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Role of SpoVG in Asymmetric Septation in *Bacillus subtilis*

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Deletion of the *citC* gene, coding for isocitrate dehydrogenase, arrests sporulation of *Bacillus subtilis* at stage I after bipolar localization of the cell division protein FtsZ but before formation of the asymmetric septum. A spontaneous extragenic suppressor mutation that overcame the stage I block was found to map within the *spoVG* gene. The suppressing mutation and other *spoVG* loss-of-function mutations enabled *citC* mutant cells to form asymmetric septa and to activate the forespore-specific sigma factor σ^F. However, little induction of mother cell-specific, σ^E-dependent sporulation genes was observed in a *citC spoVG* double mutant, indicating that there is an additional defect(s) in compartmentalized gene expression in the *citC* mutant. These other defects could be partially overcome by reducing the synthesis of citrate, by buffering the medium, or by adding excess $MnCl_2$. Overexpression of the *spoVG* gene in wild-type cells significantly delayed σ^F activation. Increased expression and stability of SpoVG in *citC* mutant cells may contribute to the *citC* mutant phenotype. Inactivation of the *spoVG* gene caused a population of otherwise wild-type cells to produce a small number of minicells during growth and caused sporulating cells to complete asymmetric septation more rapidly than normal. Unlike the case for inactivation of the cell division inhibitor gene *minD*, many of these minicells contained DNA and appeared only when the primary sporulation signal transduction pathway, the Spo0A phosphorelay, was active. These results suggest that SpoVG interferes with or is a negative regulator of the pathway leading to asymmetric septation.

Spore formation in *Bacillus subtilis* is a developmental pathway in which sequential, compartmentalized gene expression is achieved by interlocking cascades of regulatory factors and morphological cues (67). The primary environmental signal for initiation of sporulation is nutrient limitation (63), but this same condition also induces other adaptive responses (e.g., genetic competence, degradative enzyme synthesis, chemotaxis and motility, and antibiotic production) characteristic of slowly growing or stationary-phase cells.

The earliest morphological change that distinguishes a sporulating from a nonsporulating stationary-phase cell is the formation of an asymmetrically disposed division septum (48). During exponential growth, *B. subtilis* cells, like those of most other rod-shaped bacteria, divide exclusively at mid-cell. Mid-cell division requires the assembly at the septation site of a protein complex that includes FtsZ (4, 6), a tubulin-like GTPase (11, 50). Formation of a ring of FtsZ at the site of future septation is a prerequisite for association of other proteins with this site (25) and for septation itself. When a *B. subtilis* cell initiates sporulation, rings of FtsZ protein form at the two poles of the cell rather than at mid-cell (36). One of these rings becomes the site of asymmetric (polar) septation; the other ring dissociates. Bipolar localization of FtsZ is thought to be mediated by the product of a gene that depends on the Spo0A transcription factor for its expression, since in a *spo0A* mutant strain, polar rings of FtsZ do not form in stationary phase (36). Another factor required for polar septation is thought to be the product of a gene transcribed by the σ^H form of RNA polymerase, since a *spo0H* (σ^H) mutant does not form polar septa even though FtsZ rings assemble at polar sites (36).

Asymmetric septation permits forespore-specific activation of σ^F (1, 16, 42), the first step in a cascade of gene expression determined by sequentially active σ factors (67). Soon after σ^F becomes active, its activity leads to signals that activate σ^E in the mother cell (27, 32, 37), followed by activation of σ^G in the forespore (46, 68) and σ^K in the mother cell (9, 10, 39).

The nutritional signal that initiates sporulation is unknown but is assumed to be created intracellularly by normal metabolism (63). It has long been known that the enzymes of the Krebs citric acid cycle are induced as cells leave exponential growth phase; activities of the enzymes are required for successful sporulation (18, 21, 54, 72). To investigate the specific roles of Krebs cycle enzymes in spore formation, we have analyzed the stages of sporulation blockage in mutants deficient in various steps of the cycle (8, 29, 31). We found that the absence of the third enzyme, isocitrate dehydrogenase (ICDH), causes a specific block at stage I (31); mutant cells enter stationary phase, organize their chromosomes in an axial filament (as in wild-type cells), and assemble apparently normal rings of FtsZ protein at both poles (31). No polar septation occurs, however. In the accompanying paper (41), we show that abnormally high accumulation of citrate is responsible, at least in part, for this phenotype.

To understand the basis for this stage I block and to identify proteins that may participate in or regulate asymmetric septation, we sought suppressor mutations that would restore polar septation to an ICDH (*citC*) mutant strain. To do so, we searched for spontaneous pseudorevertants of a *citC* null mutant in which expression of the σ^F-dependent gene *spoIIQ* (38) was restored. One such mutation proved to be in the *spoVG* gene (53, 56, 58), whose product was not previously known to affect the pathway leading to asymmetric septation.

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MATERIALS AND METHODS

Strains and growth conditions. The *B. subtilis* strains and plasmids used in this study are listed in Table 1. Cells were grown in nutrient broth sporulation (DS) medium (19) supplemented with antibiotics (5), if necessary. Sporulation was induced either by nutrient exhaustion in DS broth or by a resuspension method (65). LacZ indicator plates contained 5-bromo-4-chloro-3-indolyl-β-D-galacto-

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

Strain or plasmid	Genotype or description	Source, reference, or construction
Strains		
E. coli		
JM107	*endA1 gyrA96 thi hsdR17* (r_K^- m_K^+) *supE44 relA1* λ^- Δ(*lac-proAB*) e14$^-$/F′ *traD36 proAB lacI*^{q} *lacZ*ΔM15	Laboratory stock
B. subtilis		
JH642	*trpC2 pheA1*	J. A. Hoch
FJS107	*trpC2* SPβ^s	62
AG918	*trpC2 pheA1 sof-1 cat*	A. D. Grossman
DZR143	Δ*spo0A*::*erm trpC2 pheA1*	28
KS265	*spoVG*::Tn*917*ΩHU265	56
MB284	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::[Φ(*spoVG′-lacZ*) *cat*]	43
MO1657	Δ*amyE*::[Φ(*sspE′-lacZ*) *cat*] *trpC2 pheA1*	20
MO1778	Δ*spoVG*::*tet* Δ*spoIIB*::*kan trpC2 pheA1*	66
PL33	*minD*::pPL6 (*cat*)	35
SJB78	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::[Φ(*spoVG′-lacZ*) *cat*] *trpC2 pheA1*	MB284 DNA→JH642[a]
SJB219	Δ*citC*::*spc trpC2 pheA1*	31
SJB225	Φ(*spoIID′-lacZ*) *cat trpC2 pheA1*	31
SJB229	Φ(*spoIID′-lacZ*) *cat* Δ*citC*::*spc trpC2 pheA1*	31
SJB231	Δ*citZC*::*spc trpC2 pheA1*	41
SJB294	Δ*amyE*::[Φ(*spoIIQ′-lacZ*) *cat*] *trpC2 pheA1*	31
SJB295	Δ*amyE*::[Φ(*spoIIQ′-lacZ*) *cat*] Δ*citC*::*spc trpC2 pheA1*	31
SC432	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::[Φ(*cotA′-lacZ*) *cat*]	S. Cutting
SR10	Φ(*spoIID′-lacZ*) *cat*	52
AS5	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::[Φ(*cotA′-lacZ*) *cat*] Δ*citC*::*spc* Δ*spoVG*::*tet sof-1 cat trpC2 pheA1*	SJB219 DNA→KMB433
AS9	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::[Φ(*cotA′-lacZ*) *cat*] Δ*citC*::*spc* Δ*spoVG*::*tet spo0A*$^+$ *cat trpC2 pheA1*	SJB219 DNA→KMB434
KMB97	Δ*amyE*::[Φ(*spoIIQ′-lacZ*) *cat*] Δ*citC*::*spc spoVG2 trpC2 pheA1* (mutant of SJB295)	
KMB136	Δ*amyE*::[Φ(*spoIIQ′-lacZ*) *cat*] Δ*citC*::*spc spoVG*::Tn*917*ΩHU265 *trpC2 pheA1*	KS265 DNA→SJB295
KMB158	Δ*amyE*::[Φ(*spoIIQ′-lacZ*) *cat*] Δ*thrC*::(*spoVG*$^+$ *erm*) Δ*citC*::*spc spoVG2 trpC2 pheA1*	pKM56→KMB97
KMB162	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::[Φ(*spoVG′-lacZ*) *cat*] Δ*citC*::*spc trpC2 pheA1*	MB284 DNA→SJB219
KMB197	Δ*citC*::*spc* Δ*spoVG*::*tet trpC2 pheA1*	MO1778 DNA→SJB219
KMB198	Δ*amyE*::[Φ(*spoIIQ′-lacZ*) *cat*] Δ*citC*::*spc* Δ*spoVG*::*tet trpC2 pheA1*	MO1778 DNA→SJB295
KMB199	Δ*spoVG*::*tet trpC2 pheA1*	MO1778 DNA→JH642
KMB200	Δ*amyE*::[Φ(*spoIIQ′-lacZ*) *cat*] Δ*spoVG*::*tet trpC2 pheA1*	KMB199 DNA→SJB294
KMB207	Δ*citZC*::*spc* Δ*spoVG*::*tet trpC2 pheA1*	MO1778 DNA→SJB231
KMB208	Φ(*spoIID′-lacZ*) *cat* Δ*citC*::*spc* Δ*spoVG*::*tet trpC2 pheA1*	SR10 DNA→KMB197
KMB210	Δ*amyE*::[Φ(*sspE′-lacZ*) *cat*] Δ*citC*::*spc* Δ*spoVG*::*tet trpC2 pheA1*	MO1657 DNA→KMB197
KMB212	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::[Φ(*cotA′-lacZ*) *cat*] Δ*citC*::*spc* Δ*spoVG*::*tet trpC2 pheA1*	SC432→KMB197
KMB217	Δ*amyE*::[Φ(*spoIIQ′-lacZ*) *cat*] Δ*citZC*::*spc* Δ*spoVG*::*tet trpC2 pheA1*	SJB294 DNA→KMB207
KMB218	Φ(*spoIID′-lacZ*) *cat* Δ*citZC*::*spc* Δ*spoVG*::*tet trpC2 pheA1*	SR10 DNA→KMB207
KMB219	Δ*amyE*::[Φ(*sspE′-lacZ*) *cat*] Δ*citZC*::*spc* Δ*spoVG*::*tet trpC2 pheA1*	MO1657 DNA→KMB207
KMB220	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::[Φ(*cotA′-lacZ*) *cat*] Δ*citZC*::*spc* Δ*spoVG*::*tet trpC2 pheA1*	SC432→KMB207
KMB271	*minD*::pPL6 (*cat*) Δ*spoVG*::*tet trpC2 pheA1*	PL33 DNA→KMB199
KMB272	*minD*::pPL6 (*cat*) *trpC2 pheA1*	PL33 DNA→JH642
KMB352	Δ*spo0A*::*erm* Δ*spoVG*::*tet trpC2 pheA1*	DZR143 DNA→KMB199
KMB353	Δ*spo0A*::*erm minD*::pPL6 (*cat*) *trpC2 pheA1*	DZR143 DNA→KMB272
KMB367	Δ*citZC*::*spc* Δ*spoVG*::*tet spo0A cat trpC2 pheA1*	AG918 DNA→KMB207
KMB377	Δ*citZC*::*spc* Δ*spoVG*::*tet sof-1 cat trpC2 pheA1*	AG918 DNA→KMB207
KMB398	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::[Φ(*cotA′-lacZ*) *cat*] Δ*citZC*::*spc* Δ*spoVG*::*tet spo0A*$^+$ *cat trpC2 pheA1*	SC432 DNA→KMB367
KMB399	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::[Φ(*cotA′-lacZ*) *cat*] Δ*citZC*::*spc* Δ*spoVG*::*tet sof-1 cat trpC2 pheA1*	SC432 DNA→KMB377
KMB422	Δ*spoVG*::*tet sof-1 cat trpC2 pheA1*	MO1778 DNA→KMB415
KMB423	Δ*spoVG*::*tet spo0A*$^+$ *cat trpC2 pheA1*	MO1778 DNA→KMB416
KMB433	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::[Φ(*cotA′-lacZ*) *cat*] Δ*spoVG*::*tet sof-1 cat trpC2 pheA1*	SC432 DNA→KMB422
KMB434	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::[Φ(*cotA′-lacZ*) *cat*] Δ*spoVG*::*tet spo0A*$^+$ *cat trpC2 pheA1*	SC432 DNA→KMB423
Plasmids		
pHP13	Vector	
pJL52	Helper plasmid[b]	
pKM98	*spoVG*$^+$	
pKM157	*spoVG2*	

[a] MB284 DNA was introduced into strain JH642.
[b] Plasmid pJL52 was used to increase the stability of pHP13-derived plasmids in *B. subtilis* by the method of LeDeaux and Grossman (33).

side (X-Gal) at 80 μg/ml. *Escherichia coli* JM107 (71) was used for construction of plasmids.

DNA manipulation and transformation. Plasmid DNA was isolated from *E. coli* cells by a modified version of the method of He et al. (26). Other DNA manipulations were done by standard protocols (55). Preparation of electroporation-competent cells of *E. coli* and transformation with a Bio-Rad GenePulser apparatus (Bio-Rad Laboratories) were performed by the method of Dower et al. (14). Chromosomal DNA from *B. subtilis* was prepared as described previously (19). Transformation of *B. subtilis* cells with chromosomal DNA or plasmids was done by the procedure of Dubnau and Davidoff-Abelson (15).

Construction of an integrative genomic library of *B. subtilis*. A *B. subtilis* integrative plasmid, pPS34, and a *B. subtilis* genomic library were constructed by Pascale Serror (59). pPS34 was created by ligating the 2.8-kb *Ssp*I fragment of pBluescript SK(−) (pSK−) (Stratagene, Inc.) to a 1.35-kb erythromycin resistance gene (*erm*) excised from pJPM9 (43) as a blunt-ended *Eco*RI-*Hin*dIII fragment. The erythromycin cassette was inserted in the same orientation as the *lacZ* gene of pSK−. For construction of a *B. subtilis* genomic library, chromosomal DNA from strain FJS107 (Table 1) was digested separately with *Alu*I, *Hin*pI, and *Hpa*II. After *Hin*pI and *Hpa*I fragments were blunt ended, they and the *Alu*I fragments were inserted in separate ligations in the *Eco*RV site of pPS34. The cloned DNAs were pooled and, in a subsequent transformation, integrated into the chromosome of JH642 by homologous recombination. DNA from the pooled Erm^r transformants was prepared for use as a random insertion library.

Transfer of a suppressor mutation. Competent cells of strain SJB219 (Δ*citC*::*spc*) (Table 1) were transformed with the chromosomal DNA from a spontaneous pseudorevertant, KMB77S3 (Δ*citC*::*spc* Δ*amyE*::[*spoIIQ'-lacZ cat*] *spoVG2*), and chloramphenicol-resistant (Cam^r) transformants were selected. Most were slightly blue on DS plates containing X-Gal, but some colonies were as dark blue as KMB77S3, indicating that the unlinked *spoVG2* mutation and the *spoIIQ'-lacZ* fusion were cotransferred by congression. One of the congressants, KMB97, was used for subsequent mapping experiments (see Results).

Cloning of the *spoVG2* mutant allele. The *spoVG* locus of KMB97, located within a 680-bp *Hin*dIII fragment, was amplified by PCR with the following primers: 5′-AAGTGATTCTGGGAGAGCCGGGATC-3′ (which anneals about 170 bp upstream of the *spoVG* initiation codon) and 5′-AGGCTTACCGCAA ACTGGATGAAGG-3′ (which anneals about 280 bp downstream of the *spoVG* termination codon). The amplified DNA fragment (770 bp) was cloned directly into the modified *Eco*RV site of pT7Blue(R) (Novagen, Inc.). The resulting plasmid (pKM73) was sequenced to confirm that there were no additional mutations other than *spoVG2* in the *spoVG* open reading frame. In order to eliminate possible PCR-derived mutations in the *spoVG* promoter, the *Bst*XI-*Sac*I fragment containing the *spoVG2* mutation was excised and fused to the 5′ end of wild-type *spoVG* in pKM70 which had been digested with the same enzymes, creating pKM89. To create pKM70, the *spoVG*-containing 680-bp *Hin*dIII fragment from pLS5 (a pBR322 derivative) (64) was inserted at the *Hin*dIII site of a version of pSK− with a disrupted *Bst*XI site in its multicloning site.

Overexpression of *spoVG* and preparation of anti-SpoVG antibodies. A strain producing a maltose-binding protein (MBP)-SpoVG fusion protein was constructed as follows. A *Bam*HI site was introduced by PCR just upstream of the initiation codon of *spoVG*, and the amplified fragment was cloned in pT7Blue(R). A 300-bp *Bam*HI fragment from the resulting plasmid was inserted in the proper orientation at the *Bam*HI site of pMAL-c2 (New England Biolabs), a plasmid for construction of MBP fusions, to create pKM102. *E. coli* JM107 harboring pKM102 was used for overexpression of MBP-SpoVG.

Cells were grown to mid-exponential phase (optical density at 600 nm of 0.5) in 500 ml of L broth supplemented with 0.2% glucose and 100 μg of ampicillin per ml; 1 mM isopropyl-thio-β-D-galactosidase (IPTG) was added to the culture, and incubation was continued for 2.5 h. Cells were harvested, washed once with column buffer (20 mM Tris-HCl buffer [pH 7.4] containing 200 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, and 0.5 mM phenylmethylsulfonyl fluoride [PMSF]), and resuspended in 10 ml of the same buffer. Cells were disrupted by sonication and centrifuged to remove cell debris. Supernatant fluid was applied to an amylose column (≈5 ml) that had been equilibrated with column buffer. After extensive washing of the column, MBP-SpoVG was eluted with column buffer containing 10 mM maltose. Fractions containing MBP-SpoVG were collected and dialyzed against 20 mM Tris-HCl buffer (pH 8) containing 100 mM NaCl and 2 mM $CaCl_2$. About 30 μg of factor Xa (Boehringer Mannheim) was incubated overnight at 4°C with 9 mg of MBP-SpoVG. The reaction mixture was dialyzed against 20 mM Tris-HCl buffer (pH 8) containing 25 mM NaCl and 0.5 mM PMSF and then centrifuged. The supernatant fluid was subjected to DEAE-Sephacel chromatography to remove factor Xa. SpoVG and MBP-SpoVG coeluted from the DEAE column in a gradient of NaCl (25 to 500 mM). Fractions containing SpoVG and MBP-SpoVG were pooled and subsequently applied to an amylose column to separate SpoVG from residual MBP-SpoVG fusion protein. The flowthrough fraction from each preparation typically contained about 0.75 mg of SpoVG polypeptide.

SpoVG polypeptide (1 mg) was mixed with adjuvant and injected into a female New Zealand White rabbit. Injections and bleedings were done with the assistance of Sada Yaser, Tufts University Animal Care Facility. To remove nonspecific antibodies, the antisera against SpoVG were mixed with a crude extract of a *spoVG* null mutant and kept on ice for 1 h. After centrifugation, antibodies in the supernatant fluid were partially purified by differential ammonium sulfate precipitation (35 to 50% of saturation).

Immunoblot analysis. Cells were harvested from a 50-ml culture in DS medium, washed once with 50 mM HEPES buffer (pH 8) containing 1 mM EDTA, 150 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, and 0.5 mM PMSF, and resuspended in 2 ml of the same buffer. Cells were disrupted by sonication followed by centrifugation to remove cell debris. Supernatant fluid (crude extract; 10 μg protein) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electrotransferred to an Immobilon-P (Millipore Corp.) membrane. The membrane was exposed to anti-SpoVG antibodies, and the immune complexes were visualized by using color-forming substrates of an alkaline phosphatase-linked secondary antibody (30).

Microscopic analysis. Fixation of cells and DNA staining with 4,6-diamidino-2-phenylindole (DAPI) were done essentially by the method of Wu et al. (70). Cells were harvested before and after induction of sporulation by the resuspension method (65) and fixed in 70% ethanol. Cell suspensions were kept at 4°C overnight, and subsequently subjected to nucleoid staining (1 μg of DAPI per ml). Samples were observed by using an Olympus BX60 microscope equipped with a C4742-95 digital camera (Hamamatsu). Images were processed with Image Pro Plus version 3.0 (Media Cybernetics) and Adobe Photoshop.

Samples for thin-section electron microscopic analysis were prepared as described previously (31) and visualized by A. Brown-Cormier, Electron Microscopy Unit, Department of Anatomy and Cellular Biology, Tufts University.

Construction of other plasmids. pKM56 was constructed by inserting the 680-bp *Hin*dIII fragment of pLS5 (see above) in the *Hin*dIII site of pDG1664, a *B. subtilis* integration vecter for the *thrC* locus (22).

Plasmid pKM82, a derivative of pSK−, contains the *spoVG* gene within a *Hin*dIII fragment excised from pLS5 (see above). The orientation of the *spoVG* gene in pKM82 is such that transcription is in the direction opposite that of *lacZ*. pKM98 was constructed by ligating a *Pst*I-*Sal*I fragment of pKM82 to pHP13 (23), which had been digested with the same enzymes. The *spoVG2* allele was removed from pKM89 as a *Bam*HI-*Sal*I fragment and inserted at the corresponding sites of pHP13, creating pKM157.

Other methods. DNA sequencing was done by using a Sequenase reagent kit (U.S. Biochemical Corp.) or by M. Berne, Tufts University Protein and Nucleic Acid Analysis Facility, using an ABI Automated Sequencer. Direct sequencing of PCR-amplified fragments was performed after treatment of the fragments with shrimp alkaline phosphatase and exonuclease I (U.S. Biochemical Corp.) according to the manufacturer's instructions. DNA sequences were analyzed with the DNA Strider and BLAST programs (2). β-Galactosidase activity was measured and expressed as Miller units as described previously (61). All oligonucleotides used in this study were synthesized by M. Berne.

RESULTS

Isolation of spontaneous suppressor mutations that restore expression of *spoIIQ-lacZ* in a Δ*citC* mutant. Sporulation of an ICDH (Δ*citC*::*spc*) null mutant is blocked at stage I after expression of Spo0A-phosphate-dependent genes and bipolar localization of FtsZ rings early in stationary phase but before the formation of an asymmetric septum at one of the cell poles (31). Because of the failure of asymmetric septum formation, neither forespore-specific, σ^F-dependent genes nor mother cell-specific, σ^E-dependent genes are turned on in the Δ*citC* mutant (31). To investigate the basis of the stage I block, extragenic suppressor mutations were sought by looking for spontaneous variants of the Δ*citC* mutant in which expression of σ^F-dependent genes was restored. For this purpose, we used a Δ*citC* mutant strain (SJB295) carrying a fusion of the *E. coli lacZ* gene to the σ^F-dependent promoter of the *spoIIQ* gene (38). In independent trials, two spontaneous mutants were isolated as dark blue colonies on plates of DS medium containing X-Gal. These colonies were as translucent as those of the Δ*citC* mutant, indicating that they were still defective in sporulation. In transformation crosses, the suppressing mutations proved to be unlinked to *citC* or to the *spoIIQ'-lacZ* fusion integrated at the *amyE* locus. The *spoIIQ'-lacZ* fusion and the suppressing mutation of one of the strains were simultaneously moved by congression into strain SJB219 (Δ*citC*::*spc*), creating strain KMB97 (see Materials and Methods). As shown in Fig. 1a, *spoIIQ'-lacZ* expression in strain KMB97 was increased in stationary phase to almost 50% of that of the wild-type strain.

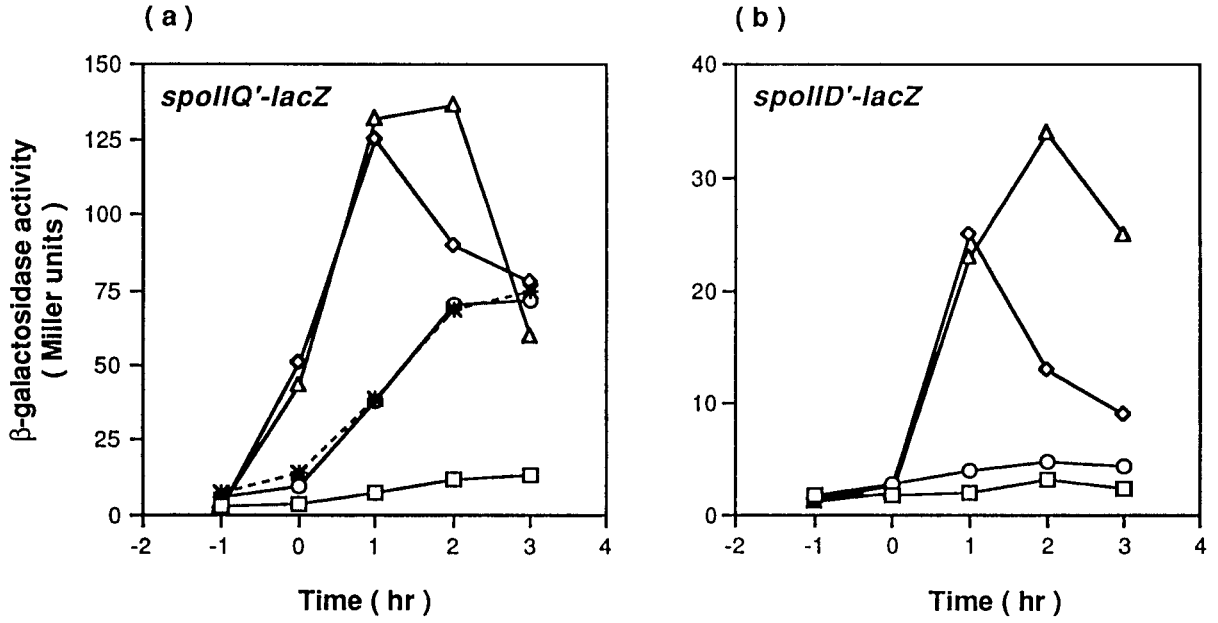

FIG. 1. Expression of *spoIIQ'-lacZ* (a) and *spoIID'-lacZ* (b) fusions in *citC spoVG* double mutants. Cells were grown in DS broth and harvested at the indicated times for measurement of β-galactosidase activity. Symbols: △, SJB294 (a) and SJB225 (b) (*citC*$^+$); □, SJB295 (a) and SJB229 (b) (Δ*citC*); ✳, KMB97 (Δ*citC spoVG2*); ○, KMB198 (a) and KMB208 (b) (Δ*citC* Δ*spoVG*); ◇, KMB200 (a) and KMB209 (b) (Δ*spoVG*). Time zero is defined as the onset of stationary phase.

Identification of the *spoVG2* mutation. To map the suppressing mutation in KMB97, we initially transformed strain KMB97 with a chromosomal library of random integrations of pPS34 (Erm^r; see Materials and Methods). DNA from the pooled transformants was then used to transform strain SJB295. Erm^r transformants that formed blue colonies on plates of DS medium containing X-Gal were tested individually for linkage of the suppressing mutation to *erm*. The location of an integrated plasmid showing about 50% linkage to the suppressing mutation was determined by cleavage of chromosomal DNA with *Eco*RI, ligation at a low DNA concentration, transformation of *E. coli*, extraction of plasmid, and sequencing of the cloned insert. The site of plasmid insertion was in the 5′ end of the *mfd* gene (44), located at 7° on the chromosomal map. Further genetic crosses demonstrated that the mutation is 30% linked to *abrB* (47), located about 15 kb upstream of the *mfd* gene, and 70% linked to each of two insertion mutations previously shown to flank the *spoVG* gene (24). The *spoVG* locus of KMB97 was amplified by PCR and direct sequence analysis of the PCR-amplified fragments showed a point mutation (CCT to CTT), causing a single amino acid substitution from proline to leucine at residue 63. This mutation was designated *spoVG2*. Subsequently, the *spoVG2* mutant allele was cloned and the presence of the mutation was confirmed by sequence analysis. The second, independently isolated, suppressor mutant was found to have the same mutation, perhaps because this mutation causes the same phenotype as a null mutation (see below).

Proof that *spoVG2* is the suppressing mutation and is recessive. When the wild-type *spoVG* gene was introduced at the *thrC* locus in KMB97, the suppressor mutant phenotype disappeared (Fig. 2), indicating that *spoVG2* is required for suppression and that the *spoVG2* mutation is recessive to the wild-type *spoVG*$^+$. The recessiveness of *spoVG2* suggests that it is a loss-of-function mutation. To confirm this point, we tested the ability of null alleles of *spoVG* to suppress the Δ*citC* mutation. When *spoVG*::Tn*917*ΩHU265 (56) or Δ*spoVG*::*tet* (66) was introduced into the Δ*citC* mutant (creating strain KMB136 or KMB198, respectively), the expression of the *spoIIQ* gene in each of the *citC spoVG* mutants was indistinguishable from that in KMB97 (Fig. 1a and data not shown), implying that the *spoVG2* mutation abolishes a negative effect of SpoVG on σ^F activation in the Δ*citC* mutant.

Expression of *spoVG* in a *citC* mutant. Although Δ*citC* mutant cells were known to express the *spoVG* gene and have no defect in σ^H activity (31), we reexamined *spoVG* gene expression in detail. As shown in Fig. 3a, *spoVG* expression was

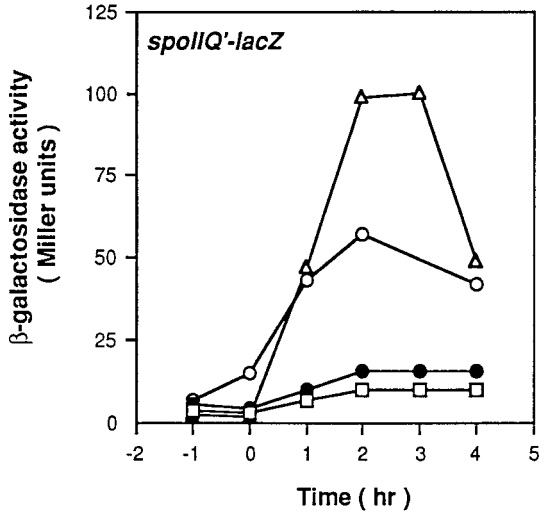

FIG. 2. *spoVG2* is a recessive mutation. Cells carrying a *spoIIQ'-lacZ* fusion were grown in DS broth and harvested at the indicated times for measurement of β-galactosidase activity. Symbols: △, SJB294 (*citC*$^+$); □, SJB295 (Δ*citC*); ○, KMB97 (Δ*citC spoVG2*); ●, KMB158 (Δ*citC spoVG2* Δ*thrC*::*spoVG*$^+$). Time zero is defined as the onset of stationary phase.

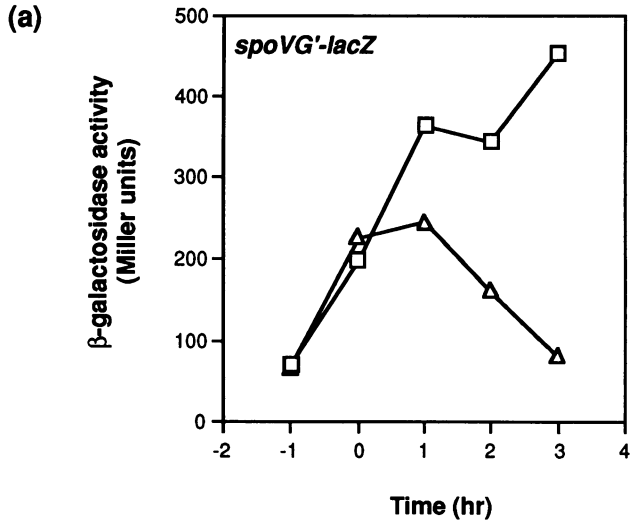

FIG. 3. Expression of a *spoVG'-lacZ* fusion in a Δ*citC* mutant. (a) Wild-type (SJB78; △) and Δ*citC* mutant (KMB162; □) strains were grown in DS broth and harvested at the indicated times for measurement of β-galactosidase activity. (b) Samples of wild-type (SJB294) and Δ*citC* mutant (SJB295) cultures were harvested at the 1-h intervals after the onset of stationary phase indicated over the gels and disrupted by sonication. Proteins (10 μg) in the crude extract were separated in sodium dodecyl sulfate-polyacrylamide gels and subjected to immunoblot analysis with anti-SpoVG antibodies.

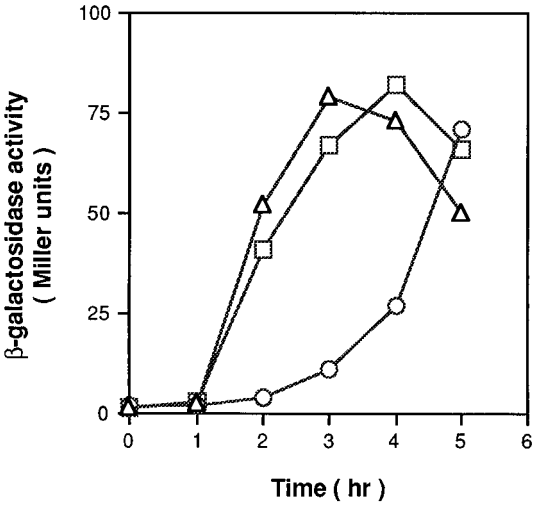

FIG. 4. Expression of *spoIIQ'-lacZ* in strains harboring the *spoVG* gene and the *spoVG2* allele on a multicopy plasmid. Cells having pHP13 (vector only; △), pKM98 (*spoVG*$^+$; ○), or pKM157 (*spoVG2*; □) were grown in DS broth and harvested at the indicated times for measurement of β-galactosidase activity. Time zero is defined as the onset of stationary phase.

induced at the normal time in the mutant at the onset of sporulation but, unlike the case for the wild-type strain, failed to shut off after T_1 (1 h after the onset of stationary phase), reaching a maximum level approximately twofold higher than that seen in the wild-type strain.

Consistent with the results of *spoVG'-lacZ* expression, the level of SpoVG protein in the wild-type strain was high in early stationary phase, at least until T_1, but decreased significantly thereafter (Fig. 3b). In the Δ*citC* mutant, the level of SpoVG was constant throughout stationary phase, remaining at nearly the maximal level seen in the wild-type strain (Fig. 3b), raising the possibility that the phenotype of a Δ*citC* mutant is due, at least in part, to prolonged expression or stability of SpoVG.

Overexpression of *spoVG* in *B. subtilis*. To assess further the potential negative effect of SpoVG on asymmetric septation, we measured the effect on wild-type cells of overexpression of *spoVG*. We used a low-copy-number plasmid, pHP13, for this purpose, since it is known that high dosage of the σ^H-dependent *spoVG* promoter results in severe inhibition of sporulation at an early stage (3, 73). Figure 4 shows that, in wild-type cells harboring *spoVG*$^+$ on pHP13 (pKM98), σ^F-dependent *spoIIQ* expression was significantly delayed. This inhibitory effect was not caused by titration by the *spoVG* promoter of any regulatory factor, since no detectable difference in *spoIIQ* expression was found in cells carrying the plasmid-borne *spoVG2* allele compared to cells carrying the vector only.

Sporulation gene expression in a *citC spoVG* double mutant. Since colonies of a Δ*citC*::*spc* Δ*spoVG*::*tet* double mutant were as translucent as those of the original Δ*citC* mutant and the number of heat-resistant spores, measured at about T_{20}, was nearly identical to that of the Δ*citC* mutant (Table 2), we examined later stages of the sporulation pathway to identify the defect. The expression of the mother cell-specific, σ^E-dependent *spoIID* gene (52) is normally induced following activation of σ^F in the forespore. However, in the Δ*citC* Δ*spoVG* double mutant, there was relatively little induction of *spoIID'-lacZ* (Fig. 1b). As expected from the low level of σ^E activity in the mother cell, there was little or no expression of the later sporulation genes *sspE* (17) and *cotA* (57), which are transcribed by RNA polymerases containing σ^G and σ^K, respectively (data not shown). These defects were not due to the *spoVG* mutation, since a *spoVG* single mutant had no detectable deficit in *spo* gene expression (data not shown) and sporu-

TABLE 2. Sporulation of *citC spoVG* double mutant[a]

Strain (relevant genotype)	Viable cells (no./ml)	Spores (no./ml)
JH642 (wild type)	6.3×10^8	5.0×10^8
KMB199 (Δ*spoVG*)	1.7×10^8	1.2×10^8
SJB219 (Δ*citC*)	2.6×10^7	3.3×10^3
KMB197 (Δ*citC* Δ*spoVG*)	1.2×10^7	2.4×10^3

[a] Cells were grown in DS broth. At T_{20}, viable-cell titers were determined by plating and spore titers were determined by plating after heating at 80°C for 10 min.

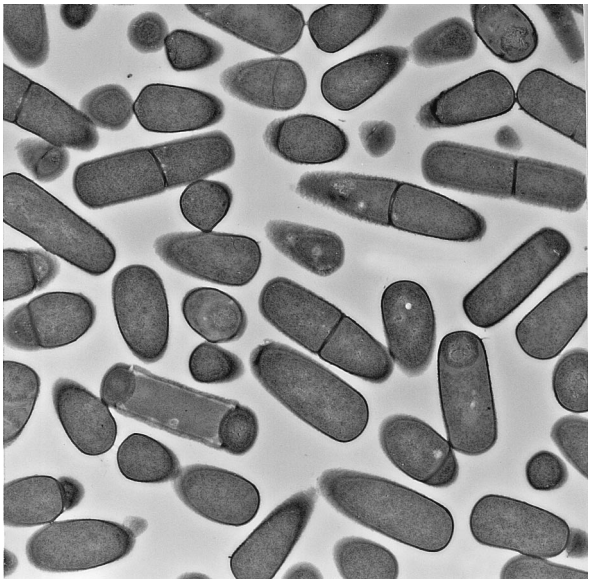

FIG. 5. Thin-section electron microscopic analysis. A sample of a culture of Δ*citC* Δ*spoVG* mutant cells (KMB197) was harvested at T_6 and prepared for transmission electron microscopy as described in Materials and Methods.

TABLE 3. Expression of sporulation genes in tricarboxylic acid cycle mutants

Relevant genotype	Supplement to medium[b]	Relative expression of sporulation genes (%)[a]			
		spoIIQ	*spoIID*	*sspE*	*cotA*
Δ*citC*	None	<10	<10	<10	<10
Δ*citC* Δ*spoVG*	None	43	19	<10	<10
	HEPES	81	20	29	<10
	$MnCl_2$	62	36	27	<10
	HEPES + $MnCl_2$	170	43	30	37
Δ*citC* Δ*spoVG sof-1*	None				<10
Δ*citZC*	None	26	13	<10	<10
Δ*citZC* Δ*spoVG*	None	76	36	29	<10
Δ*citZC* Δ*spoVG sof-1*	None				110

[a] *B. subtilis* strains carrying a promoter of a sporulation gene (σ^F-dependent *spoIIQ*, σ^E-dependent *spoIID*, σ^G-dependent *sspE*, or σ^K-dependent *cotA*) fused to the *E. coli lacZ* gene were grown in DS broth and harvested every hour after onset of stationary phase for measurements of β-galactosidase activity. The levels of expression of the *lacZ* fusions in mutants were estimated relative to the maximum level of expression of the same fusions in the wild-type strain. The average maximal β-galactosidase activities (in Miller units) of the various fusions in the wild-type strains were: *spoIIQ-lacZ*, 120; *spoIID-lacZ*, 27; *sspE-lacZ*, 250; and *cotA-lacZ*, 100.

[b] In some experiments, 50 mM HEPES (pH 8) or 0.75 mM $MnCl_2$ or both were added to DS medium.

lated at close to the wild-type frequency (reference 53 and Table 2).

Thin-section electron microscopy of the Δ*citC* Δ*spoVG* mutant revealed that approximately 20% of the population of cells formed the sporulation-specific asymmetric septum, i.e., overcame the stage I block in sporulation characteristic of the Δ*citC* single mutant, and proceeded further in the developmental pathway (Fig. 5). At T_6, engulfment of the forespore (normally seen at T_3 to T_4) may have been completed in a few cells, but no further development was seen. It seems that post-stage I defects remain unsuppressed in the *citC spoVG* double mutant.

In the accompanying paper, we show that the stage I block in a Δ*citC* mutant can be partially overcome by a reduction in citrate accumulation (41). To see whether the citrate and *spoVG* effects are additive, we created a triple mutant strain from which *citZ* (the major citrate synthase gene), *citC*, and *spoVG* had been deleted. In this strain, expression of *spoIIQ'-lacZ* was nearly as high as in wild-type cells, and expression of both *spoIID'-lacZ* and *sspE'-lacZ* was increased (Table 3). The inhibitory effect of excess citrate is due to a decrease in pH and chelation of divalent cations (41). Thus, the effect of the *citZ* mutation on a *citC spoVG* double mutant could be mimicked by supplementing the medium with HEPES buffer (pH 8) or excess $MnCl_2$ (Table 3). Simultaneous addition of HEPES and excess Mn^{2+} to *citC spoVG* double-mutant cells allowed significant restoration of expression of all *spo* genes tested (Table 3), yet the cells still did not form heat-resistant spores efficiently (7.2×10^6 per ml). This result presumably indicates that the absence of ICDH activity causes additional physiological changes that we have yet to identify.

The only combination of conditions known to suppress the sporulation defect of a *citC* mutant nearly completely is to reduce citrate production (as in a *citZ* mutant) and modify the *spo0A* gene by the *sof-1* mutation (41). When the *sof-1* allele of *spo0A* was introduced into *citZC spoVG* mutant cells, late *spo* gene expression and spore formation were restored to near-normal levels (Table 3 and data not shown).

Minicell formation in *spoVG* null mutant. Since the phenotype of the *spoVG* mutant suggests that the wild-type protein interferes with asymmetric septation, we tested the effects on cell division and sporulation of a *spoVG* mutation alone or in combination with a mutation in *minD*, a gene whose product inhibits polar septation during exponential growth (34, 35, 69). We confirmed that single null mutations in either *spoVG* or *minD* had relatively small effects on sporulation frequency (34, 35, 53). In our hands, the numbers of viable cells and spores obtained with either strain were decreased to about 50% of those of the wild-type strain after growth overnight in DS broth (Table 2 and data not shown). The *minD spoVG* double mutant suffered further losses in viability and spore titer to 30 and 10%, respectively (data not shown). The impaired ability of the double mutant to maintain viability and produce spores was reflected in the production of minicells by single and double mutants. O. Resnekov (51) had noted previously that a *spoVG* null mutant produces minicells. We estimated the minicell fraction at 2 to 3% of total *spoVG* mutant cells during vegetative growth (Fig. 6 and Table 4). A *minD* mutant produced about 13% minicells.

We examined minicell formation in a mid-exponential-phase culture of the Δ*spoVG* mutant in more detail by visualization of septa and nucleoids. Surprisingly, in addition to typical anucleate minicells, the Δ*spoVG* mutant culture also included cells having unusual distributions of nucleoids, such as minicells containing a condensed nucleoid or cells with a disporic phenotype (60), both occurring at a very low but significantly higher frequency than in the wild-type strain (Fig. 6 and Table 4). When mid-exponential-phase cells of the *spoVG* mutant were induced to sporulate by the resuspension method to synchronize the initiation of sporulation, significant numbers of anucleate minicells, minicells containing nucleoids, and disporic cells were found in the mutant culture at T_1, and stage II cells appeared earlier in the mutant culture than in the wild-type strain culture (Fig. 6 and Table 4).

Since polar septation concomitant with condensation of the nucleoid in the forespore compartment is the cell division event unique to initiation of sporulation in *B. subtilis*, we tested the contribution of the Spo0A phosphorelay (7), the essential signal transduction system for initiation of sporulation, to the formation of these kinds of minicells. When *spo0F* and *spo0B*

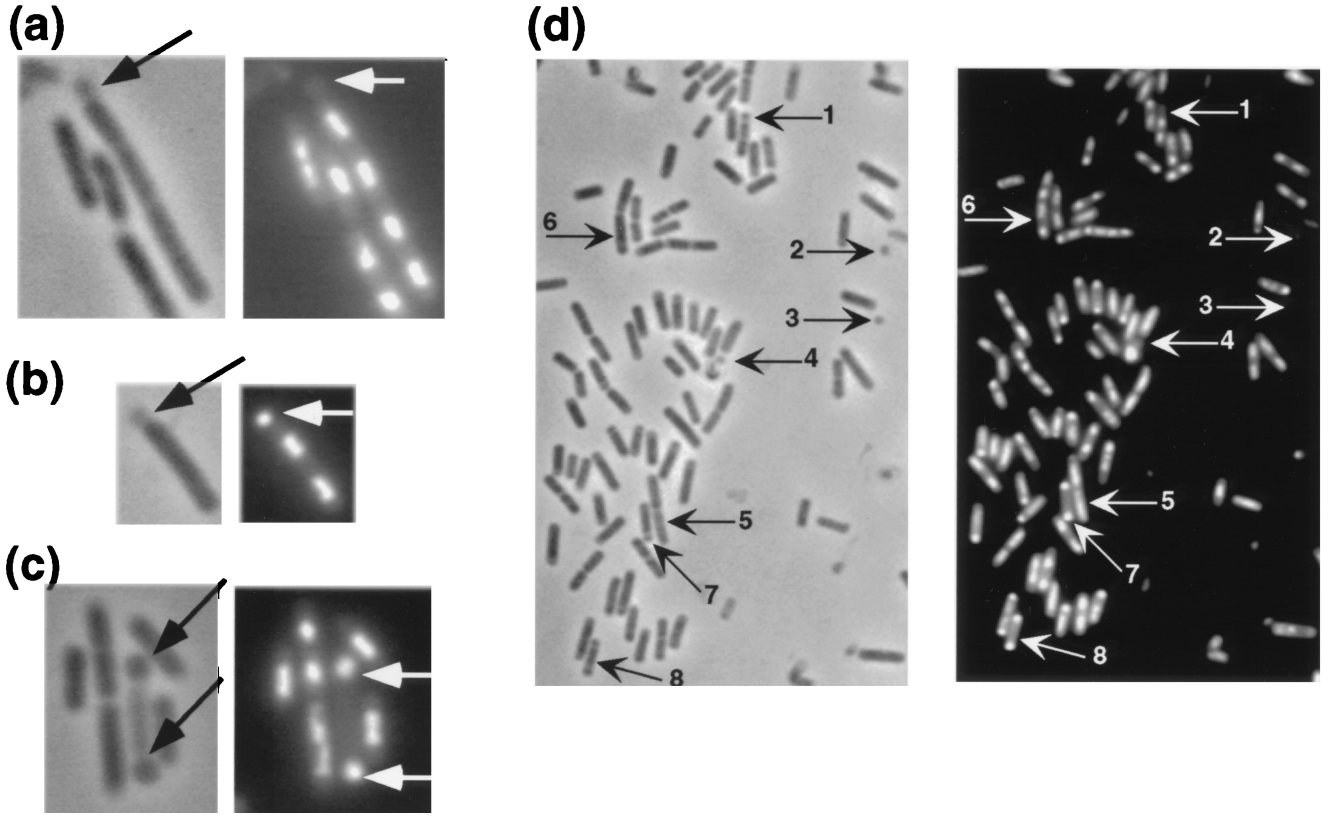

FIG. 6. Minicell formation in a Δ*spoVG* mutant. Cells of strain KMB200 (Δ*spoVG*) were grown to mid-exponential phase and induced to sporulate by the resuspension method of Sterlini and Mandelstam (65). Ethanol-fixed cells were observed under phase-contrast microscopy (left-hand panels), and nucleoids were visualized by DAPI staining (right-hand panels). (a to c) Exponential-phase cells, showing an anucleate minicell (a), a minicell containing a condensed nucleoid (b), and a disporic cell (c). (d) Cell population of the mutant at 1 h after resuspension. Arrows 1 to 4 indicate anucleate minicells, arrow 5 indicates a sporulating cell at stage 0-I, arrow 6 indicates a sporulating cell at stage II, and arrows 7 and 8 indicate disporic cells.

mutations were introduced into the Δ*spoVG* mutant, few minicells of any type were observed in a vegetative cell culture of the triple mutant. Similarly, a *spo0A* deletion greatly decreased the frequency of minicells in the Δ*spoVG* mutant culture (<0.13%). In contrast, formation of anucleate minicells by a *minD* mutant was not affected by a *spo0A* mutation.

To see whether the effects of a *citC* mutation would be suppressed by a *minD* mutation, we tested *spoIIQ'-lacZ* expression and sporulation in a *citC minD* double mutant. A *minD* mutation had no effect on sporulation frequency or expression of *spoIIQ* in a *citC* mutant (data not shown).

DISCUSSION

Null mutations in the *spoVG* gene partially relieve the stage I block of a Δ*citC* mutant, allowing a significant fraction of the Δ*citC* mutant cell population to form an asymmetric septum and activate expression of σ^F-dependent genes in the forespore compartment. This evidence suggests that a normal function of ICDH overcomes the activity of SpoVG, which is itself an inhibitor or a negative regulator of the σ^H-dependent pathway to the asymmetric septum. In other work, we have shown that it is the enzymatic activity of ICDH that is critical for completing stage I (31, 41).

TABLE 4. Minicell formation in Δ*spoVG* mutant

Type of cell[a]	No. of cells[b] at:					
	Mid-exponential phase		1 h[c]		2 h[c]	
	Δ*spoVG*	WT	Δ*spoVG*	WT	Δ*spoVG*	WT
Vegetative cell or stage 0-I cell	1,223	1,442	1,003	1,009	741	652
Anucleate minicell	40	1	15	1	13	1
Minicell containing a nucleoid	2	0	9	0	2	0
Sporulating cell at stage II	2	1	55	16	122	126
Disporic cell	3	0	3	0	9	0

[a] Examples of vegetative cells, anucleate minicells, minicells containing a nucleoid, disporic cells, and sporulating cells at stages 0-I and II are shown in Fig. 6.
[b] Cells of wild-type (WT) and Δ*spoVG* mutant strains were grown in Casamino Acids-containing rich medium of Sterlini and Mandelstam (65) to mid-log phase and induced to sporulate by resuspension into poor medium. Cells were fixed with 70% ethanol to visualize septa, and nucleoid distribution was analyzed by DAPI staining (see Materials and Methods for details). Numbers represent total counts obtained from four individual experiments.
[c] After induction of sporulation.

spoVG was the first developmentally regulated gene cloned from *B. subtilis* (58). Close homologs are now known to exist in *Bacillus megaterium*, *Clostridium acetobutylicum*, *Borrelia burgdorferi*, and *Archaeoglobus fulgidus*, organisms which collectively represent gram-positive and -negative eubacteria and the archaea. Proline-63, the residue mutated in our suppressor strains, is conserved in all five SpoVG proteins.

The absence of SpoVG in sporulating cells of *B. subtilis* is known to cause aberrations in the germ cell wall and the cortex, defects seen at T_5 to T_6 (stage V) (53), and to make the stage II defect of a *spoIIB* mutant more severe (40). However, extensive gene expression studies have shown that the *spoVG* gene is a very early stationary phase gene, expressed at a significant level even during exponential growth phase and further induced at the end of exponential phase (45, 74). This transcription depends on the σ^H form of RNA polymerase and is partially repressed during early exponential phase by a global negative regulator, AbrB (74). The concentration of SpoVG protein reaches a maximum level at the onset of stationary phase and decreases substantially after T_2, indicating that SpoVG most likely has its primary function early in stationary phase. In the Δ*citC* mutant, *spoVG* expression is induced at the normal time but continues to increase even after several hours in stationary phase. Moreover, the level of SpoVG protein remains high at a time when it is greatly diminished in wild-type cells. Thus, the dependence of asymmetric septation on ICDH activity might be attributed to an effect on SpoVG synthesis or stability. Asymmetric septation, however, is an early event in stationary phase; it is uncertain whether prolonged expression of SpoVG (seen after T_1) is in fact responsible for the primary effect of the *citC* mutation. On the other hand, overexpression of *spoVG* in wild-type cells interferes with asymmetric septation.

The MinCD complex is a cell division inhibitor in exponential-phase cells of *B. subtilis* (34, 35, 69), as well as in *E. coli* (12, 13). Cells lacking these proteins form septa at potential division sites near cell poles and produce anucleate minicells during vegetative growth (13). The Min proteins appear to mask the polar cell division sites in normal growing cells, restricting FtsZ ring formation to medial sites (49). (Under some growth conditions, other proteins can carry out this function in *B. subtilis* [36a].) The masked sites, however, must become accessible to FtsZ when *B. subtilis* cells enter the spore formation program, implying that polar septation inhibitors (e.g., MinCD) lose activity in stationary phase. Our finding that deletion of the *spoVG* gene increases the rate and extent of asymmetric septation in both wild-type and *citC* mutant cells suggests that a normal role of SpoVG in early stationary phase is to temporarily prevent asymmetric septation after the Min proteins cease to function. Perhaps the subtle defects observed in late sporulating cells of a *spoVG* mutant (40, 53) are due to the loss of precise timing of asymmetric septum formation.

Although the primary function of SpoVG seems to be to regulate asymmetric septation in stationary-phase cells, the *spoVG* gene is also expressed, albeit at a lower level, in a population of exponential-phase cells. The fact that an exponential-phase culture of the *spoVG* mutant contains some minicells with condensed nucleoids and cells with a disporic phenotype is consistent with a heterogeneous population in which some cells initiate sporulation while others grow. This supposition is consistent with our finding that appearance of the aberrant cells is dependent on the Spo0A phosphorelay.

While inactivation of SpoVG partially overcomes the stage I block of a Δ*citC* mutant, the cells do not complete the sporulation process or even proceed beyond stage III. Moreover, only a subpopulation (<30%) of the *citC spoVG* double mutant cells form asymmetric septa and the level of σ^F-dependent gene expression reaches only 30 to 50% of the level in wild-type cells. Further suppression of the stage I block in a *citC* mutant requires reducing citrate synthesis or raising the pH of the medium and supplementation with $MnCl_2$ to compensate for overaccumulation of citrate (41).

The mechanism by which SpoVG regulates the extent and timing of asymmetric septation is unknown. SpoVG may interact directly with the septation apparatus or may act indirectly by regulating the synthesis or stability of proteins required for septation. Future experiments will be directed toward identifying the mechanism by which SpoVG regulates asymmetric septation and the means by which ICDH activity intervenes in this process. A working model has the following elements. As cells make the transition from exponential growth to stationary phase, *citC* and *spoVG* are simultaneously induced. At the same time, polar sites for assembly of FtsZ-containing cell division complexes become unmasked, but polar septation is still prevented in an unknown way by newly accumulated SpoVG. As Krebs cycle enzymes, including ICDH, begin to function, acidic metabolites of the glycolytic pathway are assimilated, raising extracellular pH, inactivating SpoVG, and inducing asymmetric septation, a process that requires one or more Mn^{2+}-dependent proteins. Our data, however, do not rule out the possibility that low pH, Mn^{2+} limitation, and SpoVG affect polar septation by independent mechanisms.

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