -*gfp*'-*dgkB* allele was not simultaneously introduced. We conclude, therefore, that the disrupted *dgkB*::pMUTINCC (*erm*) allele was intro-

duced into the strains with disruption of either or both of *yflE* and *yfnI*, demonstrating that these strains with abolished *dgkB* expression are viable.

Subcellular localization of YflE, YfnI, and DgkB

Since PgsA, a key enzyme for the synthesis of PG, is localized to the division septum in *B. subtilis* cells (Nishibori et al., 2005), LTA synthases that utilize PG as a substrate were expected to show similar subcellular localization. We expressed N-terminally GFP-fused YflE and YfnI proteins at low levels (by inducing the P_{xyl} promoter with 0.05% xylose) from the fusion genes integrated in the *amyE* locus and observed that they were localized to the septum under a fluorescence microscope, as expected (Fig. 6, a and b). Septal enrichment of YflE at a higher expression level (with 0.5% xylose) was reported by Schirner et al. (2009).

Since YflE and YfnI produce a large amount of DG, we expected that DG kinase would show a subcellular localization similar to these LTA synthases. However, an N-terminally GFP-fused DgkB protein expressed from the fusion gene integrated in the *amyE* locus was found not only in the septal region but also along the cell periphery (Fig. 6c). Although DgkB is a soluble protein (Jerga et al., 2007), it should reside on the membrane surface to phosphorylate DG in the membrane. *S. aureus* DgkB was demonstrated to have an affinity for anionic phospholipids and to bind to phospholipid vesicles *in vitro* (Jerga et al., 2009), and perhaps *B. subtilis* DgkB moves around for similar reasons.

Conclusion In *E. coli*, the major source of DG is the transfer of the G1P moiety of PG to generate mature membrane-derived oligosaccharides (MDOs) (Raetz and Newman, 1979). DG kinase of *E. coli* is encoded by the *dgkA* gene. It is a membrane protein (Sanders et al., 1996) and belongs to a different protein family than the

soluble DG kinase family, which includes *B. subtilis* DgkB (Jerga et al., 2007). Although *dgkA* is not an essential gene, *dgkA*-defective *E. coli* mutants do not grow well at low osmolarity (Raetz and Newman, 1978) or in the presence of arbutin (Jackson et al., 1984). At low osmolarity MDO synthesis is greatly enhanced (Kennedy, 1982). Arbutin is an analog of MDO in terms of G1P transfer from PG, and in its presence the transferase shows high activity even at high osmolarity (Bohin and Kennedy, 1984). Consequently, under these conditions, a large amount of DG accumulates. Thus it would appear that DG accumulation is deleterious to cell growth. It is, however, yet to be elucidated why DG accumulation is deleterious to bacterial growth.

We show here that disruption of *yflE* and *yfnI*, or of one of these LTA synthesis genes suppresses the lethality of *dgkB* repression and that the accumulation of DG after *dgkB*-repression was suppressed by the disruption of *yfnI*, indicating that the DG accumulation is involved in the lethality after *dgkB* repression. However, *yflE* disruption did not suppress the DG accumulation while it did suppress the growth defect, indicating that DG accumulation is not deleterious to the *yflE*-disrupted cells. The DG accumulation in *yflE*-disrupted cells, presumably resulted from the efficient synthesis of LTA by YfnI freed from a possible control by YflE (Wörmann et al., 2011), but we cannot explain the reason for the suppressive effect of the *yflE* disruption. Further disruption of *ugtP*, whose product consumes DG as a substrate (see Fig. 1), partially weakened the suppression by *yflE* disruption. We conclude that DgkB is required to keep the cellular level of DG below a lethal level and further that disruption of either or both of *yflE* and *yfnI* makes *dgkB* dispensable. Indeed, a disrupted *dgkB* allele was successfully introduced into these disruptants.

Only minor DG accumulation was detected in cells doubly disrupted for *yflE* and *yfnI*, indicating that YqgS and YvgJ make an even smaller contribution to DG production than YflE and YfnI during vegetative growth. LTA synthesis by YflE and YfnI is most likely a major source of DG production in the vegetative cells of *B. subtilis*. The role of YflE is believed to be that of a “housekeeping” enzyme, while YfnI may be a “stress-responding” enzyme as the latter is possibly sigma M-dependent (Wörmann et al., 2011; Sutcliffe, 2011). YfnI becomes more efficient in LTA synthesis and produces longer polymers ($\times 1.6$ in chain length) than YflE when YflE is absent, suggesting that the activity of YfnI is under some sort of control by YfnE. The exact roles the enzymes play *in vivo* and the precise relationships between them remain to be elucidated.

DG accumulation in the *dgkB*-repressed cells has a deleterious effect on *B. subtilis* growth as it does in the case of *E. coli*. However, DG accumulation in *yflE*-disrupted cells does not. This obviously contradictory result indi-

cates that we are far from a full understanding of the essential role that DgkB plays in the DG cycle and that of LTA on the cell surface of *B. subtilis*. The longer LTA polymer on the surface of *yflE*-disrupted cells may have a function that mitigates the deleterious effect of DG accumulation. The analysis with two-dimensional TLC has revealed an imbalance of phospholipid composition in *dgkB*-repressed cells (Table 3). The imbalance induced after the interrupted reintroduction of DG into the phospholipid synthetic pathway and simultaneous consumption of PG through LTA synthesis, which causes DG accumulation, might be the cause for the growth stasis of *dgkB* repressed cells.

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